

Elimination of *cdc2* Phosphorylation Sites in the *cdc25* Phosphatase Blocks Initiation of M-Phase

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The *cdc25* phosphatase is a mitotic inducer that activates $p34^{cdc2}$ at the G2/M transition by dephosphorylation of Tyr15 in $p34^{cdc2}$. *cdc25* itself is also regulated through periodic changes in its phosphorylation state. To elucidate the mechanism for induction of mitosis, phosphorylation of *cdc25* has been investigated using recombinant proteins. *cdc25* is phosphorylated by both cyclin A/ $p34^{cdc2}$ and cyclin B/ $p34^{cdc2}$ at similar sets of multiple sites in vitro. This phosphorylation retards its electrophoretic mobility and activates its ability to increase cyclin B/ $p34^{cdc2}$ kinase activity three- to fourfold in vitro, as found for endogenous *Xenopus cdc25* in M-phase extracts. The threonine and serine residues followed by proline that are conserved between *Xenopus* and human *cdc25* have been mutated. Both the triple mutation of Thr48, Thr67, and Thr138 and the quintuple mutation of these three threonine residues plus Ser205 and Ser285, almost completely abolish the shift in electrophoretic mobility of *cdc25* after incubation with M-phase extracts or phosphorylation by $p34^{cdc2}$. These mutations inhibit the activation of *cdc25* by phosphorylation with $p34^{cdc2}$ by 70 and 90%, respectively. At physiological concentrations these mutants cannot activate cyclin B/ $p34^{cdc2}$ in *cdc25*-immunodepleted oocyte extracts, suggesting that a positive feedback loop between *cdc2* and *cdc25* is necessary for the full activation of cyclin B/ $p34^{cdc2}$ that induces abrupt entry into mitosis in vivo.

INTRODUCTION

The molecular mechanism that regulates the initiation of nuclear division has been investigated intensively in recent years (Murray, 1993, for review). The central cell cycle engine that induces mitosis is maturation-promoting factor (MPF), a complex of B-type cyclins and a serine/threonine protein kinase, $p34^{cdc2}$. The protein kinase activity of this complex is tightly regulated by phosphorylation and dephosphorylation of $p34^{cdc2}$, which ensures the proper timing of mitosis. Phosphorylation of Thr161 on $p34^{cdc2}$ by $p34^{cdc2}$ -activating kinase (CAK) is required for its activity (Solomon *et al.*, 1992). In contrast, phosphorylation on Thr14 and Tyr15 is inhibitory to $p34^{cdc2}$ and has a dominant inhibitory effect even if Thr161 is also phosphorylated. Recently the kinase that phosphorylates Tyr15 has been demonstrated to be the product of the *wee1* gene (Parker *et al.*, 1992; McGowan and Russell, 1993), which was originally identified as a negative regulator of mitosis by yeast genetics (Russell and Nurse, 1987a). Human *wee1* kinase cannot phosphorylate Thr14 in vitro, and the ki-

nase responsible for this phosphorylation remains unknown (Parker *et al.*, 1992; McGowan and Russell, 1993). Recent evidence in several laboratories demonstrates that the phosphatase that dephosphorylates Tyr15, and probably Thr14, is the *cdc25* gene product (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b; Strausfeld *et al.*, 1991; Lee *et al.*, 1992) that was originally identified as a mitotic inducer by yeast genetics (Russell and Nurse, 1986). Therefore the activity of cyclin B/ $p34^{cdc2}$ is determined by the balance between the activities of *wee1* and *cdc25*.

To understand the mechanism of entry into mitosis, it is important to determine how *wee1* and *cdc25* are regulated in the cell cycle. Both *wee1* and *cdc25* seem to be involved in G2/M checkpoint controls that ensure mitosis is not initiated until DNA is fully replicated (Enoch and Nurse, 1990; Enoch *et al.*, 1992). Recently the *nim1/cdr1* kinase, identified genetically as a negative regulator of *wee1* (Russell and Nurse, 1987b; Feil-otter *et al.*, 1991), has been shown to phosphorylate and inactivate *wee1* directly (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). On the other

hand, *cdc25* activity has been shown to be high at mitosis and low in interphase, and *cdc25* has been shown to be activated by phosphorylation in M-phase. In addition, *cdc25* activity and its phosphorylation state are maintained at the low interphase level in the presence of incompletely replicated DNA (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). Recently, it has been reported that anti-cyclin B immunoprecipitates from mammalian cells have kinase activity toward human *cdc25* and that M-phase extracts immunodepleted with anti-p34^{cdc2} or anti-cyclin B antibodies fail to phosphorylate *cdc25*, whereas cyclin A/p34^{cdc2} appears able to phosphorylate only degraded *cdc25* (Hoffmann *et al.*, 1993). Therefore both *wee1* and *cdc25*, which function as key upstream regulators of p34^{cdc2}, seem to be regulated themselves by phosphorylation and dephosphorylation, although the effects of phosphorylation on their activity are opposite. It is clearly important to identify the kinases and phosphatases that act on *wee1* and *cdc25* to elucidate the biochemistry of the G2/M checkpoint and the induction of mitosis.

Here we report that both cyclin A/p34^{cdc2} and cyclin B/p34^{cdc2} can phosphorylate *Xenopus cdc25* in vitro and cause a mobility shift similar to that found in vivo. This phosphorylation also activates *cdc25* in vitro. Mutations in *cdc25* that eliminate conserved p34^{cdc2} phosphorylation sites prevent the mobility shift and activation by phosphorylation with p34^{cdc2}. Furthermore, these mutants fail to induce M-phase when expressed at a physiological level in cell-free extracts.

MATERIALS AND METHODS

Site-directed Mutagenesis of *Xenopus cdc25* cDNA

The amino acid sequence KLYPYDVPDYAS, containing the hemagglutinin (HA) epitope recognized by the 12CA5 monoclonal antibody (mAb) (Field *et al.*, 1988), was attached as follows to the C-terminus of the *Xenopus cdc25C1* protein (originally named *cdc25A* (Izumi *et al.*, 1992) but renamed here to avoid confusion with human *cdc25A*). Two oligonucleotides, 5'-AGCTTTATCCATATGACGTCCAGATACGCTAGCTTGT-3' and 3'-AATAGGTATACTGCAGGGTCTAATGCGATCGAACATCGA-5', were treated with T4 polynucleotide kinase, heated at 65°C for 2 min, annealed by slow cooling, and then ligated into partially *HindIII*-cut pBluescript SK+ (Stratagene, La Jolla, CA) containing full-length *Xenopus cdc25C1* cDNA (*pcdc25C1*). Clones containing the epitope fragments at the C-terminal *HindIII* site of the *cdc25C1* gene were screened by *EcoRI* and *NdeI* digestion. Proper orientation of the fragment was ascertained by DNA sequencing (*pcdc25C1HA*). The resultant C-terminal sequence is MKLYPYDVPDYASL instead of the original C-terminal sequence MKL. Then the full-length *Xenopus cdc25C1* gene with this epitope tag was cloned into the *SalI* and *XbaI* sites of the M13mp19 plasmid. Site-directed mutagenesis to introduce Ser-to-Ala, Thr-to-Val, or Cys-to-Ala mutations was performed as described previously (Izumi and Maller, 1991). The following oligonucleotides were used: T48V, 5'-GTCAGT-CACAGGTACCAAAGGCTGTCC-3' (*pcdc25C1HA-T48V*); T67V, 5'-AGGCAGCGTTTGGGTACCTACCCTGAA-3' (*pcdc25C1HA-T67V*); T138V, 5'-TTTAAACTTGGTACCCTGCAAAGAAGG-3' (*pcdc25C1HA-T138V*); S205A, 5'-CACTTATGGGGGCTCCCAAG-ATATT-3' (*pcdc25C1HA-S205A*); S285A, 5'-TGGCATAGAAGGTGCACGGTAAAGGCGGCT-3' (*pcdc25C1HA-S285A*); C457A, 5'-CCTCTTTCAGAAGAGAATTCGGCATGAAAGATTATAATTA - 3'

(*pcdc25C1HA-C457A*). Each mutant was then cloned into the *SalI* and *XbaI* sites of pBluescript SK+, which was named as in parentheses above. The *SalI-ScaI* fragment (0.25 kilobase [kb]), the *SalI-StyI* fragment (4.8 kb) of *pcdc25C1HA-T48V*, and the *ScaI-StyI* fragment (0.05 kb) of *pcdc25C1HA-T67V* were ligated, resulting in *cdc25C1HA* mutated at both T48V and T67V (*pcdc25C1HA-T2*). Similarly, the *SalI-ScaI* fragment (0.25 kb) of *pcdc25C1HA-T48V*, the *ScaI-StyI* fragment (0.05 kb) of *pcdc25C1HA-T67V*, and the *SalI-StyI* fragment (4.8 kb) of *pcdc25C1HA-T138V* were ligated, resulting in *cdc25C1HA* mutated at T48V, T67V, and T138V (*pcdc25C1HA-T3*). The *HindIII-XbaI* fragment (3.4 kb) of *pcdc25C1HA-T138V*, the *HindIII-ApaI* fragment (0.34 kb) of *pcdc25C1HA-S205A*, and the *ApaI-XbaI* fragment (1.4 kb) of *pcdc25C1HA-S285A* were also ligated to make *cdc25C1HA* mutated at T138V, S205A, and S285A (*pcdc25C1HA-T1S2*). Furthermore, the *StyI-XbaI* fragment (3.2 kb) of *pcdc25C1HA-T3* and the *StyI-XbaI* fragment (1.9 kb) of *pcdc25C1HA-T1S2* were ligated to make *cdc25C1* mutated at all five of the sites described above (*pcdc25C1HA-T3S2*).

