Interactions between Protein Kinase CK2 and Pin1

EVIDENCE FOR PHOSPHORYLATION-DEPENDENT INTERACTIONS*

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The peptidyl-prolyl isomerase Pin1 interacts in a phosphorylation-dependent manner with several proteins involved in cell cycle events. In this study, we demonstrate that Pin1 interacts with protein kinase CK2, an enzyme that generally exists in tetrameric complexes composed of two catalytic CK2α and/or CK2α′ subunits together with two regulatory CK2β subunits. Our results indicate that Pin1 can interact with CK2 complexes that contain CK2α. Furthermore, Pin1 can interact directly with the C-terminal domain of CK2α that contains residues that are phosphorylated in vitro by pS4–sce and in mitotic cells. Substitution of the phosphorylation sites of CK2α with alamines resulted in decreased interactions between Pin1 and CK2. The other catalytic isoform of CK2, designated CK2α′, is not phosphorylated in mitotic cells and does not interact with Pin1, but a chimeric protein consisting of CK2α′ with the C terminus of CK2α was phosphorylated in mitotic cells and interacts with Pin1, further implicating the phosphorylation sites in the interaction. In vitro, Pin1 inhibits the phosphorylation of Thr-1342 on human topoisomerase IIα by CK2. Topoisomerase IIα also interacts with Pin1 suggesting that the effect of Pin1 on the phosphorylation of Thr-1342 could result from its interactions with CK2 and/or topoisomerase IIα. As compared with wild-type Pin1, isomerase-deficient and WW domain-deficient mutants of Pin1 are impaired in their ability to interact with CK2 and to inhibit the CK2-catalyzed phosphorylation of topoisomerase IIα. Collectively, these results indicate that Pin1 and CK2α interact and suggest a possible role for Pin1 in the regulation of topoisomerase IIα. Furthermore, these results provide new insights into the functional role of the mitotic phosphorylation of CK2 and provide a new mechanism for selectively regulating the ability of CK2 to phosphorylate one of its mitotic targets.

The human peptidyl-prolyl isomerase Pin1 was first isolated for its ability to interact with NIMA protein kinase in the two-hybrid system (1). Pin1 is an 18-kDa protein with orthologs that have been identified in yeast, Drosophila, Xenopus, fungi, mice, and plants (2–8). Pin1 belongs to the parvin family of isomerases, which is distinct from the cyclophilin and FK506 binding protein peptidyl-prolyl isomerase families. Studies on Pin1 provide evidence for its involvement in the G2/M transition and in mitosis. Pin1 can interact with many mitotic regulators including Myt1, Wee1, Pkl1/Pxl1, and Cdc25, although not with Cdc2 (8–10). Overexpression of Pin1 in HeLa cells resulted in a G2 arrest, whereas depletion of Pin1 in HeLa cells and yeast led to a mitotic arrest (1). Two groups found that the addition of recombinant Pin1 to cycling Xenopus extracts in vitro stopped extracts from entering mitosis (8, 9). Additionally, Winkler et al. (11) observed that interphase Xenopus egg extracts depleted of the Xenopus Pin1 homolog, xPin1, entered mitosis more rapidly than controls. They also clearly demonstrated that xPin1 is required for the execution of the DNA replication checkpoint. Collectively, these results link Pin1 to the G2/M transition and events regulating mitosis.

Another important protein implicated in cell cycle events and in cell viability is the highly conserved Ser/Thr protein kinase CK2,1 a tetrameric enzyme that is composed of two catalytic (CK2α and/or CK2α′) subunits and two regulatory (CK2β) subunits (reviewed in Refs. 12–16). Genetic studies in yeast suggest a role for CK2 at distinct stages during cell cycle progression including the G2/M transition where it is required for the phosphorylation of topoisomerase II (12, 17). In mammalian cells, CK2 activity increases when quiescent cells are stimulated to proliferate (reviewed in Refs. 13–16). Studies performed using selective CK2 inhibitors, antisense down-regulation, or kinase-inactive mutants of CK2 also illustrate that CK2 is required during various stages of cell cycle progression including the G2/M transition and in checkpoint control (18–20). Moreover, CK2 activity levels are elevated in a number of tumors and leukemic cells. Targeted overexpression of CK2 in transgenic mice results in the development of T cell lymphoma and mammary tumorigenesis (21, 22). Additionally, accelerated lymphomagenesis is observed when mice with elevated expression of CK2 in T cells are crossed with transgenic mice overexpressing c-Myc or Tal-1 or in mice that are deficient in p53 (23, 24). Collectively, these studies implicate CK2 as an

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1 The abbreviations used are: CK2, protein kinase CK2 or casein kinase II; HA, the YPYDVPDY epitope of influenza virus hemagglutinin; Myc, the MASMEQKLISEEDLNN epitope of the c-Myc protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; Ni-NTA, nickel-nitrilotriacetic acid; FPLC, fast protein liquid chromatography.
CK2 is a participant in a p34 Cdc2-mediated protein kinase responsible for the mitotic phosphorylation of CK2 and that Thr-1342 on topoisomerase II.

Interestingly, the other isoform of the CK2 catalytic subunit (i.e. CK2α) is selectively phosphorylated in mitotic cells at 4 sites localized within its C-terminal 60 amino acids. Interestingly, the other isoform of the CK2 catalytic subunit (i.e. CK2α') has a distinct C-terminal domain that lacks these phosphorylation sites and is not phosphorylated in mitotic cells (26).

The mitotic phosphorylation sites on CK2α can all be phosphorylated *in vitro* by p34CK2, suggesting that this enzyme is responsible for the mitotic phosphorylation of CK2 and that CK2 is a participant in a p34CK2-mediated protein kinase cascade. Identification of the sites that are phosphorylated by p34CK2 revealed that the mitotic phosphorylation sites on CK2α (Thr-344, Thr-360, Ser-362, and Ser-370) can all be classified as proline-directed phosphorylation sites because each of the phosphorylated residues is immediately N-terminal to a proline (28). The regulatory β subunit of CK2 is also phosphorylated at autophosphorylation sites and at a site that is maximally phosphorylated in mitotic cells (26). Despite the identification of these phosphorylation sites, no clear effect of phosphorylation of CK2 on CK2 activity has yet been observed (29).

Because the mitotic phosphorylation sites on CK2 are all Ser-Pro or Thr-Pro sites that resemble the optimal binding motif for Pin1 (30), we hypothesized that CK2 may interact with Pin1 in a phosphorylation-dependent manner as a part of events regulating the cell cycle. Experiments described in this paper were undertaken to test for an interaction between Pin1 and CK2 and for the phosphorylation dependence of this interaction. Furthermore, having demonstrated interactions between CK2 and Pin1, we examined the effects of Pin1 on CK2 activity toward several substrates. Of particular interest was the CK2α isoform, a protein that is important for cell cycle progression. At the non-permissive temperature, yeast with temperature-sensitive CK2 fail to enter mitosis (12, 27, 31). In mammalian cells that CK2 phosphorylates residues on topoisomerase II, topoisomerase II is hypo-phosphorylated and inactivity is observed in transformation.

