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Periodic Changes in Phosphorylation of the *Xenopus* cdc25 Phosphatase Regulate Its Activity

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The cdc25 tyrosine phosphatase is known to activate cdc2 kinase in the G2/M transition by dephosphorylation of tyrosine 15. To determine how entry into M-phase in eukaryotic cells is controlled, we have investigated the regulation of the cdc25 protein in *Xenopus* eggs and oocytes. Two closely related *Xenopus* cdc25 genes have been cloned and sequenced and specific antibodies generated. The cdc25 phosphatase activity oscillates in both meiotic and mitotic cell cycles, being low in interphase and high in M-phase. Increased activity of cdc25 at M-phase is accompanied by increased phosphorylation that retards electrophoretic mobility in gels from 76 to 92 kDa. Treatment of cdc25 with either phosphatase 1 or phosphatase 2A removes phosphate from cdc25, reverses the mobility shift, and decreases its ability to activate cdc2 kinase. Furthermore, the addition of okadaic acid to egg extracts arrested in S-phase by aphidicolin causes phosphorylation and activation of the cdc25 protein before cyclin B/cdc2 kinase activation. These results demonstrate that the activity of the cdc25 phosphatase at the G2/M transition is directly regulated through changes in its phosphorylation state.

INTRODUCTION

In eukaryotic cells, replication of the genetic material and segregation of duplicated sets of chromosomes between daughter cells must be regulated in a faithful and punctual manner. For example, entry into M-phase occurs only after DNA replication is completed (for review, see Hartwell and Weinert, 1989). A central control mechanism in the transition from interphase to mitosis is the activation of the cyclin B/cdc2 complex called maturation-promoting factor (for review, see Maller, 1991). During S- and G2-phase, cdc2 is phosphorylated on tyrosine 15, and this phosphorylation leads to suppression of its kinase activity (Gould and Nurse, 1989). Genetic and biochemical studies have suggested that phosphorylation/dephosphorylation of tyrosine 15 in cdc2 plays a role in coupling M-phase to completion of S-phase and that the cdc25 protein has a central role in the process of cdc2 activation (Russell and Nurse, 1986; Dasso and Newport, 1990; Enoch and Nurse, 1990; Kumagai and Dunphy, 1991). In fact, recent evidence has demonstrated that the cdc25 protein itself is the tyrosine phosphatase responsible for the dephosphorylation of tyrosine 15 in cdc2 (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991b; Strausfeld *et al.*, 1991; Lee *et al.*, 1992). To further understand the G2/M restriction point in the cell cycle and the dependence of mitosis on completion of S-phase, it is necessary to elucidate how the cdc25 protein is regulated at the molecular level during the cell cycle. Here we present the molecular cloning of *Xenopus* cdc25 and show that the activity of the protein product oscillates in the meiotic and mitotic cell cycles. Furthermore, this oscillation is regulated through changes in the phosphorylation state of the cdc25 protein itself.

MATERIALS AND METHODS

Molecular Cloning of the cDNA of the Xenopus cdc25 Homologs

Taking into account the homology between several cdc25 homologs previously described, highly degenerate primers corresponding to the consensus cdc25 protein sequence were designed as follows: 5′ primer, 5′-CTGGATCCATHATHRTNTTYCAYTGYGA-3′, corresponding to the amino acid sequence Ile-Ile-(Ile or Val or Met)-Phe-His-Cys-(Glu or Asp); 3′ primer, 5′-CCTCTAGAAGCTTCNGGRTARTAN-ARNGCNGGRTA-3′, corresponding to the amino acid sequence Tyr-Pro-Ala-Leu-Tyr-Tyr-Pro-Glu (Y = C + T, R = A + G, H = A + C + T, N = A + C + G + T). The underlined sequence contains a *Bam*HI site in the 5′ primer and *Xba* I and *Hin*dIII sites in the 3′ primer. Poly(A)$^+$ mRNA from *Xenopus* oocytes was prepared according to standard methods using phenol extraction, LiCl precipitation, and

two cycles of oligo (dT) chromatography by E. Erikson in this lab. 3′ primer-specific single-stranded cDNA was prepared at 37°C for 1 h in a 20-μl reaction containing 2.5 μg of poly(A)$^+$ mRNA, 1 μg of 3′ primer, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 units of RNasin, and 300 units of Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). The reaction was stopped by heating at 95°C for 5 min. The 0.1-ml polymerase chain reaction (PCR) mixture consisted of 50 mM KCl, 10 mM tris (hydroxymethyl)aminomethane (Tris)-HCl, pH 8.3, 1.8 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT), 10 μg of each oligonucleotide set, and 20 μl of the M-MLV reverse transcriptase mixture described above. The PCR incubations were, for the first 5 cycles, 94°C for 1 min, 37°C for 1.5 min, and 72°C for 2.5 min with 2.5 min ramp time between 37 and 72°C and, for further 35 cycles, 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min in a DNA Thermal Cycler (Perkin Elmer-Cetus). The reaction products were separated on a 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME), and fragments of the expected size (~130 base pair) were purified, digested with *Bam*HI and *Xba* I, and subcloned into the *Bam*HI and *Xba* I sites of pBluescript SK (+) vector (Stratagene, La Jolla, CA). Five clones were sequenced to confirm similarity with the human cdc25 homolog. A *Xenopus* oocyte cDNA library in the lambda ZAP II vector (Stratagene) was constructed using oligo dT primers and the mRNA described above. Two different *Xenopus* cdc25 PCR clones were used as a probe to obtain cDNA clones. Several positive clones were purified, and pBluescript SK (−) phagemids were derived from λ phage by in vivo excision. Two different clones, called cdc25A and cdc25B, were obtained; however, cdc25A did not contain an entire open reading frame, whereas cdc25B did. Another *Xenopus* oocyte cDNA library in the λgt10 vector (Rebagliati *et al.*, 1985) (kindly provided by D. Melton, Harvard University, Cambridge, MA) was then screened with probes made from the partial cdc25A clones. A complete cdc25A cDNA clone was obtained that gave rise to two *Eco*RI fragments (0.5 and 1.8 kilobase [kb]). The sequence of the region around an internal *Eco*RI site was determined after subcloning an 0.8-kb *Hin*dIII fragment from λ DNA that covered an internal *Eco*RI site. An entire insert representing a complete cdc25A cDNA (2.3 kb) was subcloned in the right direction into a pBluescript SK (+) vector. Both cdc25A and cdc25B clones were sequenced on both strands.

Production of Antibodies to Xenopus cdc25

Anti-peptide antibodies were prepared as follows. A peptide corresponding to the amino acid sequence Cys-Lys-Thr-Ser-Val-Gly-Asp-Arg-Lys-Arg-Arg-Glu-Gin-Val-Ala, which lies at the C-terminus of the deduced *Xenopus* cdc25A sequence (Figure 1), was synthesized, conjugated to keyhole lympet hemocyanin, and injected into rabbits. Antibodies were purified by affinity chromatography on a column of peptide coupled to Affi-gel 10 (Bio-Rad, Richmond, CA).

Antibodies against bacterially produced cdc25 were prepared as follows. An *Apa* I-*Pst* I fragment of cdc25A cDNA was blunted with T4 DNA polymerase. After ligation of *Bam*HI linkers (8-mer) and *Bam*HI digestion, the 1.0-kb fragment was inserted into the *Bam*HI site of pET3c (Novagen, Madison, WI). The plasmid was transformed into *Escherichia coli* BL21 (DE3) (Studier *et al.*, 1990), and proteins were expressed as described previously (Izumi and Maller, 1991). The expressed protein (~37 kDa) represents the C-terminal half of the cdc25 protein. Antibodies were raised in rabbits by injection of gel-purified protein as described previously (Izumi and Maller, 1991), and antibodies were purified by affinity chromatography on a column of bacterially synthesized protein coupled to activated CH-Sepharose 4B.

In these studies, immunoblotting was performed with the antibodies against the C-terminal half of cdc25 expressed in bacteria, whereas immunoprecipitation was done with the antipeptide antibodies. For immunoblotting, an enhanced chemiluminescence Western blotting detection kit (Amersham, Arlington Heights, IL) was used.

