DNA topoisomerase IIα is required for chromatin condensation during prophase. This process is temporally linked with the appearance of mitosis-specific phosphorylation sites on topoisomerase IIα including one recognized by the MPM-2 monoclonal antibody. We now report that the ability of mitotic extracts to create the MPM-2 epitope on human topoisomerase IIα is abolished by immunodepletion of protein kinase CK2. Furthermore, the MPM-2 phosphopeptide on topoisomerase IIα can be generated by purified CK2. Phosphorylation of C-truncated topoisomerase IIα mutant proteins conclusively shows, that the MPM-2 epitope is present in the last 163 amino acids. Use of peptides containing all conserved CK2 consensus sites in this region indicates that only the peptide containing Arg-1466 to Ala-1485 is conserved CK2 consensus sites in this region indicates that only the peptide containing Arg-1466 to Ala-1485 is able to compete with topoisomerase IIα for binding of the MPM-2 antibody. Replacement of Ser-1469 with Ala abolishes the ability of the phosphorylated peptide to bind to the MPM-2 antibody while a peptide containing phosphorylated Ser-1469 binds tightly. Surprisingly, the MPM-2 phosphopeptide influences neither the catalytic activity of topoisomerase IIα nor its ability to form molecular complexes with CK2 in vitro. In conclusion, we have identified protein kinase CK2 as a new MPM-2 kinase able to phosphorylate an important mitotic protein, topoisomerase IIα, on Ser-1469.

Precise coordination of cell cycle progression is critical not only for normal cell division but also under conditions of stress leading to DNA damage or incomplete DNA synthesis. Derepression of cell cycle control has been shown to be a leading cause of genetic instability in human cancers (1–3) for which regulation of cell cycle control has been shown to be a leading cause of genetic instability in human cancers (1–3) for which reason considerable effort is invested toward the identification and characterization of the surveillance mechanisms that control cell cycle progression ("check points"). To prevent damaged cells to divide, the G2 checkpoint is activated in response to DNA damage or incomplete DNA synthesis leading to cell cycle arrest at the G2/M interphase (2, 4, 5). In addition, vertebrate cells can activate a checkpoint during early prophase in response to DNA damage resulting in return of damaged cells to G2 (6). Traditionally defined, the prophase stage of mitosis starts with the first visible sign of chromosome condensation and ends at nuclear envelope breakdown. While the exact biochemical mechanisms controlling the onset of prophase are incompletely understood (for recent review, see Ref. 7), chromosome condensation is associated with extensive phosphorylation of proteins involved in the regulation of chromatin structure. For example, the nuclear enzyme DNA topoisomerase IIα which is known to play an important role in chromosome condensation (8–12) is subject to complex phosphorylation during mitosis including phosphorylation by the mitotic kinase cdc2 (also known as p34cdc2-cyclin B or CDK1). These events lead to the generation of mitosis-specific phosphorylation sites which are recognized by the monoclonal MPM-2 and 3F3/2 antibodies (13–15). Among these phosphorylation sites, the MPM-2 epitope appears to be particularly important, since its presence on mitotic chromosomes is closely associated with the condensed state (6). The MPM-2 monoclonal antibody was originally raised against mitotic HeLa cells. Subsequent studies show, that it specifically recognizes a cell cycle-regulated phosphopeptide present in mitotic and meiotic proteins from a wide variety of species (16). These proteins become phosphorylated at the G2/M transition and are dephosphorylated at the end of mitosis (17). In addition to topoisomerase IIα, more than 50 other phosphorylated proteins are recognized by the MPM-2 antibody including microtubule-associated proteins, components of the anaphase-promoting complex, phosphatases, and a number of protein kinases including protein kinase CK2 (16, 18–26). Multiple kinases are able to generate MPM-2 epitopes including mitotic kinases such as cdc2 kinase as well as kinases which are also active during interphase such as MAP1 kinase (22, 27–32). Interestingly, some MPM-2 kinases as, for example, NIMA are themselves activated by other MPM-2 kinases indicating the complexity of the signaling pathways which regulate mitotic entry (22, 33).

Protein kinase CK2 is a serine/threonine kinase which has shown to be dramatically phosphorylated in mitotic cells (34, 35). CK2 is the major kinase phosphorylating topoisomerase II in yeast (36) and a stable topoisomerase II-CK2 molecular complex has been demonstrated (12). Interestingly, CK2 differentially phosphorylates topoisomerase II in a cell cycle-dependent manner: some phosphoacceptor sites are preferentially phosphorylated in G2, while others are preferentially phosphorylated in mitosis (36).

