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Chk1 Kinase Negatively Regulates Mitotic Function of Cdc25A Phosphatase through 14-3-3 Binding

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The order and fidelity of cell cycle events in mammals is intimately linked to the integrity of the Chk1 kinase-Cdc25A phosphatase pathway. Chk1 phosphorylation targets Cdc25A for destruction and, as shown here, inhibits interactions between Cdc25A and its mitotic substrate cyclin B1-Cdk1. Phosphorylation of Cdc25A on serine 178 and threonine 507 facilitates 14-3-3 binding, and Chk1 phosphorylates both residues in vitro. Mutation of T507 to alanine (T507A) enhanced the biological activity of Cdc25A. Cdc25A(T507A) was more efficient in binding to cyclin B1, activating cyclin B1-Cdk1, and promoting premature entry into mitosis. We propose that the Chk1/Cdc25A/14-3-3 pathway functions to prevent cells from entering into mitosis prior to replicating their genomes to ensure the fidelity of the cell division process.

The Cdc25A protein phosphatase positively regulates the cell cycle by activating cyclin-dependent protein kinases (Cdks). *Cdc25A* was identified as a potential human oncogene based on its ability to transform primary mouse embryo fibroblasts in cooperation with activated Ras or loss of *RB* (14). In addition, *Cdc25A* is overexpressed in a variety of human cancers (5, 6, 15, 34), and in many cases, *Cdc25A* overexpression is a poor prognostic indicator. *Cdc25A* is an E2F target gene, and Cdc25A is required for efficient induction of S-phase entry by E2F-1 (47). Overexpression of *Cdc25A* accelerates S-phase entry (4, 44), and microinjection of antibodies against Cdc25A arrests cells in G₁ (23, 25). Although these findings indicate an important role for Cdc25A in regulating the G₁-to-S phase transition, Cdc25A also functions at other cell cycle stages (13, 30, 52, 57). Cdc25A is capable of binding to cyclins D1, E, A, and B1 and can dephosphorylate Cdks bound to each of these cyclins in vitro (13, 43, 52). In addition, Cdc25A is present and active in all stages of the cell cycle and Cdc25A levels rise as cells progress from G₂ phase into mitosis (3, 4, 23, 25, 30, 31, 44).

The activity and abundance of Cdc25A is regulated by reversible phosphorylation, protein-protein interactions, and ubiquitin-mediated proteolysis (3, 8, 23, 29, 31). Regulation of Cdc25A is essential not only for progression of cells through an unperturbed cell division cycle but also for cells to arrest in response to checkpoint activation (57). Cdc25A is rapidly degraded in a proteasome-dependent manner in cells exposed to UV light, hydroxyurea, or ionizing radiation (IR) (11, 29, 31). In addition, overexpression of Cdc25A causes bypass of the IR-induced S- and G₂-phase checkpoints and the DNA replication checkpoint (11, 29–31).

Human Cdc25A is phosphorylated at several sites in vivo, and Chk1 is required for the majority of these phosphorylations in vivo during interphase (57). Chk1 phosphorylates Cdc25A to promote its ubiquitin-mediated proteolysis (11, 16, 20, 46, 57). Chk1 also promotes the degradation of Cdc25A in *Xenopus laevis* (26, 45). In vitro, Chk1 phosphorylates human Cdc25A at several sites, and phosphorylation of these residues requires Chk1 in vivo (46, 57). These findings argue that Chk1 is a major regulator of Cdc25A phosphorylation in vivo. Here we identify additional Cdc25A phosphorylation sites and report a novel mechanism of Chk1-mediated Cdc25A regulation involving 14-3-3 proteins.

MATERIALS AND METHODS

Antibodies. Cdc25A was detected with antibodies purchased from Santa Cruz and Upstate Biotechnology, Inc. (UBI). Cdc25A fusion proteins were precipitated with anti-c-Myc (9E10)-agarose conjugate and detected by Western blotting with c-Myc polyclonal antibody (Santa Cruz). Anti-14-3-3, -cyclin B1, -Cdk1, -glutathione *S*-transferase (GST), and -His antibodies, as well as anti-cyclin B1 conjugated to agarose, were purchased from Santa Cruz. Anti-phospho-histone H3 antibodies were purchased from UBI, and β -catenin antibodies were purchased from Transduction Laboratories. Antibodies specific for Cdc25A phosphorylated at S178 and T507 were generated by immunizing rabbits with the phosphopeptides C-TQRQNPAPAR and C-RTKSRPTWAGE coupled to keyhole limpet hemocyanin.

Generation of tTA-regulated adenoviruses. An *XhoI/HindIII* fragment of pUHD10-3 (17, 40), containing seven repeats of the tetracycline operator linked to a cytomegalovirus minimal immediate-early promoter, a polylinker for cloning, and a simian virus 40 polyadenylation signal, was subcloned into pAdTrack (21). The resulting plasmid (pAdTrackTRE) was digested with *XbaI* and ligated to a linker containing *SalI*, *NotI*, *EcoRV*, and *XhoI* restriction sites to generate pAdTrackTRE-SX. pAdTrackTRE-SX carrying the genes for Myc-tagged forms of Cdc25A was cotransformed with pAdEasy-1 into *Escherichia coli* BJ5183 to achieve homologous recombination. Recombinant adenoviruses were generated and propagated in 293 cells using the pAdEasy system as described previously (21). Expression of Cdc25A is regulated by the tetracycline transactivator (tTA), with recombinant adenovirus encoding tTA (24).

14-3-3 binding assays. Transfected Cosm6 or 293 cells infected with recombinant adenoviruses were suspended in buffer A (10 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 1 mM EGTA, 25 mM NaF) supplemented with 1 mM dithiothreitol (DTT) and the following inhibitor cocktail: 1 μ M microcystin, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 U of aprotinin per ml, 20 μ M leupeptin, and 20 μ M pepstatin. Cells were lysed by passage through a 25-gauge needle 30 times. Precleared lysates were incubated with

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anti-c-Myc(9E10)-agarose, and Cdc25A precipitates were washed in buffer A. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed for Myc-Cdc25A and 14-3-3 proteins by Western blotting.

Far-Western analysis. HeLa cells coinfecting with recombinant adenoviruses encoding tTA and Myc-tagged versions of wild-type (wt) and mutant Cdc25A proteins were lysed in mammalian cell lysis buffer (MCLB; 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 2 mM DTT, 1 mM sodium orthovanadate, 10 mM β -glycerophosphate, 1 mM NaF, 1 μ M microcystin, 1 mM PMSF, 5 μ g of leupeptin per ml, and 10 μ g of aprotinin per ml). Clarified supernatants were incubated with anti-c-Myc(9E10)-agarose conjugate. Precipitates were resolved by SDS-PAGE and transferred to Hybond-P membranes (Amersham). Membranes were blocked in 5% milk-TBS (10 mM Tris [pH 8.0], 150 mM NaCl) overnight at 4°C and then washed in TBS. Washed membranes were incubated in 0.1 μ g of GST-14-3-3e per ml in 1-mg/ml bovine serum albumin (BSA) in TBS. Membranes were washed in TBS and then incubated in GST antibody. Membranes were washed in TBS and then incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. Proteins were visualized by the ECL system (Amersham). Membranes were stripped and re-probed using anti-Myc antibodies to detect Myc-tagged Cdc25A. For peptide competition assays, membranes were washed and blocked as described above. Peptide (0.1 mM) was incubated with 0.1 μ g of GST-14-3-3e per ml in TBS containing 1 mg of BSA per ml for 2 h at room temperature. Membranes were then incubated in the peptide-GST-14-3-3e mixture overnight at 4°C. Membranes were washed in TBS, incubated with antibodies specific for GST, and processed as described above. The peptides used for the competition assays were as follows: PHCVPRDLSWLDLEANMCLP (38, 48) and phosphorylated and unphosphorylated forms of C-TQRQNS₁₇₈APAR and C-RTKSRT₅₀₇WAGE. In addition, two phosphopeptides lacking 14-3-3 consensus sequences were used. The pT68 phosphopeptide from Chk2 consists of the sequence C-ETVSpT₆₈QELYS, and the pS123 phosphopeptide from Cdc25A consists of the sequence C-LKRSHpS₁₂₃DSL D.