Preparation of Xenopus Oocyte Extracts

Oocyte extracts were prepared exactly as described previously (Izumi and Maller, 1991). Germinal vesicles were collected manually with a Pasteur pipette by squeezing equatorially with forceps oocytes that had received a 0.5-mm incision in the center of the animal pole 30 min earlier.

Preparation of Xenopus Egg Extracts

Cytostatic factor (CSF) (metaphase-arrested) extracts were prepared as described previously (Murray *et al.*, 1989), except that leupeptin was omitted from all buffers. Control extracts were incubated at 23°C with sufficient sperm chromatin to form ~500 nuclei/μl, and 0.4 mM $CaCl_2$ was added to induce exit from metaphase arrest. To arrest extracts in interphase with DNA and aphidicolin, sperm chromatin to form 2000 nuclei/μl and 50 μg/ml of aphidicolin (Calbiochem, San Diego, CA) were added to the extracts at the same time as the $CaCl_2$. Sperm chromatin was isolated by the method described previously (Lohka and Maller, 1985). Extracts were routinely inspected for nuclear formation and nuclear envelope breakdown at various times by both phase contrast and fluorescence microscopy using the DNA stain 4′, 6-diamidine-2-phenylindole dihydrochloride.

Phosphorylation and Immunoprecipitation of cdc25 in CSF-Arrested Extracts

These were performed basically according to the methods described previously, except that DNA was added to the extracts (Izumi and Maller, 1991). One millicurie of ^{32}Pi and 1 mCi of [γ-^{32}P]ATP were added to 100 μl of CSF-arrested extracts at indicated intervals after $CaCl_2$ addition. At appropriate times, judged by the morphology of sperm nuclei, samples were frozen in a dry ice bath. For immunoprecipitation, extracts diluted sixfold with lysis buffer (30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.5, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 200 mM NaCl, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 50 mM β-glycerophosphate, 5 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM dithiothreitol [DTT], 10 μg/ml of each aprotinin, leupeptin, chymostatin, and pepstatin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) were centrifuged at 15 000 × *g* for 10 min. The supernatant was precleared with protein A-Sepharose beads, and the supernatant was incubated with 10 μg/ml of affinity-purified anti-cdc25 antibodies at 4°C for 2 h. The antibodies were collected by agitation with protein A-Sepharose at 4°C for 1 h, and immunoprecipitates were washed once with the lysis buffer, three times with the lysis buffer containing 1 M NaCl, and then eluted with SDS sample buffer for analysis by SDS polyacrylamide gel electrophoresis (PAGE). Phosphoamino acid analysis and tryptic phosphopeptide mapping of the cdc25 protein eluted from gel were performed as described previously (Izumi *et al.*, 1991).

Assay of Endogenous Xenopus cdc25 Activity from CSF Extracts

cdc25 activity was assayed by activation of the histone H1 kinase activity of the cyclin B/cdc2 complex. At various times, extracts were prepared, and cdc25 immunoprecipitated as described above. To make substrates for the cdc25 protein, cyclin B/cdc2 complexes were immunoprecipitated from CSF extracts arrested in interphase with DNA and aphidicolin for ~90 min after $CaCl_2$ addition using a mixture of sheep antisera generated against either cyclin B1 or B2 as described previously (Izumi and Maller, 1991) followed by immobilization with protein G-Sepharose. After immunoprecipitation, both cdc25 beads and cyclin B/cdc2 beads were washed separately with phosphatase buffer (50 mM Tris, pH 7.4, 30 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM DTT, 10 μg/ml of each aprotinin, leupeptin, chymostatin, and pepstatin, and 0.5 mM PMSF). For each assay, cdc25 immunocomplexes derived from 10 to 20 μl of original extract and cyclin B/cdc2

immunocomplexes derived from 3 to 10 μl of extract, depending on the extent of cyclin B synthesis in each extract, were mixed and incubated at 23°C for 30 min with agitation every 5 min. Mixed beads were then washed with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 2 mM Na_3VO_4) and incubated at 23°C for 10 min in the kinase buffer with 100 μM [γ-^{32}P]ATP and 0.5 mg/ml of histone H1. The samples were analyzed by SDS-PAGE followed by autoradiography and Cerenkov counting of the excised H1 band.

Phosphatase 1 (PP1) and phosphatase 2A (PP2A) catalytic subunit were prepared from rabbit skeletal muscle as described previously (Tung *et al.*, 1984). cdc25 immunocomplexes prepared as described above were treated with a final concentration of either 0.2 units/ml of PP1 or 0.1 units/ml of PP2A with or without 8 μM microcystin-LR (Bethesda Research Laboratories) at 23°C for 30 min (1 unit of activity is that amount dephosphorylating 1 nmol/min of ^{32}P-histone H1 phosphorylated by cdc2 kinase). When ^{32}P-labeled H1 was used as substrate, 5 μM of microcystin completely inhibited the same amount of PP1 and PP2A used in the cdc25 activity assay. Microcystin was added to all the cdc25-containing samples with or without PP1 or PP2A, to a final concentration of 16 μM, and incubated further on ice for 30 min. Then, an equal volume of cyclin B/cdc2 beads was added and incubated at 23°C for 30 min. H1 kinase assays were performed as described above, except for the presence of 8 μM microcystin in the kinase buffer. In some experiments, we used 20 μM okadaic acid (a kind gift of P. Cohen, University of Dundee, Scotland) instead of microcystin.

RESULTS

Isolation of Xenopus cdc25 cDNA Clones

Protein sequence comparison of the known cdc25 homologs (Russell and Nurse, 1986; Edgar and O'Farrell, 1989; Russell *et al.*, 1989; Sadhu *et al.*, 1990) revealed that the C-terminal portions of the proteins are highly conserved. We designed a pair of degenerate oligonucleotides corresponding to two of the most conserved regions for use in the PCR. The 5′ oligonucleotide set was 576-fold degenerate over 28 nucleotides, and the 3′ set was 4096-fold degenerate over 35 nucleotides. Amplification of mRNA from *Xenopus* oocytes produced an expected 130-nucleotide-long DNA fragment. Sequence analysis of this fragment revealed an open reading frame encoding a peptide having ~77% identity to the human cdc25 homolog (Sadhu *et al.*, 1990) and established that a cdc25-related fragment had been cloned.

The inserts from two different PCR-derived clones were used to screen a *Xenopus* oocyte cDNA library. Two different clones, termed cdc25A and cdc25B, were obtained. The region of each open reading frame is shown (Figure 1). The open reading frame of clone cdc25A begins at the AUG codon 57 bases upstream of the initiation codon shown in Figure 1. However, it is very unlikely that translation begins here because there is a stop codon just upstream (Figure 1, left, underline). The presumed initiation codons of cdc25A and cdc25B shown in Figure 1 are flanked by a Kozak consensus sequence (Kozak, 1986). Therefore, both cdc25A and cdc25B are predicted to encode a protein of 550 amino acids. The calculated molecular weight and isoelectric point for cdc25A are 62 000 and 5.7, and for cdc25B, 62 000 and 6.0, respectively. The cdc25A and cdc25B gene products are 94% identical at the amino acid level.

These proteins share a conserved C-terminal region with the other known cdc25 homologs (Figure 2A). Most of the homology is located in the region that starts at amino acid position 362 and extends for 189 residues to the C-terminus. These regions of the cdc25A and cdc25B protein are 67 and 68%, respectively, identical to the equivalent C-terminal region of the human cdc25 homolog (Sadhu *et al.*, 1990), and contain the motif, HCXXXXXR, which is found in all phosphotyrosine phosphatases, including cdc25. These cysteine and arginine residues are essential for phosphatase activity of the cdc25 protein (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b) and other tyrosine phosphatases (Guan and Dixon, 1990; Streuli *et al.*, 1990; Guan *et al.*, 1991).

