Molecular Biology of the Cell
Vol. 5, 323–338, March 1994

Inhibition of cdc2 Activation by INH/PP2A

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Submitted November 11, 1993; Accepted January 26, 1994
Monitoring Editor: Tim Hunt

INH, a type 2A protein phosphatase (PP2A), negatively regulates entry into M phase and the cyclin B-dependent activation of cdc2 in *Xenopus* extracts. INH appears to be central to the mechanism of the trigger for mitotic initiation, as it prevents the premature activation of cdc2. We first show that INH is a conventional form of PP2A with a Bα regulatory subunit. We next explore the mechanism by which it inhibits cdc2 activation by examining the effect of purified PP2A on the reaction pathways controlling cdc2 activity. Our results suggest that although PP2A inhibits the switch in tyrosine kinase and tyrosine phosphatase activities accompanying mitosis, this switch is a consequence of the inhibition of some other rate-limiting event. In the preactivation phase, PP2A inhibits the pathway leading to T161 phosphorylation, suggesting that this activity may be one of the rate-limiting events for transition. However, our results also suggest that the accumulation of active cdc2/cyclin complexes during the lag is only one of the events required for triggering entry into mitosis.

INTRODUCTION

Entry into M phase in eukaryotic cells depends upon a cytoplasmic factor called maturation-promoting factor (MPF) (Miake-Lye *et al.*, 1983; Gerhart *et al.*, 1984). This activity was originally defined by its ability to induce M phase events when injected into G2-arrested immature *Xenopus* oocytes (Masui and Markert, 1971; Smith and Ecker, 1971). MPF was unique among other inducers of oocyte maturation in its ability to rapidly induce maturation in the absence of new protein synthesis. In addition, upon the induction of meiosis, much more MPF could be extracted from the oocyte than the amount initially injected, suggesting that the injected MPF had activated a latent pool of MPF, which was termed preMPF (Wasserman and Masui, 1975; Cyert and Kirschner, 1988). MPF was later demonstrated to be a protein kinase complex composed of $p34^{cdc2}$ and cyclin B (Evans *et al.*, 1983; Arion *et al.*, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Lohka *et al.*, 1988; Booher *et al.*, 1989; Labbé *et al.*, 1989), and preMPF was shown to correspond to a pool of cyclin/cdc2 complexes phosphorylated on sites that inhibit its activity (Dunphy and Newport, 1989; Gautier *et al.*, 1989).

To study the mechanism of this posttranslational activation of MPF, Cyert and Kirschner (1988) developed an in vitro system that mimicked the amplification observed in vivo. They demonstrated that a small amount of active MPF can activate preMPF in the oocyte extract. However, removal of an inhibitor fraction from oocyte extracts allowed the activation of preMPF even in the absence of added MPF. Adding back this inhibitor fraction to preMPF blocked the spontaneous activation of preMPF, demonstrating the presence of a critical factor in the oocyte that prevented the activation of MPF and thus locked the oocyte in interphase. This putative inhibitor was called INH.

We purified INH and identified it as a type 2A protein phosphatase, PP2A (Lee *et al.*, 1991), one of the four major classes of serine-threonine protein phosphatases (reviewed in Cohen, 1989). Okadaic acid, a potent and highly specific inhibitor of PP2A (Bialojan and Takai, 1988), induces premature entry into both mitosis and meiosis when added to interphase cells or extracts (Goris *et al.*, 1989; Felix *et al.*, 1990), supporting the hypothesis that INH/PP2A is a key negative regulator of M phase induction. However, because PP2A has a broad substrate specificity in vitro, the target of PP2A crucial for M phase regulation has not yet been identified. Because the cyclin B/$p34^{cdc2}$ protein kinase complex is the key regulator of M phase, PP2A must directly or indirectly regulate its activity. Consistent with this hypothesis, PP2A was shown to delay the activation of cdc2 after the addition of cyclin B to *Xenopus* extracts (Solomon *et al.*, 1990).

Several key features of the activation of cdc2 in interphase *Xenopus* extracts have been elucidated biochemically. Unlike oocyte extracts, which contain a stockpile of cyclin B in a complex with $p34^{cdc2}$ (Dunphy and Newport, 1989; Gautier *et al.*, 1989), interphase extracts from activated eggs contain monomeric $p34^{cdc2}$ and are devoid of cyclin B unless protein synthesis is allowed to occur (Minshull *et al.*, 1989; Murray and Kirschner, 1989). Addition of a threshold level of cyclin B protein is both necessary and sufficient to drive the extract into mitosis, providing a powerful in vitro system to study the biochemical events involved in the activation of cdc2 (Minshull *et al.*, 1989; Murray and Kirschner, 1989).