siRNA experiments. The small interfering RNA (siRNA) oligonucleotide corresponding to nucleotides 127 to 147 of the human Chk1 coding region (57) was synthesized by Dharmacon Research, Inc. The control siRNA duplex was designed based on a target sequence of 5'-GCGCGCTTTGTAGATTCCG-3'. siRNA duplexes were transfected into HeLa cells using Oligofectamine (Invitrogen) according to the manufacturer's recommendations. Twenty-four hours after transfection, cells were coinfecting with adenoviruses encoding tTA and Myc-tagged versions of wt and mutant Cdc25A proteins. Cells were harvested 9 h after infection by lysing in MCLB. Proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Double thymidine block and release protocol. To synchronize HeLa cells at the G₁/S border, cells were treated with 2 mM thymidine for 16 to 19 h. Cells were released from the block by washing with phosphate-buffered saline (PBS), followed by trypsinization and reseeding into complete growth medium containing 24 μ M (each) thymidine and 2'-deoxycytidine. After 8 to 9 h, thymidine was added to the medium to a final concentration of 2 mM, and cells were cultured for an additional 16 to 18 h. Cells were then rinsed twice with PBS and cultured in complete growth medium.

Overproduction of Cdc25A proteins in S phase. HeLa cells were synchronized using a double thymidine block and release protocol. Four hours before release from the second block, cells were coinfecting with adenoviruses encoding tTA and GFP (control) or tTA and Myc-tagged versions of wt and mutant Cdc25A proteins. Infections were carried out at a multiplicity of infection of 5 to 10. Cells were harvested by trypsinization 3 h after release from the second block. A portion of the cells was analyzed for chromosomal integrity by performing chromosome spreads (22) and for percent mitotic cells by costaining with propidium iodide (PI) and anti-phospho-histone H3 antibody (50). Cell cycle profiles were obtained by fluorescence-activated cell sorting (FACS) using a FACSCaliber machine (Becton-Dickinson Instruments). The data were analyzed with Cellquest software (Becton-Dickinson). The remaining cells were lysed in MCLB and analyzed for Cdc25A by Western blotting or were incubated with anti-cyclin B1 agarose. Cyclin B1/Cdk1 precipitates were washed in incomplete kinase buffer (20 mM Tris-HCl [pH 7.5], 15 mM MgCl₂, 1 mM DTT), and kinase reactions were carried out in buffer consisting of 20 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 1 mM DTT, 0.5 mg of histone H1 per ml, 0.2 mM ATP, and 10 μ Ci of [³²P]ATP. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose. Radiolabeled histone H1 was visualized by autoradiography and quantitated by scintillation counting.

Cyclin B1 binding assays. HeLa cells were synchronized using a double thymidine block. Four hours prior to release, cells were coinfecting with adenoviruses encoding tTA and GFP or tTA and wt or mutant Cdc25A. Infected cells

were collected 3 h after release (7 h of infection). Alternatively, HeLa cells were transfected for 24 h with plasmid DNA encoding Myc-tagged versions of Cdc25A using Lipofectamine 2000 (Invitrogen). Cells were lysed in buffer A supplemented with 1 mM DTT and inhibitor cocktail by passage through a 27-gauge needle 30 times. Myc-Cdc25A proteins were precipitated using anti-c-Myc(9E10)-agarose. Precipitates were resolved by SDS-PAGE followed by Western blotting with anti-Myc or anti-cyclin B1 antibodies.

Purification of cyclin B1/Cdk1 complexes phosphorylated at T14 and Y15. Sf9 insect cells were coinfecting with recombinant baculoviruses encoding *Xenopus* Myt (32), GST-cyclin B1 (human), and human Cdk1 (1, 36). *Xenopus* Myt efficiently phosphorylates Cdk1 on both threonine 14 and tyrosine 15. Cells were collected 40 h later, washed in PBS, and then lysed for 15 min at 4°C in NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with 2 mM DTT, 1 μ M microcystin, 1 mM sodium orthovanadate, 2 mM PMSF, 0.15 U of aprotinin per ml, 20 μ M leupeptin, and 20 μ M pepstatin. Clarified lysates were incubated with glutathione-agarose beads (Sigma Chemical Co.). Beads were washed four times with NETN, three times with LiCl buffer (0.5 M LiCl, 50 mM Tris, pH 8.0), and three times with thrombin cleavage buffer (TCB; 25 mM Tris [pH 7.5], 1 mM EDTA, 100 mM NaCl, 2.5 mM CaCl₂, 0.01% Brij 35). Cyclin B1/Cdk1 complexes were cleaved from GST and eluted from the beads in TCB containing 30 mM glutathione and 50 U of thrombin in TCB for 1 h at room temperature. Supernatants were loaded on 2-ml Q Sepharose columns (Pharmacia) that had been washed several times with buffer B (25 mM Tris [pH 7.5], 1 mM EDTA, 0.01% Brij 35). Columns were washed with 10 volumes of buffer B, and complexes were eluted in buffer B containing 1 M NaCl. Fractions containing phosphorylated Cdk1/cyclin B1 complexes were pooled, dialyzed in a solution containing 20 mM Tris (pH 7.4), 2 mM DTT, and 10% glycerol, and frozen at -80°C.