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In the present study, we have identified CK2 as a topoisomerase II-directed MPM-2 kinase and characterized the phosphorylation site which leads to generation of the MPM-2 epitope on human topoisomerase IIα. We have also investigated the influence of this phosphorylation on the catalytic activity of topoisomerase II as well as on the ability of topoisomerase IIα to form molecular complexes with CK2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nocodazole, leupeptin, pepstatin A, CHAPS, EGTA, Trizma, HEPES, ATP, GTP, heparin, Tween 20, IGEPAL CA-630, penicillin G, streptomycin sulfate, 100 μM sodium fluoride, 0.5 mM DTT, and protease inhibitor tablets were purchased from Sigma-Aldrich. DNase I, protein A-Sepharose, and Pefabloc SC were obtained from Roche Molecular Biochemicals. Immunoblot polyvinylidene difluoride membranes, Tris-Tricine, 10–20% linear gradient ready gel, Tris-Tricine SDS-PAGE sample buffer, 10 × Tris/Tricine/SDS, and peptide molecular weight markers were supplied from Bio-Rad. λ-Phosphatase and protein molecular weight markers were purchased from New England Biolabs, Inc. Protein Phosphatase 2A was purchased from Upstate Biotechnology, PIPES was obtained from Research Organics Inc. and protein G-Sepharose was supplied by Zymed Laboratories Inc. Immuno- blot maxisorp surface (96 well) were supplied by Nalge Nunc International. Microcon centrifugal filters were purchased from Millipore while Western blots were obtained from Amersham Pharmacia Biotech. Peptides were synthesized, purified, and characterized by mass spectrometry by Neosys- tem Laboratories.

**Antibodies**—Mouse monoclonal anti-HPM-2 antibodies were purchased from Upstate Biotechnology. Rabbit polyclonal anti-CK2 α and anti-CK2 β antibodies were prepared as described (37, 38). Anti-topoisomerase IIα mouse monoclonal antibodies SWTS3D1 and SWR1C2, herein designated T3D1 and RC12, were generous gifts from Gary Gorsky (University of Oklahoma, Oklahoma City, OK). Peroxidase-conjugated goat anti-rabbit and donkey anti-mouse antibodies were supplied by Jackson Immunoresearch Laboratories, Inc.

**Enzymes**—The YepWOB6 plasmid containing hTopoIIα cDNA under the Gal1 promoter (39) was kindly provided by James C. Wang (Harvard University, MA). The plasmid was overexpressed in Saccharomyces cerevisiae DBY 745 strain and purified as described (40). Purified enzyme preparations contained no detectable DNA topoisomerase I activity as determined by relaxation of supercoiled plasmid DNA in the absence of ATP. C-terminal truncated forms of hTopoIIα were constructed from the YepWOB6 plasmid. Recombinant CK2 was expressed in baculovirus-infected insect cells and purified as described (32). Cd3 kinase was generously provided by Laurent Meijer (Station Biologique, Roscoff, France).

**Cell Culture**—HeLa S3 cells were grown in 0.5-liter spinner flasks in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 60 μM MgCl2, 1 mM DTT, 100 μM ATP, 0.1% Tween 20 (TBST), 100 μM Nac, and 0.5% Tween 20 with PBS prior to extraction with 200 μl of a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% CHAPS, 200 mM microcystin-LR (Calbiochem), 200 μM okadaic acid (Sigma) and a mixture of protease inhibitors (5 μg/ml of pepstatin A, leupeptin, and Pefabloc SC) was added. Extraction mixtures were incubated 20 min at 4 °C with intermittent vortexing followed by centrifugation at 4 °C for 15 min at 10,000 x g. Supernatants were saved, and protein concentrations determined. For CK2 immunodepletion or heparin depletion experiments, protein concentrations were adjusted to 500 μg/ml prior to addition of anti-CK2 antibodies or heparin-Sepharose. In addition, the lysis buffer also contained 300 mM NaCl.

**CK2 Immunodepletion**—Mitotic cell extracts were prepared as described and incubated for 3 h at 4 °C under rotation in the absence of presence of a 1/100 dilution of a polyclonal antibody directed against the regulatory β subunit. Extracts were incubated with protein A-Sepharose beads for 30 min at 4 °C. Supernatants were saved and the precipitation repeated overnight with or without the CK2 β-directed antibodies. After incubation with protein A-Sepharose, supernatants were saved and new precipitations were carried out for 3 h at 4 °C in the absence or presence of a 1/100 dilution of a polyclonal antibody directed toward the catalytic α-subunit of CK2 in order to eliminate CK2 activity which is not associated with the β subunit. Supernatants were collected after incubation with protein A-Sepharose and their remaining MPM-2 kinase activity was determined.

**Determination of MPM-2 Kinase Activity**—Depleted and non-depleted mitotic extracts were diluted to 20 ng/μl with TEM (20 μl final volume), containing phosphatase and protease inhibitors. The reaction buffer was supplemented with 1 mM DTT, 0.5 mM ATP or GTP as indicated. Three hundred ng of purified mutant or wild type hTopoIIα were incubated with diluted extracts for 25 min at 37 °C. Heparin at 25 μg/ml was included in some assays to determine if this would inhibit the MPM-2 kinase. Reactions were stopped with 2 × SDS-PAGE loading buffer (30% glycerol, 4% sodium dodecyl sulfate, 187 mM Tris-HCl, pH 6.8, and bromphenol blue).