Solomon *et al.* found that there is an ~20-min lag between cyclin accumulation and $p34^{cdc2}$ activation (Solomon *et al.*, 1990). During the lag, $p34^{cdc2}$ binds to cyclin. This event is a prerequisite for further posttranslational modifications on two independent sets of phosphorylation sites. One is an activating phosphorylation on threonine 161, catalyzed by CAK ($p34^{cdc2}$ activating kinase) kinase (Solomon *et al.*, 1992; Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993), that is absolutely required for the kinase activity of $p34^{cdc2}$ (Booher and Beach, 1986; Gould *et al.*, 1991). The other is a pair of inhibitory phosphorylations on threonine 14 and tyrosine 15 (Labbe *et al.*, 1988; Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989). In fission yeast, the tyrosine 15 phosphorylation is controlled by cdc25 (reviewed in Millar and Russell, 1992) and wee1/mik1 (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren *et al.*, 1991). cdc25 dephosphorylates both of the inhibitory sites (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Strausfield *et al.*, 1991), whereas wee1 appears to phosphorylate only tyrosine 15 (Parker and Piwnica-Worms, 1992; Booher *et al.*, 1993; McGowan and Russell, 1993). Therefore, there may be a kinase distinct from wee1 in *Xenopus* extracts that phosphorylates threonine 14, although the identity of the kinase is not known (though it could be mik1). During interphase, the tyrosine kinase activity (wee1/mik1) is high and the phosphatase activity (cdc25) is low (Solomon *et al.*, 1990; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Smythe and Newport, 1992), allowing $p34^{cdc2}$ to accumulate predominantly in the tyrosine-phosphorylated, inactive state. During the lag, some process opposing the negative regulation by PP2A triggers the activation of the tyrosine phosphatase and the inactivation of the tyrosine kinase. These changes lead to the sudden dephosphorylation of $p34^{cdc2}$ on the inhibitory sites, resulting in its abrupt conversion to the mitotic form, which is unphosphorylated on threonine 14 and tyrosine 15 but retains threonine 161 phosphorylation (Solomon *et al.*, 1990).

Addition of okadaic acid to the interphase extract drastically alters the kinetics of cdc2 activation by cyclin (Felix *et al.*, 1990; Solomon *et al.*, 1990). In the presence of okadaic acid, cdc2 activation occurs at subthreshold levels of cyclin without a lag phase. Conversely, increasing the level of PP2A in the interphase extract delays cdc2 activation and raises the threshold for cyclin (Solomon *et al.*, 1990). These observations demonstrate the importance of PP2A in allowing cdc2 kinase to accumulate in an inactive state until a threshold level of cyclin has accumulated. What remains to be elucidated is how PP2A prevents the immediate and premature activation of cdc2 and how cyclin overcomes the negative regulation by PP2A.

One possibility is that cyclin and PP2A antagonize one another during the lag to control the activities of cdc25 and wee1, which cooperate to keep the cyclin B/$p34^{cdc2}$ complex inactive. PP2A might prevent the activation of cdc25 and the inactivation of wee1, whereas cyclin would promote these processes. A threshold level of cyclin would normally be required to overcome the inhibition by PP2A. However, in the absence of PP2A activity, low levels of cyclin could suffice to turn on cdc25 and turn off wee 1 relatively quickly. Studies on the regulation of cdc25 and wee 1 have, in fact, pointed to an essential role for PP2A in maintaining the low level of cdc25 activity as well as the high level of wee1/mik1 activity (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Smythe and Newport, 1992). According to this proposal, the target of PP2A would be cdc25 or wee1, or a regulator of cdc25 or wee1.

An alternative explanation is that PP2A regulates the state of threonine 161 phosphorylation, either directly or indirectly. Because threonine 161 phosphorylation is absolutely essential for the kinase activity of cdc2, the extent of phosphorylation on this site could be a rate-limiting step. To better define the target(s) of PP2A, we needed to distinguish among these possibilities. In this study, we examined the effect of PP2A on the rate of phosphorylation and dephosphorylation of both the inhibitory and activating phosphorylation sites of cdc2. Because the level of PP2A activity appears to determine the length of the lag, the initial event, which opposes PP2A and eventually overcomes PP2A, must occur during the lag. Therefore, we examined the effect of PP2A on these potential targets immediately after cyclin addition, up to the time of $p34^{cdc2}$ activation. The results presented here indicate that PP2A affects neither cdc25 nor wee1/mik1 activities during the lag, but does affect threonine 161 phosphorylation. Furthermore, in a test of the hypothesis that a critical threshold level of active cdc2 kinase can activate a positive feedback loop turning on cdc25 and turning off wee1, we demonstrate the insufficiency of active cdc2 for this process.

MATERIALS AND METHODS

Extracts and H1 Kinase Assays

Interphase extracts were prepared from eggs lain into MMR (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA pH 7.8). After washing and dejellying, eggs were incubated in 100 μg/ml cycloheximide in MMR for 30 min before activation with

the calcium ionophore A23187 (2 μg/ml) in MMR. Cycloheximide was included at 100 μg/ml in all subsequent buffers. Ten to twenty minutes after activation, the eggs were washed in MMR, followed by washes in XB (100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM sucrose, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] pH 7.7), and finally into XB + protease inhibitors (10 μg/ml each of pepstatin, leupeptin, and chymostatin). All subsequent steps were carried out exactly as previously described (Murray *et al.*, 1989; Solomon *et al.*, 1990), and extracts were stored in aliquots at −80°C. All of the activation reactions were carried out at room temperature. H1 kinase assays were performed by adding 6 μl of H1-ATP mix (200 μM ATP, 0.5 μci/μl ^{32}P-γ-ATP, and 500 μg/ml histone H1) to 10-μl beads or 12 μl of reaction mixtures diluted 1:25 into EB (80 mM β-glycerophosphate, 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 15 mM $MgCl_2$ pH 7.3). One micromolar okadaic acid was added to the ^{32}P-ATP-H1 mix to prevent dephosphorylation of H1 by PP2A during the kinase assay. Assays were carried out at room temperature for 5–10 min (within the linear range).