Phosphatase assays using bacterial GST fusion proteins and Cdk1/cyclin B1 complexes. DH5 α cells were transformed with plasmids encoding GST fusions of Cdc25A (wt and mutants), cultures were grown at 37°C to an A₆₀₀ of 0.6, and isopropyl-1-thio- β -D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After growing for an additional 2 h at 37°C, cells were pelleted by centrifugation. Cell pellets were washed with PBS and then suspended in STE (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) containing 1.25% N-lauryl sarcosine supplemented with 2 mM DTT, 1 mg of lysozyme per ml, and proteinase inhibitors (4 mM PMSF, 20 μ g of aprotinin per ml, 40 μ M leupeptin). After rocking at 4°C for 20 min, cells were lysed by sonication. Lysates were clarified by centrifugation (13,000 \times g for 15 min), and Triton X-100 was added to a final concentration of 1%. Proteins were precipitated with GST-agarose beads (Sigma Chemical Co.) and washed twice with NETN buffer, twice with NETN buffer containing 1 M NaCl, and twice with phosphatase buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 2 mM DTT). The levels of GST fusion proteins were estimated by comparison to a BSA standard after SDS-PAGE and Coomassie blue staining or were quantified by using GST antibodies, enhanced chemifluorescence (ECF) reagents, and the Storm 860 imaging system (Amersham). Recombinant Cdc25A immobilized on 10 μ l of GST-agarose was suspended in 17 μ l of phosphatase buffer. Reactions were incubated at 30°C for 10, 15, or 20 min after the addition of 3 μ l of cyclin B1/Cdk1 substrate. Reactions were stopped by the addition of 1 μ l of 50 mM sodium vanadate and were placed on ice. Activation of cyclin B1/Cdk1 was determined by performing histone H1 kinase assays. Ten microliters of 4 \times kinase buffer (80 mM Tris-HCl [pH 7.5], 60 mM MgCl₂, 4 mM DTT, 0.8 mM ATP, 2 mg of histone H1 per ml, and 40 μ Ci of [³²P]ATP) was added, and reactions were incubated at 30°C for 10 min. Proteins were resolved by SDS-PAGE on a 12% acrylamide gel and then transferred to nitrocellulose. Radiolabeled histone H1 was excised, and radioactivity was quantitated in a scintillation counter. The linearity of each assay was verified by comparing counts obtained at each time point. Levels of Cdc25A in each reaction were quantified using ECF reagents and the Storm 860 imaging system. Histone H1 kinase activities were normalized for levels of Cdc25A in each reaction.

Phosphatase assays using HeLa cell-derived Cdc25A and Cdk1/cyclin B1 complexes. Approximately 3 \times 10⁶ cells per 100-mm-diameter tissue culture dish were coinfecting with recombinant adenoviruses encoding Myc-tagged Cdc25A (wt and mutants) and tTA at a multiplicity of infection of 5 for a total of 8 h. A portion of the infected cells was harvested for FACS analysis to ensure that cell cycle profiles were similar, and the remaining cells were suspended in buffer A and lysed by passage through a 27-gauge needle 30 times. After centrifugation at 16,600 \times g for 5 min, supernatants were further clarified by centrifugation at 100,000 \times g for 1 h. Lysates were incubated with 25 μ l of packed protein A-agarose (Pierce) at 4°C for 1 h. Cdc25A immunoprecipitations were performed with 20 μ l of anti-c-Myc(9E10)-agarose conjugate at 4°C for 2 h. Precipitates were washed four times in buffer A and twice with phosphatase buffer

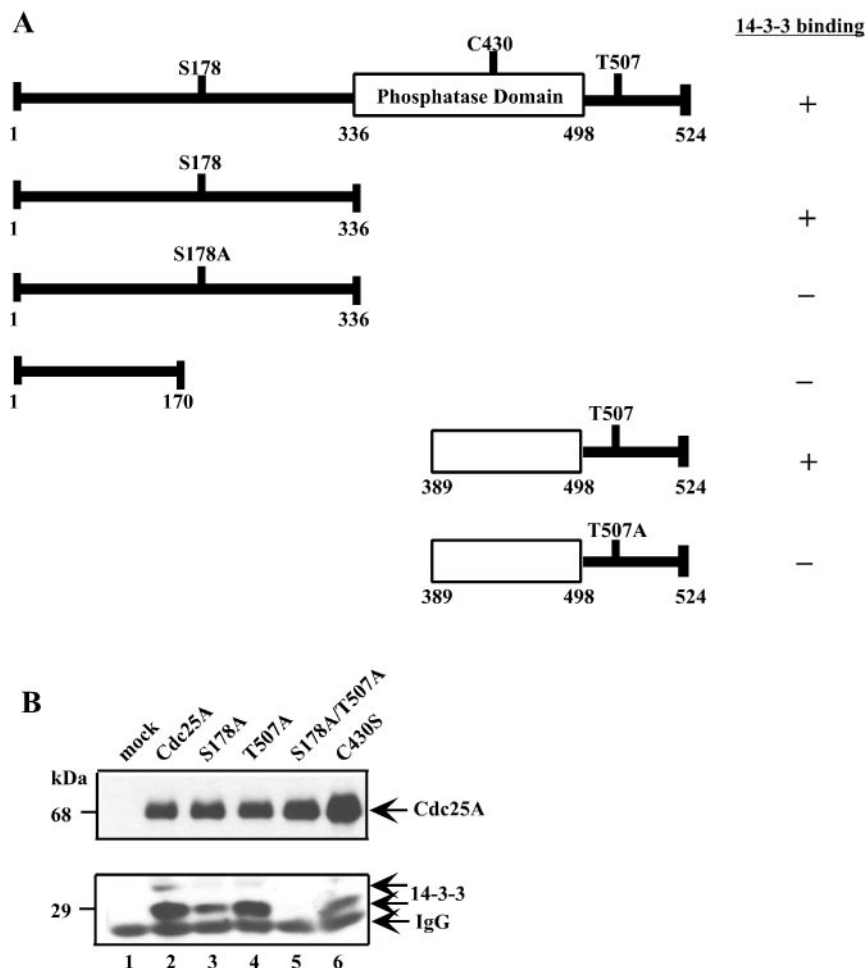


FIG. 1. Dependency of 14-3-3 binding to Cdc25A on S178 and T507. (A) Cosm6 cells transfected with vector alone (mock) or with plasmids encoding the indicated forms of Myc-Cdc25A were lysed and incubated with anti-c-Myc-agarose. Precipitates were resolved by SDS-PAGE and monitored for Cdc25A and 14-3-3 proteins by immunoblotting. +, coprecipitation of 14-3-3 with Cdc25A; -, absence of 14-3-3 in Cdc25A precipitates. (B) 293 cells mock infected or infected with recombinant adenovirus encoding the indicated forms of Cdc25A were lysed and then incubated with anti-c-Myc-agarose. Precipitates were resolved by SDS-PAGE and monitored for Cdc25A and 14-3-3 proteins by immunoblotting.

and then were suspended in 75 μ l of phosphatase buffer. Five microliters of Cdc25A immobilized on Myc-agarose was suspended in 20 μ l of phosphatase buffer. Reactions were incubated at 30°C for 10, 15, or 20 min after the addition of 5 μ l of cyclin B1/Cdk1 substrate. Samples were processed for histone H1 kinase assays as described above. The remaining Cdc25A precipitates were immunoblotted and quantified by using Myc antibodies, ECF reagents, and the Storm 860 imaging system. Histone H1 kinase activities were normalized for levels of Cdc25A in each reaction.

Phosphatase assays using mFP. The levels of GST fusion proteins were estimated by comparison to a BSA standard after SDS-PAGE and Coomassie blue staining or were quantified by using ECF reagents and the Storm 860 imaging system. Immunoblotting was performed with antibody specific for GST together with alkaline phosphatase (AP)-conjugated anti-rabbit immunoglobulin G (Jackson ImmunoResearch). Proteins were visualized by chemifluorescence. Approximately 10 μ g of purified GST-Cdc25A (wt and mutants) was incubated in 500 μ l of buffer consisting of 50 mM Tris, 50 mM Bis-Tris, 100 mM sodium acetate (pH 6.5), 0.01% Tween 20, 1 mM DTT, and 62.5 μ M 3-*O*-methyl fluorescein phosphate (mFP) (Sigma) at room temperature as described previously (49). Reactions were followed by spectroscopy at 477 nm. All experiments were done in triplicate.