**Preparation of Extracts from Isolated Chromosomes and Mitotic Cells**—Nocodazole-blocked HeLa S3 cells (1 × 10⁶) were centrifuged 5 min at room temperature at 200 × g. Pellets were washed three times with PBS prior to extraction with 200 μl of a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 750 mM NaCl, 2 mM EDTA, 0.75% IGEPAL CA-630, 200 μM microcystin-LR, 200 μM okadaic acid and protease inhibitor mixture. The salt concentration of this buffer is elevated in order to precipitate any chromatin proteins such as hTopoIIα. Heparin (25 μg/ml) was included in some of these assays to determine if this would inhibit the MPM-2 kinase. Reactions were stopped with 2 × SDS-PAGE loading buffer (30% glycerol, 4% sodium dodecyl sulfate, 187 mM Tris-HCl, pH 6.8, and bromphenol blue).

**Gel Electrophoresis and Immunoblotting**—Proteins were separated by electrophoresis using 5–20% gradient SDS-polyacrylamide gels and transferred to Immunoblot polyvinylidene difluoride membranes. For Western blot analysis, membranes were first blocked for 45 min at room temperature with 20 μg Tris-HCl, pH 7.9, 137 mM NaCl (TBS) containing 5% bovine serum albumin (Sigma-Aldrich). For immunoblotting, the MPM-2 mouse monoclonal antibody was diluted to 2 μg/ml, the rabbit polyclonal anti-CK2 α antibody diluted 1000 times and the anti-hTopoIIα mouse monoclonal antibody 1:500 times in TBS containing 0.1% Tween 20 (TBST). Incubation with the first antibody was carried out for 90 min at room temperature. Membranes were then washed twice for 10 min with TBST followed by 1 h incubation with the peroxidase-conjugated secondary antibodies. The goat-anti-rabbit antibodies were diluted 80,000 times in TBST whereas the donkey anti-mouse antibodies were diluted 4000 times in the same buffer. Membranes were washed again with TBST and the results were revealed with the ECL chemiluminescence kit.

**Phosphorylation of hTopoIIα by Purified CK2 and cd2c Kinase**—Purified hTopoIIα (150 ng) was incubated for 25 min at 30 °C with either 25 ng of purified CK2 or cd2c kinase or with both enzymes together in a 15-μl final volume containing 20 μM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, 100 μM βSA, 50 mM NaCl, and 20 μM ATP.

Reactions were stopped by addition of 15 μl of 2 × SDS-PAGE loading buffer.

**Cell Extract Preparation for MPM-2 Kinase Assay**—Nocodazole-blocked HeLa S3 cells (5 × 10⁶) were centrifuged for 5 min at room temperature at 200 × g. Pellets were washed three times with phosphate-buffered saline (PBS) and 1 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EGTA, 4 mM MgSO4 (TEM), 1% CHAPS, 200 mM microcystin-LR (Calbiochem), 200 μM okadaic acid (Sigma) and a mixture of protease inhibitors (5 μg/ml of pepstatin A, leupeptin, and Pefabloc SC) was added. Extraction mixtures were incubated 20 min at 4 °C with intermittent vortexing followed by centrifugation at 4 °C for 15 min at 10,000 x g. Supernatants were saved and the protein concentrations determined. For CK2 immunodepletion or heparin depletion experiments, protein concentrations were adjusted to 500 μg/ml prior to addition of anti-CK2 antibodies or heparin-Sepharose. In addition, the lysis buffer also contained 300 mM NaCl.

**CK2 Immunodepletion**—Mitotic cell extracts were prepared as described and incubated for 3 h at 4 °C under rotation in the absence or presence of a 1/100 dilution of a polyclonal antibody directed against the regulatory β subunit. Extracts were incubated with protein A-Sepharose beads for 30 min at 4 °C. Supernatants were saved and the precipitation repeated overnight with or without the CK2 β-directed antibodies. After incubation with protein A-Sepharose, supernatants were saved and new precipitations were carried out for 3 h at 4 °C in the absence or presence of a 1/100 dilution of a polyclonal antibody directed toward the catalytic α-subunit of CK2 in order to eliminate CK2 activity which is not associated with the β subunit. Supernatants were collected after incubation with protein A-Sepharose and their remaining MPM-2 kinase activity was determined.

**Determination of MPM-2 Kinase Activity**—Depleted and non-depleted mitotic extracts were diluted to 20 ng/μl with TEM (20 μl final volume), containing phosphatase and protease inhibitors. The reaction buffer was supplemented with 1 mM DTT, 0.5 mM ATP or GTP as indicated. Three hundred ng of purified mutant or wild type hTopoIIα were incubated with diluted extracts for 25 min at 37 °C. Heparin at 25 μg/ml was included in some assays to determine if this would inhibit the MPM-2 kinase. Reactions were stopped with 2 × SDS-PAGE loading buffer (30% glycerol, 4% sodium dodecyl sulfate, 187 mM Tris-HCl, pH 6.8, and bromphenol blue).