MATERIALS AND METHODS

DNA Constructs—CK2 constructs had HA or Myc tags to allow the identification or isolation of transfected CK2 subunits from endogenous CK2 subunits. As described previously (35), the HA tag consists of three repeats of the influenza hemagglutinin epitope YPYDVPDYD, and the Myc tag consists of the Myc epitope MASMEQKLISEEDLNN. Constructs encoding CK2α and with a C-terminal HA tag were generated using pReCMV (Invitrogen). The following CK2α constructs were employed: wild-type c-CK2α, c-4A-HA, c-4D-HA, and c-4E-HA. In the latter three constructs, the four mitotic phosphorylation sites on CK2α, (i.e. Thr-344, Thr-360, Ser-362, and Ser-370) were mutated to alanines, aspartic acids, and glutamic acids, respectively, by using sequential PCR. All constructs were verified by sequencing.

The CK2α' subunit was HA-tagged at its N terminus and was also expressed using pReCMV (Invitrogen). In addition to wild-type CK2α', a construct encoding a chimera consisting of the N-terminal domain of CK2α' with the C-terminal domain of CK2α, as described previously (35), was utilized. The latter chimera construct encodes residues 1–296 of CK2α' together with the C-terminal fragment of CK2α (i.e. residues 299–391 of CK2α) instead of the natural C terminus of CK2α' (i.e. residues 297–350 of CK2α'). Generation of the chimera construct was described previously (35) and was achieved using a BsuBI restriction site that is conserved between CK2α and CK2α'. Importantly, the four mitotic phosphorylation sites on CK2α (i.e. Thr-344, Thr-360, Ser-362, and Ser-370) are all located with the C-terminal portion of CK2α that was transferred to CK2α' to generate the chimera. Constructs encoding CK2αβ subunits had N-terminal Myc tags and were also in pReCMV. In addition to wild-type Myc-CK2αβ, constructs were generated in which Myc-CK2αβ was replaced with Myc-CK2β with mutations at its mitotic phosphorylation site at Ser-209. For these constructs, Ser-209 was mutated to alanine (i.e. Myc-βS209A) or to aspartic acid (i.e. Myc-βS209D). All constructs were verified by sequencing.

Several DNA constructs were made for use in the production of recombinant GST-Pin1 fusion proteins. GST fusion proteins were generated using the pGEX-KG (36) vector. The Pin1 cDNA was obtained from American Type Culture Collection (ATCC 928227). It was amplified by PCR to introduce NcoI and HindIII restriction sites to the 5' and 3' ends, respectively, of the Pin1 coding region to facilitate subcloning. Primers for PCR were as follows: GGA TCC CCA TGG CAG CCG AGA AGA AGC TG (forward primer designated p1) and GGA TCC AAG CTT CCT CGC GAC CTC CC (reverse primer designated p2). The PCR product (~490 bp) was ligated into PCR-Blunt (Invitrogen) and sequenced. The modified Pin1 cDNA was then subcloned into pGEX-KG using the NcoI and HindIII sites to generate pGEX-KG-Pin1.

Four mutant GST-Pin1 constructs were also generated. GST-Pin1Y23A and GST-Pin1R68A,R69A were full-length GST-Pin1 fusion proteins with point mutations resulting in the loss of WW binding ability (28). In fact, in light of its predicted WW interaction motif for Pin1 (30), we hypothesized that CK2 may interact with GST-Pin1-(47–230) as described previously (35) and was achieved using a BsuBI restriction site that is conserved between GST-Pin1-(47–230) and GST-Pin1-(47–280). In this study, we demonstrated interactions between CK2 and Pin1, we examined the effects of Pin1 on CK2 activity toward several substrates. Of particular interest was the CK2α isoform, a protein that is important for cell cycle progression. At the non-permissive temperature, yeast with temperature-sensitive CK2 fail to enter mitosis (12, 27, 31). In mammalian cells that CK2 phosphorylates residues on topoisomerase II, topoisomerase II is hypo-phosphorylated and inactivity is observed in transformation.

Protein Production and Purification—GST constructs were generated using the pGEX-KG vector (36). The vector had a N-terminal Myc tag consisting of the Myc epitope MASMEQKLISEEDLNN. Con- structs encoding the N-terminal 198 amino acids of c-Myb (41), were generous gifts of Dr. D. BigDye Terminator method (PerkinElmer Life Sciences) at the Robarts Research Institute (Ontario, Canada). Sequencing and restriction digests were used to verify all constructs.

Plasmids encoding GST-C92, a GST fusion protein encoding the C-terminal 92 amino acids of e-Myc (39), GST-Max, a GST fusion protein encoding Max (40), and GST-631, a GST fusion protein encoding the N-terminal 198 amino acids of e-Myb (41), were generous gifts of Dr. B. Luscher (Hannover, Germany).

Protein Production and Purification—GST, GST-Pin1, and GST-Pin1 mutants were expressed in BL21 or DH5α bacteria grown in 2× YT with 100 mg/µl ampicillin (Roche Molecular Biochemicals) at 37 °C. After cultures reached an absorbance of greater than 0.6 at 600 nm, isopropyl-β-D-thiogalactoside (Indolene Chemical Corp.) was added to a final concentration of 0.1. One milliliter of a bacterial culture was added to each tube. Cells were resuspended in 0.5 ml of PBS (130 mM NaCl, 3 mM KCl, 10 mM NaHPO4, 2 mM KH2PO4, pH 7.4), and then resuspended in PBS containing 1.5 mg/ml apronin, 10 µl/ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma) before being lysed using a French press or by sonication. Triton X-100 was added to this bacterial lysate to a final concentration of 1%, and the solution was incubated with continual mixing for 15–25 min at 4 °C. Cell debris was pelleted by centrifugation at 6000 × g. The supernatant...
was incubated with glutathione immobilized on agarose beads (Sigma) for 20–60 min at 4 °C or room temperature with continuous mixing. After thorough washing with PBS, the beads were used directly, or the protein was eluted using free glutathione (10 mM reduced glutathione (Sigma), 50 mM Tris-HCl, pH 8.0, 1 mM DTT). Eluted protein was dialyzed against PBS, and was added to the GST fusion protein encoding the N-terminal 198 amino acids of c-Myb. GST-CK2α encoding the C-terminal 92 amino acids of c-Myc, GST-Max (40), a GST bovine serum albumin as a standard. GST was not expressed, purified, and phosphorylated with purified p34 Cdc2 using 100 units/ml penicillin (Invitrogen). Standard calcium phosphate transfections (47) were performed using 34–40 µg of DNA per 10-cm plate. Specifically 17 µg of α or α′ constructs, 17 µg of β construct, and ~3.4 µg of pEGFP-C2 (CLONTECH), pEGFP-C3 (CLONTECH), or β-galactosidase in pBluescript CMV were used per 10-cm plate. The pEGFP or β-galactosidase plasmids were transfected into cells to monitor transfection efficiency by fluorescence microscopy or by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-glai) staining, respectively. The following sequence of events was then performed: 14–16 h after the DNA was added to the cells, the precipitate was washed and fresh media were added; 18 h later new media with a final concentration of 0.1 µg/ml nocodazole was added to the cells. After addition of 16–18 h later okadac acid (Calbiochem) was added to media to a final concentration of 1 µM; and 2 h later the cells were harvested. For interphase cell populations the nocodazole and okadac acid were not added.