Proteins and Reagents

Okadaic acid was purchased from Calbiochem. Expression of wild-type and mutant HA epitope-tagged *Xenopus* $p34^{cdc2}$ in reticulocyte lysates has been described (Gautier *et al.*, 1991; Solomon *et al.*, 1992). The 12CA5 ascites (against the HA epitope) was generated at University of California, San Francisco (UCSF) by Jim Berkhart with permission from Ian Wilson at the Scripps Clinic, San Diego. Immunoprecipitations using the ascites were performed essentially as described (Solomon *et al.*, 1992). The glutathione S-transferase (GST) cyclin B1 fusion construct containing an N-terminal fusion of GST to *Xenopus* B1 cyclin was cloned into the *Eco*RI site of pGEX-1 and was kindly provided to us by D. Kellog at UCSF. The expression and purification of the fusion protein followed their procedure (Kellogg and Murray, unpublished data). The purified *Drosophila* cdc25 protein, generously provided by Y. Gu and D. Morgan at UCSF, was expressed in *E. coli*, purified from inclusion bodies, and renatured as described in Gautier *et al.* (1991). ^{32}P-γ-ATP (150 mci/ml, crude) used in the labeling reactions was purchased from New England Nuclear (Boston, MA). Free glutathione and glutathione agarose beads were from Sigma (St. Louis, MO). The beads were collected for washing typically by spinning in a microcentrifuge at 14 000 × *g* for ~5 s.

Partial Purification of INH

INH was purified from egg extracts arrested in interphase. The initial steps of the purification, up to the first DEAE step, were carried out as in Solomon *et al.* (1993). M. Solomon (Yale University) most kindly provided us with 500 mg of the 150 mM to 1 M NaCl eluate from his DEAE column. The eluate was concentrated with ammonium sulfate, resuspended in buffer A (20 mM tris(hydroxymethyl)-aminomethane [Tris] pH 8.0, 7 mM EGTA, 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol [DTT], and protease inhibitors as described in Lee *et al.* (1991), and rerun on a second 100-ml DEAE sepharose column equilibrated in the same buffer. Bound proteins were eluted with a 500 ml 50–500 mM NaCl gradient. INH activity, assayed as described in Lee *et al.* (1991), eluted between 240–300 mM NaCl. Peak fractions were precipitated with 80% ammonium sulfate, and the resulting precipitates were collected in a 30-min spin at 10 000 rpm in a JA 20 rotor (Beckman Instruments, Palo Alto, CA). The pellets were resuspended in buffer A, adjusted to 1 M ammonium sulfate, and loaded onto a 10-ml tyrosine agarose column. Bound proteins were eluted with a 400 ml 1–0 M ammonium sulfate gradient. INH activity eluted between 550 and 450 mM ammonium sulfate. Peak fractions were concentrated by ammonium sulfate precipitation as described above and dialyzed against XB + 1 mM DTT + protease inhibitors (10 μg/ml each of pepstatin, leupeptin, and chymostatin). The tyrosine agarose step yielded an ~30-fold purification over the DEAE step (see Lee *et al.*, 1991). The materials used for the experiments in this paper were ≥1000-fold purified.

Microsequencing

For final purification, the protein mixture in the tyrosine agarose fractions containing the peak of INH activity was resolved further by a 5–20% sucrose density gradient. The peak INH fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10%), and proteins were visualized by a brief Coomassie blue staining. The major band corresponding to the ~54-kDa B subunit was recovered by electroelution, precipitated with acetone, and redissolved in 0.1 M NH_4HCO_3. Cleavage of the protein was then carried out by the addition of 1 μg trypsin and incubation at 37°C for 12 h. Tryptic cleavage fragments were separated on a C_{18}-reversed phase column, and selected peaks were subjected to protein sequence analysis with an Applied Biosystems Sequencer, Model 475A (Foster City, CA).

Generation of the T161 Phosphorylated cdc2/cyclin Complex

CAK activity, purified >1800-fold, as described in Solomon *et al.*, 1993) and generously provided by M. Solomon, was used to phosphorylate reticulocyte-translated cdc2 (as described in Solomon *et al.*, 1992). Briefly, 134 μl of reticulocyte lysate cdc2, containing the lys 33 to arg 33 mutation at ~10 ng/μl, was mixed with 15 μl of a 50 μg/ml solution of CAK (purified over DEAE, ammonium sulfate, hydroxyapatite, and mono Q) (see Solomon *et al.*, 1993) in the presence of 134 μl ^{32}P-γ-ATP (150 μci/μl, New England Nuclear), 15 μl of GST-cyclin B (50 nM, final concentration), and 30 μl of 10× XB (100 mM HEPES pH 7.7, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 880 mM KCl). After incubation at room temperature for 30 min, the material was mixed with 300 μl of glutathione agarose beads for 20 min at room temperature with rotation. After binding, the beads were washed extensively with XB^{++} (XB + 0.5 M NaCl + 0.1% NP40) + 1 mM DTT until the washes were free of counts (~10 × 15 ml), followed by ~2 × 15-ml washes in XB + 1 mM DTT. The complex was then eluted with 2 ml of XB containing 5 mM free glutathione + 0.1 mg/ml bovine serum albumin (BSA) + 1 mM DTT. The eluate was concentrated to 40 μl in a C-30 Amicon microconcentrator (Dancers, MA).