**Mitotic Cell Extract Preparation and Rephosphorylation Assay**—Isolated mitotic chromosomes (prepared from 2.5 × 10⁶ HeLa S3 cells)
were suspended in 25 μl of 60 mM PIPES, 25 mM HEPES, pH 7.5, 10 mM EGTA, 4 mM MgSO₄, 1 mM DTT, 1% CHAPS, supplemented with protease and phosphatase inhibitors and incubated for 5 min at 37 °C with 2.5 μl of DNase I. To determine the overall presence of MMP-2 phosphopeptides, chromosomes were diluted 1:1 with 2× SDS-PAGE loading buffer and analyzed by gel electrophoresis and Western blot analysis with the MMP-2 antibody. For repophosphorylation experiments, 2 μl of DNase-treated chromosomes were dephosphorylated by 50 units of λ-phosphatase in a 10-μl final volume. Then, chromosomes were incubated with 200 ng of purified CK2 for 25 min at 30 °C in a 20-μl final volume containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 40 μM GTP, 100 mM NaCl, 2% vanadate, and protease inhibitor mixture.

**Peptide Phosphorylation by CK2.—** Peptides (250 μM) were incubated for 25 min at 30 °C with 100 ng of purified CK2 in a 10-μl final volume containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 40 μM ATP in the presence or absence of 1 μl of [γ-32P]ATP and 100 μM NaCl. The ability of the peptides to compete with hTopoIIα for binding of the MMP-2 antibody was determined as described below.

**Competition of Peptides for Binding of the MMP-2 Antibody to hTopoIIα.—** Two hundred μg of purified hTopoIIα was incubated either overnight at 4 °C or alternatively, for 2 h at 37 °C in 96-well immunoplates followed by blocking with 200 μl of 2% BSA/BSA/PBS for 1 h at 37 °C. CK2-phosphorylated peptides were diluted in PBS containing 50 μg/ml heparin and 0.5 μg/ml anti-MMP-2 antibody to 100 μl final volume. Mixtures were incubated for 1 h at room temperature, added to hTopoIIα-coated wells, washed three times with 0.1% Tween 20-PBS (PBST), and incubated at 37 °C for 1.5 h. Wells were washed 5 times with PBST prior to incubation with secondary antibodies (1/2000 dilution in 2% BSA/PBST) for 1 h at 37 °C. Wells were then washed 5 times with PBST and MMP-2 binding revealed with soluble peroxidase substrate. Reactions were stopped after 15 min at room temperature by addition of H₂SO₄ followed by measurement of the optical density at 490 nm with a Dynex MRX microtiter plate reader (Dynex Technologies, Inc.).

**Enzyme-linked Immunosorbent Assay Test on CK2-phosphorylated Peptides.—** Peptides were phosphorylated by CK2 as described above. Reactions loaded on Microcon centrifugal filters (molecular weight cut-off: 10 kDa) and centrifuged for 12 min at 10,000 g. Eluates, containing only peptides, were saved and the final concentration adjusted to 15 μg/ml with PBS. One hundred μl of diluted peptides were loaded in 96-well immunoplates and kept 2 h at 37 °C. After three washes with 200 μl of PBS, each well was incubated with 200 μl of 2% BSA/PBS for 1 h at 37 °C. Wells were washed 5 times with PBST and MMP-2 binding revealed with soluble peroxidase substrate. Reactions were stopped after 15 min at room temperature by addition of H₂SO₄ followed by measurement of the optical density at 490 nm with a Dynex MRX microtiter plate reader (Dynex Technologies, Inc.).

**Co-immunoprecipitation of hTopoIIα and CK2.—** Purified hTopoIIα (250 ng) was incubated for 25 min at 30 °C with 40 ng of purified CK2 in 15 μl final volume of phosphorylation buffer in the absence or presence of ATP. Then, 10 μl of anti-topoisomerase II α antibody-conjugated protein G beads were added, the mixture was diluted in immunoprecipitation buffer followed by incubation for 2 h at 4 °C. The beads were washed three times with 500 μl of immunoprecipitation buffer and resuspended in 50 μl of 1× SDS-PAGE loading buffer. Samples were then subjected to electrophoresis and Western blot analysis.