Phosphorylation of S178 and T507 as a function of the cell cycle. HeLa cells synchronized by a double thymidine block were infected with adenoviruses encoding Myc-tagged Cdc25A and tTA. G₁/S-arrested cells were collected prior to release from the second block, S-phase cells were collected at 3 h after release from the block, and G₂-phase cells were collected 7 h after release from the

block. Mitotic cells were obtained by infecting cells immediately after release from the block in medium containing nocodazole for 10 h.

Additional methods. Cdc25A mutants were generated by a PCR-based strategy or by use of the QuickChange XL site-directed mutagenesis kit (Stratagene) and were verified by sequencing.

Chk1 and Chk2 kinase reactions were carried out *in vitro* with bacterially produced His-Cdc25A and insect cell-produced GST-tagged Chk1 and Chk2 as described previously (19, 57).

RESULTS

Phosphorylation of Cdc25A at S178 and T507 mediates the binding of 14-3-3 proteins to Cdc25A. 14-3-3 proteins were isolated in a two-hybrid screen using human Cdc25A as bait (8). A mutagenesis strategy was employed to identify residues in Cdc25A that confer 14-3-3 binding (Fig. 1A). 14-3-3 bound independently to both the N (amino acids 1 to 336) and C (amino acids 389 to 524) termini of Cdc25A. Substitution of alanine for serine 178 (S178A) eliminated binding to residues 1 to 336 and substitution of alanine for threonine 507 (T507A) eliminated 14-3-3 binding to residues 389 to 524. Full-length Cdc25A proteins substituting alanine for S178, T507, or both

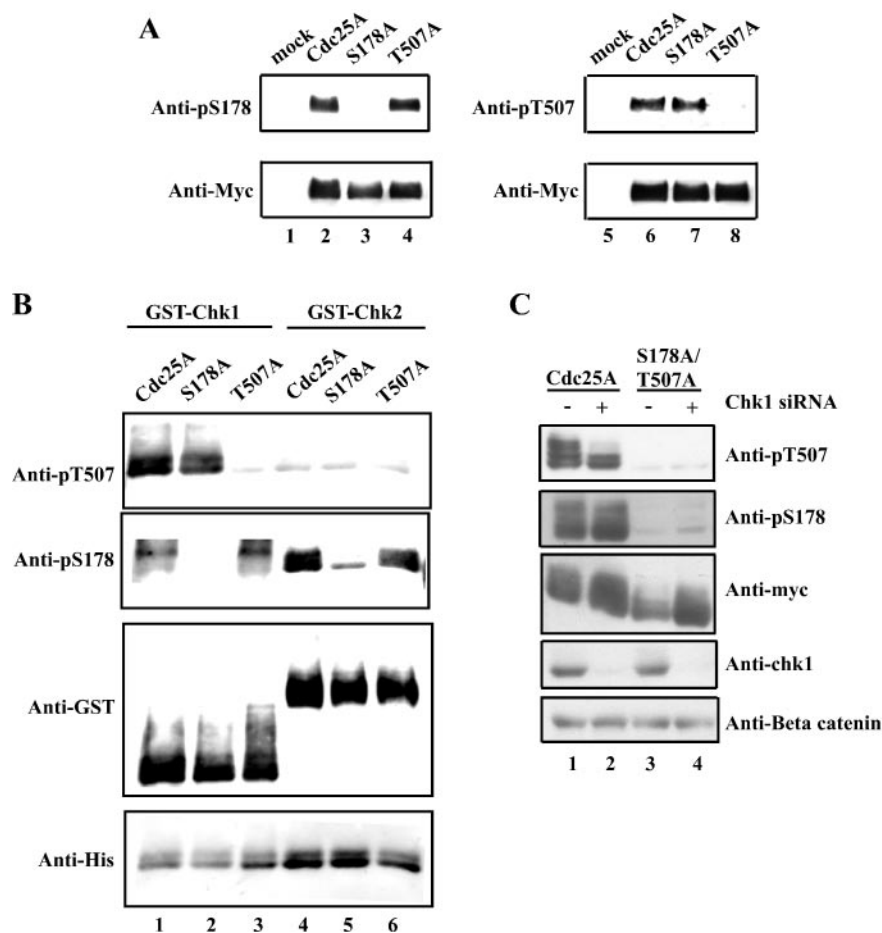


FIG. 2. Phosphorylation of Cdc25A at S178 and T507 in vivo and in vitro. (A) Lysates from HeLa cells infected with adenoviruses encoding the indicated Myc-Cdc25A proteins were resolved by SDS-PAGE, and proteins were detected by immunoblotting with phospho-specific antibodies followed by antibodies specific for the Myc epitope (anti-Myc). (B) Kinase assays were performed in vitro in the presence of His₆Cdc25A proteins and either GST-Chk1 or GST-Chk2. Reaction products were resolved by SDS-PAGE and subjected to Western blotting to monitor phosphorylation of Cdc25A at T507 (anti-pT507) and S178 (anti-pS178) and to monitor levels of His₆Cdc25A (anti-His), GST-Chk1, and GST-Chk2 (anti-GST). (C) HeLa cells were treated with control or Chk1-specific siRNAs followed by infection with recombinant adenovirus encoding the indicated forms of Cdc25A. Lysates were prepared and analyzed by Western blotting with the indicated antibodies.

were generated and tested for 14-3-3 binding. Complete loss of 14-3-3 binding was seen when both S178 and T507 were replaced with alanine (Fig. 1B, lane 5). Thus, both S178 and T507 are required for interactions between Cdc25A and 14-3-3 proteins. The phosphatase activity of Cdc25A was not absolutely required for 14-3-3 binding, as a catalytically inactive form of Cdc25A (C430S) was capable of binding to 14-3-3 proteins (Fig. 1B, lane 6).

14-3-3 binding to target proteins is, in general, mediated by phosphorylation (33, 54). Phosphorylation of Cdc25A at S178 and T507 in vivo was confirmed using phospho-specific antibodies (Fig. 2A). In addition, mutation of S178 did not affect phosphorylation of T507 nor did mutation of T507 affect phosphorylation of S178. Given that phosphorylation of Cdc25A in vivo is absolutely dependent on Chk1, we predicted that Chk1 would phosphorylate Cdc25A at S178 and T507 in vitro (57). As seen in Fig. 2B, Chk1 phosphorylated both residues in vitro (lanes 1 to 3). Interestingly, the Chk2 protein kinase, which has been proposed to regulate Cdc25A following IR (11), was incapable of phosphorylating Cdc25A at T507 in vitro (Fig. 2B,

lanes 4 to 6). Next, the phosphorylation of Cdc25A at T507 and S178 was examined in cells in which Chk1 levels had been reduced by Chk1-specific siRNAs. As seen in Fig. 2C, Chk1 but not β -catenin levels were significantly reduced in cells treated with Chk1-specific siRNAs (lanes 2 and 4). Levels of both Cdc25A (lane 2) and Cdc25A(S178A/T507A) (lane 4) were higher in cells in which Chk1 levels had been reduced by siRNA treatment. This result is consistent with our previous work demonstrating that Chk1 negatively regulates the stability of Cdc25A during an unperturbed cell cycle (57). Importantly, even though Cdc25A levels were elevated in cells lacking Chk1, reactivity of Cdc25A with the pT507 antibody was significantly reduced (lane 2) relative to reactivity of Cdc25A in cells containing Chk1 (lane 1). Loss of Chk1 did not affect S178 phosphorylation as dramatically as T507 phosphorylation. These results support the conclusion that Chk1 is the major T507 kinase but that kinases in addition to Chk1 regulate S178 phosphorylation. This is consistent with the results in Fig. 2B showing that Chk2 is capable of phosphorylating Cdc25A at S178 but not T507 in vitro.