Production and Purification of GST-*cdc25*

pGEX2TΔ*EcoNI-XbaI* (Gabrielli *et al.*, 1992b), in which the *EcoNI* site of the *Schistosoma japonicum* glutathione S-transferase (GST) gene (Smith and Johnson, 1988) was mutated to a *XbaI* site in the pGEX-2T vector (Pharmacia, Piscataway, NJ), was a generous gift of Margaret S. Lee and Helen Piwnica-Worms (Harvard University). *pcdc25C1* was digested by *StuI* and partially digested by *EcoRI*. The resultant 2.0-kb fragment was ligated into the *SmaI* and *EcoRI* sites of pGEX2TΔ*EcoNI-XbaI* (pGEX2T-*cdc25C1*). Then the *XbaI-PstI* fragment (2.4 kb) of pGEX2T-*cdc25C1* was cloned into the *XbaI* and *PstI* sites of pVL1393 (Invitrogen, San Diego, CA) (pGST*cdc25C1-W*). The resultant plasmid vector along with wild-type baculovirus DNA was transfected into Sf9 cells by cationic liposome treatment to produce recombinant virus encoding a GST-fused *cdc25C1* protein that lacks the first 18 N-terminal amino acids of *cdc25C1*. pGST*cdc25C1HA-W*, pGST*cdc25C1HA-T3*, and pGST*cdc25C1HA-T3S2* were constructed similarly, using *pcdc25C1HA*, *pcdc25C1HA-T3*, and *pcdc25C1HA-T3S2*, respectively. Recombinant plaques were isolated and purified as described previously (Piwnica-Worms, 1990).

Approximately 2 g of packed Sf9 cells harvested 48 h after infection was suspended in 5 ml of NET buffer (50 mM Tris(hydroxymethyl)aminomethane (Tris) pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and proteinase inhibitors mix (10 μg/ml of leupeptin, pepstatin, chymostatin, and aprotinin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The cell suspension was sonicated (30" × 8 times) and incubated at 4°C for 20 min with rocking after addition of 0.5% of Nonidet P-40 (Sigma, St. Louis, MO). A supernatant fraction was prepared by centrifugation at 12 000 × g at 4°C for 20 min. The extract was mixed with 1.5 ml of glutathione-agarose beads (Sigma) at 4°C for 2 h with agitation and washed twice with NET buffer containing 0.5% Nonidet P-40 and 0.5 M NaCl, twice with NET buffer plus 0.5% Nonidet P-40, and finally twice with 40 mM Tris pH 8.0 containing 2 mM DTT. The GST-*cdc25* protein was eluted with elution buffer (40 mM Tris pH 8.0, 2 mM DTT, and 10 mM glutathione) at 4°C for 30 min. The protein concentration was quantitated by densitometric scanning of a Coomassie blue-stained gel with an ImageQuant v3.0 system (Molecular Dynamics, Sunnyvale, CA) with bovine serum albumin (BSA) as a standard.

Phosphorylation of GST-*cdc25* In Vitro

Mitogen-activated protein kinase (MAPK) (Barrett *et al.*, 1992), S6 kinase II (Erikson *et al.*, 1991), and cAMP-dependent kinase (Beavo *et al.*, 1974) were purified in this laboratory as described. Cyclin B/*cdc2* was purified by elution from p13^{suc1}-Sephacrose as described (Kusubata *et al.*, 1992). This preparation has a high specific activity for H1 phosphorylation but contains other bands in addition to cyclin B and p34^{cdc2}. Western blotting of the preparation confirmed the absence of detectable cyclin A or cdk2. In other experiments, the p13^{suc1}-eluted

preparation was purified further by chromatography on a Mono S column (Pharmacia), and this preparation, consisting largely of cyclin B and p34^{cdc2} as judged by silver staining, was also able to phosphorylate, shift, and activate cdc25 in vitro. Bacteria transformed with expression vectors containing protein A or protein A-bovine cyclin A fusion protein were a generous gift of Jörg P. Adamczewski and Tim Hunt (ICRF Clare Hall Laboratories, South Mimms, England) (Kobayashi *et al.*, 1992). Active cyclin A/p34^{cdc2} was prepared by incubation of 1.2 µg of protein A-cyclin A with 40 µl of a metaphase-arrested extract from unfertilized eggs (Murray *et al.*, 1989) at 23°C for 50 min. Protein A was treated in the same way as a control. The protein A-cyclin A- and protein A-associated proteins were purified with rabbit IgG-Sepharose, followed by washing as described for immunoprecipitation (Izumi *et al.*, 1992).

Phosphorylation assays were performed in kinase buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES] pH 7.4, 10 mM MgCl₂) containing 100 µM ATP and 10 µCi of [γ -³²P]ATP for 15 min at 30°C unless otherwise specified. Reactions were terminated by addition of sodium dodecyl sulfate (SDS) polyacrylamide gel sample buffer and analyzed by electrophoresis (Laemmli, 1970).

Two-dimensional (2-D) tryptic phosphopeptide maps were performed as described previously (Izumi *et al.*, 1992). Briefly, the phosphoproteins were eluted from gels, trypsin-digested, spotted onto cellulose thin-layer plates (Kodak, Rochester, NY), and subjected to electrophoresis at 1 kV for 25 min at pH 8.9 (1% NH₄HCO₃). Plates were dried and subjected to ascending chromatography (n-butanol/pyridine/acetic acid/water, 75:50:15:60).

Assay of cdc25 Activity

The assay of cdc25 took advantage of the GST tag to reisolate cdc25 after phosphorylation reactions. This assay used soluble cdc25, an improvement over the previously described assay (Izumi *et al.*, 1992) that used immobilized cdc25. cdc25 activity is monitored by activation of the H1 kinase activity of cdc2 after dephosphorylation of tyrosine 15 in an immobilized, low activity, cyclin B/p34^{cdc2} preparation from *Xenopus* stage VI oocytes. Soluble recombinant *Xenopus* GST-cdc25 (1 µg) was phosphorylated by cyclin B/p34^{cdc2} in vitro in the presence of 0.5 mM ATP in a final volume of 30 µl for 30 min at 23°C. Controls were incubated with buffer and ATP alone. The reaction was stopped by 200 µl of ice-cold lysis buffer (30 mM HEPES pH 7.5, 1% Triton X-100, 200 mM NaCl, 100 mM NaF, 100 mM Na₄P₂O₇, 2 mM Na₃VO₄, 50 mM β -glycerophosphate, 5 mM EDTA, 5 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA], 1 mM DTT, proteinase inhibitors mix as described above) containing 1 µM microcystin. The samples were incubated with 25 µl of glutathione-agarose beads for 1 h at 4°C to reisolate GST-cdc25. After washing the beads five times with lysis buffer containing 1 M NaCl and twice with phosphatase buffer (50 mM Tris pH 7.4, 30 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM DTT, proteinase inhibitors mix), GST-cdc25 was eluted with 100 µl of elution buffer at 4°C for 30 min. To measure cdc25 activity, 80 µl of the supernatant after microcentrifugation containing soluble GST-cdc25 was incubated for 1 h at 23°C with protein G-Sepharose beads containing tyrosine-phosphorylated, low activity cyclin B/p34^{cdc2} that had been immunoprecipitated from an extract of 10 stage VI oocytes using a mixture of sheep antibodies against *Xenopus* cyclin B1 and B2 (Izumi and Maller, 1991). Cyclin B/p34^{cdc2}-containing beads were microcentrifuged to remove GST-cdc25 and washed twice with lysis buffer containing 1 M NaCl and once with kinase buffer containing 2 mM Na₃VO₄. p34^{cdc2} activity was then measured by incubation at 30°C for 15 min in kinase buffer containing 2 mM Na₃VO₄, 500 µM [γ -³²P]ATP, and 0.5 mg/ml of histone H1. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography and Cerenkov counting of the excised H1 band.

cdc25 activity after phosphorylation by protein A-cyclin A/p34^{cdc2} in vitro was assayed with the following modifications. After phosphorylation of GST-cdc25 by protein A-cyclin A/p34^{cdc2} beads, prepared as described above, 1 volume (30 µl) of stop buffer (phosphatase

buffer containing 0.2 M EDTA, 4 mM DTT, 2 µM microcystin) was added to the reaction mix. Forty-five microliters from the 60 µl supernatant were transferred to a new tube after microcentrifugation. After recentrifugation 30 µl was again transferred into a new tube. These procedures remove all protein A-cyclin A beads, which would interfere with the subsequent assay of cdc25 activity using activation of the H1 kinase activity of p34^{cdc2}. The supernatant containing cdc25 was then incubated with tyrosine-phosphorylated cyclin B/p34^{cdc2} derived from stage VI oocytes. Beads were washed and assayed for H1 kinase activity as described above.