**Relaxation Assay.—** Purified topoisomerase II (400 ng) was first preincubated in the presence or absence of 100 units of λ-phosphatase in a dephosphorylation buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 mM Na₃EDTA, 5 mM DTT, 0.01% Brij 35, and 2 mM MnCl₂ for 30 min at 30 °C. Alternatively, dephosphorylation was carried out with 0.1 unit of protein phosphatase 2A in a buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 0.2 mM MnCl₂, 0.5 mM DTT, and 50 mM KCl. These conditions result in complete dephosphorylation of topoisomerase II. Different amounts of topoisomerase II (1–5 ng/μl), preincubated with or without phosphatase, were then added to relaxation buffer (150 ng of pBR322 DNA, 125 mM KCl, 7.5 mM MgCl₂, 0.75 mM ATP, 20 mM Tris-HCl, pH 7.5) and reaction mixtures incubated for 10 min at 37 °C. Samples were subjected to electrophoresis in 0.8% agarose gels with 1× TBE buffer (2 mM EDTA, 90 mM Tris borate, pH 8.3) for 4 h at 6 V/cm at room temperature followed by staining with 0.5 μg/ml ethidium bromide.

**RESULTS**

**Protein Kinase CK2 Can Generate the MMP-2 Epitope on Human Topoisomerase IIα—** Both protein kinase CK2 and cdc2 kinase are able to phosphorylate human topoisomerase IIα during mitosis (14, 15). We therefore wished to determine if one of these two protein kinases was also able to generate the MMP-2 epitope. The results show that the MMP-2 epitope is created on purified, recombinant human topoisomerase IIα after phosphorylation with CK2, but not after phosphorylation with cdc2 kinase (Fig. 1A, lanes 2 and 3). Since CK2 is extensively phosphorylated by cdc2 kinase during mitosis (34, 35), the influence of the simultaneous presence of both CK2 and cdc2 kinase was also determined. The results show that the presence of cdc2 kinase greatly stimulates the ability of CK2 to generate the MMP-2 phosphopeptide on topoisomerase IIα (Fig. 1A, lanes 4).

**Mitotic Cell Extracts Can Create the MMP-2 Epitope on Topoisomerase IIα Using GTP and Is Inhibited by Heparin—** The ability of extracts from mitotic cells to generate the MMP-2 phosphopeptide was determined. The results show that the MMP-2 epitope can be created both in the presence of ATP and GTP (Fig. 1B, lanes 2 and 3). In contrast, no MMP-2 epitope is created if low concentrations of heparin (25 ng/μl) are included in the suspension buffer (Fig. 1B, lanes 4 and 5). These data are consistent with a role for CK2 as a topoisomerase II-directed MMP-2 kinase.

**CK2 Immunodepletion or Treatment with Immobilized Heparin Abolish the Ability of Mitotic Cell Extracts to Create the MMP-2 Epitope—** To further confirm the role of CK2 as the kinase creating the MMP-2 phosphopeptide on topoisomerase IIα, mitotic extracts were either depleted with heparin-Sepharose beads, which selectively remove proteins with strong affinity to heparin such as CK2 or immunodepleted with a CK2-directed antibody. Whereas treatments with Sepharose or protein A-Sepharose beads by themselves have little effect on formation of the MMP-2 epitope (Fig. 1C, lanes 2 and 4), both depletion of heparin-binding proteins and CK2 immunodepletion result in complete loss of MMP-2 kinase activity (Fig. 1C, lanes 3 and 5). Together, these results strongly suggest that the mitotic MMP-2 epitope on topoisomerase II is generated by CK2.

**CK2 Specifically Creates One MMP-2 Reactive Band of 170 kDa on Isolated Dephosphorylated Chromosomes—** Recent results show that although all proteins which carry MMP-2 phosphopeptides contain a loose MMP-2 consensus motif, peptides carrying the same sequences are only phosphorylated by a limited number of MMP-2 kinases (42). This suggests that...
Additional interaction domains may be required for the specific phosphorylation of MPM-2 proteins by a given MPM-2 kinase. We therefore wished to determine how many of the MPM-2 reactive proteins present in isolated mitotic chromosomes might be substrates for CK2. Immunoblot analysis of isolated human prometaphase chromosomes with a MPM-2 directed antibody reveals the presence of seven major MPM-2 reactive bands (lane 1). Dephosphorylation of isolated chromosomes leads to almost complete loss of MPM-2 reactivity (lane 2) which cannot be restored by reincubation of the chromosomes in the presence of ATP or GTP (lanes 4 and 6). Rephosphorylation of chromosomes by purified CK2 in the presence of ATP or GTP (lanes 3 and 5) results in the recreation of a single MPM-2 reactive band with a molecular size of 170 kDa. The migration of molecular size markers is indicated to the right. B, total mitotic extracts were dephosphorylated (lane 1) followed by rephosphorylation with purified CK2 in the presence of GTP (lane 2). This is accompanied by the creation of a limited number of MPM-2 reactive bands with molecular weights as indicated to the right. C, dephosphorylated mitotic extracts (lane 1) were immunodepleted with a topoisomerase II-directed antibody (lane 2) followed by rephosphorylation with purified CK2 of native and immunodepleted extracts (lanes 3 and 4). The results show that topoisomerase II immunodepletion leads to the selective disappearance of the 170-kDa MPM-2 reactive band (indicated by a closed arrow). Open arrowheads, heavy and light chain of the antibody used for immunodepletion of topoisomerase II.