**Extract Preparation for GST and GST-Pin1 Binding Assays**—After removing the last wash, sample buffer and washings twice with PBS, interphase cells were harvested by scraping in 500 µl per 10-cm plate of Tris Lysis Buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 150 mM NaCl) with 1 mM DTT, 1.5 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml PMSF, and 1 µM microcystin LR (Sigma). Mitotic cells were shaken off the plates by repeated pipetting of the media over the plate surface. The media were collected; the cells were pelleted by centrifugation, and the pellet was washed once with PBS. The cell pellet was then resuspended in a volume equivalent to 500 µl of Tris Lysis Buffer per 10-cm plate harvested. Both interphase and mitotic cells were then sonicated for 30 s and then centrifuged at 107,700 × g for 20 min at 4 °C. The cell extract supernatants were then stored until use at −80 °C. Protein determinations were performed using the BCA protein assay (Pierce) using bovine serum albumin as a standard.

Interphase nuclear extracts and mitotic chromosome preparations to be used as a source of topoisomerase IIa for GST and GST-Pin1 binding assays were isolated as described (33). Prior to use in the binding assays, these preparations were subjected to DNase (Roche Molecular Biochemicals) treatment in the presence of 10 mM MgCl2 and 5 mM CaCl2 for 10–20 min at room temperature.

**Chromatographic Fractionation of Cell Extracts**—Untransfected cells were used to prepare interphase and mitotic fractions containing CK2α to be used in kinase assays. To obtain mitotic extracts, cells were grown in media with 0.1 µg/ml nocodazole for 16–21 h. Interphase and mitotic cells were harvested as described above except using Buffer A (50 mM Tris-Cl, pH 7.5, 2 mM EDTA, 1 mM DTT, 50 mM NaCl) with 1.5 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml PMSF, 1 µM microcystin, and 10 mM NaF instead of Tris Lysis Buffer. Equal amounts of protein, as determined by Coomassie protein assay (Pierce), were loaded on an FPLC column. Chromatographic fractionation was performed with either a Amersham Biosciences Mono Q column 5 × 50 mm on a Amersham Biosciences FPLC system or a Waters Protein Pak Q-8HR column on a Waters 625LC system. Protein was eluted using a 25-ml salt gradient starting with Buffer A and ending with Buffer B (50 mM NaPO4, 0.1 mM Tris-Cl, pH 8.0, and incubated at room temperature for 15–60 min with gentle mixing. After centrifugation at 6,600 × g for 20–30 min, the supernatant was incubated with Ni-NTA Superflow beads for 1 h with vigorous mixing. Beads were washed repeatedly with 8 ml urea, 0.1 mM NaH2PO4, 0.01 mM Tris-Cl, pH 6.3. After removal of the last wash, sample buffer (108 mM Tris, pH 6.8, 3.6% SDS, 18% glycerol, 0.01% bromphenol blue, 10% β-mercaptoethanol) was added to beads to release the Y2C proteins.

Pin1 with a N-terminal His tag and a fusion protein designated N70 with residue 1342 were described previously (33). The Y2C fragment encodes residues 1158–1362 of topoisomerase IIα and has a pI of 9.5 indicating an overall basic character. These proteins were expressed in BL21 bacteria grown in LB or for experiments involving His-Pin1 or N70 with Ni-NTA Superflow beads (Qiagen). Briefly, pelleted on ice for 15 min, resuspended in 8 ml urea, 0.1 mM NaH2PO4, 0.01 mM Tris-Cl, pH 8.0, and incubated at room temperature for 15–60 min with gentle mixing. After centrifugation at 6,600 × g for 20–30 min, the supernatant was incubated with Ni-NTA Superflow beads for 1 h with vigorous mixing. Beads were washed repeatedly with 8 ml urea, 0.1 mM NaH2PO4, 0.01 mM Tris-Cl, pH 6.3. After removal of the last wash, sample buffer (108 mM Tris, pH 6.8, 3.6% SDS, 18% glycerol, 0.01% bromphenol blue, 10% β-mercaptoethanol) was added to beads to release the Y2C proteins.

GST-Pin1 and His-Pin1 Binding Assays—For binding assays with GST fusion proteins, equal amounts of GST-Pin1 or GST were incubated with 20 µl of a 1:1 slurry of glutathione-immobilized agarose beads to PBS for 20 min at 4 °C with continual mixing. The beads were then washed 3 or 4 times with PBS and/or Tris Lysis Buffer. An aliquot of the cell extract (prepared as described in section 2.5) was mixed 1:1 with sample buffer and set aside for the gel. In each binding assay, 20 µl of 1:1 beads was added to 350–600 µl of the cell extract (typically 0.1–2 mg/ml) for 1 h at 4 °C with continuous mixing. The beads were then washed three times with Tris Lysis Buffer. After the removal of the last wash, a 50-µl sample buffer was added to the beads to elute bound proteins.

Binding assays with His-Pin1 were similarly performed by incubating His-Pin1 or N70 with Ni-NTA resin (Qiagen) according to the manufacturer’s recommendations.

**SDS-PAGE and Immunoblots**—Proteins were separated on 6 or 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane and probed with antibodies described by Wadhams et al. (49), over 1 h at 100 V in Blotting Buffer (25 mM Tris-Cl, pH 7.5, 190 mM Tris gels, 20% methanol). Prestained molecular weight markers (broad range from New England Biolabs) were used for reference. Markers and their molecular masses are as follows: 175-kDa MBP-β-galactosidase, 83-kDa MBP-paramyosin, 62-kDa glutamic dehydrogenase, 47.5-kDa aldolase, 32.5-kDa trisascose isomerase, 25-kDa β-lactoglobulin A, 16.5-kDa lysozyme, and 6.5-kDa aprotinin.

Protein Kinase CK2 Interacts with Pin1
Immunoblots for colorimetric detection were treated in the following way: after washing in TBS (20 mM Tris-Cl, pH 7.5, 500 mM NaCl) the membranes were blocked for 30–60 min with 5% gelatin in TBS, followed by a 1-h incubation in the primary antibody solution and then 30–60 min in the secondary antibody solution. TBST (TBST with 0.05% Tween 20) and PBS (phosphate-buffered saline) buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 0.2 mM DTT, 100 μM PMSF, 1 mM DTT, and 200 or 400 μM microcystin with or without 50 μM leupeptin, 100 μM pepstatin A, and 25 μM leupeptin were used to wash blots between incubations. Both primary and secondary antibodies were diluted in 1% gelatin in TBST for 1–4 h. After this the blot was washed extensively, air-dried, and visualized using a PhosphorImager (Molecular Dynamics).