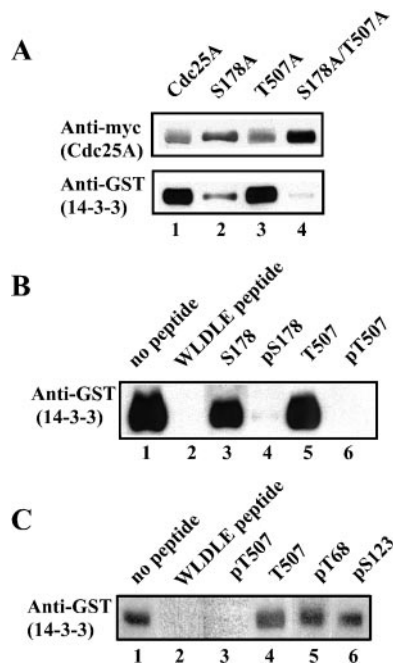


FIG. 3. Interactions between Cdc25A and 14-3-3 proteins are direct. (A) Far Western analysis was performed to monitor the binding of 14-3-3 protein to wt and mutant forms of Cdc25A. Membranes containing the indicated form of Myc-Cdc25A were incubated with purified GST-14-3-3 ϵ , and 14-3-3 bound to Cdc25A was detected by Western blotting with GST antibodies. (B and C) Specific phosphopeptides compete with 14-3-3 proteins for binding to Cdc25A. Membranes containing the indicated forms of Myc-Cdc25A were incubated with GST-14-3-3 ϵ in the presence of the indicated phospho- and nonphosphopeptides. 14-3-3 bound to Cdc25A was detected by Western blotting with GST antibodies.

Far Western analysis was performed to determine if binding of 14-3-3 to Cdc25A was direct. As seen in Fig. 3A, 14-3-3 bound directly to Cdc25A (lane 1) but poorly to the S178A (lane 2) and S178A/T507A (lane 4) mutants. Furthermore, peptides containing phosphorylated S178 (Fig. 3B, lane 4) or T507 (Fig. 3B, lane 6, and Fig. 3C, lane 3) competed for 14-3-3 binding, as did an unphosphorylated peptide (Fig. 3B and C, lanes 2) that binds to 14-3-3 proteins (38, 48). The unphosphorylated S178 and T507 peptides did not compete efficiently in this assay, nor did phosphopeptides lacking 14-3-3 consensus motifs (Fig. 3C). These results demonstrate that 14-3-3 binds directly to Cdc25A and that phosphorylation of S178 and T507 mediates this interaction.

14-3-3 binding mutants induce a mitotic-like state in S-phase cells. A high percentage of abnormal chromosome spreads were observed in S-phase cells expressing the T507A or S178A/T507A mutant (Fig. 4B and C). In addition, cyclin B1-associated kinase activity was approximately threefold higher in cells expressing Cdc25A(T507A) or Cdc25A(S178A/T507A) than in cells expressing wt Cdc25A, and the activity was approximately 1.5-fold higher in cells expressing Cdc25A(S178A) (Fig. 4D). Furthermore, S-phase cells expressing the T507A or S178A/T507A mutant stained positive for histone H3 phosphorylation, a modification normally seen only in mitotic cells (Fig. 5) (50, 51). PI staining confirmed that cells were in S phase. Approximately 8 to 9% of cells expressing wt

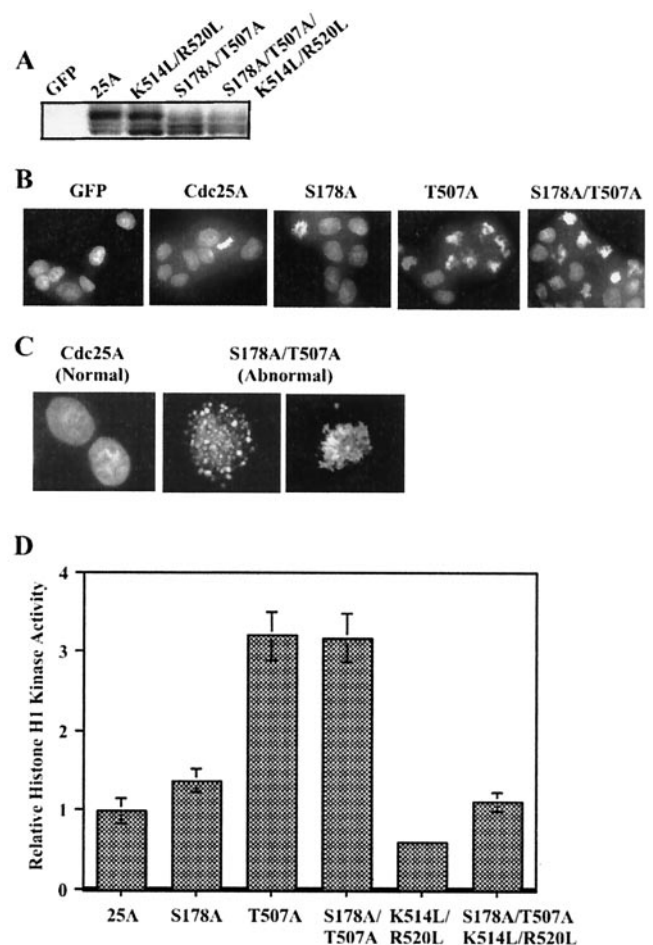


FIG. 4. 14-3-3 binding mutants activate cyclin B1/Cdk1 and induce a mitotic-like state when expressed in S phase. S-phase HeLa cells expressing GFP and various forms of Myc-Cdc25A were analyzed for Cdc25A levels by Western blotting (A) and for chromosomal integrity by examining DAPI-stained nuclei (B and C). Magnification, $\times 40$ (B) and $\times 100$ (C). Cells were also analyzed for cyclin B1-associated histone H1 kinase activity (D). Standard errors of the means ($n = 4$) are shown as error bars along the y axis.

Cdc25A or Cdc25A(S178A) stained positive for phospho-histone H3, whereas the percentage staining positive for phospho-histone H3 increased significantly in cells expressing the T507A (29.1%) or S178A/T507A (28.0%) mutant. Failure to observe cells with DNA contents of less than 2N indicates that the 14-3-3 binding mutants did not induce S-phase cells to undergo apoptosis.