The N-terminal halves of the cdc25 homologs from different species share little sequence similarity. However, several short stretches of amino acids are conserved. For example, putative nuclear targeting sequences are found in both *Xenopus* cdc25 clones as well as those from other species (Figure 2B). The two basic domains separated by 10 intervening spacer amino acids were first identified in *Xenopus* nucleoplasmin, and both basic domains have been found to be required for nuclear targeting (Robbins *et al.*, 1991). Furthermore, similar motifs have been found in other nuclear targeting sequences, such as those of the N1 protein, p53, and the steroid receptor superfamily (Robbins *et al.*, 1991). However, these motifs in yeast cdc25 homologs are not as strictly conserved as in cdc25 homologs from higher eukaryotes. Interestingly, several potential phosphorylation sites for cdc2 kinase are found in the vicinity of this motif. The human cdc25 protein is suggested to be localized primarily in the nucleus (Millar *et al.*, 1991a). In addition, wheat germ agglutinin, an inhibitor of nuclear transport, has been found to inhibit tyrosine dephosphorylation of cdc2 by the cdc25 protein in *Xenopus* egg extracts (Kumagai and Dunphy, 1991). Therefore, if the cdc25 protein enters the nucleus at some phase of the cell cycle, these sequences are likely to be important for the translocation.

Mobility Shifts of the cdc25 Protein During Xenopus Cell Cycles

To characterize the endogenous cdc25 protein, we prepared polyclonal antibodies against the bacterially produced C-terminal half of the cdc25A protein. First, immunoblotting was done during the process of oocyte maturation (Figure 3A). For up to 2 h after progesterone treatment, the level of cdc25 remained constant with an apparent M_r of 76 000, slightly larger than expected from the predicted sequence. However, in correlation with increased cdc2 histone H1 kinase activity before germinal vesicle breakdown (GVBD), cdc25 began to

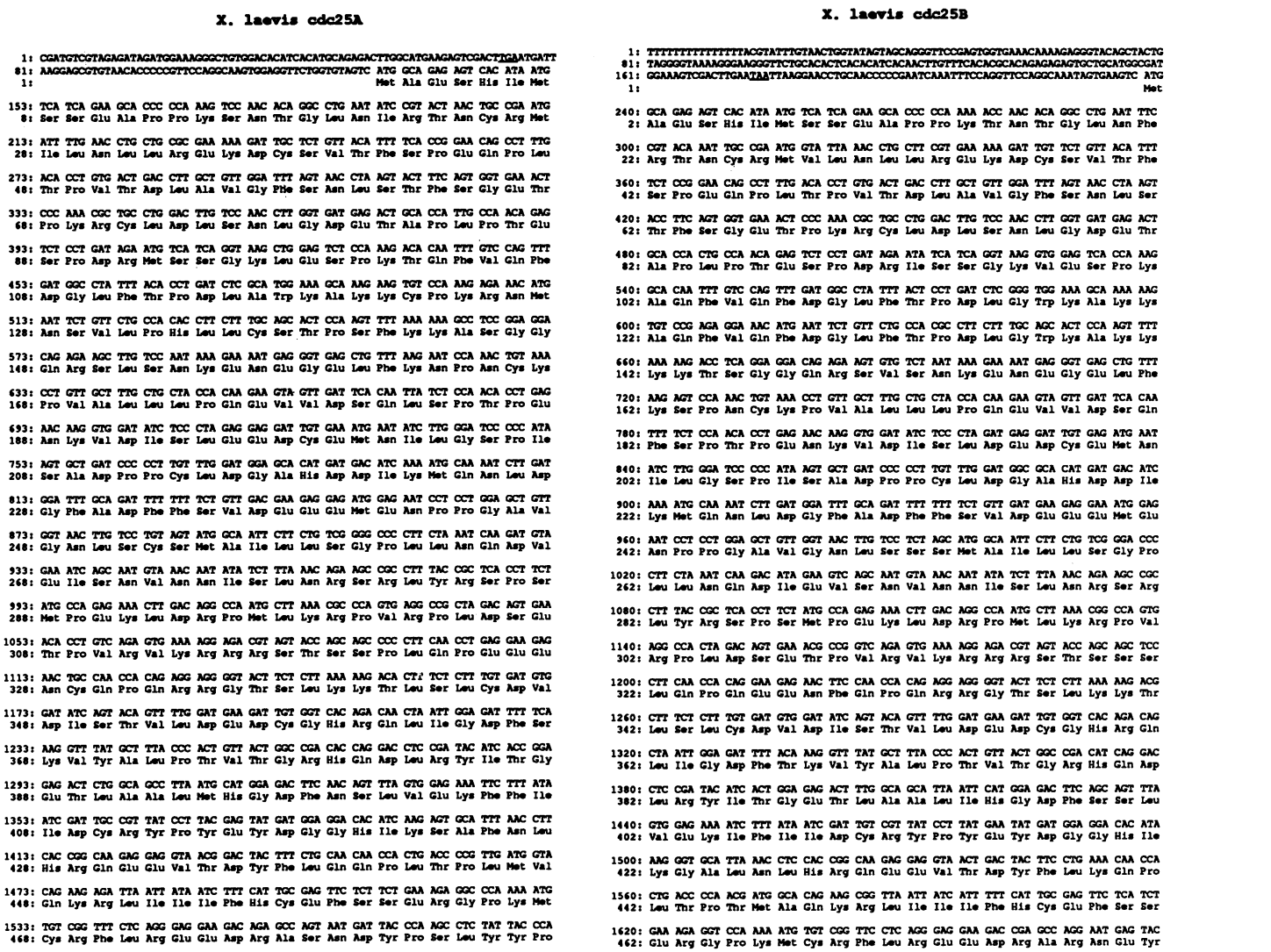

Figure 1. Nucleotide and amino acid sequences of *Xenopus* cdc25A and cdc25B. (Left) Sequence of cdc25A cDNA. (Right) Sequence of cdc25B cDNA. In-frame stop codons upstream of the presumed initiation codons are underlined and termination codons are marked by asterisks. The GenBank accession numbers for cdc25A and cdc25B are M96857 and M96858, respectively.

shift in electrophoretic mobility to 92 kDa at 2.5 h, and was completely shifted by 3.5 h. During the transition from meiosis I to II at 4.5–5 h, cdc25 partially shifted down coincident with reduced cdc2 kinase activity. Because cdc25 contains a putative nuclear targeting sequence as described above, we determined whether cdc25 was in the germinal vesicle (nucleus) or the cytoplasm. Surprisingly, cdc25 was not detected in the germinal vesicles from as many as 10 oocytes, whereas it was easily detected in the cytosol fraction from two oocytes (Figure 3B).

Next, similar experiments were done in CSF metaphase-arrested extracts from *Xenopus* eggs (Lohka and Maller, 1985; Murray *et al.*, 1989) (Figure 3C). Meta-

A

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X.l. cdc25A     1-361  LIGDFSKVYALPTVTGRHQDLRYITGETLAALMHGDFNSLVEKFFIIDCRYPYEYDGGHIKSAFNLHRQEEV
X.l. cdc25B     1-361  .....T.........................I....S.....I................G.L........
Human cdc25C    1-281  ........C.....S.K....K.VNP..V...LS.K.QG.I...YV.........L....QG.L..YS...L
string          1-276  .......A....LME...R..KS.SS..V.R.LK.E.SDK.ASYR.........FE....EG.K..YTT.QI
S.pombe cdc25   1-389  F.SSHVEDLS..CFAVKEDS.KR..Q...LG.LD.K.KDIFD.CI.....FE...L....ST.V..NTKQAI
MIH1            1-227  IPYYYDDRN------SMTFS.EFLQKRLKNI.QNNMCE.FYNSCR.....FE...T....INSV.I.SRD.L

X.l. cdc25A           TDYFLQQPLTPLMVQ-------KRLIIIFHCEFSSERGPKMCRFLREEDRASND--YPSLYYPELYLLKGGY
X.l. cdc25B           .....K.....T.A.-------.....*.....*.............R.E--..............
Human cdc25C          FNF..KK.IV..DT.-------..I..V.*.....*..R...C......SL.Q--..A.......I.....
string                L.E..TVQQ.E.QQ.QNAESGH..N.....*.....*....S....NL..ER.TNA..A.H...I...HN..
S.pombe cdc25         V.A..SK...------------H.VALV.*.H.AH*A.HLALHF.NT..RM.SHR..F.....V.I.H...
MIH1                  EYE.IHKV.HSDTSNNNTLP----TLL.I*....H*..SLASH..NC..II.QDH..K.F..DILI.D...