Identification of Regions Likely to Contain the MPM-2 Phosphorylation Site—To identify motifs likely to contain the MPM-2-phosphorylation site, we first determined which serine and threonine residues corresponded to the CK2 consensus motif. It is generally held, that the minimum consensus for CK2 is (S/T)XXE/D, where the carboxylic determinants, Glu and Asp, may be replaced by phosphorylated Ser or Tyr. Additional positive determinants include multiple acidic residues surrounding the Ser/Thr residues at position −3 to +7 (46–50). Next, sequence analysis between different mammalian topoi-
The MPM-2 epitope is present in the last 163 amino acids of topoisomerase IIα. A series of C-truncated topoisomerase IIα mutants were constructed as shown. The positions of major CK2 phosphorylation sites (43), the mitotic SF3/2 phosphopeptide (15), the mitotic MPM-2 phosphopeptide (45), and the active site tyrosine are indicated. B, the upper part shows the electrophoretic migration of full-length topoisomerase IIα (lane 1) as well as the three truncated forms, T1, T2, and T3 (lanes 2–4). Only wild type and the T1 mutant are phosphorylated by CK2 as revealed by autoradiography after incubation with labeled ATP (middle) while only wild type topoisomerase IIα is recognized by a monoclonal antibody directed against the MPM-2 epitope (bottom). C, wild type topoisomerase IIα (lane 1) and the three truncated forms of topoisomerase II (lanes 2–4) were incubated with mitotic extracts in the presence of ATP and the generation of the MPM-2 phosphopeptide revealed by Western blot analysis.

To determine if this is also the case for the human enzyme, CK2 was extensively phosphorylated with CK2. This agreement with results reported by others (51, 52). More surprisingly, dephosphorylation of topoisomerase II as measured by relaxation of supercoiled DNA. Phosphorylation has no effect on the ability of topoisomerase IIα for binding of the MPM-2 antibody was determined. The results (Fig. 5E) show that R20A containing a phosphorylated Ser-1469 is able to compete with topoisomerase IIα for binding of the MPM-2 antibody. In contrast, neither unphosphorylated R20A nor R20A where Ser-1469 is replaced with an Ala were able to compete for generation of the MPM-2 phosphopeptide on topoisomerase IIα.

Phosphorylation Has No Effect on the Catalytic Activity of Topoisomerase IIα—To determine if creation of the MPM-2 site may influence the catalytic activity of topoisomerase IIα, topoisomerase II was extensively phosphorylated with CK2. This did not affect the catalytic activity (results not shown), in agreement with results reported by others (51, 52). More surprisingly, dephosphorylation of topoisomerase IIα by λ phosphatase (Fig. 6A) or protein phosphatase 2A (results not shown) also had no effect on the catalytic activity of topoisomerase II as measured by relaxation of supercoiled DNA. These results suggest that the catalytic activity of human topoisomerase IIα is not regulated by phosphorylation, whether mediated by CK2 or by other protein kinases.

Phosphorylation Has No Effect on the Ability of Topoisomerase I to Form Molecular Complexes with CK2—We have previously reported that topoisomerase II from yeast is able to form stable molecular complexes with CK2 which are independent of the phosphorylation status of topoisomerase II (12). To determine if this is also the case for the human enzyme, topoisomerase IIα and CK2 were incubated in the presence or absence of ATP followed by immunoprecipitation with an an-
Western blot analysis with an antibody directed toward the catalytic subunit of CK2 shows that despite clear differences in the level of the MPM-2 phosphoepitope on topoisomerase II, equal amounts of CK2 were recovered in topoisomerase IIα immunoprecipitates (Fig. 6B). Therefore, like previously described for the yeast enzyme, human topoisomerase IIα forms stable molecular complexes with CK2 which are not affected by the phosphorylation state of the topoisomerase.

**DISCUSSION**

Formation of MPM-2 reactive epitopes is a biochemical hallmark of mitosis in a wide variety of animal species ranging from nematodes to human (16). The distribution of MPM-2 reactive epitopes during mitosis displays a dynamic localization pattern which parallels that of the ongoing mitotic process. Furthermore, microinjection of MPM-2 antibodies into mitotic or meiotic cells leads to growth arrest, strongly suggesting that...
at the G2/M transition. Consistent with this observation, it has been observed that the number of proteins which are substrates for the MPN-2 kinase activity of CK2 seems to be quite limited since phosphorylation of isolated mitotic chromosomes only leads to the formation of a single MPN-2 reactive protein while less than 10 substrates are present in total mitotic extracts. The two proteins which most consistently are substrates for the MPN-2 kinase activity of CK2 are topoisomerase IIα and a protein with a molecular mass of ~110 kDa. Although the identity of this protein is not known, its molecular weight corresponds to another major CK2 substrate, nucleolin, which like topoisomerase IIα also forms stable molecular complexes with CK2 (66) and undergoes mitosis-specific phosphorylation (67).