Generation of the Y15-phosphorylated cdc2/cyclin Complex

cdc2/cyclin complexes labeled only on the inhibitory phosphorylation sites (Y15 and most likely also T14) were generated in a two-stage reaction. In the first stage, 18 μl of interphase extract was diluted to 60 μl with XB containing 50 nM GST-cyclin B (final concentration). After incubation for 30 min at room temperature, the activated complexes, phosphorylated on T161 (Solomon *et al.*, 1990), were bound to 60 μl of glutathione agarose beads for 30 min at room temperature with rotation, followed by two washes in 15 ml of XB^{++} + 1 mM DTT and two washes in 15 ml of XB + 1 mM DTT. The bead-bound active complexes were labeled on the inhibitory sites by a second stage incubation with 36 μl of interphase extract + 67 μl of ^{32}P-γ-ATP (150 μci/μl) + 12 μl of 10× XB. After extensive washing, the labeled inactivated complexes were eluted and concentrated to 50 μl exactly as described above for the ^{32}P-T161–phosphorylated substrate. In addition, we confirmed the 34-kDa labeled protein to be cdc2 by demonstrating its high affinity to $p13^{suc1}$ beads (Solomon *et al.*, 1990).

Gel Electrophoresis and Immunoblotting

SDS–PAGE for all of the experiments (except the T161 phosphorylation assay) were performed on 5–15% gradient gels. Separation of the T161 phosphorylated species from unphosphorylated $p34^{cdc2}$ was performed on 10% polyacrylamide gels. An LKB semidry blotting apparatus (Piscataway, NJ) was used to transfer proteins onto 0.22-μm nitrocellulose membranes (0.9 milliamp h/cm^2). The anti-cdc2 blot was performed using antiserum generated against a *Xenopus* cdc2 C-terminal peptide, diluted 1:200. Antibody incubations were in 5% BSA + 0.1% Tween in 10 mM Tris pH 8.0, 150 mM NaCl; washes

were in Tris/NaCl. Anti-phosphotyrosine blots were performed in the continuous presence of 5% nonfat dry milk + 0.1% Tween, in 10 mM Tris pH 8.0, 150 mM NaCl. Five hundred millimolar NaCl was included in the washes after the primary antibody (4G10 at 1 μg/ml, UBI, Lake Placid, NY) incubation. Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Cappel, Cochraneville, PA) was used at a 1:10 000 dilution. Immunodetection was performed using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Twofold dilutions of tyrosine-phosphorylated $p34^{cdc2}$ were used to assess the linear range for the detection system.

RESULTS

INH Is a Conventional PP2A Holoenzyme

Our previous studies raised the possibility that INH might be a special form of PP2A. The finding that PP2A holoenzyme preparations from porcine cardiac tissue appeared to possess relatively little INH activity, whereas the catalytic subunit from the same tissue was active, led us to hypothesize that INH might be a special embryonic form of PP2A that enabled the embryo to undergo rapid cleavage divisions (see Cohen, 1989 for review; Lee *et al.*, 1991). We took two approaches to this question: 1) determining whether INH activity is unique to the oocyte and early embryo and 2) determining the molecular composition of INH. To address the first question, we prepared a crude extract from oocyte and adult liver tissue, fractionated each by DEAE sepharose chromatography, and determined the peak of PP2A activity using a conventional substrate (myosin light chain). We then assayed the peak fractions for INH activity. If INH in the oocyte were a special form of PP2A, we would have expected oocyte PP2A fractions, when compared to adult liver PP2A fractions, to possess a higher level of INH activity relative to myosin light chain phosphatase activity. The comparison indicated that adult liver PP2A was just as active in the INH assay as the oocyte PP2A. In addition, INH activity in both extracts copurified with the single major peak of PP2A activity on DEAE chromatography. These results suggested that INH activity is not unique to the oocyte and probably occurs throughout development; furthermore, INH appears to be the same as the major form of PP2A in oocytes as well as in the adult liver.

To address the second point, we purified INH from activated eggs by DEAE and tyrosine agarose chromatography, followed by sucrose density gradient centrifugation. Three major polypeptides copurified with INH (Figure 1A) and corresponded in molecular weight to the known A (~64 kDa) and B (~54 kDa) regulatory subunit and the catalytic subunit C (~37 kDa) of conventional PP2A (see Cohen, 1989 for review). The identity of the catalytic subunit was confirmed by immunoblotting with a monoclonal antibody against the catalytic subunit of PP2A (as in Lee *et al.*, 1991), and the identity of the polypeptide corresponding to the B subunit was confirmed by immunoblotting with a polyclonal sera directed against an N-terminal peptide of the Bα isoform of mammalian PP2A (Mayer *et al.*, 1991).