X.l. cdc25A           KDFFPEYKELCEPQSYCPMHHQDFREDLLKFRTKCKTSVGDRKRREQVARLMKL          550
X.l. cdc25B           .........................E.....................I..I...          550
Human cdc25C          R......M..............HKTE..RC.SQS.VQE.E.QL...I.L.V.DMSP        473
string                .E..ESHV.....HA.RT.LDPAYN.AYRH..A.S.SWN..-GLGGATG..K.SRSRLML    479
S.pombe cdc25         .S.YENH.NR.D.IN.V..NDRSHVMTCT.AMNNF.RNATF-M.TKSYTFWP.CVSFPRR    580
MIH1                  .AVL-TFP...Y.RQ.VG.NS.ENLLNCEQEMD.FRRESKR-FATKNNSFR-..ASPSNP    419-474.
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B

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X.l.nucleoplasmin  148-170  ESPPKAVKRPAATKKAGQAKKKK
X.l.cdc25A and B   294-316  RPMLKRPVRPLDSETPVRVKRRR
Human cdc25A       269-291  RSVLKRPERSQEESPPGSTKRRK
Human cdc25B       316-338  RPILKRLERPQDRDTPVQNKRRR
Human cdc25C       223-245  RPRLKQVEKFKDNTIPDKVKKKY
string             172-225  RDCFKRPEPPASANCSPIQSKRHR
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Figure 2. Homology between cdc25 proteins. (A) The C-terminal region of cdc25 homologs from *Xenopus*, human, *Drosophila* (*string*), *S. pombe*, and *Saccharomyces cerevisiae* (MIH1) are compared. Identical matches with *Xenopus* cdc25A are dotted. Dashes indicate individual amino acid gaps to generate optimal alignment. The HC and R residues conserved in all phosphotyrosine phosphatases are highlighted. Recently, another two human cdc25 homologs have been cloned (Galaktionov and Beach, 1991). (B) The nuclear targeting sequence of *Xenopus* nucleoplasmin is aligned with putative nuclear targeting sequences of several cdc25 clones. Residues important for the nuclear targeting of nucleoplasmin are marked by asterisks (Robbins *et al.*, 1991). Basic amino acids, arginine (R), and lysine (K) are written in bold. In the case of human cdc25 clones, A, B, and C, are named according to Galaktionov and Beach (1991).

phase-arrested unfertilized eggs showed cdc25 migrating as a 92-kDa polypeptide. Shortly after the addition of calcium to release metaphase arrest, the 92-kDa protein shifted down to 76 kDa, and at 120 min after calcium addition, just before entry into first mitosis, cdc25 again shifted up to 92 kDa. Then cdc25 again shifted down to 76 kDa at exit from first mitosis. In contrast, in egg extracts arrested in interphase with DNA and aphidicolin (Dasso and Newport, 1990), cdc2 kinase activity remained low and cdc25 exhibited a M_r of 76 000 at 120 min (Figure 3C, first lane, marked by asterisk) and remained stable for ≥3 h. Because the shift of the cdc25 protein in polyacrylamide gels was dramatic, we obtained additional evidence that these bands represent modified forms of the same protein. cdc25 translated in a reticulocyte lysate containing ^{35}S-methionine was incubated with either an interphase or a metaphase egg extract and then immunoprecipitated using an antibody to a peptide encoding 15 residues at the C-terminus of cdc25. Although the radiolabeled cdc25 incubated with an interphase extract remained at its original position of 76–78 kDa in gels, cdc25 incubated with a metaphase extract had shifted up to 92 kDa (Figure 3D). It is interesting that microcystin, a specific inhibitor of both PP1 and PP2A, prevented a partial shift down of metaphase cdc25 during the incubation (Figure 3D, lanes d and e). This experiment demonstrates that both the 76- and 92-kDa bands are derived from the same protein and that the shift is due to posttranslational modification, most likely phosphorylation. In agreement with this, immunoprecipitation experiments showed no detectable newly synthesized cdc25 in ^{35}S-methionine-labeled egg extracts.

Phosphorylation Causes the Shift of the cdc25 Protein

The dramatic shift of the cdc25 protein suggests that it is modified covalently, most likely by phosphorylation. To determine directly if this was the case, egg extracts were labeled with [γ-^{32}P]ATP, and the cdc25 protein was immunoprecipitated. The anti-peptide antibody