It is not known how the different MPN-2 kinases recognize their specific substrates. It is likely that at least two factors are involved, the motif around the MPN-2 phosphorylation site and the presence of additional sequence motifs (42). The second factor may not play an important role in the case of CK2, since the R20A polypeptide by itself is a good substrate for the MPN-2 kinase activity of CK2. Rather, the choice of substrates may be due to the unique sequence requirements of CK2. In contrast to most Ser/Thr protein kinases, CK2 is extremely acidic (46–48, 50). Furthermore, in contrast to proline-directed protein kinases such as cdc2 kinase, CDK2, and MAP kinases, a proline at the +1 position is a strong negative determinant for CK2-mediated phosphorylation (50). This may be especially important since both MAP kinases and cdc2 kinase also have MPN-2 kinase activities. Thus, the generation of the MPN-2 epitope on a given substrate by a specific MPN-2 kinase may, at least in part, be due to differences in consensus requirement among different MPN-2 kinases.

Further analysis of the MPN-2 epitope on topoisomerase IIα reveals that this sequence motif shows some unusual features both with respect to most MPN-2 phosphoproteins and with respect to typical CK2 phosphorylation sites. While the residues downstream from Ser-1469 are highly acidic and typical for CK2 sites, the presence of a proline in the –1 position as well as the cluster of basic residues further upstream is quite unusual (50). The MPN-2 site on topoisomerase IIα is also unusual compared with most other MPN-2 sites since the proline residue is N-terminal rather than C-terminal to the phosphorylated Ser/Thr residue (26, 29). While this variation clearly does not prevent the MPN-2 antibody from recognizing phosphorylated Ser-1469, it is not yet clear if this motif is functionally similar to more classical MPN-2 sites in other proteins.

It is puzzling that some of the MPN-2 kinases also are active during interphase, raising the question how they are able to generate mitosis-specific phosphorylation sites. The simplest explanation would be that substrate and enzyme are present in different cellular subcompartments during interphase. However, this is clearly not the case for CK2 and topoisomerase IIα, since CK2 is the major kinase targeting topoisomerase II during interphase in a variety of organisms ranging from yeast to man (36, 43, 51, 68). A possible clue is that both subunits of CK2 are extensively phosphorylated by cdc2 kinase during mitosis (34). This is particularly dramatic for the catalytic α subunit where 4 residues in the C-terminal domain are phosphorylated by cdc2 kinase resulting in substantial conformational modifications (35). This is consistent with our in vitro findings that the ability of CK2 to create the MPN-2 epitope is greatly stimulated in the presence of cdc2 kinase, although this
kinase by itself has no MPM-2 kinase activity toward topoisomerase II. Cdc2-dependent phosphorylation of topoisomerase II could enhance the accessibility of the MPM-2 epitope for its CK2-catalyzed phosphorylation. Alternatively, CK2 activity could be stimulated upon phosphorylation by cdc2 or, as previously suggested, by a mechanism that does not imply the phosphorylation of CK2 (69). Further experiments will be required to discriminate between these different mechanisms.

It is widely believed that formation of MPM-2 epitopes is functionally important for orderly mitotic progression. However, despite almost two decades of active research, the mechanism by which phosphorylation of the MPM-2 phosphoepitope affects its various substrates remains unclear. Our results show that the MPM-2 activity of CK2 has no effect on the catalytic activity of topoisomerase IIa. More surprisingly, the observation that dephosphorylation of topoisomerase IIa by two different phosphatases also have no influence on the catalytic activity of the enzyme suggests that the catalytic activity of human topoisomerase IIa is, at least in vitro, not regulated by phosphorylation. This may not be restricted to the human enzyme since similar results have been reported for topoisomerase II from fission yeast (70).

Even without changing the catalytic activity of topoisomerase IIa, phosphorylation of Ser-1469 may still alter other properties of the enzyme such as its intracellular localization or its interactions with certain molecular partners. Protein phosphorylation in the vicinity of nuclear localization signal sequences may influence the cellular localization of proteins (71). While multiple potential bipartite nuclear localization signals have been identified in the C-terminal part of human topoisomerase IIa, only one sequence corresponding to amino acids 1454 to 1497 was shown to confer strong nuclear localization to a reporter protein (72). This suggests that the formation of the MPM-2 phosphoepitope on Ser-1469 may influence the localization of topoisomerase IIa during mitosis. Interestingly, while the CK2 motif is highly conserved among topoisomerase IIa from different mammalian species, it is not present on topoisomerase IIb. The two different topoisomerase II isoforms show different cellular localization during mitosis, since topoisomerase IIa remains tightly associated with the mitotic chromosomes whereas the β isofrom dissociates from the chromatin during this step of the cycle (73).