Phosphorylation of Cdc25A on T507 negatively regulates the ability of Cdc25A to dephosphorylate Cdk1. Phosphatase assays were performed to monitor the ability of each Cdc25A mutant to dephosphorylate and activate cyclin B1/Cdk1 in vitro. To verify that mutation of S178 and/or T507 did not affect the intrinsic phosphatase activity of Cdc25A, the activity of bacterially produced Cdc25A was examined, because bacterial Cdc25A is neither phosphorylated nor bound to 14-3-3 proteins. Mutation of S178 and T507 either alone or together did not affect the activity of bacterially produced Cdc25A (Fig. 6A). Next, HeLa cells were infected with adenoviruses encod-

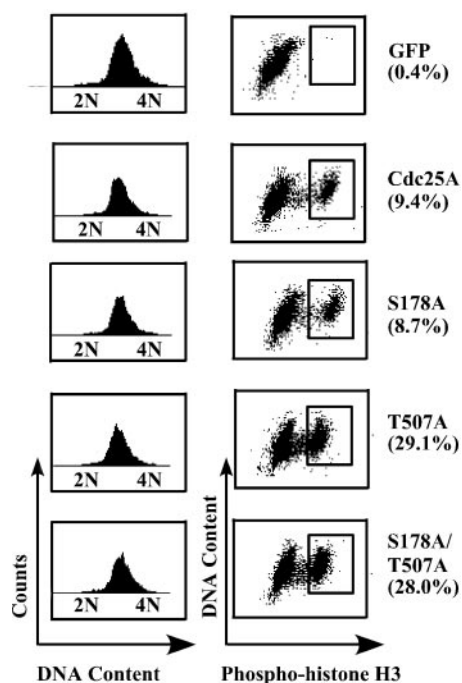


FIG. 5. 14-3-3 binding mutants induce histone H3 phosphorylation in S-phase cells. S-phase HeLa cells expressing GFP and various forms of Myc-Cdc25A (same cells as for Fig. 4) were costained for DNA content and histone H3 phosphorylation and analyzed by flow cytometry (50). Percentages of mitotic cells are indicated.

ing GFP, Cdc25A, or mutant forms of Cdc25A. Cells were subjected to FACS analysis at the time of harvest to verify that cell cycle profiles were identical (data not shown). wt and mutant forms of Cdc25A were isolated from infected cells and assayed for the ability to activate Cdk1 in vitro. HeLa cell-derived Cdc25A, unlike bacterially derived protein, is phosphorylated and bound to 14-3-3 proteins. The T507A and S178A/T507A mutants consistently showed an approximately twofold increase relative to wt Cdc25A in the ability to activate cyclin B1/Cdk1 in vitro (Fig. 6B). The activity of Cdc25A(S178A) was modestly increased over that of wt Cdc25A. Furthermore, higher levels of cyclin B1/Cdk1 were observed to coprecipitate with the 14-3-3 binding mutants of Cdc25A than with wt Cdc25A (Fig. 7B).

The C terminus of Cdc25A contains a cyclin B1/Cdk1 docking site. Taken together, these results suggest that phosphorylation of T507 and subsequent 14-3-3 binding to the C terminus of Cdc25A negatively regulates interactions between Cdc25A and cyclin B1/Cdk1. Cdc25B has a cyclin/Cdk docking site within its C terminus (49), and Cdc25A, but not Cdc25C, conserves many of these residues (Fig. 7A). To test whether the C terminus of Cdc25A conferred cyclin B1/Cdk1 binding, mutations in the putative binding domain were generated and mutants were tested for the ability to bind to cyclin B1/Cdk1 (Fig. 7C). Mutation of K514 and R520 severely impaired the ability of Cdc25A to bind to cyclin B1/Cdk1.

If the T507A and S178A/T507A mutants obtain their biological potency by enhanced binding to cyclin B1/Cdk1, mutation of residues in the binding domain is expected to reduce this potency. To test this, S-phase cells expressing wt and mu-

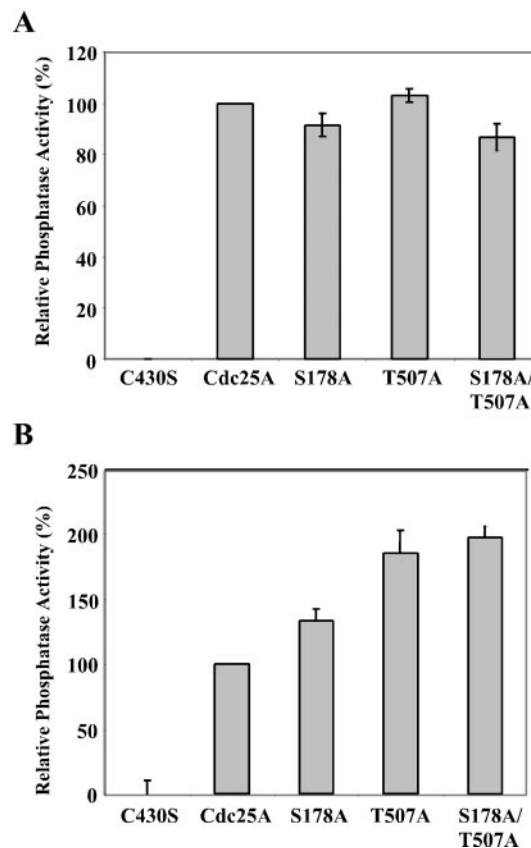


FIG. 6. Threonine 507 negatively regulates phosphatase activity of Cdc25A. wt and mutant forms of Cdc25A were purified as GST fusion proteins from bacteria (A) or as Myc fusion proteins from HeLa cells (B). Phosphatase assays were carried out in the presence of Cdc25A and purified cyclin B1/Cdk1 containing Cdk1 phosphorylated on both T14 and Y15. Phosphatase reactions were terminated and histone H1 kinase assays were performed to measure the activation of cyclin B1/Cdk1. ^{32}P incorporation into histone H1 was determined by scintillation counting. Cdc25A precipitates were immunoblotted and quantified by use of Myc antibodies, ECF reagents, and the Storm 860 imaging system (Amersham). Histone H1 kinase activities were normalized for levels of Cdc25A in each reaction. Values obtained for wt Cdc25A were set at 100, and the mutant values are shown relative to this. Standard errors of the means ($n \geq 3$) are shown as error bars along the y axis.

tant forms of Cdc25A were monitored for cyclin B1/Cdk1-associated histone H1 kinase activity. As seen in Fig. 4D, the binding domain mutants of Cdc25A exhibited reduced capacities to activate cyclin B1/Cdk1 in vivo. To verify that the binding domain mutants retained phosphatase activity, their ability to dephosphorylate the artificial substrate mFP in vitro was tested (49). As seen in Fig. 7E, mutation of the binding domain did not affect the intrinsic activity of Cdc25A toward mFP.

Interactions between Cdc25A and 14-3-3 proteins are cell cycle regulated. These results suggest that phosphorylation of Cdc25A on T507 and subsequent 14-3-3 binding prevent Cdc25A from binding to cyclin B1/Cdk1. This model predicts that Cdc25A should be phosphorylated on T507 and bound to 14-3-3 proteins until cells approach mitosis, at which point loss of 14-3-3 binding would promote activation of the Cdk1 kinase.