Immunoblots and radioactive detection were performed as described above for colorimetric blot except that the secondary antibody used was the rabbit anti-mouse antibody diluted 1:11,000, and there was a third incubation after this with protein A-gold (specific activity 70–100 μCi/mg, ICN) at 50,000–100,000 cpm/ml in 1% gelatin in TBST for 1–4 h. After this the blot was washed extensively, air-dried, and visualized using a PhosphorImager (Molecular Dynamics).

Topoisomerase II Phosphorylation Assay—The topoisomerase II phosphorylation assay was performed as described in Daum and Gorbach (33). Briefly, the Y2C fragments of topoisomerase IIα were run on SDS-PAGE gel and blotted as described above. The blots were cut into strips along the edge of the lanes. These blot strips were blocked in 3% gelatin in TBS for 15–30 min. Blots were washed once in TBST and again in TBST or TEM (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 4 mM MgSO₄). The blots were equilibrated in TEM with inhibitors (1.5 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM PMSF, 1 mM DTT, and 200 or 400 μM microcystin), and then the kinase assay was performed. Blot strips were incubated with and without 0.1 mM peptide substrate in 1% Triton X-100 and TEM with inhibitors (1.5 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM PMSF, 1 mM DTT, and 200 or 400 μM microcystin) for 15,000 min at 1% gelatin in TBST for 1–30 °C. To halt the reaction, blot strips were washed four times with TBST. Blot strips were incubated with a 1:5000 dilution of 3F3/2 antibody in 1% BSA in TBST for 1 h, washed, incubated with goat anti-mouse horseradish peroxidase 1:15,000 in 1% BSA in TBST for 1 h, and finally incubated in the SuperSignal solution (Pierce) for the activity of CK2 from FPLC fractions used in these experiments. Antibody concentrations used in these experiments was from 0 to 2 μM.

CK2 Kinase Assays—CK2 kinase assays were performed using a synthetic peptide substrate, RRRDDDSDDD, as described previously (19, 48). In brief, small aliquots (6–12 μl) of CK2 from the FPLC fractions were incubated with and without 0.1 μM peptide substrate in 20 mM Tris-Cl, pH 7.5, 60 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 100 μM γ-[³²P]ATP (specific activity 50–100 cpm/pmol) for 5 and 10 min. The total reaction volume was 30 μl. An aliquot of the mix was then spotted on P81 phosphocellulose paper, washed extensively with 1% phosphoric acid, washed once with 95% ethanol, and quantified using a scintillation counter or PhosphorImager. To examine the effects of Pin1 on CK2 activity, peptide phosphorylation assays were similarly performed in the presence of the indicated amount of GST or GST-Pin1 using FPLC fractions as the source of CK2. Alternatively, these kinase assays were performed using casein or GST fusion proteins encoding known protein substrates of CK2 in the presence of the indicated amounts of Pin1. For the latter assays, kinase assays were performed in buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 0.2 mM DTT, 100 μM PMSF, 0.5 μM DTT, 100 μM γ-[³²P]ATP (specific activity 400–1000 cpm/pmol) for 10 min at 30 °C, using each of the indicated proteases as substrate. Following incubation for 10 min at 30 °C, kinase reactions were terminated by the additional SDS-PAGE sample buffer and boiling. Following SDS-PAGE, phosphorylated proteins were detected using a PhosphorImager.

Incorporation of ³²P into Topoisomerase II—The wild-type Y2C fragment of topoisomerase IIα and the mutant form with the Thr-1342 to alanine substitution T1342A were produced in bacteria and then purified and blotted to polyvinylidene difluoride membrane as described above (33). The membranes were blocked in 5% BSA in TBST. The blots were then treated in the following manner: washed twice with TBST, once with TEM, once with TEM with 0.5% Triton X-100, once with TEM with 25 μM ATP and 200 μM microcystin LR, and then again with TEM. The blots were then incubated for 35 min at 37 °C with mixing in TEM with 25 μM ATP, 5 μCi (γ-[³²P]ATP, 80 μl of CK2-enriched FPLC fraction, 1.5 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM PMSF, 1 mM DTT, and 200 μM microcystin without 50 μM leupeptin) in a total volume of 3 ml. To terminate the reaction, the blots were washed three times with TEM with 10 mM EDTA, 20 mM NaF, and 0.1% Triton X-100 and once in TEM with 10 mM EDTA, 20 mM NaF. The blots were air-dried and visualized using a PhosphorImager.

RESULTS Pin1 Interacts with Protein Kinase CK2—As a first step toward testing whether Pin1 could interact with CK2, recombinant Pin1 proteins were generated. GST and GST-Pin fusion proteins were produced in bacteria and purified using glutathione-agarose beads (not shown). GST-Pin1 or GST binding assays (i.e. pulldowns) were performed using U2OS cell lysates. To test whether CK2 interacts with Pin1, the proteins that bound to GST-Pin1 or GST were examined by immunoblotting with antibodies against CK2α (Fig. 1A). CK2α was readily detected in both interphase and mitotic extracts (Fig. 1A). In the mitotic extract, multiple α- and β- bands are present with the bands of recombinant electrophoretic mobility representing the phosphorylated forms of α that have been characterized previously (26, 28, 29). Because CK2α can be phosphorylated at up to 4 sites in mitotic cells, individual bands represent different phosphorylated forms of CK2 with the bands exhibiting the slowest electrophoretic mobility representing the most heavily phosphorylated forms of CK2α. In binding assays, GST-Pin1

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**Fig. 1. Pin1 interacts with protein kinase CK2α.** A, extracts from unsynchronized U2OS cells (marked I) and from cells arrested in mitosis (marked M) were used in GST and GST-Pin1 binding assays as described under “Materials and Methods.” Extracts as well as the proteins bound to GST or to GST-Pin1 (designated as Pin1) were separated by 12% SDS-PAGE, transferred to membranes, and analyzed on immunoblots using anti-CK2α antibodies with colorimetric detection. The position of endogenous CK2α is indicated (α) as is the phosphorylated form of endogenous CK2α (pα). B, to test whether interactions between Pin1 and CK2 can be observed with transfected CK2, GST and GST-Pin1 binding assays were performed as in A using extracts from U2OS cells that had been transfected with epitope-tagged CK2 subunits. The immunoblot was probed with anti-HA antibodies to detect the HA-tagged CK2α subunits. As in A, lanes I are derived from unsynchronized cells, and lanes M are derived from cells arrested in mitosis with nocodazole. The position of CK2α with a C-terminal HA tag is indicated (α-HA) as is the phosphorylated form of HA-tagged CK2α (pα-HA). The positions of molecular weight markers are also illustrated to the right of each panel.
interacted strongly with mitotic CK2α but not with interphase CK2α (Fig. 1A). It is also apparent that the most heavily phosphorylated form of CK2α exhibits the greatest interactions (Fig. 1A, last lane). CK2α was not detected in the GST binding assays (Fig. 1A) indicating that the Pin1 portion of the GST-Pin1 proteins is that which is required for interactions with CK2. Overall, these results indicate that Pin1 interacts with CK2.