Another possibility is that the MPM-2 phosphoepitope may influence the association of topoisomerase IIα with other cellular proteins. A particular attractive candidate is the novel mitotic regulator, Pin1. This protein is an essential peptidyl-prolyl-cis-trans isomerase, which is able to catalyze rotation around the peptide bond adjacent to a proline residue thereby influencing the conformation of certain proteins (74). Pin1 binding is dependent on mitosis-specific phosphorylation of target proteins and shows almost the same substrate specificity as the MPM-2 antibody (75). Preliminary results in our laboratory suggest that topoisomerase II forms molecular complexes with Pin1 in a phosphorylation-dependent manner, as has been described for other MPM-2 epitopes.2

In conclusion, these data suggest that CK2 in addition to its numerous functions during interphase may play an important role in mitosis. Our results show that CK2 has MPM-2 kinase activity toward Ser-1469 of topoisomerase IIa, an important mitotic protein required for chromosome condensation and for segregation of intertwined sister chromatids. These findings provide a framework for further investigation into the role of the MPM-2 phosphoepitope on the mitotic functions of topoisomerase IIa. In addition, identification of other CK2 sub-

2 A. E. Escargueil and A. K. Larsen, unpublished observations.
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49. Marchiori, F., Meggio, F., Marin, O., Berin, G., Calderan, A., Ruzza, P., and Pinna, L. A. (1988) Biochim. Biophys. Acta 971, 332–338
50. Meggio, F., Marin, O., and Pinna, L. A. (1994) Cell Mol. Biol. Res. 40, 401–409
51. Kimura, K., Sajo, M., Tanaka, M., and Enomoto, T. (1996) J. Biol. Chem. 271, 10990–10995
52. Redwood, C., Davies, S. L., Wells, N. J., Fry, A. M., and Hickson, I. D. (1998) J. Biol. Chem. 273, 3635–3642
53. Davis, F. M., Wright, D. A., Penkala, J. E., and Rao, P. N. (1989) Cell Struct. Funct. 14, 271–277
54. Kimura, K., Saijo, M., Tanaka, M., and Enomoto, T. (1996) J. Biol. Chem. 271, 10990–10995
55. Larsen, A. K., Skladanowski, A., and Bojanowski, K. (1996) Prog. Cell Cycle Res. 2, 229–239
56. Krek, W., Maridor, G., and Nigg, E. A. (1992) J. Cell Biol. 116, 43–55
57. Filhol, O., Cochet, C., and Chambaz, E. M. (1996) Biochemistry 35, 9928–9936
58. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
59. Allende, J. E., and Allende, C. C. (1995) FASEB J. 9, 313–323
60. Pinna, L. A., and Meggio, F. (1997) Prog. Cell Cycle Res. 3, 71–97
61. Padmanabha, R., Chen-Wu, J. L., Hanna, D. E., and Glover, C. V. (1990) Mol. Cell. Biol. 10, 4089–4099
62. Retinaswamy, A., Birnbaum, M. J., and Glover, C. V. (1998) J. Biol. Chem. 273, 5869–5877
63. Snell, V., and Nurse, P. (1994) EMBO J. 13, 2066–2074
64. Kikkawa, U., Mann, S. K., Firtel, R. A., and Hunter, T. (1992) Mol. Cell. Biol. 12, 5711–5723
65. Hansa, D. E., Retinaswamy, A., and Glover, C. V. (1995) J. Biol. Chem. 270, 25905–25914
66. Li, D., Dobrowolska, G., and Krebs, E. G. (1996) J. Biol. Chem. 271, 15662–15668
67. Gulli, M. P., Faubladier, M., Sicard, H., and Caizergues-Ferrer, M. (1997) Chromosoma 105, 532–541
68. Ackerman, P., Glover, C. V., and Osheroff, N. (1988) J. Biol. Chem. 263, 12653–12660
69. Meggio, F., Boldyreff, B., Marin, O., Issinger, O. G., and Pinna, L. A. (1995) Eur. J. Biochem. 230, 1025–1031
70. Shiosaki, K., and Yanagida, M. (1992) J. Cell Biol. 119, 1023–1036
71. Schwab, M. S., and Dreyer, C. (1997) Eur. J. Cell Biol. 73, 287–297
72. Mirski, S. E., Gerlach, J. H., Cummings, H. J., Ziraghi, R., Greer, P. A., and Cole, S. P. (1997) Exp. Cell Res. 237, 452–455
73. Meyer, K. N., Kjeldsen, E., Straub, T., Knudsen, B. R., Hickson, I. D., Kikuchi, A., Kreipe, H., and Boege, F. (1997) J. Cell Biol. 136, 775–788
74. Lu, K. P., Hanes, S. D., and Hunter, T. (1996) Nature 380, 544–547
75. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) Genes Dev. 12, 706–720