Preparation of pre-MPF Fractions from Ovaries

The pre-MPF fraction (tyrosine-phosphorylated cyclin B/p34^{cdc2}) from oocytes was prepared as described (Cyert and Kirschner, 1988) with modifications. Whole ovaries from one or two unprimed frogs were removed under anesthesia with Tricaine (Sigma) and cut into small fragments. Ovaries were incubated with 1 mg/ml Dispase (Boehringer, Indianapolis, IN) in buffer (88 mM NaCl, 33 mM Ca(NO₃)₂, 1 mM KCl, 0.41 mM CaCl₂, 0.82 mM MgSO₄, and 10 mM HEPES pH 7.8) for 2 h at room temperature with gentle stirring. The partially digested ovaries were then incubated with 0.6 mg/ml of collagenase type 1A (Sigma) for 30 min at room temperature with gentle stirring. Oocytes were rinsed extensively with buffer to remove most of the small oocytes, then rinsed with EB (80 mM β -glycerophosphate, 15 mM MgCl₂, and 20 mM EGTA pH 7.3), lysed in EB containing 10 mM DTT and proteinase inhibitors as described previously (Cyert and Kirschner, 1988), and centrifuged at 4°C for 1 h at 50 000 rpm in an SW55 rotor. Ammonium sulfate fractionation (33%) was performed as described previously (Cyert and Kirschner, 1988). After centrifugation at 12 000 \times g for 20 min at 4°C, the pellet was dissolved in approximately one-tenth of the initial volume of the soluble fraction with EB containing 10 mM DTT and proteinase inhibitors. After dialysis, samples were frozen at -80°C. For cdc25 immunodepletion experiments, a sample (240 µl) was incubated at 4°C for 1 h with either 40 µl of control protein A-Sepharose or 100 µg of anti-cdc25 antibodies raised against the C-terminal half of *Xenopus* cdc25 expressed in *Escherichia coli* (Izumi *et al.*, 1992) that had been previously bound to 40 µl of protein A-Sepharose. After the beads were removed by centrifugation, the cdc25-depleted or control supernatants were stored at -80°C.

RESULTS

cdc25 Is Phosphorylated by Both Cyclin A/p34^{cdc2} and Cyclin B/p34^{cdc2} In Vitro

To analyze the mechanism of phosphorylation of cdc25 in detail, it is essential to have purified and active recombinant cdc25 protein. Because initial attempts to express active *Xenopus* cdc25 in bacteria were unsuccessful, cdc25 was expressed in baculovirus-infected Sf9 cells as a fusion protein with GST encoded by *S. japonicum* (Smith and Johnson, 1988). GST-cdc25 eluted from glutathione beads migrated with the predicted molecular weight of 102 000 upon SDS-PAGE (Figure 1, lane a). Because the histone H1 kinase activity and cdc25 activity are very closely correlated in *Xenopus* oocytes and eggs (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992) and the major H1 kinase is cyclin B/p34^{cdc2}, it was first examined whether recombinant GST-cdc25 was a substrate for cyclin B/p34^{cdc2}. GST-cdc25 was phosphorylated by cyclin B/p34^{cdc2} in vitro and exhibited two radiolabelled bands upon SDS-PAGE, one at 103 kDa (slightly higher than the unphosphorylated form) and one at 113 kDa (11 kDa higher than the unphosphor-

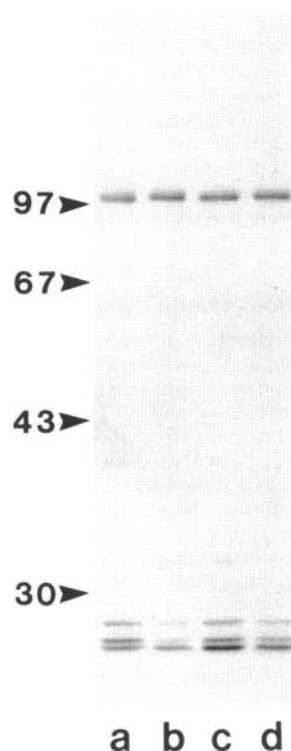


Figure 1. Purification of GST-cdc25 proteins produced in Sf9 cells. GST-cdc25C1-W (lane a), GST-cdc25C1HA-W (lane b), GST-cdc25C1HA-T3 (lane c), and GST-cdc25C1HA-T3S2 (lane d) were purified by glutathione-agarose affinity chromatography from Sf9 cells producing each product, subjected to SDS-PAGE, and stained by Coomassie blue. Proteins (26–28 kDa) are endogenous or pGEX2T-derived GST. The positions of the molecular weight standards: phosphorylase b, 97 400; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000 are shown on the left.

ylated form) (Figure 2A). The large mobility shift found after *in vitro* phosphorylation is reminiscent of the phosphorylation-dependent shift of endogenous cdc25 during M-phase in egg extracts and intact oocytes (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). This shift was also detected in Coomassie blue-stained gels, and phosphorylation did not occur in the GST domain. Other experiments showed that the relative proportion of cdc25 present as the upper band increased as the ratio between kinase and substrate was increased. Phosphoamino acid analysis of cdc25 revealed that both serine and threonine, but not tyrosine, were phosphorylated (data not shown), as found in *Xenopus* egg extracts (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992).

To test whether this shift was specifically associated with phosphorylation at cdc2-consensus sites, other kinases that are active in certain *Xenopus* cell cycles were also examined for phosphorylation of cdc25. Although MAPK, S6 kinase II, and cAMP-dependent kinase could phosphorylate cdc25 *in vitro*, none of these kinases caused a mobility shift (Figure 2B). Therefore the shift found after phosphorylation by cyclin B/p34^{cdc2} is site-specific.

2-D phosphopeptide mapping was performed to compare the peptides phosphorylated in cdc25 by cyclin B/p34^{cdc2} with those phosphorylated in extracts of eggs that can carry out cell cycle transitions *in vitro*. Ideally *in vitro*-phosphorylated cdc25 should be compared with endogenous cdc25 phosphorylated *in vivo* or in extracts. However, there are multiple *Xenopus* cdc25 genes whose

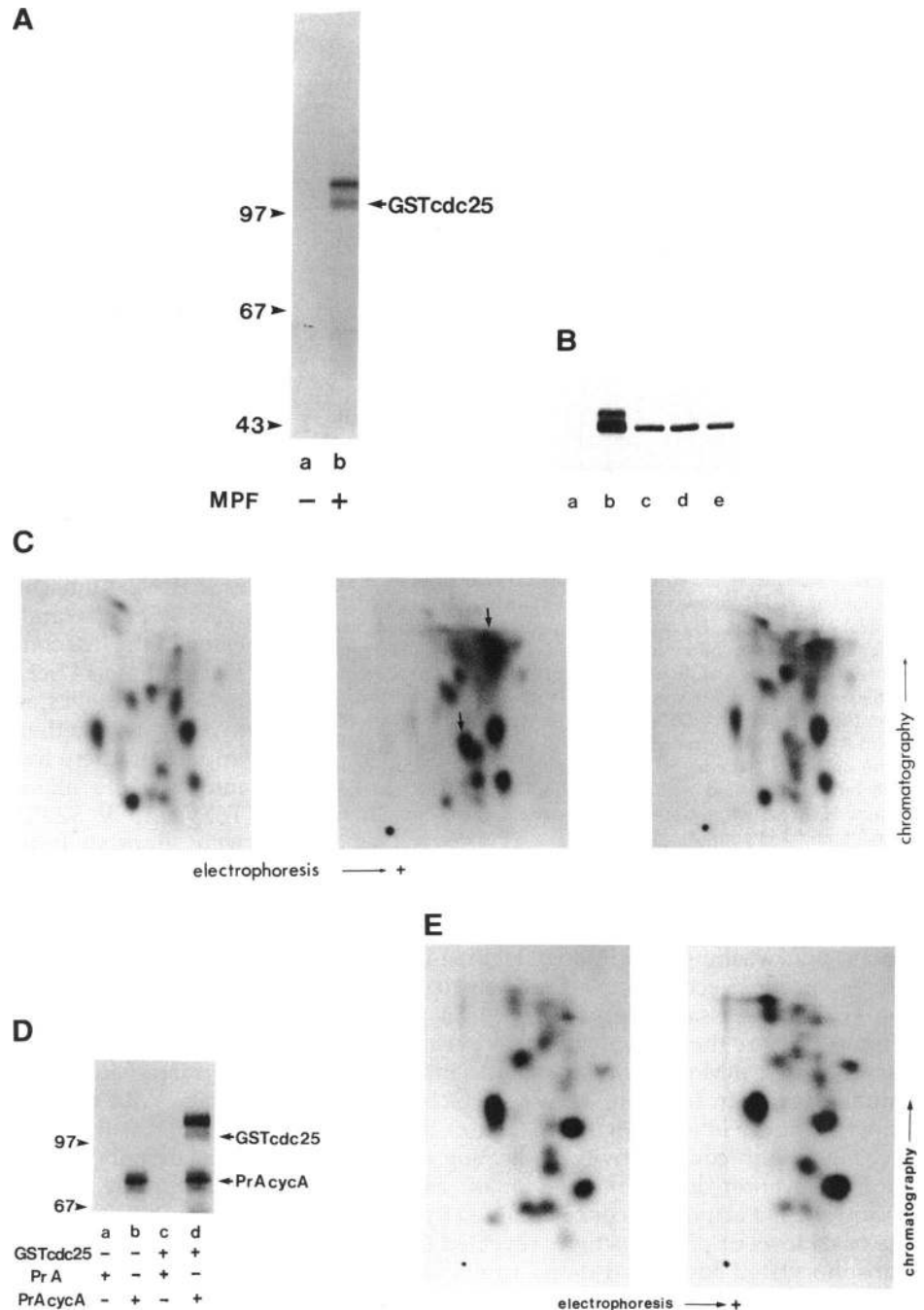
primary sequences are very similar but distinct (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), and antibodies that discriminate among these gene products are currently not available. Furthermore, the phosphopeptide maps of endogenous *Xenopus* cdc25 are very complex (Izumi *et al.*, 1992; Kumagai and Dunphy 1992). Therefore functional recombinant GST-cdc25 protein radiolabeled in egg extracts was analyzed by peptide mapping. Most of the phosphopeptides found in cdc25 phosphorylated by cyclin B/p34^{cdc2} *in vitro* were found to also be phosphorylated in extracts (Figure 2C). However, the latter contained several additional phosphopeptides, which may suggest the existence of a kinase other than cyclin B/p34^{cdc2} that can phosphorylate cdc25 *in vivo*.