Interactions between CK2 and Pin1 were also examined using transfected CK2. Cells were transfected with HA-tagged CK2α (designated α-HA) together with Myc-tagged wild-type CK2β and extracts prepared for examination of interactions with Pin1 (Fig. 1B). As seen with CK2α from untransfected cells, Pin1 interacts with transfected CK2α-HA with the greatest interaction observed with the CK2α-HA from mitotic cells (Fig. 1B, last lane). However, in contrast to the results observed with the endogenous CK2, transfected CK2α-HA exhibits the appearance of multiple a-bands in interphase extracts in the transfected cell extracts suggesting that the CK2α-HA is phosphorylated even without mitotic synchronization (Fig. 1B, 1st lane). We do not know the precise reason for the presence of these phosphorylated forms of CK2α-HA, but we speculate that these extra interphase bands may be the result of aberrant cell cycle events induced by overexpression of CK2 or by the transfection procedure. Because some of the CKα-HA that is present in interphase extracts is phosphorylated, it is therefore not surprising that some CK2α-HA is observed in the GST-Pin1 pulldown from interphase extracts (Fig. 1B, 2nd last lane). In this pulldown, there is an obvious enrichment of the mostly heavily phosphorylated form of CK2α-HA. Overall, the transfection experiments reinforce the suggestion that Pin1 preferentially interacts with phosphorylated CK2α.

Comparison of Interactions between Pin1 and CK2α Versus CK2α’—Significant differences between CK2α and CK2α’ are found only within their C-terminal regions (50), and the mitotic phosphorylation sites on α all lie within this region (26, 28). CK2α’ does not have any of the mitotic phosphorylation sites and is not phosphorylated in mitotic cells. To determine whether CK2α’ could be transformed into a Pin1 interactor if it gained the mitotic phosphorylation sites of CK2α, a chimeric construct of CK2α’ (designated α’/α) with the C terminus of α’ replaced by that of α was used in GST-Pin1 binding assays (Fig. 2). The addition of the C-terminal domain of CK2α to CK2α’ resulted in the appearance of multiple bands as seen with phosphorylated α (Fig. 2) indicating that the α’/α chimera undergoes mitotic phosphorylation reminiscent of that seen with CK2α. In pulldown assays, the CK2α’/α chimera clearly exhibits interactions with GST-Pin1, whereas negligible interactions between GST-Pin1 and CK2α’ are observed under these conditions (Fig. 2B, compare last 2 lanes). As well, no CK2 subunits were retained in GST binding assays (Fig. 2). Clearly, the addition of the C-terminal domain of CK2α is capable of converting CK2α’ into a Pin1 interactor. It is also noteworthy that the transfections of CK2α, CK2α’, and CK2α/α were all performed in the presence of Myc-Ck2β. Consequently, because CK2α’ was not retained by GST-Pin1, it would appear that the ability of CK2 to interact with Pin1 resides in CK2α and not CK2β.

Pin1 Interacts with a GST Fusion Protein Encoding the C-terminal 126 Amino Acids of CK2α—Results shown in Figs. 1 and 2 demonstrate that phosphorylated forms of CK2α can be isolated from cell extracts using GST-Pin1. However, because the source of CK2 was soluble cell extracts, it was not possible to determine whether CK2α interacts directly with GST-Pin1 or whether the interaction between CK2 and Pin1 is mediated by other proteins that are present in cell extracts. Consequently, we performed experiments to determine whether CK2α can interact directly with Pin1 and to determine whether the C-terminal domain of CK2α is sufficient for interactions with Pin1. Pulldown assays were performed using a bacterially expressed GST fusion protein encoding the C-terminal 126 amino acids of CK2α. This bacterially expressed fusion protein was purified and phosphorylated at the mitotic phosphorylation sites of CK2α by p34Cdc2 using [γ-32P]ATP (28). As illustrated in Fig. 3, this phosphorylated fusion protein exhibits interactions in pulldown assays with His-tagged Pin1 that had also been expressed and purified from bacteria. Notably, the amount of 32P-labeled fusion protein that is retained by His-tagged Pin1 immobilized on Ni-NTA resin is ~4–5-fold above the background binding of this fusion protein that is observed using Ni-NTA resin alone or an unrelated His-tagged protein immobilized on Ni-NTA (Fig. 3, C and D). These results demonstrate that the C-terminal domain of CK2α is sufficient for interactions with Pin1 and indicate that CK2α can interact directly with Pin1.

Examination of Interactions between Pin1 and Phosphorylation Site Mutants of CK2—By having demonstrated that the C-terminal domain of CK2α is sufficient for interactions with Pin1, further experiments were undertaken to examine directly the importance of phosphorylation for interactions of CK2 with Pin1. Cells were transfected with wild-type CK2α (α-HA) or a mutant CK2α (αA-HA). CK2αA-HA is a mutant where each of the four mitotic phosphorylation sites of CK2α have been substituted with non-phosphorylatable alanines. Although wild-type CK2α-HA appears as several bands exhibiting shifts in electrophoretic mobility resulting from phosphorylation, the αA-HA mutant appears as a single band that co-migrates with
non-phosphorylated CK2α. Consistent with the prediction that phosphorylation is important for the interaction, α4A-HA was minimally retained on GST-Pin1 beads in comparison with mitotic wild-type α in binding assays (Fig. 4A, compare last 2 lanes). Neither α-HA nor α4A-HA were retained by GST beads (Fig. 4A). In other experiments, this interaction was quantified using iodinated protein A as described under “Materials and Methods.” There was 5–40-fold more wild-type α-HA than α4A-HA retained on GST-Pin1 beads. These results indicated that at least one of the mitotic phosphorylation sites of CK2α is important for its interactions with Pin1.

Next, the effect of replacing the phosphorylation sites of CK2α with glutamic acid or aspartic acid was examined. It was hypothesized that the negative charge of these amino acids might mimic the negative charge of phosphate groups and allow for interactions with Pin1. Cells were transfected with αE-HA or αD-HA together with Myc-β, and again GST and GST-Pin1 binding assays were performed (Fig. 4B). These two phosphorylation site mutants of CK2α bound far less effectively to the Pin1 beads relative to wild-type mitotic CK2α (Fig. 4B). None of the proteins bound to GST beads (Fig. 4B, lanes 5–7). Therefore, the negative charges provided by the glutamic and aspartic acids were not sufficient to mimic completely the phosphorylated serines and threonines on CK2α for Pin1 recognition.