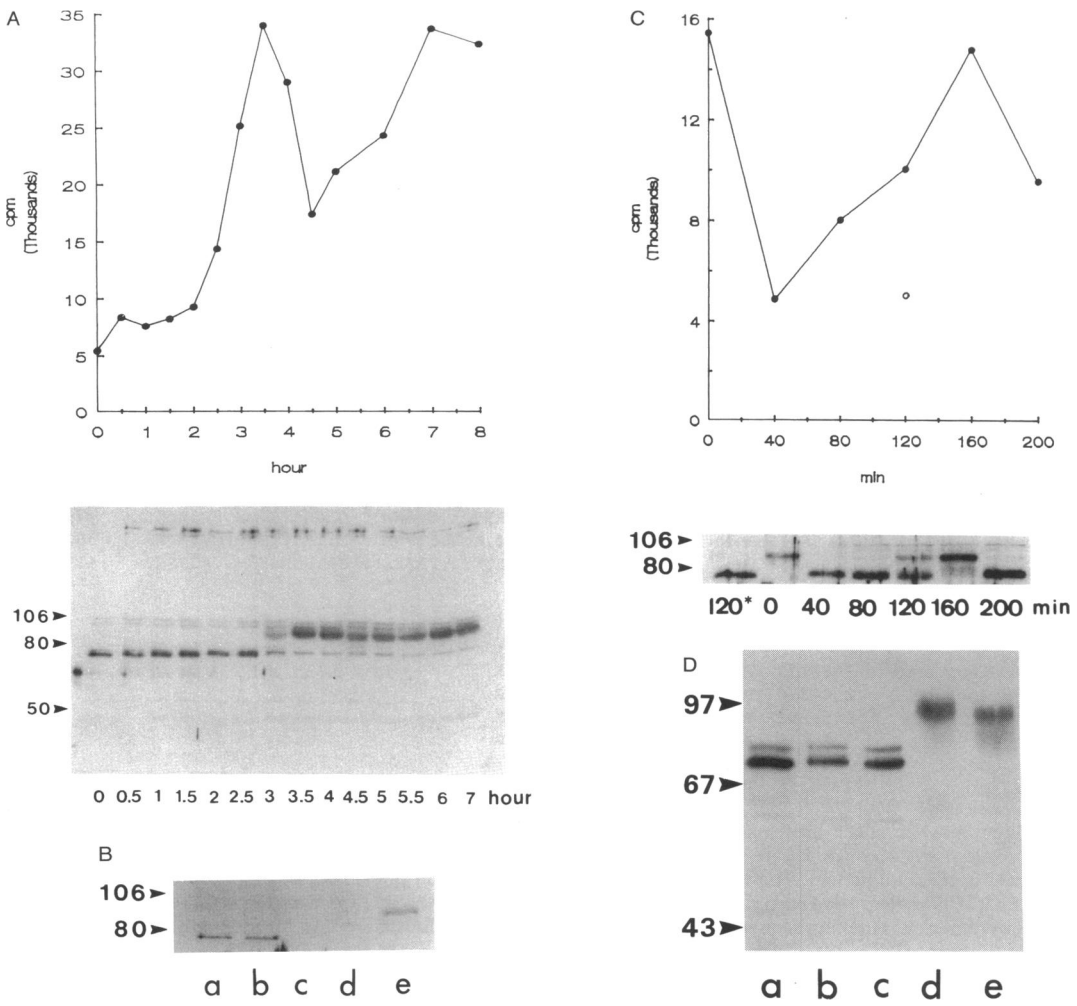

Figure 3. Electrophoretic shift of the cdc25 protein in *Xenopus* oocytes and eggs. (A) Stage VI oocytes were isolated and lysed at the indicated times after incubation with 10 μM progesterone. Samples of the extract corresponding to 0.3 and 1.2 oocytes were assayed for histone H1 kinase activity (upper) and for cdc25 by immunoblotting (lower), respectively. Although the 92-kDa band at 2.5 h is not evident in this figure, it could be detected after longer exposure of the film. In this experiment, GVBD began at 3 h. (B) Extracts from whole prophase oocytes (lane a), the enucleated cytoplasm (lane b), the germinal vesicle (lane c), or whole M-phase oocytes (lane e) were immunoblotted with anti-cdc25 antibodies. Each fraction was derived from two oocytes. Pooled germinal vesicles from 10 oocytes were also blotted (lane d). (C) At various times after calcium-induced release into interphase of a metaphase-arrested *Xenopus* egg extract, samples were removed and assayed as in A. H1 kinase activity of a DNA/aphidicolin extract arrested in S-phase at 120 min is shown by the open circle. (D) cdc25A mRNA was translated in a rabbit reticulocyte lysate as described previously (Izumi and Maller, 1991) (lane a). Autoradiography of the ^{35}S-methionine-labeled translation product revealed a minor 78-kDa protein and a major 76-kDa protein. The 78-kDa protein may reflect a product whose translation begins at the AUG codon 57 bases upstream of the presumed initiation codon shown in Figure 1. After incubation of 3.5 μl of the labeled translation product with 10 μl of either an interphase (lanes b and c) or a metaphase (lanes d and e) egg extract with (lanes b and d) or without (lanes c and e) 1 μM microcystin at 23°C for 30 min in the presence of 150 μM emetine, which completely inhibits protein synthesis, cdc25 was immunoprecipitated with anti-cdc25 antibodies, and analysed by SDS-PAGE and fluorography.

immunoprecipitated cdc25 as a 76-kDa phosphoprotein from interphase extracts (Figure 4A, lane a). In contrast, cdc25 was a highly phosphorylated 80- to 92-kDa phosphoprotein when immunoprecipitated from an M-phase extract (Figure 4A, lane c). Immunoprecipitation of both the 76-kDa phosphoprotein in interphase and the 80- to 92-kDa phosphoprotein in M-phase was specifically blocked by the presence of the bacterially expressed C-terminal half of cdc25. The bulk of the cdc25 polypeptide in M-phase was in the maximally phosphorylated form at 92 kDa as judged by immunoblotting (Figure 3C). We believe that the most highly phosphorylated form of the cdc25 protein is characteristic of M-phase, and intermediate ^{32}P-labeled forms may be a consequence of dephosphorylation occurring during immunoprecipitation even in the presence of multiple phosphatase inhibitors (see Figure 5B). The cdc25 protein from extracts arrested in S-phase with DNA/

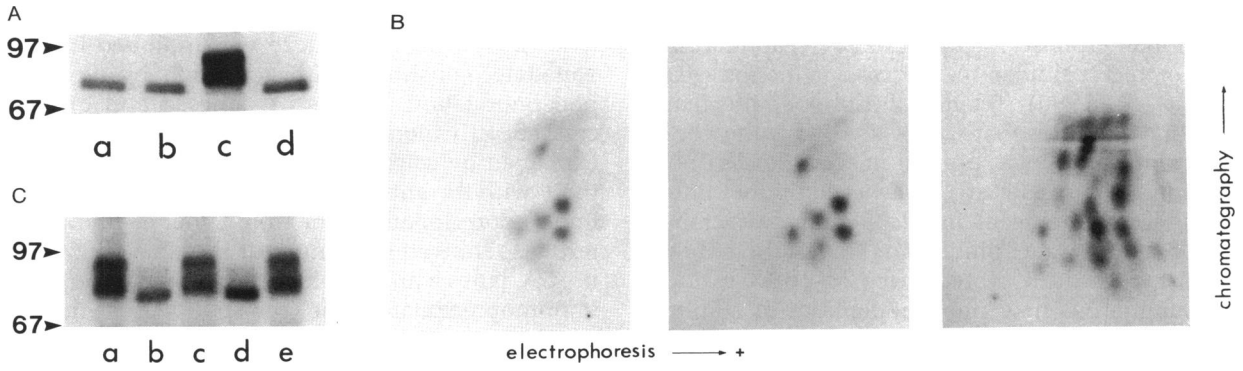

Figure 4. Phosphorylation of the cdc25 protein in *Xenopus* egg extracts. (A) Metaphase II-arrested extracts were induced to re-enter the cell cycle by the addition of calcium. From 30 to 70 min (lanes a and b) and 70 to 140 min (lane c and d) after calcium treatment, ^{32}Pi and [γ-^{32}P]ATP were added. The cdc25 protein was immunoprecipitated from interphase (lanes a, b, and d) or M-phase (lane c) extracts and analyzed by SDS-PAGE. In lanes b and d, extracts were arrested in S-phase with 2000 sperm nuclei/μl and 50 μg/ml of aphidicolin. An autoradiogram of the gel is shown. (B) Two-dimensional phosphopeptide mapping was performed on cdc25 from an interphase extract (left), a DNA/aphidicolin S-phase extract (middle), or a M-phase extract (right). In the case of M-phase, only the upper band of the cdc25 protein at 92 kDa was excised from the gel. (C) Extracts were labeled with ^{32}Pi and [γ-^{32}P]ATP at 70 min after calcium addition to release metaphase II arrest. Labeling was stopped at 140 min when 70% of the nuclei exhibited NEBD. The cdc25 protein was immunoprecipitated, and the immunoprecipitates were treated with buffer alone (lane a), PP1 (lanes b and c), or PP2A (lanes d and e) in the absence (lanes a, b, and d) or in the presence (lanes c and e) of 8 μM microcystin and analyzed by SDS-PAGE.

aphidicolin was phosphorylated to the same extent as a control interphase extract and showed no change in electrophoretic mobility (Figure 4A, lanes b and d), as expected from the immunoblotting data (Figure 3C). Phosphoamino acid analysis of immunoprecipitated cdc25 revealed the major phosphoamino acid was

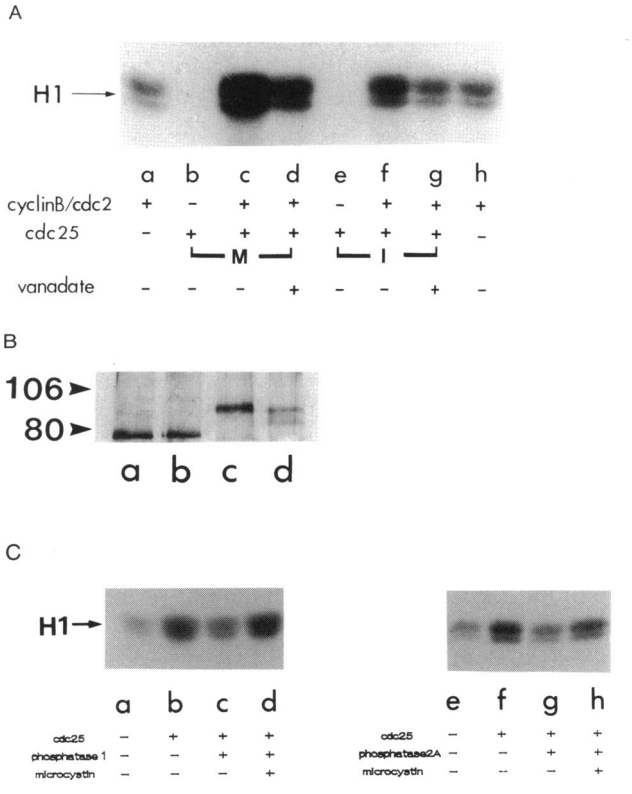

Figure 5. cdc25 activity in *Xenopus* egg extracts. (A) cdc25 immunoprecipitates from metaphase II-arrested extracts before (lanes b–d, in metaphase; M) or 40 min after calcium addition (lanes e–g, in interphase; I) were incubated with (lanes c, d, f, and g) or without (lanes b and e) cyclin B/cdc2 complexes isolated from a DNA/aphidicolin-arrested interphase extract for 30 min at 23°C in the absence (lanes b, c, e, and f) or in the presence (lanes d and g) of 2 mM sodium vanadate. In lanes a and h, cyclin B/cdc2 complexes were incubated with control protein A-Sepharose beads instead of cdc25 beads. The radiolabel in histone H1 was as follows: 2991 cpm (lane a); 573 cpm (b); 14 620 cpm (c); 6815 cpm (d); 303 cpm (e); 5788 cpm (f); 3407 cpm (g); and 3502 cpm (h). (B) cdc25 protein was immunoprecipitated by an anti-peptide antibody from an interphase (lanes a and b) or a metaphase (lanes c and d) egg extract in the presence (lanes a and c) or in the absence (lanes b and d) of 1 μM microcystin. After electrophoresis, it was immunoblotted using an antibody toward bacterially expressed cdc25 protein. Note that metaphase cdc25 immunoprecipitates in the absence of microcystin were partially dephosphorylated and shifted down. (C) cdc25 immunoprecipitates from M-phase were divided equally into aliquots and treated with PP1 or PP2A with or without 8 μM microcystin as in Figure 4C. Then microcystin and cyclin B/cdc2 complexes were added to all the samples, and H1 kinase activity was measured. The radiolabel in histone H1 was as follows: 5291 cpm (lane a); 12 420 cpm (b); 7640 cpm (c); 12 258 cpm (d); 4148 cpm (e); 8154 cpm (f); 4750 cpm (g); and 7893 cpm (h).