Next, cyclin A/p34^{cdc2} complexes were examined for the ability to phosphorylate cdc25. Recombinant protein A-cyclin A was incubated with metaphase-arrested egg extracts and purified with IgG-Sepharose beads. Cyclin A/p34^{cdc2} could also phosphorylate and cause a mobility shift of cdc25 to the same extent as observed with cyclin B/p34^{cdc2} (Figure 2D), contrary to a recent report with bacterially-expressed human cdc25C (Hoffmann *et al.*, 1993). 2-D mapping revealed that the sites phosphorylated by cyclin A/p34^{cdc2} and cyclin B/p34^{cdc2} were very similar with some additional minor phosphopeptides phosphorylated by cyclin A/p34^{cdc2} (Figure 2E). Although the major cyclin A-associated kinase in *Xenopus* eggs is p34^{cdc2} (Minshull *et al.*, 1990; Clarke *et al.*, 1992; Devault *et al.*, 1992; Gabrielli *et al.*, 1992a), cyclin A can associate with cdk2 *in vitro* (Kobayashi *et al.*, 1992). Therefore the cyclin A-associated kinase activity from *Xenopus* egg extracts used in this study could contain small amounts of cdk2, which might explain these minor additional phosphorylation sites. However, it has been reported that human cyclin A/cdk2 is unable to phosphorylate human cdc25C *in vitro* (Hoffmann *et al.*, 1993).

cdc25 Is Activated by Phosphorylation with p34^{cdc2} In Vitro

Because endogenous *Xenopus* cdc25 is activated by phosphorylation in *Xenopus* egg extracts (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), it was important to determine whether phosphorylation of cdc25 by p34^{cdc2} *in vitro* causes activation of the enzyme. As recombinant *Xenopus* cdc25 protein shows very poor activity toward artificial substrates such as *p*-nitrophenyl phosphate, the activity of recombinant cdc25 was assayed in a way similar to that previously described except that the cdc25 enzyme is soluble (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). This assay measures cdc25-dependent activation of the histone H1 kinase activity of tyrosine-phosphorylated cyclin B/p34^{cdc2}, which is a physiological substrate for the cdc25 phosphatase. The tyrosine-phosphorylated cyclin B/p34^{cdc2}

Figure 2. Phosphorylation of *Xenopus* GST-cdc25 in vitro. (A) GST-cdc25C1-W (0.3 μ g) was phosphorylated in the absence (lane a) or presence (lane b) of purified cyclin B/p34^{cdc2}. An autoradiogram of the gel is shown. The arrow indicates the position of Coomassie blue-stained unphosphorylated GST-cdc25C1-W. (B) GST-cdc25C1-W (0.3 μ g) was phosphorylated without any addition (lane a) or with cyclin B/p34^{cdc2} (2.8 u toward histone H1) (lane b), MAP kinase (3 u toward myelin basic protein) (lane c), S6 kinase II (1.1 u toward S6) (lane d), and cAMP-dependent kinase (3.1 u toward histone H1) lane e (1 unit is the amount that catalyzes incorporation of 1 picomole of phosphate per min into each substrate). An autoradiogram of the gel is shown. (C) GST-cdc25C1-W (2 μ g) was incubated in 100 μ l of metaphase-arrested egg extract in the presence of ³²Pi (3 mCi) for 40 min at 23°C. After addition of 500 μ l of lysis buffer and microcentrifugation, ³²P-labeled GST-cdc25 was recovered with 50 μ l of glutathione-agarose and washed extensively as described previously for immunoprecipitation of endogenous cdc25 (Izumi *et al.*, 1992). After excision from an SDS gel, 2-D tryptic phosphopeptide mapping was performed on the upper band at 115 kDa of GST-cdc25C1-W phosphorylated by purified cyclin B/p34^{cdc2} (left), in metaphase egg extracts (middle), or on a mixture of both (right). The spots detected only in extracts are shown by arrows. (D) Protein A-cyclin A/p34^{cdc2} on IgG beads (lanes b and d) or protein A alone on beads (lanes a and c) were incubated in the absence (lanes a and b) or in the presence (lanes c and d) of GST-cdc25C1-W (0.3 μ g). The products of the reaction were resolved by SDS-PAGE and visualized by autoradiography. The arrows indicate the positions of Coomassie blue-stained unphosphorylated GST-cdc25C1-W and protein A-cyclin A. (E) GST-cdc25C1-W protein was phosphorylated by protein A-cyclin A/p34^{cdc2} as in D (left) or by cyclin B/p34^{cdc2} as in A (right). The upper 115-kDa band of GST-cdc25 was excised from the gel and subjected to 2-D tryptic phosphopeptide mapping as in C.



was isolated from stage VI oocytes by immunoprecipitation as described under MATERIALS AND METHODS. Although cdc25 without prior phosphorylation could activate cyclin B/p34^{cdc2} less than twofold (Figure 3A, lane b), cdc25 that had been phosphorylated by MPF (a complex of active cyclin B/p34^{cdc2}) caused a three- to fourfold activation (Figure 3A, lane c and see also Figure 6). It should be pointed out that only one-third of the GST-cdc25 protein was shifted up after

phosphorylation by MPF in this experiment as judged by immunoblotting, suggesting full phosphorylation could increase the activity of the cdc25 preparation further. Although we found some affinity of cdc25 toward cyclin B/p34^{cdc2} as reported by Jesus and Beach (1992), the increased activity of phosphorylated cdc25 cannot be explained by coimmunoprecipitation of H1 kinase activity derived from the initial phosphorylation step, because recombinant cdc25 phosphorylated by cyclin



Figure 3. Activation of *cdc25* by phosphorylation with cyclin A/p34^{cdc2} or cyclin B/p34^{cdc2} in vitro. (A) GST-*cdc25C1-W* (1 μ g) phosphorylated without any additions (lane b) or with active cyclin B/p34^{cdc2} (lanes c and d) was incubated with (lanes b and c) or without (lane d) tyrosine-phosphorylated cyclin B/p34^{cdc2} isolated from stage VI oocytes, which was then assayed for activation of H1 kinase activity as described in MATERIALS AND METHODS. In lane a, the cyclin B/p34^{cdc2} was incubated with buffer instead of GST-*cdc25C1-W*. The products of the H1 kinase reaction were resolved by SDS-PAGE, and radiolabel in histone H1 was quantitated by autoradiography and Cerenkov counting containing the excised gel bands. The radiolabel in histone H1 was as follows: 12 214 cpm (lane a), 20 477 cpm (b), 39 850 cpm (c), and 528 cpm (d). (B) Similar experiments were done except that the GST-*cdc25C1-W* was phosphorylated by protein A-cyclin A-associated kinase on IgG beads (lanes c and d) or protein A alone on beads (lane b). In lane a, cyclin B/p34^{cdc2} was incubated with buffer pretreated with protein A on beads. The radiolabel in histone H1 was as follows: 4286 cpm (lane a), 8849 cpm (b), 16 074 cpm (c), and 233 cpm (d).

B/p34^{cdc2} itself showed no significant H1 kinase activity under our washing conditions (Figure 4A, lane d). Moreover, *cdc25* activation was unlikely to occur during the H1 kinase assay, because the *cdc25* protein was removed before the assay, which was performed in the presence of 2 mM sodium vanadate to inhibit any residual *cdc25*. In addition, MAPK, which can phosphorylate *cdc25* in vitro (Figure 2B, lane c), did not cause any increase in *cdc25* activity, indicating that residual *cdc25* activation during the H1 kinase assay did not occur. The net activity of *cdc25* obtained by subtracting the basal level of p34^{cdc2} activity revealed that in vitro-phosphorylated *cdc25* had three- to fivefold more activity (Figure 4A, see also Figure 7). These values are similar to those found for endogenous *cdc25* in M-phase compared to interphase (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992).

cdc25 phosphorylated by cyclin A/*cdc2* also showed more activity than *cdc25* without phosphorylation (Figure 3B). After phosphorylation *cdc25* did not contain any H1 kinase activity derived from the protein A-cyclin A/p34^{cdc2}, which remained tightly associated with the IgG beads that could be removed by centrifugation (Figure 3B, lane d). Activation by cyclin A/p34^{cdc2} is consistent with the similarity of the phosphopeptides

generated from *cdc25* phosphorylated by cyclin A/p34^{cdc2} or by cyclin B/p34^{cdc2} (Figure 2E). Thus both cyclin A/p34^{cdc2} and cyclin B/p34^{cdc2} can phosphorylate and activate *Xenopus cdc25* in vitro.