The regulatory CK2β subunit of CK2 is also phosphorylated in mitotic cells at a Ser-Pro site (25, 27). Therefore, to test whether phosphorylation of CK2β has an impact on interactions with Pin1, a similar strategy was used utilizing cells transfected with Myc-tagged wild-type β, or mutants of β where serine 209 had been replaced with either alanine or with aspartic acid (i.e., β S209A and β S209D, respectively). Each of these constructs was transfected into cells along with CK2α-HA, and pulldowns were performed. As seen in Fig. 4C (last 3 lanes), there was no striking difference in the amount of β and α detected when GST-Pin1 binding assays were performed using these three different β constructs, suggesting that phosphorylation of β does not affect interactions of CK2 with GST-Pin1. Of note is the predominance of the β-band with the higher mobility in GST-Pin binding assays. This band is autophosphorylated and is indicative of β that is part of a tetrameric CK2 complex (19, 51, 52), suggesting that β is being pulled down as a part of a complex with α. Collectively, these results demonstrate that it is unlikely that the mitotic phosphorylation site on β is important for interactions with Pin1.
isomerase activity (9, 31) did bind CK2 domain of Pin1, GST-Pin1 Y23A which encodes full-length Pin1 with an activating mutation within its WW domain, and GST-Pin1 Y23A which encodes full-length Pin1 with an inactivating mutation within its WW domain, and GST-Pin1 H9251 which encodes full-length Pin1 with an inactivating mutation within its isomerase domain. Pulldowns were performed as in previous figures using extracts of cells that had been transfected with HA-tagged CK2α and arrested in mitosis with nocodazole. HA-tagged CK2α was detected using chemiluminescence. The pulldown with GST-Pin1 represents half as much volume as was loaded for each of the other pulldowns.

In summary, the importance of phosphorylation of CK2 for Pin1 interaction was demonstrated. Elimination of mitotic phosphorylation sites on α diminished its interactions with Pin1, and the elimination of the mitotic phosphorylation site on β did not have a significant effect. Furthermore, α could be transformed into a Pin1-interacting protein with the addition of the C-terminal domain of α containing the mitotic phosphorylation sites.

**Examination of Interactions between CK2αα and Pin1 Using Pin1 Mutants**—Pin1 is composed of a WW domain that exhibits phosphorylation-dependent interactions with target proteins as well as a peptidylprolyl isomerase domain that exhibits phosphorylation-dependent catalytic activity (30, 37). To examine the role of each of these domains for binding CK2αα, binding assays were performed with various Pin1 mutants (Fig. 5). By itself, the isomerase domain of Pin1 designated GST-Pin1 Y23A did not bind CK2αα-HA (Fig. 5, 4th lane). By comparison, GST-Pin1 Y23A encoding only the WW domain of Pin1 did exhibit interactions with CK2αα-HA (Fig. 5, 5th lane). Similarly, GST-Pin1 Y23A, a mutant that abolishes the binding activity of the WW domain (37) did not bind CK2αα-HA, whereas GST-Pin1 H9251, a mutant reported to diminish significantly isomerase activity (9, 31) did bind CK2αα-HA (Fig. 5). Overall, these results suggest that the WW domain is most important for binding CK2αα-HA. However, the interactions between the WW domain and CK2αα-HA appeared to be much weaker than those observed with wild-type full-length Pin1 with CK2αα-HA. Although we have not rigorously excluded the possibility that other regions of Pin1 are important for interactions with CK2αα, the importance of the WW domain is consistent with evidence indicating that this protein interaction module is essential for the cellular functions of Pin1.

**Examination of the Effects of Pin1 on CK2 Activity**—To determine whether Pin1 affects the catalytic activity of CK2, we examined the effect of Pin1 on the ability of CK2 to phosphorylate peptide and protein substrates. As seen in Fig. 6, Pin1 at various concentrations does not exert any dramatic effect on the in vitro activity of CK2 toward an optimized peptide substrate (Fig. 6A) nor toward known CK2 substrates such as casein (Fig. 6B), GST-Max (Fig. 6C), or a GST fusion protein encoding a portion of c-Myb that contains residues known to be phosphorylated by CK2 (Fig. 6D) (53). Similarly, Pin1 had no effect on the phosphorylation of a GST fusion protein encoding a portion of c-Myc that contains known CK2 phosphorylation sites (data not shown). Collectively, these results indicate that Pin1 is not a general inhibitor of CK2.

Although CK2 is active at all stages in the cell cycle (29), there is evidence that the activity of CK2 toward specific substrates could be modulated at specific stages during the cell cycle. For example, CK2 was recently shown to phosphorylate residues on topoisomerase IIα that are maximally phosphorylated in mitotic cells (33, 34). These observations strongly suggest that topoisomerase IIα is a mitotic target of CK2 in mammalian cells. Studies with temperature-sensitive mutants of CK2 in yeast provide similar indications that topoisomerase IIα is also a mitotic target for CK2 in yeast (31). Because we have previously shown that CK2 is phosphorylated in mitotic cells (26, 28) and because Pin1 interacts preferentially with mitotic CK2 and has been implicated in the control of mitotic events, we were thus interested in examining the effects of Pin1 on the CK2-catalyzed phosphorylation of the mitotic phosphorylation sites on topoisomerase IIα. To achieve this objective, we utilized a recombinant fragment of topoisomerase IIα, designated Y2C, as a substrate for CK2. The Y2C fragment of topoisomerase II used in this study is the fragment of topoisomerase IIα encoding residues 1158–1362. This fragment was previously utilized for the identification of Thr-1342 as the phosphorylated residue on topoisomerase IIα that is recognized by the 3F3/2 monoclonal antibodies (33). Furthermore, topoisomerase IIα can be immunoprecipitated from mitotic cells using 3F3/2 monoclonal antibodies, and CK2 phosphorylation can generate 3F3/2 reactivity on topoisomerase IIα that is associated with isolated chromosomes (33). Consequently, it appears that Thr-
of topoisomerase II used to phosphorylate wild-type Y2C or T1342A mutant Y2C fragment