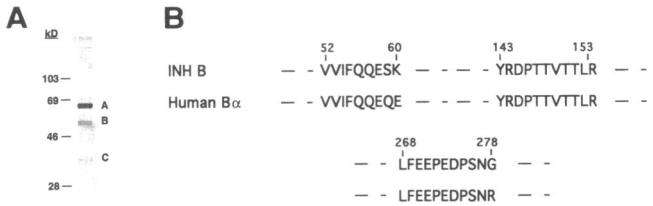

Figure 1. Sequence comparison of the B subunit of INH with the Bα subunit of human PP2A. (A) INH purified from *Xenopus* eggs as described in RESULTS and MATERIALS AND METHODS, resolved on SDS-PAGE, and silver stained. Polypeptides corresponding to the A, B, and C subunits are indicated. (B) The sequences of three tryptic fragments obtained from the B subunit of INH are aligned with the amino acid sequence of the Bα isoform of human PP2A (Mayer *et al.*, 1991).

Gel-purified material corresponding to the B subunit was obtained and cleaved with trypsin. The tryptic fragments were resolved by high-performance liquid chromatography and subjected to amino acid sequence analysis (see MATERIALS AND METHODS). Sequence information from three tryptic fragments was obtained, and comparison to the cloned sequences of the B subunit isoforms of human PP2A (Mayer *et al.*, 1991) indicated a high degree of amino acid sequence identity between the *Xenopus* egg B subunit and the Bα isoform of human PP2A (Figure 1B). In conclusion, INH appears to be a conventional PP2A holoenzyme.

PP2A Does Not Inhibit the Tyrosine Phosphatase During the Lag

PP2A most likely prevents the premature activation of cdc2 by modulating one or more steps that are part of the trigger for mitotic initiation. In this and the following sections, we examine the reaction pathways that are known to control cdc2 activity for those that are modulated by PP2A during the lag with the intent of identifying the rate determining step(s) for the initiation of cdc2 activation. Increasing the level of PP2A in the interphase extract by the addition of purified PP2A results in the inhibition of cdc2 activation (Solomon *et al.*, 1990; Clarke *et al.*, 1993). $p34^{cdc2}$ accumulates in the threonine 14 tyrosine 15 (T14Y15) phosphorylated form (Clarke *et al.*, 1993; Lee and Kirschner, unpublished data), suggesting that the inhibition by PP2A is because of a failure to activate the T14Y15 phosphatase and/or a failure to inactivate the T14Y15 kinase. To assess whether this is because of direct regulation of the T14Y15 kinase(s) and phosphatase(s) by PP2A, we asked whether these enzyme activities are modulated by PP2A.

To measure the rate of tyrosine dephosphorylation on Y15 of cdc2, we generated a substrate that was radiolabeled stoichiometrically only on the inhibitory sites, Y15 and T14 (MATERIALS AND METHODS). To generate this labeled substrate, we first activated cdc2 in an interphase extract with a nondegradable GST fusion of X. *laevis* cyclin B1 (generously provided by Doug

Kellogg), and we then isolated the active complex of $p34^{cdc2}$ and cyclin on glutathione agarose beads. The active form of the kinase is phosphorylated on threonine 161 (T161) but not on the inhibitory T14Y15 sites as assessed by peptide mapping (Gould *et al.*, 1989, 1991; Solomon *et al.*, 1990; Krek and Nigg, 1992) and by probing here with an antiphosphotyrosine antibody (Figure 2A, lane 1). As we will demonstrate below, the phosphate in T161 (unlabeled here) is resistant to the action of phosphatases in the extract, and therefore T161 cannot be labeled in subsequent incubations. To label the inhibitory sites, the active cdc2 kinase complex was mixed with an interphase extract in the presence of ^{32}P-γ-ATP but in the absence of additional free cyclin. This incubation resulted in the rapid inactivation of the glutathione agarose bound cdc2/cyclin complex and tyrosine phosphorylation of $p34^{cdc2}$, as determined by Western blotting with the antiphosphotyrosine antibody (Figure 2A, lane 2). Finally, the phosphorylated substrate was reisolated on glutathione beads and eluted with free glutathione. The ^{32}P label in cdc2 (compare lanes 4 and 5 in Figure 2B), as well as the immunoreactivity to the phosphotyrosine antibody (Figure 2A, lane 3), was removed by incubating the complex with bacterially expressed and purified *Drosophila* cdc25 (Edgar and O'Farrell, 1989; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Strausfield *et al.*, 1991). Because cdc25 is highly specific in its ability to dephosphorylate cdc2 on T14 as well as Y15 (see Millar and Russell, 1992 for review), the removal of phosphate by cdc25 is indicative of phosphorylation on either or both T14 and Y15. Furthermore, because cdc25 is most likely responsible for both T14 and Y15 dephosphorylation (Millar and Russell, 1992), the rate of removal of the ^{32}P label from cdc2 should reflect the rate of removal of phosphotyrosine.

As expected from previous studies (Solomon *et al.*, 1990; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), the phosphorylated substrate is relatively stable in interphase extracts ($k \sim 0.009\ min^{-1}$) and is more rapidly dephosphorylated in mitotic extracts ($k \sim 0.052\ min^{-1}$, Figure 2C). The addition of okadaic acid to an interphase extract in the absence of cyclin does not enhance the dephosphorylation of the substrate ($k \sim 0.007\ min^{-1}$, Figure 2C), suggesting that modulation of cdc25 activity requires the presence of cyclin in the extract. To measure the rate of tyrosine dephosphorylation during the normal course of mitotic activation, we added GST-cyclin B to an interphase extract that contained the labeled substrate. Thus the labeled cyclin/cdc2 complex was a tracer for the events occurring on the unlabeled cdc2. At various time points, an aliquot was taken to assay H1 kinase activity, and another aliquot was removed to measure the phosphorylation state of the substrate after quenching with SDS sample buffer. As shown in Figure 3, the rate of tyrosine dephosphorylation during the lag is low ($k \sim 0.006\ min^{-1}$), reflecting the interphase rate (the difference in this rate compared to the