Mutation of Conserved Thr/Pro and Ser/Pro Sites in *cdc25* Prevents Electrophoretic Mobility Shifts and Specific Phosphorylation by p34^{cdc2}

In both oocyte and egg cell cycles, the *cdc25* mobility shift and activation are closely correlated with H1 kinase activity presumably representing p34^{cdc2} activity (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). Given that p34^{cdc2} can phosphorylate and activate *cdc25* in vitro as described above (Figures 2 and 3), we searched for conserved Ser/Pro and Thr/Pro sites in human and frog *cdc25*. Such sites, usually with a basic residue nearby, form the minimal consensus motif for phosphorylation by p34^{cdc2} (Nigg, 1993, for review). As is evident from the sequence reported previously (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), *Xenopus cdc25* has 14 Ser/Pro and Thr/Pro sites, 13 of which are located in the less conserved N-terminal half (Figure 4A). Five of these 14 Ser/Pro or Thr/Pro sites are conserved between *Xenopus* and human *cdc25C* (Figure 4A). To investigate whether phosphorylation of these sites is important, these five serine or threonine residues were mutated into alanine or valine, respectively (T48V, T67V, T138V, S205A, and S285A). The mutant proteins were translated in a reticulocyte lysate containing [³⁵S]methionine and incubated with a metaphase egg extract to examine M-phase mobility shifts. The radiolabeled wild-type *cdc25* incubated with an interphase extract remained at its original position with a major band at 78 kDa and a minor one at 80 kDa (Figure 4B, lanes a and m). All the radiolabeled mutants described here when incubated with an interphase extract also migrated with the same molecular weight as wild-type *cdc25*. Wild-type *cdc25* shifted up to 98 kDa after incubation with a metaphase egg extract (Figure 4B, lane b) as described previously (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). None of the mutants showed complete inhibition of the mobility shift, including a C457A mutant in which a critical cysteine residue required for phosphatase activity had been replaced (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b) (Figure 4B, lanes c-h). Among the single-site mutations, the T138V mutation inhibited the shift most strongly, about 33% (Figure 4B, lane e). Although each of T48V, T67V, and S205 mutants also exhibited partial inhibition of the shift, there still remained detectable amounts of the form with the highest mobility (Figure 4B, lane c, d, and f). Similar experiments were then carried out with mutants carrying mutations in more than one site. The mutations that abolished multiple Ser or Thr/Pro sites inhibited the mobility shift more severely (Figure 4B, lanes i-l). The shift of the T2 mutants (both Thr48

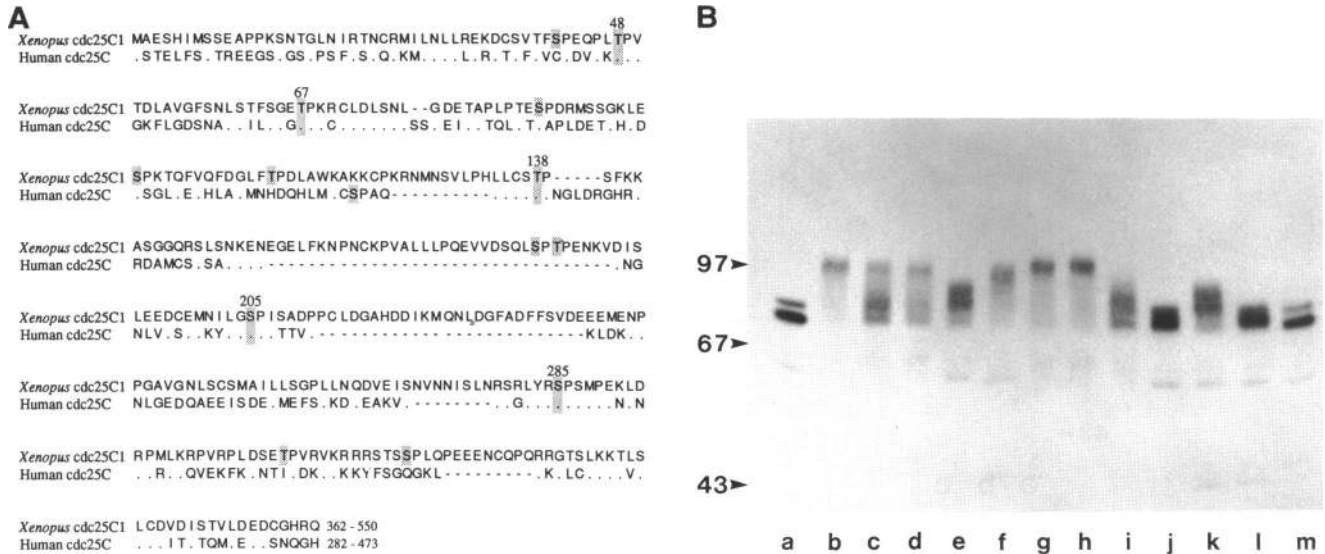


Figure 4. Electrophoretic shift of wild-type and mutant cdc25 in *Xenopus* egg extracts. (A) The N-terminal regions of *Xenopus* cdc25C1 (previously termed cdc25A, Izumi *et al.*, 1992) and human cdc25C (Sadhu *et al.*, 1990) were aligned. Identical matches with *Xenopus* cdc25C1 are dotted. ---, gaps inserted to generate optimal alignment. Thr and Ser residues followed by Pro are highlighted, and conserved residues are indicated by the amino acid number of *Xenopus* cdc25C1. (B) Wild-type and mutant cdc25C1 cDNAs subcloned in pBluescript SK+ were directly transcribed and translated in the T_NT coupled reticulocyte lysate system (Promega, Madison, WI) using T7 RNA polymerase in the presence of [³⁵S]methionine. Reticulocyte lysate containing labeled protein (4 μl) was incubated with 36 μl of either interphase (lanes a and m) or M-phase egg extracts (lanes b–l) for 15 min at 23°C. cdc25 was immunoprecipitated using antibodies directed against the C-terminal catalytic domain of cdc25 (Izumi *et al.*, 1992) in the presence of 1.5 μM microcystin and analyzed by SDS-PAGE and fluorography. Without incubation with extracts, both wild-type and mutant cdc25C1HA protein labeled with [³⁵S]methionine migrated on SDS-PAGE as a minor 80-kDa band and a major 78-kDa band. The 80-kDa protein and the 78-kDa protein reflect products whose translation begins at the AUG codon 57 bases upstream of the presumed initiation codon and at the presumed initiation AUG codon, respectively. The 78-kDa protein is not a degradation product, because both 80- and 78-kDa products were recognized by both N-terminal peptide antibodies (MAESHIMSEAFPK), and the 12CA5 mAb directed against the C-terminal HA tag (Berkeley Antibody, Richmond, CA). The molecular weights of these products were slightly larger than endogenous cdc25 because of the presence of the HA tag. The protein products were as follows: wild-type (lanes a, b, and m), T48V (c), T67V (d), T138V (e), S205A (f), S285A (g), C457A (h), T2 (T48V + T67V) (i), T3 (T48V + T67V + T138V) (j), T1S2 (T138V + S205A + S285A) (k), T3S2 (T48V + T67V + T138V + S205A + S285A) (l). Electrophoretic shifts of mutant cdc25 were quantified by comparing the relative mobility of the most shifted molecules to that of fully shifted M-phase wild-type cdc25. Similar results were obtained in at least two independent experiments with each mutant.

and Thr67 were mutated) was inhibited by 40–50%, and the highest mobility form was not evident, in contrast to mutation of either T48V or T67V alone (Figure 4B, lanes c, d, i). Furthermore, in T3 mutants (Thr48, Thr67, and Thr138 were mutated) the shift was prevented by >90% (Figure 4B, lane j, see also Figure 7B, lane h). The T1S2 mutant construct (Thr138, Ser205, and Ser285 were mutated) revealed no further inhibition compared with the T138V mutation alone (Figure 4B, lanes e and k). Finally, the T3S2 mutant (all five residues were mutated) showed inhibition of the shift similar to that found in the T3 mutant (Figure 4B, lanes j and l, see also Figure 7B, lane j). Thus the shift found in M-phase is not because of phosphorylation of a single site but rather to multiple phosphorylation events, and the three threonine residues, Thr48, Thr67, and Thr138, are responsible for 90% of the electrophoretic mobility shift of the cdc25 protein in M-phase.

To investigate functional effects of phosphorylation at these sites, we expressed mutant GST-cdc25 proteins in Sf9 cells infected with baculovirus encoding GST-

cdc25C1-T3 and GST-cdc25C1-T3S2, whose mobility shifts were most deficient among all the mutants described above (Figure 4B). These constructs in Sf9 cells also contained a HA tag at the C-terminus. Both wild-type and mutant GST-cdc25C1HA migrated on SDS-PAGE with molecular mass of 104 000, slightly larger than GST-cdc25C1-W because of the presence of the HA tag (Figure 1). Cyclin B/p34^{cdc2} was able to phosphorylate both wild-type and mutant GST-cdc25 in vitro significantly (Figure 5A). However the effects of phosphorylation on the M-phase mobility shift were very different between wild-type and mutants. Wild-type exhibited the dramatic shift described before, migrating with a *M_r* of 115 000 and 105 000 (Figure 5A, lane a), whereas both mutants showed only a slight shift with a single 105 000 form (Figure 5A, lanes b and c). These findings are consistent with the shifts of [³⁵S]methionine-labeled mutant products found after incubation with metaphase extracts (Figure 4B). The radioactivity incorporated into mutant T3 and T3S2 after phosphorylation by cyclin B/p34^{cdc2} varied from 50 to