CK2 was partially purified by FPLC from extracts derived from cells

tonation on immunoblots with 3F3/2 antibodies. For this experiment, CK2

partially purified CK2 was performed as in

mitotic CK2 was prepared as in

immunoblot using the 3F3/2 monoclonal antibody. Partially purified

ing amounts of GST or GST-Pin1 (as indicated) and detected on an

ected (Fig. 7

phorylated by CK2, whereas the same topoisomerase II frag-

erylation by CK2. The 3F3/2 monoclonal antibody was utilized to assay for

Pin1 inhibits the phosphorylation of Thr-1342 on topoisomerase IIα by CK2. Phosphorylation of Thr-1342 within the Y2C fragment of topoisomerase IIα was monitored using the phospho-specific 3F3/2 antibody as described under “Materials and Methods.” A, wild-type Y2C fragment of topoisomerase II (wt) as well as a mutant form of the Y2C fragment that harbors a substitution of Thr-1342 with alanine (i.e. T1342A) were incubated with CK2 and ATP prior to detection on immunoblots with 3F3/2 antibodies. For this experiment, CK2 was partially purified by FPLC from extracts derived from asynchronously growing cells as described under “Materials and Methods.” B, CK2 was partially purified by FPLC from extracts derived from cells arrested in mitosis as described under “Materials and Methods” and used to phosphorylate wild-type Y2C or T1342A mutant Y2C fragment of topoisomerase IIα using [32P]ATP in the presence or absence of the CK2 inhibitor heparin as indicated. Phosphate incorporation into Y2C fragments was detected using a PhosphorImager. C, phosphorylation of Thr-1342 within the wild-type Y2C fragment of topoisomerase IIα by partially purified CK2 was performed as in A in the presence of increasing amounts of GST or GST-Pin1 (as indicated) and detected on an immunoblot using the 3F3/2 monoclonal antibody. Partially purified mitotic CK2 was prepared as in B.

1342 is indeed phosphorylated in mitotic cells and that CK2 can phosphorylate this residue in intact topoisomerase IIα and in the Y2C fragment.

The 3F3/2 monoclonal antibody was utilized to assay for phosphorylation of one of the mitotic sites (i.e. Thr-1342) of topoisomerase IIα by partially purified mitotic CK2 (Fig. 7). The CK2 utilized in these studies was partially purified from mitotic extracts utilizing ion exchange chromatography as described under “Materials and Methods.” Partial purification was performed to separate CK2 from many of the other protein kinase or phosphatase activities in extracts that could confound the examination of CK2 activity. It is evident that the wild-type Y2C fragment of topoisomerase IIα was readily phosphorylated by CK2, whereas the same topoisomerase II fragment with Thr-1342 mutated to alanine was negligibly detected (Fig. 7A). This result is consistent with previous findings (33). Additionally, assays were performed in the absence of CK2 or ATP. In either case, no bands were detected with the 3F3/2 antibody (data not shown). These control assays further confirmed that the antibody was sensitive to phosphorylation.

Because topoisomerase II is multiply phosphorylated and a potential target for kinases distinct from CK2 (31, 54–57), we also performed experiments to determine whether the Y2C fragment was phosphorylated by kinases other than CK2 present in the FPLC fraction. As seen in Fig. 7B, the CK2 inhibitor heparin completely abolished the incorporation of 32P into both the wild-type and Thr-1342/Ala mutant Y2C fragments. This result suggests that no other kinases were present and phosphorylating the topoisomerase II fragment to any appreciable extent. Comparison of the wild-type Y2C topoisomerase IIα fragment with the Thr-1342/Ala mutant topoisomerase IIα fragment indicates that residues in addition to Thr-1342 are phosphorylated by CK2 (Fig. 7B). By utilizing the 3F3/2 antibody to examine the phosphorylation of Thr-1342, we examined the effect of GST-Pin1 on the ability of CK2 to phosphorylate this residue. A dose-dependent inhibition of Thr-1342 phosphorylation by CK2 was clearly observed (Fig. 7C). By comparison, the addition of GST generated no inhibition of Thr-1342 phosphorylation. Thus, Pin1 can inhibit the phosphorylation of Thr-1342 on topoisomerase IIα by CK2.

Investigation of the Mechanism by Which Pin1 Inhibits Phosphorylation of Topoisomerase IIα by CK2—The peptidylprolyl isomerase activity of Pin1 is essential for its in vivo functions (9, 30). Consequently, there has been considerable speculation that Pin1 does indeed catalyze conformational changes in at least some of its target proteins. However, for the most part, direct evidence for Pin1-catalyzed conformational changes in its target proteins has not been obtained. One exception is the cell cycle regulatory phosphatase Cdc25 (58, 59). Lines of evidence demonstrating that Cdc25 undergoes conformational changes include the fact that its dephosphorylation by protein phosphatase 2A is affected by catalytic amounts of Pin1 (58). Similarly, recognition of Cdc25 by phosphospecific MPM-2 monoclonal antibodies is affected by Pin1 (59). Based on these studies, we performed experiments to examine the ability of Pin1 to alter the reactivity of CK2 toward MPM-2 monoclonal antibodies or to affect the dephosphorylation of CK2 by protein phosphatase 2A. However, unlike Cdc25, protein phosphatase 2A was ineffective at the dephosphorylation of CK2 either with or without Pin1 treatment (data not shown). In a similar vein, Cdc25 was not recognized by MPM-2 antibodies in the presence or absence of Pin1 (not shown). Because these strategies, which were employed successfully to monitor conformational changes in Cdc25, were not amenable for an examination of CK2, we performed a number of additional studies to obtain mechanistic insights into the manner in which Pin1 inhibits the phosphorylation of topoisomerase IIα by CK2.

Because Pin1 interacts with CK2 in a phosphorylation-dependent manner, we were interested in determining whether the inhibition of Thr-1342 phosphorylation resulted solely from phosphorylation-dependent interactions between CK2 and Pin1. To achieve this objective, we compared interphase CK2 to mitotic phosphorylated CK2 in the Thr-1342 phosphorylation assay. Extracts from interphase or mitotic cells were fractionated by FPLC (Fig. 8). Immunoblots were probed with anti-α antibodies to ensure that mitotic α was phosphorylated (Fig. 8A), and kinase assays were performed to determine the amount of CK2 kinase activity in each fraction (Fig. 8B). As illustrated in Fig. 8C, Pin1 exhibits a similar degree of inhibition of Thr-1342 phosphorylation with CK2 from mitotic and interphase fractions. This result suggests that the inhibition of Thr-1342 phosphorylation is not exclusively the result of phosphorylation-dependent interactions between CK2 and Pin1.

To explore further the mechanism by which Pin1 inhibits the phosphorylation of topoisomerase II by CK2, we were interested in testing the possibility that topoisomerase II also interacts with Pin1, as has been suggested previously (30, 34). To achieve this objective, pulldown assays were performed to determine whether topoisomerase IIα obtained from interphase or mitotic cells can interact with GST-Pin1. As seen in Fig. 9, a band of ~175,000 daltons recognized by anti-topoisomerase IIα antibodies is observed in interphase nuclear extracts or in mitotic chromosome preparations and in pulldowns using GST-Pin1 but not GST. It is evident that the amount of topoisomer-
Protein Kinase CK2 Interacts with Pin1

**DISCUSSION**

Pin1 interacts with a number of mitotic phosphoproteins involved in the cell cycle (8–10), and on the basis that CK2 is phosphorylated at Ser/Pro and Thr/Pro sites in mitotic cells (25–28), we hypothesized that Pin1 may interact with CK2 in a phosphorylation-dependent manner. By using GST-Pin1 binding assays, interactions between Pin1 and CK2 were indeed detected. When comparing binding of Pin1 to interphase and mitotic CK2, far more mitotic CK2 interacted with Pin1 based on the intensity of bands on blots from the binding assays. Results comparing wild-type CK2α to the CK2αA4 mutant, which has the four mitotic phosphorylation sites of CK2α mutated to alanines, showed a dramatic decrease in interactions between Pin1 and CK2. Lending further weight to the conclusion that phosphorylation is important for the interactions between CK2 and Pin1 is the observation that CK2α can be converted into a Pin1-interacting protein when its C terminus is replaced by that of CK2α (Fig. 2).