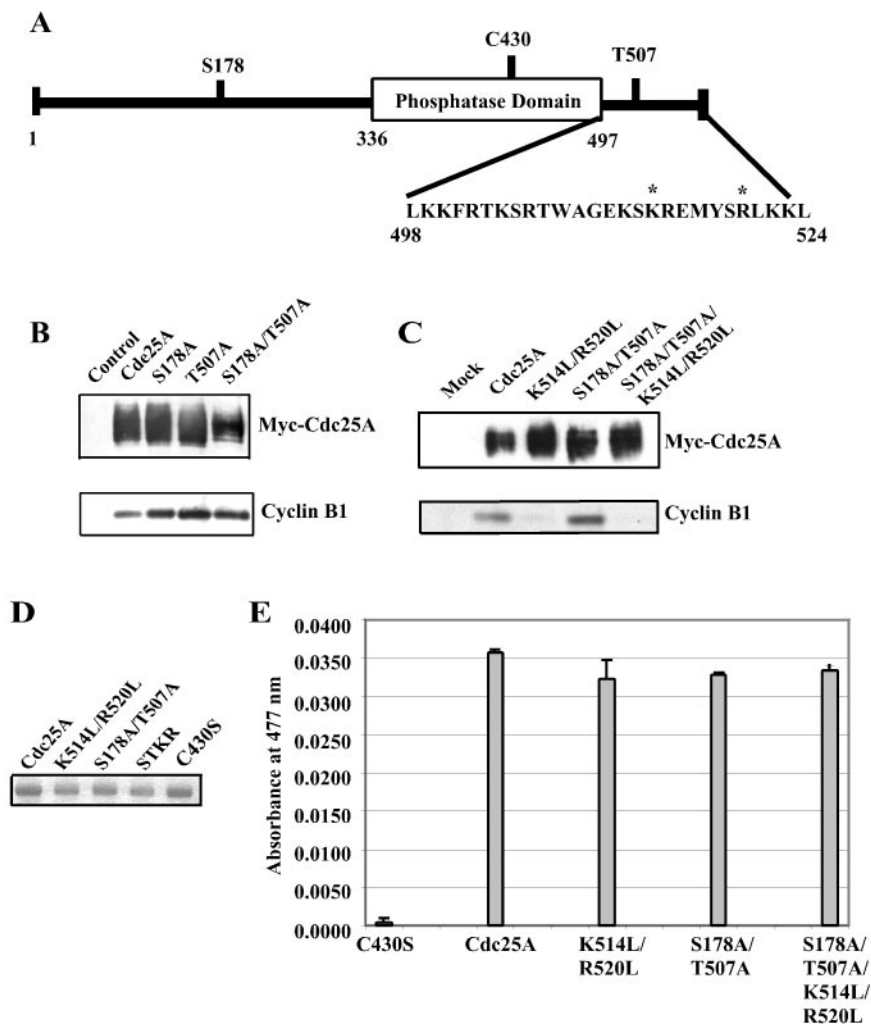


FIG. 7. The C terminus of Cdc25A contains a cyclin B1 docking site. (A) Schematic representation of Cdc25A and amino acids comprising the C-terminal cyclin B1 docking site. Asterisks denote K514 and R520. (B) Loss of 14-3-3 binding promotes interactions between Cdc25A and cyclin B1. HeLa cells synchronized at the G_1/S border were infected with recombinant adenoviruses encoding GFP (control) or with recombinant viruses encoding various forms of Myc-Cdc25A. Three hours after release from the block, S-phase cells were lysed and incubated with anti-c-Myc-agarose. Myc-Cdc25A precipitates were resolved by SDS-PAGE, and Cdc25A and cyclin B1 were visualized by immunoblotting. (C) The C terminus of Cdc25A is required for interactions between Cdc25A and cyclin B1. HeLa cells synchronized at the G_1/S border were infected with recombinant adenoviruses encoding GFP (control) or with recombinant viruses encoding various forms of Myc-Cdc25A. Three hours after release from the block, S-phase cells were lysed and incubated with anti-c-Myc-agarose. Myc-Cdc25A precipitates were resolved by SDS-PAGE, and Cdc25A and cyclin B1 were visualized by immunoblotting. (D and E) Mutation of the cyclin B1 docking site does not impair phosphatase activity of Cdc25A. wt and mutant forms of Cdc25A were purified from bacteria as GST fusion proteins, resolved by SDS-PAGE, and visualized by Western blotting (D). Purified proteins were tested for the ability to dephosphorylate mFP *in vitro* (E). Reactions were done in triplicate and followed by spectroscopy at 477 nm. Standard errors of the means are shown as error bars along the y axis.

To test this, phospho-specific antibodies were used to examine the phosphorylation of Cdc25A on S178 and T507 throughout the cell cycle. Because of its low abundance and short half-life, we were unable to monitor the binding of endogenous Cdc25A to 14-3-3 proteins *in vivo*. Therefore, cells at various stages of the cell cycle were infected with recombinant adenovirus that inducibly expresses Cdc25A, and phosphorylation of S178 and T507 was monitored using phospho-specific antibodies (Fig. 8). The phosphorylation of Cdc25A at S178 and T507 appeared constant throughout the G_1 and S phases of the cell cycle. Despite elevated levels of Cdc25A in the G_2 and M phases of the cell cycle, reactivity with the phospho-specific antibodies

(particularly pT507) was observed to decrease (Fig. 8C, lanes 4 and 5).

DISCUSSION

In this study, a new function for the Chk1 protein kinase in regulating mitotic entry is proposed (Fig. 9). We demonstrate that Chk1 phosphorylates Cdc25A at S178 and T507 *in vitro* and that phosphorylation of these residues mediates binding of 14-3-3 protein to Cdc25A *in vivo*. Because phosphorylation of Cdc25A *in vivo* is absolutely dependent on Chk1 (57) and because Chk1 phosphorylates Cdc25A at S178 and T507 in

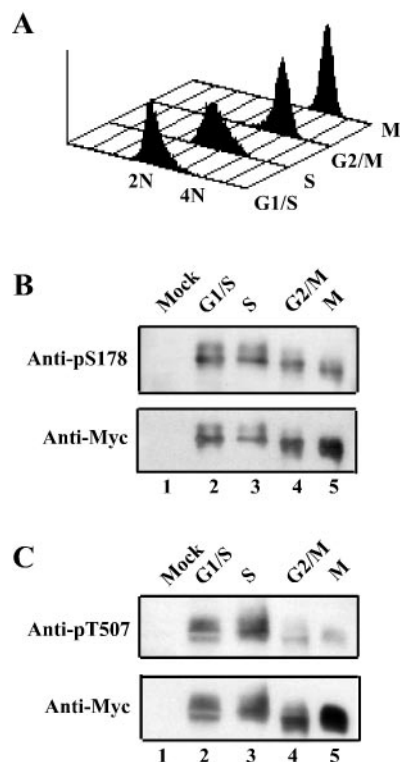


FIG. 8. Phosphorylation of Cdc25A at S178 and T507 is cell cycle regulated. HeLa cells expressing Myc-Cdc25A were synchronized at the G₁/S border by a double thymidine block protocol. Cells at the G₁/S border were collected prior to release from the block, and S- and G₂/M-phase cells were harvested at 3 and 7 h after release, respectively. Mitotic cells were obtained by incubation in nocodazole for 10 h after infection and release. Some cells were harvested and analyzed by FACS to determine DNA content (A). The remaining cells were lysed and incubated with anti-c-Myc-agarose. Precipitates were resolved by SDS-PAGE and incubated with phospho-specific antibodies followed by antibodies specific for the Myc epitope to monitor Cdc25A levels (B and C).

vitro (Fig. 2B), we infer that Chk1 regulates S178 and T507 phosphorylation in vivo. This conclusion is supported by the finding that phosphorylation of T507 and, to a lesser extent, S178 was impaired in Chk1-deficient cells (Fig. 2C). Thus, Chk1 negatively regulates entry into mitosis by preventing functional interactions between Cdc25A and the mitotic inducer Cdk1. Sorenson et al. (46) reported that both Chk1 and Chk2 phosphorylate Cdc25A to promote its turnover. Here we show that Chk1, but not Chk2, phosphorylates Cdc25A at T507 to prevent cyclin B1/Cdk1 binding. Thus, this study uncovers a unique role for Chk1 in the negative regulation of Cdc25A.