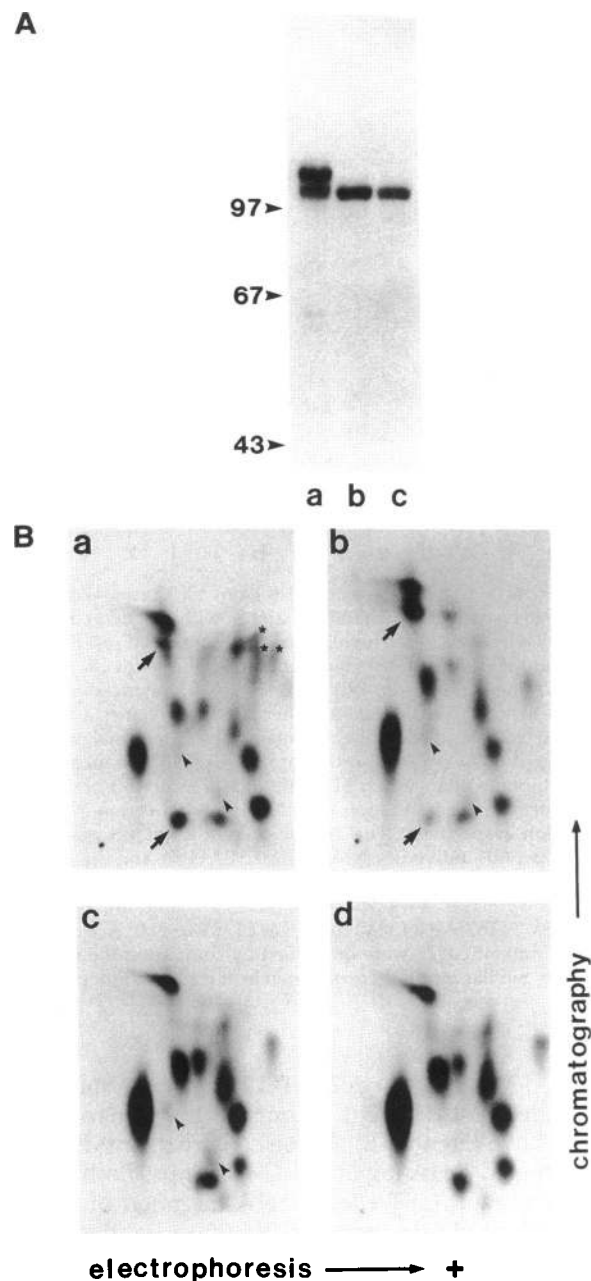


Figure 5. Phosphorylation of wild-type and mutant GST-cdc25. (A) 0.25 μ g of purified GST-cdc25C1HA-W (lane a), GSTcdc25C1HA-T3 (lane b), and GSTcdc25C1HA-T3S2 (lane c) were phosphorylated by cyclin B/p34^{cdc2} in vitro. An autoradiogram of the gel is presented. (B) After excision from an SDS-gel, 2-D tryptic phosphopeptide mapping was performed on the upper band at 115 kDa (panel a) and the lower band at 105 kDa (panel b) of GST-cdc25C1HA-W, the single GST-cdc25C1HA-T3 band (panel c), and the single GST-cdc25C1HA-T3S2 (panel d) phosphorylated by cyclin B/p34^{cdc2} as shown in A. The same amount of radioactivity (4000 cpm) was loaded onto each plate. The arrows in panels a and b indicate the spots missing in the maps of T3 and T3S2 (panels c and d). The arrowheads in panels a-c indicate the spots absent in the map of T3S2 (panel d), but not in T3 (panel c), most likely Ser205 and/or Ser285. The asterisks in panel a indicate the spots missing in the map of the lower band of wild-type (panel b).

70% and 40–50% of that of wild-type, respectively. Thus cyclin B/p34^{cdc2} can phosphorylate *Xenopus* cdc25 significantly at Ser/Pro or Thr/Pro sites other than the conserved residues that were mutated. However, phosphorylation of these sites alone had little effect on the mobility shift of cdc25. Similar results were obtained after phosphorylation with cyclin A/p34^{cdc2}.

The tryptic phosphopeptides of wild-type and mutant cdc25 after phosphorylation by cyclin B/p34^{cdc2} in vitro were compared by 2-D mapping (Figure 5B). The data, combined with the findings in Figure 4B, suggest the following conclusions:

1) Both Thr48 and Thr67 are phosphorylated by cyclin B/p34^{cdc2}, because the double T2 mutation inhibited the shift more than each single mutation alone (Figure 4B, lanes c, d, and i).

2) In addition to Thr48 and Thr67, which are predicted to be in a single tryptic phosphopeptide, Thr138 is phosphorylated by cyclin B/p34^{cdc2}, because the map of T3 lacked two major spots found in wild-type (Figure 5B, panels a and b, arrows, compared with panels c and d), and both T2 and T138V mutations did indeed inhibit the shift partially (Figure 4B, lanes e and i).

3) The fully shifted form of the wild-type cdc25 (115 kDa) after phosphorylation by p34^{cdc2} resulted from the simultaneous phosphorylation of at least two of these three threonine residues. The unshifted form of the wild-type (105 kDa) probably contains a mixture of cdc25 molecules phosphorylated at one or the other of these sites, because peptides representing the T3 sites were present in the map of this form (Figure 5B, panels a and b, arrows, compared with panel c), although the upper form contained some additional spots (Figure 5B, panel a, asterisks).

4) Phosphorylation of Ser 205 and/or Ser 285 is low, because the map of T3S2 lacked a few minor, but consistent, spots compared with that of T3 (Figure 5B, panels a–c, arrowheads, compared with panel d), and no additional effect on mobility was evident with the T3S2 mutant (Figure 4B, lanes j and l). The low level of radiolabel in these spots may reflect a lower relative rate of phosphorylation of these sites with the limiting amount of cyclin B/p34^{cdc2} present in the reaction.

5) Other Ser and/or Thr/Pro sites are also phosphorylated significantly by p34^{cdc2}, which is consistent with the findings of Figure 5A.

Mutation of cdc25 Phosphorylation Sites Prevents Activation by Cyclin B/p34^{cdc2} In Vitro

Although there is a very tight correlation between the cdc25 mobility shift and activity (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), it was important to directly compare the activities of wild-type and mutant GST-cdc25. The basal activity of GST-cdc25C1HA-W was 0.73 ± 0.23 u (mean \pm SEM, $n = 3$), whereas the activity after phosphorylation by cyclin B/p34^{cdc2} increased to

3.14 ± 0.35 u (4.3-fold activation) (Figure 6), similar to the value for GST-cdc25C1-W (Figure 3A). On the other hand, the basal activities of GST-cdc25C1HA-T3 and GST-cdc25C1HA-T3S2 were 0.54 ± 0.03 u and 0.11 ± 0.06 u, respectively. Phosphorylation by cyclin B/p34^{cdc2} still increased slightly the activities of the mutants, but activated activity was far below that of wild-type, 0.90 ± 0.19 u for T3, and 0.36 ± 0.09 u for T3S2 (Figure 6). Similar results were obtained using protein A-cyclin A/p34^{cdc2} instead of cyclin B/p34^{cdc2}. These data show that phosphorylation of the T3 and S2 sites is required for the activation of cdc25 in vitro by p34^{cdc2}, although they do not exclude the possibility that phosphorylation of other sites is also required.

The different basal activity between wild-type and mutant cdc25 proteins is likely a consequence of the wild-type cdc25 already being phosphorylated and activated to some extent in Sf9 cells. This is suggested by the peptide maps of the wild-type, which revealed that even the unshifted form contains at least one of the T3-specific phosphopeptides (Figure 5B, panel b). Another formal possibility is that multiple mutations in cdc25 cause a general disruption of the enzyme structure and lower its activity. Although this is always theoretically possible even with single point mutations, the likelihood that these mutations inhibited cdc25 by altering the overall conformation of the protein seems remote for the following reasons: both mutants were phosphorylated by cyclin B/p34^{cdc2} to the same extent as the wild-type on all the other unmutated Ser/Pro and/or Thr/Pro sites (Figure 5B, panels c and d), the antibodies toward the C-terminal domain of cdc25 could recognize mutants as efficiently as wild-type (see Figure 4B), and finally, these mutants retained some biological activity because of their ability to induce oocyte maturation when microinjected (see below).

cdc25 Phosphorylation Site Mutants Are Unable to Initiate Entry into M-phase in Oocyte Extracts

Microinjection of exogenous cdc25 from other species into *Xenopus* oocytes has previously been found to induce oocyte maturation (Gautier *et al.*, 1991; Lee *et al.*, 1992). This situation suggested oocyte injection as a means to compare wild-type and mutant cdc25 under conditions where positive feedback occurs. In testing the homologous cdc25 used in this study with this assay, it was found that either protein or mRNA of wild-type and mutant *Xenopus* cdc25 was able to induce maturation similarly in stage VI oocytes. Even the time course of germinal vesicle breakdown was indistinguishable between wild-type and mutant cdc25, at least at the protein concentration used (final concentration of the injected cdc25 in the oocyte was 10 ng/ μ l [100 nM]; this concentration is sufficient to induce about 50% germinal vesicle breakdown).

In the microinjection assay, it seemed possible that functional differences between the wild-type and mu-

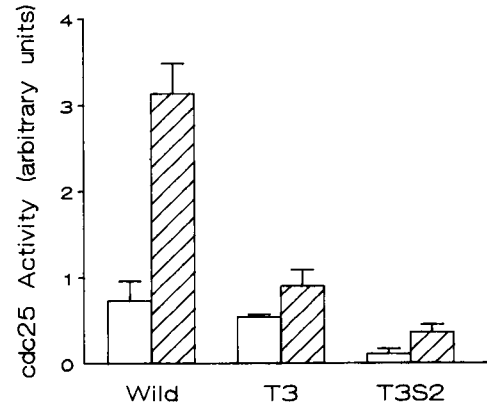


Figure 6. Comparison of wild-type and mutant GST-cdc25 activities in vitro. The activities of GST-cdc25C1HA-W, GST-cdc25C1HA-T3, and GST-cdc25C1HA-T3S2 without (□) or with (▨) prior phosphorylation by MPF were measured as described in Figure 3A. The mean \pm SEM ($n = 3$) is shown. The basal H1 kinase activity of the tyrosine-phosphorylated cyclin B/p34^{cdc2} complexes isolated from stage VI oocytes was defined as 1 arbitrary unit and has been subtracted from the cdc25 activity shown. Coimmunoprecipitation of H1 kinase activity with either wild-type or mutant cdc25 phosphorylated by MPF was always <5% of the basal activity of the tyrosine-phosphorylated cyclin B/p34^{cdc2} used as substrate.