The mutation of the mitotic phosphorylation sites on CK2α to aspartic acids and glutamic acids was performed to mimic a permanent phosphorylation state by the addition of these negatively charged residues. However, in comparison to the wild-type mitotic CK2α, these mutants were poor interactors with Pin1. Consequently, it would appear that the negative charge alone is not sufficient for Pin1 recognition. This result concurs with the findings of Yaffe et al. (30) who found that Glu-Pro- and Asp-Pro-containing peptides were isomerized by Pin1 at a slower rate than phosphorylated Ser-Pro-containing peptides. Thus, glutamic and aspartic acids do not appear to be effective substitutes for phosphorylated serines and threonines in terms of Pin1 recognition.

It is generally accepted that CK2 is a tetrameric enzyme composed of two catalytic CK2α and/or CK2α′ subunits and two regulatory CK2β subunits. Previous studies (43) indicate that tetrameric complexes can be composed of identical catalytic...
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subunits (i.e. two CK2α subunits or two CK2α’ subunits in complexes with two CK2β subunits). Alternatively, tetrameric CK2 complexes can be composed of one CK2α subunit and one CK2α’ subunit together with two CK2β subunits (43). In this study, CK2β was consistently detected in GST-Pin1 binding assays whenever CK2α was detected. The presence of CK2β in pulldowns with GST-Pin1 may simply result from the association of CK2β with CK2α. Alternatively, because CK2β is also phosphorylated at a Ser/Pro site in mitotic cells (25), it is possible that direct interactions between CK2β and Pin1 may contribute to the interactions between CK2 and Pin1. However, there are two lines of evidence that suggest that CK2β does not interact with Pin1. First of all, when CK2α’ is co-expressed with CK2β (Fig. 2), there is no evidence of interactions between CK2α’ and Pin1 indicating that CK2β is not sufficient to mediate interactions between Pin1 and CK2. Furthermore, as shown in Fig. 4, the substitution of Ser-209 of CK2β by alanine or glutamic acid resulted in no dramatic change in the amount of CK2 detected in the binding assays. Therefore, it would appear that if Pin1 does interact with CK2β directly it does so in a phosphorylation-independent manner. More likely, CK2β is involved indirectly with Pin1 via its interactions with α in tetrameric CK2 complexes, a suggestion that is supported by the observation that the autophosphorylated form of CK2β is predominantly seen in the binding assays (Fig. 4C). Importantly, autophosphorylation of CK2β appears to be an indicator that CK2β is in a complex with the CK2 catalytic subunits (35, 51, 52). Collectively these results indicate the importance of the phosphorylation of CK2α, not CK2β, for interactions with GST-Pin1. In a related vein, we have also demonstrated that the C-terminal domain of CK2α is sufficient for interactions with Pin1 (Fig. 3). Consequently, in addition to tetrameric CK2 complexes that contain CK2α, it is possible that CK2α that is not in tetrameric CK2 complexes could also be interacting with Pin1 in pulldown assays.

Our results indicate that the WW domain of Pin1 by itself, as well as full-length Pin1 deficient in isomerase activity, retained some ability to interact with CK2. The ability of the WW domain alone to bind Pin1 target proteins has been observed with a number of other proteins including Cdc25, Plk1, and Cdc27 (37). However, in this study, less CK2 was pulled down using these mutant forms of Pin1 than with the full-length functional Pin1. These results suggest that both domains are important for optimal interactions between Pin1 and CK2.

Pin1 and CK2 interact in a manner dependent on the mitotic phosphorylation of CK2. This suggests that their interaction may play a role in events during mitosis. Given the massive burst of phosphorylation of proteins in mitosis, one might propose that Pin1 may modulate how CK2 phosphorylates proteins necessary for mitotic events to occur. This line of reasoning led to an examination of the effect of Pin1 on the ability of CK2 to phosphorylate a mitotic CK2 substrate. In mammalian cells, topoisomerase II is highly phosphorylated during G2 and mitosis and isomerization, is sufficient to exert an effect. For example, Pin1 caused a decrease in the p9-mediated stimulation of Cdc25 and Myt1 phosphorylation catalyzed by the Cdc2-cyclin B complex (10). Catalytically inactive Pin1 was capable of exerting this inhibition suggesting that interactions between Pin1 and Cdc25 and Myt1 were responsible for the observed inhibition (10). In the case of CK2 and topoisomerase IIα, there is additional complexity because our results demonstrate that both of
these proteins can interact with Pin1. Furthermore, several studies have demonstrated that CK2 physically interacts with topoisomerase II in mammalian cells and in yeast, although controversy regarding the relationship of CK2 to the regulation of topoisomerase II remains (31–34, 61–63). Clearly, additional studies beyond the scope of this present work will be required to fully illuminate the precise role of Pin1 in the regulation of CK2 and topoisomerase II.

An important observation in this study is that Pin1 interacts with CK2 in a phosphorylation-dependent manner because it is the first report of an interaction involving the mitotic phosphorylated form of CK2. Additionally, these results provide further evidence for functional and perhaps regulatory differences between CK2α and CK2α*. Functional and/or regulatory differences between these two catalytic isoforms of CK2 are in keeping with the high degree of conservation of CK2α and CK2α* that is observed between species and the conservation of the mitotic phosphorylation sites on CK2α (50, 64). Clearly, the precise functional role of this interaction between CK2 and Pin1, which both have key roles in the cell cycle, is an intriguing question that requires further attention. In vitro, Pin1 can inhibit the phosphorylation of Thr-1342 of topoisomerase II by CK2. However, given the complex nature of the possible interactions of Pin1 with CK2 and/or topoisomerase II, the precise manner in which Pin1 exerts this inhibition remains to be elucidated. Nevertheless, the ability of Pin1 to modulate selectively the substrate specificity of CK2 represents an important new mechanism for the regulation of CK2. In a similar vein, the recent studies of Keller et al. (65) suggest that the FACT complex plays a similar role in selectively modulating the activity of CK2 to phosphorylate p53 in response to DNA damage. In addition to the implications for CK2, the fact that Pin1 interacts with topoisomerase II and affects its phosphorylation by CK2 may have novel regulatory significance for topoisomerase II.

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