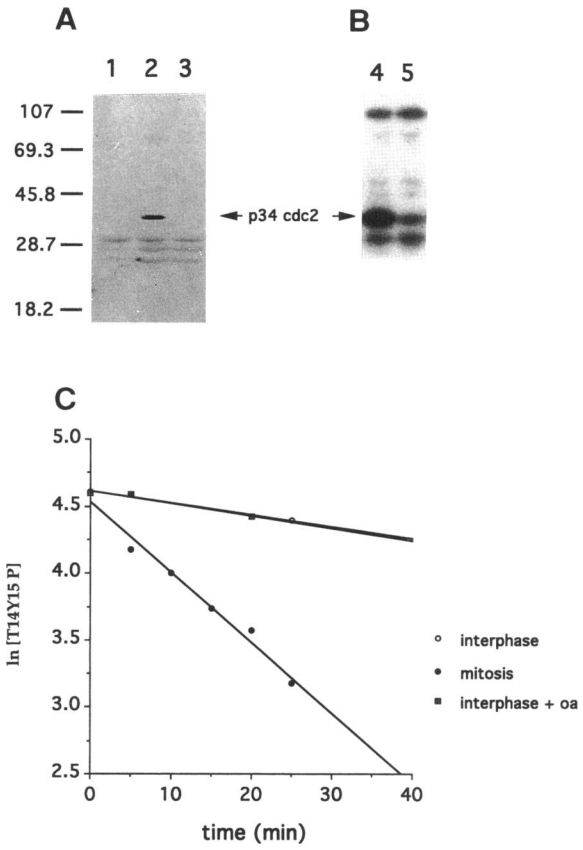

Figure 2. Generation of the tyrosine-phosphorylated cdc2 substrate (as described in MATERIALS AND METHODS). (A) Phosphotyrosine blot of (lane 1) glutathione agarose-bound cyclin/cdc2 complex in the first stage, (lane 2) the complex after the second stage incubation, and (lane 3) the complex after a 30-min incubation at room temperature with 3 μM bacterially expressed *Drosophila* cdc25 in XB. (B) The complex is ^{32}P-labeled on tyrosine 15 and probably threonine 14. The ^{32}P label in the complex after the second stage incubation (lane 4) is efficiently removed upon a 30-min incubation with 3 μM *Drosophila* cdc25 in XB (lane 5). (C) The substrate is dephosphorylated rapidly in mitosis but not in interphase or in interphase in the presence of 1 μM okadaic acid. Six microliters of the ^{32}P-labeled substrate were mixed with 9 μl of a mitotic extract (7.5 μl interphase extract + 1.5 μl of 300 nM GST-cyclin B and preincubated for 30 min at room temperature), interphase extract (7.5 μl interphase extract + 1.5 μl XB), or interphase extract in the presence of 1 μM okadaic acid (7.5 μl interphase + 1.5 μl of 20 μM okadaic acid). At the indicated times, 2 μl of the mixture was removed and quenched in SDS sample buffer. The samples were resolved by SDS-PAGE and visualized by a phosphorimager system (Molecular Dynamics, Sunnyvale, CA) for quantitation.

rate given above for the interphase extract is because of variation in extracts and does not reflect a difference in rates in the presence or absence of cyclin). At the time of cdc2 activation, the tyrosine phosphatase activity increases dramatically (to $k \sim 0.061\ min^{-1}$), as has been demonstrated previously (Solomon *et al.*, 1990; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). When the same reaction was carried out in the presence of added PP2A (Figure 3), the abrupt activation of the tyrosine phosphatase did not occur, and the H1 kinase activity

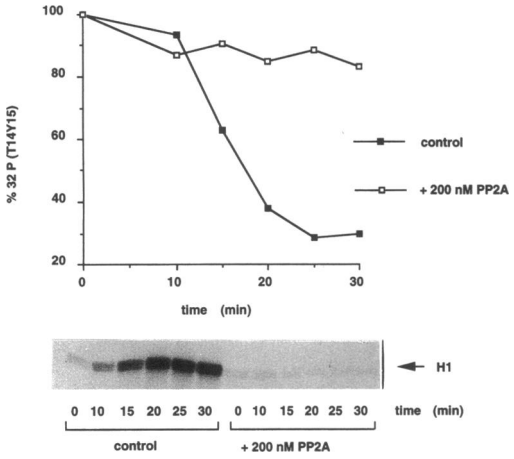

Figure 3. PP2A does not affect the tyrosine phosphatase activity during the lag. Four microliters of the tyrosine-phosphorylated substrate was mixed with 12 μl of interphase extract + 4 μl of 0.5 μM GST-cyclin B in the presence of 20 μl of either 400 nM purified PP2A in XB or XB alone. After the indicated times at room temperature, 3 μl of the mixture was removed and quenched in SDS sample buffer while 3 μl was bound to 10-μl $p13^{suc1}$ beads on ice (Solomon *et al.*, 1990) and assayed for H1 kinase activity (MATERIALS AND METHODS). The graph depicts the percentage loss of ^{32}P label from the substrate with time, whereas the lower panel shows the H1 kinase activity at each corresponding time.

of $p34^{cdc2}$ remained the same as that in the interphase extract lacking added PP2A. This result confirmed our suspicion that PP2A ultimately inhibits the activation of cdc25 (see also Clarke *et al.*, 1993). However, added PP2A had no effect on tyrosine phosphatase activity during the lag, which remained low ($k \sim 0.006$ min^{-1}) but no lower than in the control reaction ($k \sim 0.006$ min^{-1}). Thus during the lag phase, the tyrosine phosphatase activity is not further inhibited by PP2A, and therefore it appears that the elevation of tyrosine phosphatase activity during activation is a response to a different rate limiting triggering event.