phosphoserine, with phosphothreonine as a minor species either in interphase, M-phase, or DNA/aphidicolin-arrested extracts, similar to the case in fission yeast (Moreno *et al.*, 1990). Tryptic phosphopeptide maps showed that the phosphate associated with the cdc25 protein in interphase was present in six or seven phosphopeptides (Figure 4B, left), and no additional phosphopeptides were present in cdc25 from extracts arrested in S-phase with aphidicolin (Figure 4B, middle). In contrast, the cdc25 protein from M-phase extracts revealed numerous new phosphopeptides in addition to those found in interphase (Figure 4B, right). Therefore, increased phosphorylation of the cdc25 protein in M-phase occurs at different sites than those phosphorylated in interphase.

To demonstrate that the electrophoretic shift found in M-phase is due to phosphorylation, ^{32}P-labeled cdc25 was immunoprecipitated and incubated with the catalytic subunits of the serine/threonine-specific phosphatases PP1 or PP2A. Both phosphatases significantly decreased the ^{32}P content of the cdc25 protein isolated from M-phase and caused an electrophoretic shift down to the "interphase position," 76 kDa (Figure 4C, lanes b and d). It should be noted that neither phosphatase could completely dephosphorylate cdc25, and after phosphatase treatment the protein remained phosphorylated at a level characteristic of interphase. The presence of 8 μM microcystin blocked the ability of either phosphatase to dephosphorylate cdc25 (Figure 4C, lanes c and e). This concentration of microcystin was found to completely inhibit both phosphatases as judged by dephosphorylation of ^{32}P-labeled histone H1 in vitro. These results demonstrate that the electrophoretic shift found in M-phase is due to phosphorylation.

cdc25 Phosphatase Activity Oscillates During The Cell Cycle

The cdc25 protein is a tyrosine phosphatase that directly activates cyclin B/cdc2 kinase activity by dephosphorylating tyrosine 15 (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b; Strausfeld *et al.*, 1991; Lee *et al.*, 1992). We assayed the activity of the endogenous cdc25 protein at different points in the cell cycle in *Xenopus* egg extracts. This assay measured activation of cyclin B/cdc2 complexes isolated from extracts blocked in S-phase with DNA/aphidicolin, conditions that lead to accumulation of the inactive tyrosine 15-phosphorylated form of the cyclin B/cdc2 complex (Dasso and Newport, 1990). First, we compared the activity of the cdc25 protein from metaphase-arrested unfertilized eggs with that from activated interphase eggs. The interphase cyclin B/cdc2 complex used as substrate of cdc25 had low H1 kinase activity as expected (Figure 5A, lanes a and h), and the addition of cdc25 from a metaphase extract caused a four- to fivefold activation (Figure 5A, lane c). In contrast, activation by cdc25 from an interphase extract was less than twofold (Figure 5A, lane f). Activation by cdc25 was inhibited by sodium vanadate, consistent with activation by tyrosine dephosphorylation (Figure 5A, lanes d and g). The increased activation by cdc25 from M-phase cannot be explained by coimmunoprecipitation of H1 kinase activity with the anti-cdc25 antibody, because anti-cdc25 immunoprecipitates alone from either metaphase or interphase showed no significant H1 kinase activity (Figure 5A, lanes b and e). The possibility that the antibody immunoprecipitated the phosphorylated metaphase cdc25 more efficiently than the interphase form is also excluded, because immunoprecipitation followed by immunoblotting revealed that the same amount of cdc25 protein was immunoprecipitated from an interphase or a metaphase extract (Figure 5B).

Because this assay used endogenous cyclin B/cdc2 complex as the substrate, the extent of activation by cdc25 varied between assays, depending on the level of synthesized cyclin and the basal activity of cdc2 kinase in each extract. However, metaphase cdc25 was consistently able to activate cdc2 kinase activity to a higher degree than interphase cdc25 (3.2 ± 1.1-fold activation in metaphase, mean $\pm$ SD, $n = 5$, compared with 1.6 ± 0.4-fold in interphase, $n = 5$). Furthermore, by subtraction of the basal level of cdc2 kinase activity in the assay to express net cdc25 activity, metaphase cdc25 had 4.4-fold (± 2.2, $n = 5$) more activity than that of interphase. The activation assay was linear with time up to 60 min and was dose dependent for both the interphase and metaphase forms of cdc25 in the concentration range we used. The time course of changes in cdc25 activity in metaphase II extracts progressing to mitosis I after calcium addition was also investigated. The activity of cdc25 increased again to mitotic levels in correlation with the shift of cdc25 to retarded electrophoretic mobility. As expected, cdc25 from extracts blocked in S-phase with DNA/aphidicolin showed low activity, similar to that in control interphase extracts (cf. Figure 6).

Phosphorylation Increases cdc25 Activity

As described above, the ability of cdc25 to dephosphorylate and activate the cyclin B/cdc2 complex is highly correlated with its phosphorylation state. To investigate whether the increased phosphorylation was responsible for the increased activity, we assayed cdc25 activity with or without phosphatase treatment under the same conditions described in Figure 4C. After treatment with or without either PP1 or PP2A, the cdc25 preparation was incubated with 8 μM microcystin to completely inhibit any residual activity of either PP1 or PP2A, and the cyclin B/cdc2 complex was added and assayed for H1 kinase activity. Both PP1 and PP2A reduced the ability of cdc25 to activate cdc2, and treatment of either phosphatase with microcystin blocked these

effects (Figure 5C). Other experiments using 20 μM okadaic acid instead of microcystin showed similar results. In this experiment, cdc25 incubated without phosphatase showed only a twofold activation of cdc2 kinase. Because M-phase cdc25 was partially dephosphorylated during immunoprecipitation (Figures 4A and 5B) or during incubation of reticulocyte lysates with extracts (Figure 3D), we speculate that the maximally phosphorylated state of cdc25 is labile. Recent experiments show that inclusion of microcystin in the immunoprecipitation buffers increases the fraction of cdc25 recovered in the most highly phosphorylated state, and therefore the activation in vivo of cdc25 in M-phase may be considerably higher than fourfold. Additionally, we consistently found that cdc25 activity declined during the initial incubation at 23°C even without added phosphatase. In principle, this could be due to either dephosphorylation or degradation, but our data clearly show a consistent decrease in cdc25 activity attributable to dephosphorylation by PP1 or PP2A. The possibility that residual amounts of PP1 or PP2A dephosphorylated cyclin B/cdc2 or H1 directly was excluded by the absence of changes in cdc25 activity with inactivated phosphatase. In addition, the basal H1 kinase activity of cyclin B/cdc2 complexes was not affected by protein A beads that had been treated with the same amount of either phosphatase. These results demonstrate that dephosphorylation of the cdc25 phosphatase decreases its activity.

cdc25 is Activated Before Cyclin B/cdc2 Activation

The results described above show that either PP1 or PP2A can remove phosphate from mitotically activated cdc25 protein and inactivate it in vitro. To investigate whether such a regulatory mechanism occurs in extracts, we added okadaic acid, an inhibitor of both phosphatases, to *Xenopus* egg extracts competent to carry out both DNA replication and mitosis in vitro (Figure 6). It has been demonstrated that okadaic acid can cause premature activation of cdc2 in egg supernatants (Félix *et al.*, 1990), and we have found that okadaic acid can also overcome a DNA/aphidicolin block of mitosis (Walker