tant forms could have been obscured by the background level of wild-type cdc25 present in the oocyte. Therefore it was desirable to develop a method for assaying the mutants without any contribution from endogenous cdc25. Cyert and Kirschner (1988) previously developed a fractionated cell-free system from resting oocytes that would activate pre-MPF in an ATP-dependent manner. The nature of the ATP requirement is unknown but can be postulated to reflect a requirement for phosphorylation of cdc25. Pre-MPF (Cyert and Kirschner, 1988) contains the tyrosine-phosphorylated cyclin B/p34^{cdc2} complex used in the in vitro assay of cdc25 activity described earlier in this paper. To evaluate this cell-free system for assaying cdc25, the effect of immunodepleting cdc25 on activation of pre-MPF by ATP was studied. Immunodepletion of endogenous cdc25 (Figure 7B, lane b) blocked the activation of p34^{cdc2} histone H1 kinase activity by ATP (Figure 7A, panel a). The possibility that cdc25 is a target for the ATP requirement was supported further by the finding that addition of exogenous wild-type cdc25 restored activation in the presence of an ATP-regenerating system (Figure 7A, panel b). However, T3 or T3S2 induced little activation of pre-MPF when added at a concentration equivalent to that of endogenous cdc25 (Figure 7A, panels c and d). The difference in activity between wild-type cdc25 and the mutants cannot be explained simply by the difference in basal activity shown in Figure 6. Both wild-type and mutant cdc25 failed to induce pre-MPF activation without ATP (Figure 7A, panels b–d), suggesting a requirement for further activation of cdc25 through

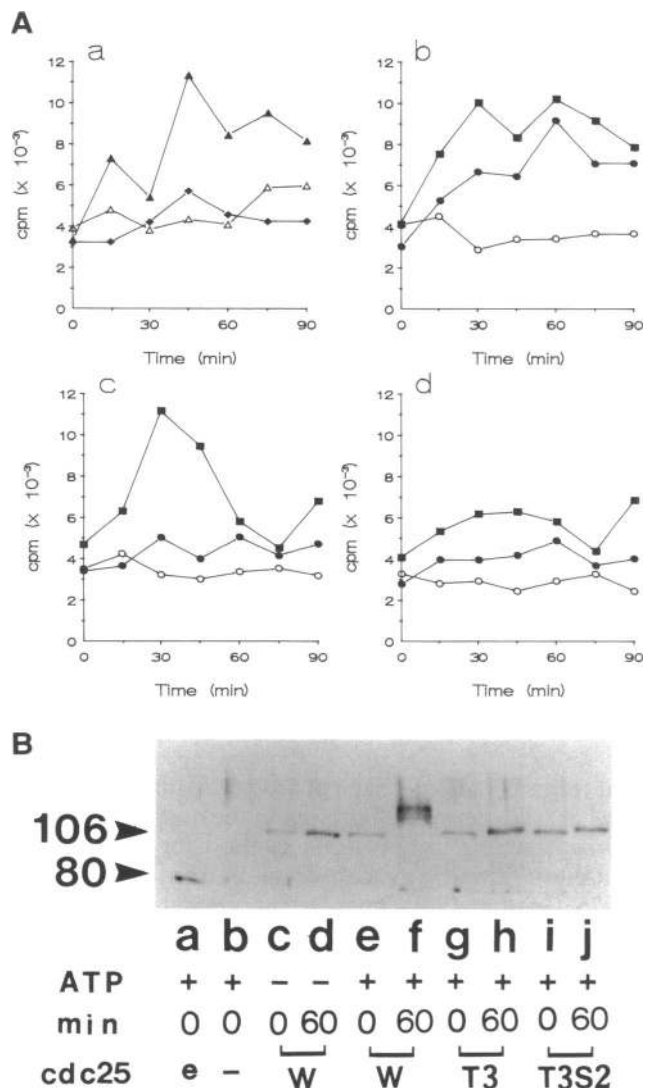


Figure 7. Phosphorylation site mutants of *cdc25* are unable to initiate M-phase (A) Pre-MPF fractions from ovaries were immunodepleted with anti-*cdc25* antibodies or treated with control protein A beads as described in MATERIALS AND METHODS. In this extract, >85% of endogenous *cdc25* was depleted, judging by the densitometric scanning of the immunoblot shown in B after prolonged exposure of the film. Either 20 ng (●, ○) or 200 ng (■) of exogenous GST-*cdc25C1HA-W* (panel b), GST-*cdc25C1HA-T3* (panel c), or GST-*cdc25C1HA-T3S2* (panel d) were added to the immunodepleted extracts in the absence (○) or presence (●, ■) of an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase), resulting in a total volume of 12 μl. At the indicated times after incubation at 23°C, 1.5 μl of the sample was taken and assayed for pre-MPF activation by H1 kinase activity or immunoblotted for *cdc25*. In panel a, H1 kinase activities are shown for control protein A bead-treated extracts without (Δ) or with (▲) an ATP-regenerating system and *cdc25*-immunodepleted extracts with an ATP-regenerating system (◆). (B) *cdc25* was immunoblotted from the following extracts at the indicated times: control protein A-treated extracts (endogenous *cdc25* only [e]) (lane a); *cdc25*-immunodepleted extracts (lane b); *cdc25*-immunodepleted extracts supplemented with 20 ng of GST-*cdc25C1HA-W* in the absence (lanes c and d) or presence (lanes e and f) of an ATP-regenerating system; and *cdc25*-immunodepleted extracts supplemented with 20 ng of GST-*cdc25C1HA-T3* (lanes g

the positive feedback between *cdc2* and *cdc25*. Furthermore, addition of a 10-fold greater amount of T3S2 still failed to induce full activation of pre-MPF, although the same amount of T3 did induce (Figure 7A, panels c and d). The 10-fold greater amounts of T3 and T3S2 expressed 7- and 1.5-fold more basal activity, respectively, than an amount of wild-type basal activity that was able to fully activate pre-MPF (Figure 6). The reduced activity in vitro of T3S2 compared with T3 after phosphorylation by cyclin B/p34^{cdc2} (Figure 6) and in the oocyte cell-free system (Figure 7A, panels c and d) suggests that phosphorylation of Ser205 and/or Ser285 contributes to the activation of *cdc25* by p34^{cdc2}.

Immunoblots of the exogenous *cdc25* added to the oocyte cell-free system indicated that GST-*cdc25C1HA-W* became phosphorylated and electrophoretically shifted when pre-MPF was activated, whereas it was not shifted in the absence of ATP (Figure 7B, lanes c-f). Mutant T3 and T3S2 were also shifted up slightly when H1 kinase was partially activated (Figure 7B, lanes g-j). This finding strongly suggests that exogenous *cdc25* molecules are phosphorylated by a *cdc25*-induced kinase, p34^{cdc2}. Therefore it is suggested that positive feedback occurred in this system and enhanced the difference in activity between wild-type and mutant *cdc25*.

DISCUSSION

cdc25 is a mitotic inducer that acts at a final stage of cyclin B/p34^{cdc2} activation by dephosphorylating Tyr15 (and probably Thr14) in p34^{cdc2} (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b; Strausfeld *et al.*, 1991; Lee *et al.*, 1992). The activity of *cdc25* increases at mitosis in cycling *Xenopus* egg extracts, a result of phosphorylation that causes a dramatic electrophoretic mobility shift. This phosphorylation event is downstream of checkpoint controls that ensure the dependence of mitosis on completion of DNA synthesis (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). These considerations make it crucial to identify the nature of the kinase(s) and phosphatase(s) working on *cdc25* to understand the mechanism of entry into mitosis. Recently it was reported that anti-cyclin B immunoprecipitates from mammalian cells have kinase activity toward human *cdc25* and that M-phase extracts immunodepleted with anti-p34^{cdc2} or anti-cyclin B antibodies fail to phosphorylate *cdc25*, whereas cyclin A/p34^{cdc2} appeared to phosphorylate only degraded *cdc25* (Hoffmann *et al.*, 1993). We report here that both cyclin A/

and h) or GST-*cdc25C1HA-T3S2* (lanes i and j) in the presence of an ATP-regenerating system. By immunoblotting, the final concentration of endogenous *cdc25* in this reaction is estimated to be 1.5–2.0 ng/μl. Note that the level of the endogenous *cdc25* in lane a was comparable to that of recombinant GST-*cdc25* in lanes c–j. Similar results shown were obtained in two other experiments.

p34^{cdc2} and cyclin B/p34^{cdc2} can phosphorylate and activate full-length *Xenopus* cdc25 in vitro. Both kinases phosphorylate cdc25 at the sites responsible for the mobility shift of cdc25 in M-phase. Multiple phosphorylation of Ser/Pro and Thr/Pro sites is required for the complete shift. Mutation of these sites diminishes the activation of cdc25 in vitro by 90%, and such mutants cannot induce p34^{cdc2} activation in oocyte extracts. The failure of Hoffman *et al.* (1993) to observe phosphorylation of cdc25 by cyclin A/p34^{cdc2} might be a result of the immunocomplex kinase assay they used, because antibody binding might affect kinase activity in vitro. Otherwise it may reflect different types of cyclin A gene products in eggs and somatic cells as found in *Xenopus* (Hunt, personal communication), or bacterially-expressed human cdc25 may be a poor in vitro substrate for cyclin A/p34^{cdc2}.