PP2A Does Not Activate the Tyrosine Kinase During the Lag

Because the tyrosine phosphatase was not affected by PP2A during the lag period, we next examined the effect of PP2A on the rate of tyrosine phosphorylation. It is sufficient to measure the rate of accumulation of phosphate if the rate of phosphorylation substantially exceeds that of dephosphorylation. During the lag, before the activation of the tyrosine phosphatase, the rate of tyrosine dephosphorylation was low enough ($k \sim 0.006$ min^{-1} compared to $k \sim 0.07$ min^{-1} for the rate of accumulation of phosphotyrosine) that the accumulation of phosphotyrosine in $p34^{cdc2}$ could be used to assess the rate of tyrosine phosphorylation. At various times after cyclin addition, we measured the rate of tyrosine phosphorylation in $p34^{cdc2}$ by immunoblotting the GST-cyclin B bound material with an anti-phosphotyrosine antibody (Figure 4A; see MATERIALS AND METHODS). We also probed the blot with a general cdc2 antibody (antiserum against the C-terminal peptide of *Xenopus* cdc2) to normalize the amount of phosphotyrosine in $p34^{cdc2}$ to the amount of total $p34^{cdc2}$ protein recovered (Figure 4B). Figure 4A shows that in the control reaction the rate of increase in tyrosine phosphate on cdc2 is approximately linear for the first 10 min ($k \sim 0.07$ min^{-1}). As can be seen by comparison of the reaction with and without added PP2A, the presence of elevated levels of PP2A does not affect the rate of tyrosine phosphorylation during the initial lag period after cyclin addition. Taken together, these results demonstrate that although PP2A eventually prevents the tyrosine dephosphorylation of $p34^{cdc2}$, PP2A does not modulate the tyrosine kinase or tyrosine phosphatase during the lag.

PP2A Does Not Affect the Rate of T161 Dephosphorylation During Cyclin Activation of $p34^{cdc2}$

We had previously shown that the isolated and active $p34^{cdc2}$/cyclin complex can be directly inactivated by PP2A with the concommitant dephosphorylation of T161 (Gould *et al.*, 1991; Lee *et al.*, 1991), suggesting that PP2A could inhibit the activation of cdc2 by preventing the accumulation of T161 phosphate. A problem in the earlier experiments, however, was that a much higher concentration of PP2A was required to remove the T161 phosphorylation than was effective at blocking cdc2 activation. To address whether PP2A is the physiological phosphatase that dephosphorylates T161, we measured the rate of T161 dephosphorylation during cyclin activation of $p34^{cdc2}$ in the extract and looked at the effects of minimal inhibitory levels of PP2A. To generate a $p34^{cdc2}$ substrate that is phosphorylated solely on T161, we took advantage of the fact that purified

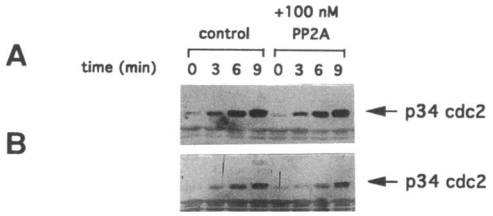

Figure 4. PP2A does not affect the tyrosine kinase activity during the lag. Twenty-four microliters of interphase extract was mixed with 16 μl of XB containing 125 nM GST-cyclin B in the presence of 40 μl of either 200 nM purified PP2A in XB or XB alone. At the indicated times, 20 μl of the mixture was removed and bound to 18 μl of glutathione agarose beads on ice for 15 min. The beads were briefly rinsed with 0.5 ml XB and mixed with SDS sample buffer. To ensure that activation occured in the control reaction but not in the PP2A-treated reaction, the H1 kinase activity was assessed after 30 min in each reaction. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with (A) the 4G10 anti-phosphotyrosine antibody and then reprobed with (B) an antiserum directed against the C-terminal peptide of *Xenopus* cdc2.

CAK activates and phosphorylates reticulocyte lysate–translated $p34^{cdc2}$ on T161 (Solomon *et al.*, 1992; Fesquet *et al.*, 1993; Poon 1993; Solomon *et al.*, 1993) in the presence of cyclin. GST-cyclin B was added to $p34^{cdc2}$ (translated in a reticulocyte lysate) in the presence of highly purified CAK (MATERIALS AND METHODS) and ^{32}P-γ-ATP. The phosphorylated complex was isolated on glutathione beads and eluted with free glutathione. To prove that the labeled 34-kDa phosphoprotein represented T161 phosphorylated $p34^{cdc2}$, we demonstrated the absence of phosphate incorporation into the alanine 161 mutant. To measure the rate of dephosphorylation, the T161 phosphorylated $p34^{cdc2}$ substrate was added into interphase extracts at a level that, by itself, had no effect on the kinetics of activation of the endogenous $p34^{cdc2}$ by cyclin B. Upon addition of cyclin B, we examined the rate of loss of labeled phosphate from the substrate during the course of activation of the endogenous $p34^{cdc2}$. As shown in Figure 5, the T161 phosphorylation is completely stable during the course of activation, even when activation is blocked by the addition of purified PP2A. Therefore, T161 dephosphorylation does not contribute to the regulation of $p34^{cdc2}$ activation, and PP2A cannot directly dephosphorylate cyclin/$p34^{cdc2}$ under these more physiological conditions.