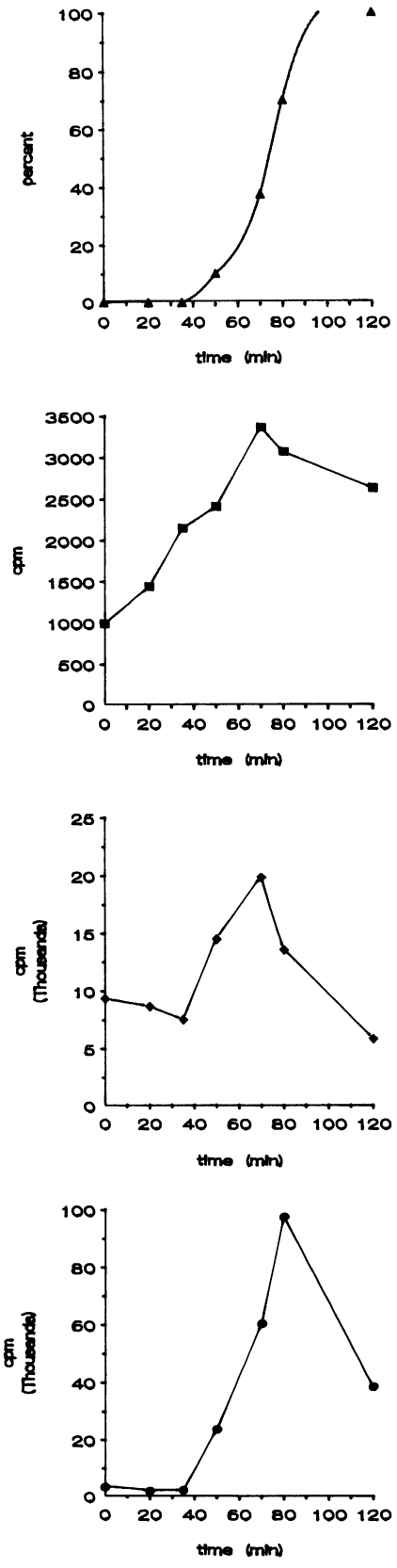

Figure 6. cdc25 is activated before cyclin B/cdc2. Metaphase II-arrested extracts were brought into interphase by calcium addition in the presence of DNA and aphidicolin as described in MATERIALS AND METHODS. At 70 min after metaphase release, the extracts were labeled with ^{32}Pi and [γ-^{32}P]ATP. At 90 min after metaphase release, the extracts were treated with 1.5 μM okadaic acid. At various times after addition of okadaic acid, samples were assayed for nuclear disassembly (▲), cdc25 activity (■), cyclin A-immunoprecipitated H1 kinase activity (♦), and cyclin B-immunoprecipitated H1 kinase activity using a mixture of anti-cyclin B1 and anti-cyclin B2 antibodies (●). cdc25 activity was expressed after subtraction of the basal H1 kinase activity of the cyclin B/cdc2 complex used as substrate (1298 cpm). Cyclin immune-complex H1 kinase assays were performed as described previously (Walker and Maller, 1991).

et al., 1992). The activity of cdc25 had increased significantly at 20 and 35 min after okadaic acid addition, whereas both cyclin A- and B-associated H1 kinase activity remained at the interphase level, and no nuclear envelope breakdown (NEBD) was detected. Before the addition of okadaic acid, there was a significant level of cyclin A-associated H1 kinase activity, as reported previously (Walker and Maller, 1991). By 50 min after okadaic acid addition, both cyclin A- and B-associated H1 kinase activity began to increase and reached maximal activity at 70 and 80 min, respectively. One hundred percent NEBD was observed at 90 min. During this period, the activity of cdc25 continued to increase, reaching a maximum by 70 min. The phosphorylation state of the cdc25 protein was also investigated in the same extracts. Before okadaic acid addition, only the lower 76-kDa protein was detected. In contrast, the shift of the cdc25 protein to an M_r of 92 000 had already begun to occur by 20 min after okadaic acid addition. The proportion of the upper band increased as time passed, and then shifted down to 80 kDa as H1 kinase activity declined on exit from mitosis. These results indicate that the cdc25 protein is phosphorylated and activated before cyclin B/cdc2 activation, as would be expected if cdc25 were an upstream effector of cdc2 kinase. Furthermore, they suggest that maintenance of the cdc25 protein in an inactive state in S-phase may reflect elevated activity of PP1 or PP2A (Walker *et al.*, 1992).

DISCUSSION

In somatic cells, entry into mitosis depends on the completion of DNA synthesis. This dependency is established by feedback controls in S-phase that arrest cell division when damaged or unreplicated DNA is present (Hartwell and Weinert, 1989). Because premature M-phase occurs without completion of S-phase in certain abnormal usually lethal situations, this dependence is highly regulated and of crucial importance in the restriction point governing the G2/M transition in the cell cycle (Nurse, 1990; Enoch and Nurse, 1991). Entry into M-phase in all eukaryotic cells is controlled by the cdc2 protein kinase complexed to cyclin B. At the onset of M-phase, this kinase is activated directly through tyrosine 15 dephosphorylation by the cdc25 tyrosine phosphatase (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b; Strausfeld *et al.*, 1991; Lee *et al.*, 1992). In fission yeast, it has been demonstrated that disruption of the cdc25 control pathway removes the dependence of mitotic initiation on completion of DNA synthesis (Enoch and Nurse, 1990). Although it has been reported that the situation is different in budding yeast (Amon *et al.*, 1992; Sorger and Murray, 1992), a cdc25-dependent mechanism is conserved in higher animal cells, because tyrosine phosphorylation and inactivation of cdc2 kinase in interphase has been documented in several vertebrate species, including *Xenopus* (Dunphy and Newport, 1989), chicken (Krek and Nigg, 1991) and human (Draetta *et al.*, 1988). Therefore, understanding how cdc25 activity is regulated is of crucial importance. From this standpoint, the cloning of *Xenopus* cdc25 genes was important, because *Xenopus* egg extracts are an ideal system to investigate biochemically the regulation of cell cycle control elements. We demonstrate here that cdc25 activity oscillates in the *Xenopus* embryonic cell cycle, and this oscillation is caused by phosphorylation and dephosphorylation of the protein itself.

It has been reported that in *Drosophila* and fission yeast, the cellular concentration of cdc25 mRNA and protein increases as cells approach M-phase, and it has been suggested that the timing of mitosis is by the periodic accumulation of the cdc25 mitotic inducer (Edgar and O'Farrell, 1989; Moreno *et al.*, 1990). However, it is unlikely that such transcriptional regulation occurs in higher eukaryotes, because the cdc25 polypeptide remains relatively constant in *Xenopus* oocytes and eggs (this study; Jessus and Beach, 1992) and in human cells (Millar *et al.*, 1991a). This finding is consistent with previous observations that translation of the mRNA for cyclin B alone is sufficient to drive the *Xenopus* embryonic cell cycle (Murray and Kirschner, 1989; Izumi and Maller, 1991).

We have found that the cdc25 protein is localized in the cytoplasm in resting oocytes. This is surprising because cdc25 contains a sequence that is highly homologous to the nuclear targeting sequence of nucleoplasmin. Therefore, if this region in cdc25 acts as a nuclear targeting sequence, it may be masked in oocytes by another protein or it may be associated with a structure that prevents its uptake into the nucleus. We do not know when cdc25 enters the nucleus, if both cdc25 and cdc2 must accumulate there before induction of M-phase. Potential phosphorylation sites around the presumptive nuclear targeting sequence are very intriguing, because phosphorylation might have a role in nuclear targeting and regulate the timing of translocation in the cell cycle, as in the case of SWI5 (Moll *et al.*, 1991).

We have shown that phosphorylation increases the activity of the cdc25 phosphatase. Phosphorylation caused a dramatic retardation in electrophoretic mobility at the G2/M transition in several different situations: oocyte maturation, cycling egg extracts, and okadaic acid-induced premature mitosis in extracts arrested in S-phase with aphidicolin. In each case, the phosphorylation and electrophoretic shift correlated with the activation of cdc25. Furthermore, the removal of phosphate with either PP1 or PP2A reduced activity and shifted the protein back to the molecular weight seen in interphase. This is the first direct evidence that phosphorylation of cdc25, and indeed of any phosphatase catalytic subunit, increases its activity, although phosphorylation of regulatory subunits or soluble inhibitor

proteins of various phosphatases have been reported to regulate phosphatase activity (for review, see Cohen, 1989). It will be of interest to determine whether phosphorylation influences activity in fission yeast, because fission yeast cdc25 is also reported to be phosphorylated (Moreno *et al.*, 1990).