In general, the binding of 14-3-3 proteins to their targets is mediated by the phosphorylation of the target protein at one of two 14-3-3-binding consensus motifs. These motifs include the RSXpSXP (mode 1) and RX(Y/F)XpSXP (mode 2) sequences, where pS denotes either phosphoserine or phosphothreonine (33, 54). Given that each monomer of the 14-3-3 dimer can simultaneously bind distinct phosphoepitopes on a single protein (35), it is possible that a single 14-3-3 dimer simultaneously binds to pS178 and pT507 on a single Cdc25A molecule. The sequences surrounding and inclusive of S178

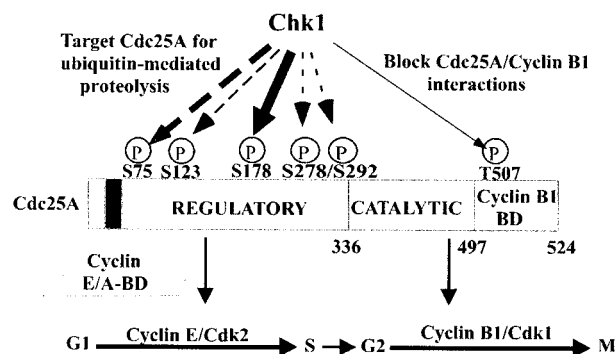


FIG. 9. Regulation of Cdc25A by Chk1. Chk1 phosphorylates Cdc25A on several N-terminal serines to promote its ubiquitin-mediated proteolysis (11, 16, 20, 46, 57). Chk1 also phosphorylates Cdc25A on S178 and T507 to facilitate 14-3-3 binding. 14-3-3 binding to the C-terminal cyclin B1 binding domain (BD) of Cdc25A blocks functional interactions between Cdc25A and cyclin B1/Cdk1 throughout interphase. These regulatory pathways prevent cells from entering into mitosis at inappropriate times during the cell cycle and following checkpoint activation. Solid arrows originating from Chk1 indicate phosphorylation sites that mediate 14-3-3 binding, whereas hatched lines represent sites that mediate Cdc25A turnover. S178 is the primary 14-3-3 binding site and S75 is the primary site regulating Cdc25A turnover, as indicated by thicker arrows.

(RQNpSAP) fit nicely with the mode 1 consensus, whereas sequences inclusive of and surrounding T507 (TKSRpTWA) do not fit either consensus very well. Thus, it is predicted that S178 is a higher-affinity 14-3-3 binding site and could serve a gatekeeper function, thereby increasing the affinity of 14-3-3 binding to the C terminus of Cdc25A when T507 is phosphorylated (53). In support of S178 serving a gatekeeper function, mutation of S178 to alanine impaired the interactions between Cdc25A and 14-3-3 to a much greater extent than did mutation of T507 (Fig. 1B and 3A). However, pT507 must be able to bind 14-3-3 independently of pS178, as the phenotype observed in cells expressing the S178 mutant was much less severe than that observed in cells expressing the T507 mutant.

T507 is located within a cyclin B1/Cdk1 docking site in the C terminus of Cdc25A, and 14-3-3 binding to this region appears to block Cdc25A from functionally interacting with cyclin B1/Cdk1. Sequences comprising the cyclin B1/Cdk1 docking domain are highly conserved among vertebrate Cdc25 homologues, particularly Cdc25A and Cdc25B family members (39, 49), and this region is disordered in the crystal structures of both Cdc25A and Cdc25B (12, 41). A motif within the N terminus of Cdc25A confers binding of Cdc25A to Cdk complexes containing cyclins A and E but not B1 (43). Mutation of this domain reduced binding of Cdc25A to cyclin A and E complexes but not to cyclin B1/Cdk1. Thus, domains within the N and C termini of Cdc25A are important for facilitating interactions between Cdc25A and various cyclin/Cdks, but the C-terminal domain appears to be the major determinant for cyclin B1/Cdk1 binding.

Taken together, these data suggest that phosphorylation of Cdc25A on T507 and subsequent 14-3-3 binding impair the ability of Cdc25A to interact with cyclin B1/Cdk1. Cdc25A is phosphorylated on T507 during the G₁ and S phases of the cell cycle, and reduced phosphorylation of T507 was noted during

G₂/M transition. This suggests that the cyclin B1/Cdk1 binding site is masked throughout much of interphase. Substitution of alanine for T507 prevented phosphorylation of Cdc25A on T507 and subsequent 14-3-3 binding at the C terminus of Cdc25A. This, in turn, is predicted to expose the cyclin B1 binding site, allowing inappropriate interactions between Cdc25A and cyclin B1/Cdk1 during S phase. This model is consistent with the observation that the single T507A and double S178A/T507A mutants were more effective in activating cyclin B1/Cdk1 both *in vivo* and *in vitro* and in promoting premature chromosome condensation and phospho-histone H3 staining during S phase.

It was recently reported that phosphorylation of S178 together with phosphorylation of serines 123, 278, and 292 regulates the turnover of Cdc25A both during interphase and in response to IR (46). This conclusion was based on studies carried out with Cdc25A phosphorylation-site mutants. In our hands, mutation of S178 alone does not impair Cdc25A turnover (data not shown). It is unclear how phosphorylation of S178 regulates the turnover of Cdc25A, given that S178 phosphorylation facilitates the binding of 14-3-3 proteins to Cdc25A. It may be that S178 needed to be mutated to block 14-3-3 binding in order to observe the effects of subsequent mutations of S123, S278, and S292 on Cdc25A stability.

14-3-3 binding regulates several important mitotic regulators. 14-3-3 binding impairs the ability of Cdc25A to interact with cyclin B1/Cdk1 (this study), 14-3-3 binding to Cdc25B and Cdc25C regulates their intracellular trafficking (9, 10, 18, 19, 27, 37, 55, 56), and 14-3-3 regulates the enzymatic activity of Wee1 (28, 42). Finally, 14-3-3 σ prevents the nuclear accumulation of cyclin B1/Cdk1 following DNA damage (7). Thus, 14-3-3 binding to mitotic regulators is a commonly employed mechanism used by cells to regulate progression through an unperturbed cell cycle as well as following checkpoint activation.

Maintenance of the Chk1/Cdc25A pathway is essential for cells to progress normally through an unperturbed cell division cycle and for cells to arrest in response to checkpoint activation. Importantly, failure to regulate Cdc25A protein levels causes bypass of the IR-induced DNA damage checkpoint and the DNA replication checkpoint, resulting in decreased cell survival (2, 11, 29, 31, 57). Thus, drugs that induce accumulation and/or inappropriate activation of Cdc25A are expected to be potent checkpoint abrogators and may prove useful in combination therapies for treating human cancers. Specific Chk1 inhibitors may be particularly useful in this regard, as they would not only lead to Cdc25A accumulation but also to activation of cyclin B1/Cdk1 in S phase to promote mitotic catastrophe.

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