The ability of cyclin B/p34^{cdc2} to phosphorylate and activate cdc25 forms the basis of a positive feedback loop between the two components. This positive feedback loop may provide an explanation for two phenomena that have been described previously. One concerns the self-amplification or "autocatalytic" nature of MPF. This was first described by Masui and Markert (1971) as basically the observation that oocytes induced to mature by microinjection of a small amount of MPF contained substantially more MPF after maturation than had been injected initially. This led to the hypothesis that oocytes contained a store of inactive pre-MPF that could be activated by injection of catalytic amounts of active MPF (Wasserman and Masui, 1975; Gerhart *et al.*, 1984). From the results in this paper, it is likely that at least one mechanism contributing to autoamplification of MPF is the p34^{cdc2}-dependent phosphorylation and activation of cdc25. A second phenomenon that may derive in part from the positive feedback is the abrupt, irreversible nature of entry into mitosis. It seems important for cell cycle transitions to be irreversible once initiated and to proceed rapidly. In the case of entry into mitosis, the abruptness of the transition may be in part a consequence of the positive feedback loop between these two components that ensures full and rapid activation of p34^{cdc2}.

However, the results in this study still do not explain the initial trigger for p34^{cdc2} activation. If cyclin B/p34^{cdc2} is the sole kinase activating cdc25, the small amount of active p34^{cdc2} that has escaped inhibitory tyrosine phosphorylation could in principle be a trigger, when cyclin B synthesis increases to levels producing threshold amounts of p34^{cdc2} activity. However, such autocatalytic activation of MPF was not seen in *Xenopus* egg extracts even after addition of activatable p34^{cdc2} in which Thr14 and Tyr15 had been mutated to nonphosphorylatable residues (Norbury *et al.*, 1991). Therefore accumulation to the threshold level of dephosphorylated cyclin B/p34^{cdc2} is unlikely to determine the timing of mitosis, at least in frog egg extracts.

In this study, we have demonstrated that cyclin A/p34^{cdc2} can phosphorylate and activate cdc25 in vitro. The phosphorylation sites are the same as those phosphorylated by cyclin B/p34^{cdc2}. Therefore, if cyclin A/p34^{cdc2} phosphorylates cdc25 in vivo, the accumulation of cyclin A might trigger the feedback loop and mitosis. Cyclin A/p34^{cdc2} is phosphorylated on Tyr15 inefficiently and indeed is active as a kinase before entry into mitosis (Walker and Maller, 1991; Clarke *et al.*, 1992; Devault *et al.*, 1992). Although the activity of cyclin A/p34^{cdc2} is usually only one-fifth to one-tenth that of the peak activity of cyclin B/p34^{cdc2}, this might be sufficient to counter the activity of either the phosphatase(s) working on cdc25 or the wee1 kinase phosphorylating Tyr15 in p34^{cdc2}. Interestingly, the addition of okadaic acid to egg extracts in the presence of unreplicated DNA causes phosphorylation and activation of cdc25 when cyclin A/p34^{cdc2} is already active but before cyclin B/p34^{cdc2} kinase activation (Izumi *et al.*, 1992). This finding may suggest that cyclin A/p34^{cdc2} complexes or other kinases phosphorylate cdc25 in vivo during initiation of mitosis. However, previous studies have shown activation of cyclin B/p34^{cdc2} in cycling extracts devoid of cyclin A (Walker and Maller, 1991), suggesting triggering of cdc25 activation by cyclin A is not an obligatory event for entry into mitosis. In these extracts cyclin A may have an important function in S-phase to monitor completion of DNA synthesis (Walker and Maller, 1991), a function that can be distinguished from any effect of cyclin A/p34^{cdc2} on the phosphorylation of cdc25 at the G2/M transition.

There are several lines of evidence suggesting the existence of kinase(s) other than cyclin B/p34^{cdc2} and cyclin A/p34^{cdc2} that can phosphorylate and activate cdc25. cdc25 was phosphorylated and activated at least partially in the presence of protein synthesis inhibitors that eliminate cyclin synthesis in frog egg extracts treated with okadaic acid (Izumi and Maller, unpublished data). The patterns of phosphopeptides found in cdc25 phosphorylated by cyclin B/p34^{cdc2} in vitro and in M-phase extracts were not identical (Figure 2C), although they have been reported to be identical in human cells (Hoffmann *et al.*, 1993). These findings suggest the existence of another cdc25 kinase that is independent of protein synthesis. This kinase could be the trigger kinase (Izumi *et al.*, 1992). Additionally, the inhibition of either the phosphatase(s) toward cdc25 or wee1 could determine the timing of mitosis. Both phosphatase 1 and phosphatase 2A are capable of dephosphorylating cdc25 in vitro (Izumi *et al.*, 1992), and phosphatase 1 activity actually declines just before entry into mitosis (Walker *et al.*, 1992). Furthermore, tyrosine kinase activity toward p34^{cdc2} is elevated in interphase and attenuated in M-phase (Smythe and Newport, 1992). The timing of entry into mitosis could be determined by these combined effects.

Genetic data suggest that *cdc25* is involved in the dependence of mitotic initiation on completion of DNA synthesis (Enoch and Nurse, 1990). It has been shown biochemically that *cdc25* activity is maintained at its low interphase level when incompletely replicated DNA is present (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). The pattern of phosphorylation of *cdc25* in interphase is the same whether unreplicated DNA is present or not (Izumi *et al.*, 1992), suggesting that there is no kinase that regulates *cdc25* negatively in the presence of unreplicated DNA. Therefore, if phosphorylation of *cdc25* is indeed regulated by a checkpoint mechanism, it is the phosphatase(s) acting on *cdc25* or the putative trigger kinase that are regulated by incompletely replicated DNA. Other studies have shown that the activity of both phosphatase 1 and phosphatase 2A remains elevated in the presence of unreplicated DNA (Walker *et al.*, 1992; Clarke *et al.*, 1993).

To elucidate the exact mechanism of entry into mitosis, the subcellular localization of *cdc25*, cyclin A/p34^{cdc2}, and cyclin B/p34^{cdc2} should be considered. The localization of *cdc25* in interphase is controversial. *cdc25* has been reported to be in the nucleus during interphase in HeLa cells (Millar *et al.*, 1991a), in rat embryonic fibroblasts REF52, and in human fibroblasts HS68 (Girard *et al.*, 1992). In contrast, *cdc25* is in the cytoplasm of prophase-arrested *Xenopus* oocytes (Izumi *et al.*, 1992) and in hamster tsBN2 cells in interphase (Seki *et al.*, 1992).

On the other hand, cyclin A is reported to be predominantly nuclear in interphase in HeLa cells, although a significant fraction is also present in the cytoplasm (Pines and Hunter, 1991). However, it should be noted that cyclin A is complexed mainly with *cdk2* in that cell (Pines and Hunter, 1991), instead of with p34^{cdc2}, which is the partner of cyclin A in *Xenopus* oocytes and eggs (Minshull *et al.*, 1990; Clarke *et al.*, 1992; Devault *et al.*, 1992; Gabrielli *et al.*, 1992a). In contrast, cyclin B/p34^{cdc2} accumulates in the cytoplasm in interphase and enters the nucleus at the beginning of mitosis in HeLa cells (Pines and Hunter, 1991) and in starfish oocytes (Ookota *et al.*, 1992). Interestingly, p34^{cdc2} has already been dephosphorylated and activated before cyclin B/p34^{cdc2} complexes move into the germinal vesicle in starfish oocytes after the addition of 1-methyladenine (Ookota *et al.*, 1992), suggesting that cyclin B/p34^{cdc2} is activated in the cytoplasm. This finding is consistent with previous reports showing that histone H1 kinase is fully activated in enucleated oocytes of starfish (Picard *et al.*, 1988) and *Xenopus* (Dabauvalle *et al.*, 1988). These results suggest that *cdc25* and its trigger kinase should be in the cytoplasm in interphase.

We have shown that *cdc25* is phosphorylated at multiple sites by p34^{cdc2}. The full mobility shift and activation of *cdc25* found in M-phase require these multiple phosphorylation events. This may explain the difficulty in isolating the fully shifted form of *cdc25* in the absence

of specific phosphatase inhibitors during in vitro procedures (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), because dephosphorylation of a single site could result in a large mobility shift of *cdc25* (see Figure 4B and Figure 5B, panels a and b). Although it is now very clear that *cdc25* is activated by periodic phosphorylation, it has previously been unclear whether phosphorylation of *cdc25* is required for initiation of mitosis by *cdc25*. *cdc25* isolated at interphase still has some activity in vitro (Izumi *et al.*, 1992; Kumagai and Dunphy 1992). Wild-type *cdc25* from bacteria or insect cells without prior phosphorylation can induce germinal vesicle breakdown (Gautier *et al.*, 1991; Lee *et al.*, 1992; and this study) and overcome the block to mitosis in egg extracts arrested in interphase with DNA and aphidicolin (Kumagai and Dunphy, 1991; Izumi and Maller, unpublished data). This result, seen in several laboratories, is at odds with the report of Hoffmann *et al.* (1993), who reported that bacterially expressed human *cdc25C* is unable to induce these events in the absence of prior thiophosphorylation. Finally, the mutants that do not undergo full phosphorylation and activation by p34^{cdc2} still retain some activity, because microinjection of either mutant protein or mRNA into oocytes can induce germinal vesicle breakdown. These results are difficult to interpret because recombinant *cdc25* may have become phosphorylated during the experiment or the endogenous wild-type *cdc25* may have become activated. Thus the failure of phosphorylation site mutants of *cdc25* to induce M-phase when expressed at a physiological level in the absence of endogenous *cdc25* suggests that phosphorylation of *cdc25* is a required event for initiation of mitosis. The ability of p34^{cdc2} to slightly activate these mutants in vitro (Figure 6) indicates that there are other Ser/Pro or Thr/Pro phosphorylation sites contributing to activation of *cdc25* by p34^{cdc2}. Final analysis of the mechanism of mitotic initiation requires understanding the roles of cyclin A/p34^{cdc2}, the putative trigger kinase, phosphatase 1, and phosphatase 2A, all of which can regulate the phosphorylation state and activity of *cdc25*.

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