It has been shown that bacterially expressed *string* protein (the *Drosophila* cdc25 homolog) can elicit the dephosphorylation and activation of cdc2 in a defined system in the absence of ATP, and it has been suggested that phosphorylation of the cdc25 protein is not absolutely required for mitosis-inducing activity (Kumagai and Dunphy, 1991). Our results are not inconsistent with this finding, because we observed that the cdc25 protein from interphase, the underphosphorylated form, is also capable of dephosphorylating and activating cdc2, albeit less efficiently than phosphorylated cdc25. Thus, it is not surprising that a relatively large amount of exogenously added unphosphorylated cdc25 protein could drive mitosis. Recently, it has been reported that purified bacterially expressed cyclin B in the absence of cdc2 can stimulate cdc25 tyrosine phosphatase activity in vitro (Galaktionov and Beach, 1991). Although it is not clear whether such regulation occurs in vivo, it is unlikely the activation of cdc25 in M-phase found in this study is due to cyclin B association, because cdc25 immunoprecipitates contained no significant H1 kinase activity and we know that cyclin B in the phosphatase assay is tightly associated with cdc2 kinase under our immunoprecipitation conditions (Izumi and Maller, 1991). Moreover, interphase cdc25 protein still had less activity than that from M-phase despite the large amount of cyclin B/cdc2 supplied as substrate in our assay. It is possible that in intact cells both mechanisms could be operative, contributing to a concerted activation of cdc25.

Recently, a 72-kDa protein in *Xenopus* oocytes was reported to react with an antibody to *Schizosaccharomyces pombe* cdc25 (Jessus and Beach, 1992). However, that antibody did not detect a shift in the 72-kDa protein during M-phase. Currently, we do not know the reason for this discrepancy. Because multiple cdc25 homologs have been found in humans (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991), that antibody might recognize the product of a different cdc25 homolog than the one described here, or the cross-reacting epitopes recognized by the antibody to *S. pombe* cdc25 might be masked when *Xenopus* cdc25 is highly phosphorylated.

In addition to the inhibitory effect of PP1 and PP2A on cdc25 activity in vitro, we found that the addition of okadaic acid, a potent inhibitor of PP1 and PP2A, leads to phosphorylation and activation of the cdc25 protein in DNA/aphidicolin S-phase-arrested extracts. This finding strongly suggests that okadaic acid-sensitive phosphatases physiologically regulate cdc25 protein activity in vivo. From our current results, we cannot determine which phosphatase is involved in cdc25 regulation. The concentration of okadaic acid required based on the different sensitivity of PP1 and PP2A is not informative, because concentrated egg extract contains high levels of both phosphatases, and differences in sensitivity to okadaic acid of PP1 and PP2A are evident only in highly diluted extracts (Cohen, 1989; Lee *et al.*, 1991).

It has been found that INH, originally identified as an endogenous activity that inhibits the spontaneous activation of pre-maturation promoting factor in *Xenopus* oocyte extracts (Cyert and Kirschner, 1988), is a form of PP2A and that both partially purified INH and PP2A can dephosphorylate a specific site on cdc2, probably threonine 161, and inactivate its kinase activity in vitro (Lee *et al.*, 1991), although the existence of such regulation in vivo is not certain. However, the release by okadaic acid from a DNA/aphidicolin block shown in this study cannot be explained simply by release of an inhibitory effect of phosphatase on cdc2 itself, because phosphorylation and activation of cdc25 protein preceded cyclin B/cdc2 activation. INH or PP2A might be an inhibitor of the cdc25 protein in vivo. Our results are consistent with previous findings (Gautier *et al.*, 1991), indicating that addition of both exogenous *string* protein and okadaic acid accelerate activation of cdc2 kinase additively, because we have shown here the phosphorylated form of the cdc25 protein induced by okadaic acid is more active than the dephosphorylated form.

Our results are also consistent with the involvement of PP1 as a potential cdc25 regulator. Involvement of PP1 in mitotic control is suggested by findings that mutations in PP1 cause mitotic abnormalities, such as defective chromosome disjoining, that might reflect G2/M uncoupling (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990). Furthermore, it has been found that PP1 activity decreases shortly before the onset of mitosis in *Xenopus* egg extracts, whereas it remains high in DNA/aphidicolin-arrested extracts (Walker *et al.*, 1992). Moreover, exogenously added inhibitor-2 causes early entry into mitosis and overcomes a DNA/aphidicolin block efficiently (Walker *et al.*, 1992).

We have found that phosphorylation of the cdc25 protein and elevation of total H1 kinase activity correlate well both in oocytes and eggs. Therefore, one candidate for a kinase that phosphorylates and activates the cdc25 protein would appear to be cdc2 kinase itself. The association of cdc25 and cyclin B/cdc2 has been reported, particularly in M-phase (Galaktionov and Beach, 1991; Gautier *et al.*, 1991; Jessus and Beach, 1992). The cdc25 sequence from all species thus far reported contains numerous potential Ser-Pro and Thr-Pro phosphorylation sites, although these are almost exclusively in its nonconserved N-terminal region. In fact, we recently found that cyclin B/cdc2 can phosphorylate and cause the electrophoretic shift in the cdc25 protein in vitro (Izumi and Maller, unpublished observation). In our experi-

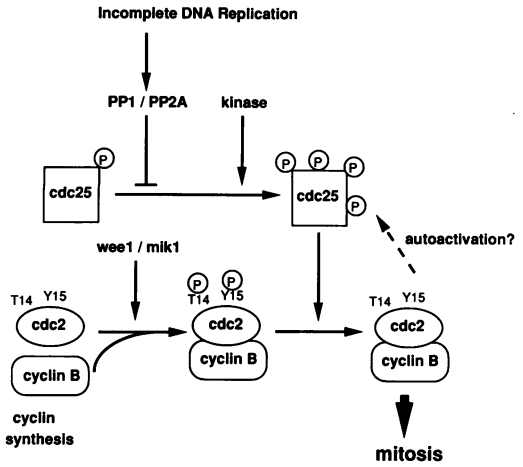

Figure 7. Model for activation of cdc25 at the G2/M transition. See text for discussion. The phosphatase responsible for inactivation of the cdc25 protein may be PP1 or PP2A or both. Kinase represents an unidentified kinase that may trigger the initial activation of the cdc25 protein by phosphorylation. Once activated, cyclin B/cdc2 complexes may contribute to further cdc25 activation in a positive (autoactivation) feedback loop.

ments, cyclin A/cdc2 H1 kinase activity was already detectable when okadaic acid stimulated cdc25 phosphorylation and activity, and thus cyclin A/cdc2 could be the kinase phosphorylating the regulatory sites in cdc25. There are also several other kinases active in interphase in *Xenopus* oocytes and eggs, including c-mos^{xe} and cdk2, that potentially could phosphorylate and activate cdc25. If kinases able to phosphorylate cdc25 are already active in interphase, this implies inhibition of PP1 or PP2A could be sufficient to trigger activation of cdc25.

The biochemical steps involved in the cdc25-dependent activation of the cdc2 protein kinase are summarized in Figure 7. The model proposes activation of cdc25 by phosphorylation is a key step in catalyzing the dephosphorylation of tyrosine 15 and threonine 14 in cdc2, leading to activation of cyclin B/cdc2 kinase activity and entry into mitosis. The phosphorylation and activation of cdc25 is blocked by the presence of incompletely replicated DNA. This could occur by persistent elevation of PP1/PP2A (Walker *et al.*, 1992) or by inhibition of the protein kinase(s) responsible for phosphorylation of cdc25, or both. For further understanding of the checkpoint ensuring mitosis is not initiated until completion of DNA synthesis, it is clearly important to characterize in detail the kinase(s) and phosphatase(s) acting on cdc25 and to evaluate potential regulation of cdc25 localization during the cell cycle.

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