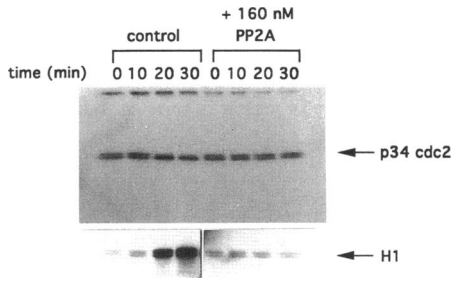

Figure 5. Threonine 161 dephosphorylation does not occur at a measurable rate in the presence or absence of added PP2A. Sixteen microliters of the ^{32}P-labeled threonine 161 phosphorylated cdc2 substrate (see MATERIALS AND METHODS) was mixed with 12 μl of interphase extract + 4 μl of 0.5 μM GST-cyclin B in the presence of 8 μl of either 500 nM purified PP2A in XB or XB alone. At the indicated times, 8 μl of the reaction mixture was removed for immunoprecipitation with the anti-HA antibody on ice (MATERIALS AND METHODS), and 1 μl was removed and diluted on ice for H1 kinase assay. The immunoprecipitated cdc2 was visualized by SDS-PAGE and autoradiography, as well as a phosphorimager system for quantitation. The top panel depicts the ^{32}P-labeled substrate with time, and the lower panel shows the H1 kinase activity at each indicated time.

T161 Phosphorylation Is Inhibited by PP2A

Because the T161 residue is not dephosphorylated during the course of activation, either in the presence or absence of PP2A, we could measure the rate of T161 phosphorylation by quantitating the level of T161 phosphorylated $p34^{cdc2}$ over time. To make this measurement, we took advantage of the fact that T161 phosphorylation confers a downward SDS gel mobility shift on $p34^{cdc2}$ (see Lorca *et al.*, 1992). By adding a trace amount of ^{35}S-methionine–labeled $p34^{cdc2}$ protein to the interphase extract, we could observe the rate of accumulation of the downward shifted form. A complicating feature of this analysis is that the downward mobility shift because of T161 phosphorylation is offset by an upward mobility shift conferred by T14Y15 phosphorylation (see Solomon *et al.*, 1990). To avoid this complication, we used the mutant in the T14 and Y15 phosphorylation sites where those sites were converted to structurally similar but nonphosphorylatable residues, alanine 14 and phenylalanine 15 (A14F15) (Gould and Nurse, 1989; Norbury *et al.*, 1991; Solomon *et al.*, 1992). The low level of AF protein (~8% of the level of the endogenous cdc2) added to the reaction had no effect on the kinetics of cdc2 activation and therefore acted simply as a marker for the state of T161 phosphorylation. At each time point in addition to taking samples for analysis of T161 phosphorylation, we also measured the H1 kinase activity in the extract. As shown in Figure 6A, the rate of T161 phosphorylation is significantly slowed by the addition of 130 nM PP2A ($k \sim 0.15$ min^{-1} to $k \sim 0.11$ min^{-1}). At higher levels of PP2A, the reaction is delayed even further (Figure 6B), suggesting that the level of PP2A in the extract determines the level of T161 phosphorylating activity. A concern raised by this result is whether the addition of PP2A to the extract, causing the level of PP2A activity to be higher than is normally experienced, reveals a physiological process. If the effect of PP2A on T161 phosphorylation were meaningful, blocking PP2A activity should lead to an acceleration of the T161 phosphorylation reaction. As shown in Figure 6C, blocking PP2A activity in the extract with 1 μM okadaic acid significantly increases the rate of T161 phosphorylation on $p34^{cdc2}$.

Because the antagonistic relationship between cyclin and PP2A is central to the mechanism of the trigger, a rate limiting event that is part of the trigger should be stimulated by cyclin in addition to being inhibited by PP2A. As shown in Figure 6D, raising the level of cyclin added to the reaction offsets the inhibitory effect of added PP2A on the initial rate of T161 phosphorylation. We can conclude from this result that cyclin and PP2A oppose one another in the regulation of T161 phosphorylation of cdc2.

Previous studies suggested that T161 phosphorylation requires prior association of $p34^{cdc2}$ with cyclin (Solomon *et al.*, 1992). In a separate experiment, we asked whether PP2A had an effect on the association of $p34^{cdc2}$ with cyclin, which could potentially account for the delay in T161 phosphorylation. To measure the rate of association, control interphase extracts or interphase extracts to which PP2A was added were spiked with ^{35}S-A14F15 $p34^{cdc2}$. GST-cyclin B prebound to glutathione agarose beads was then added. The complex could be readily isolated, and the cyclin-

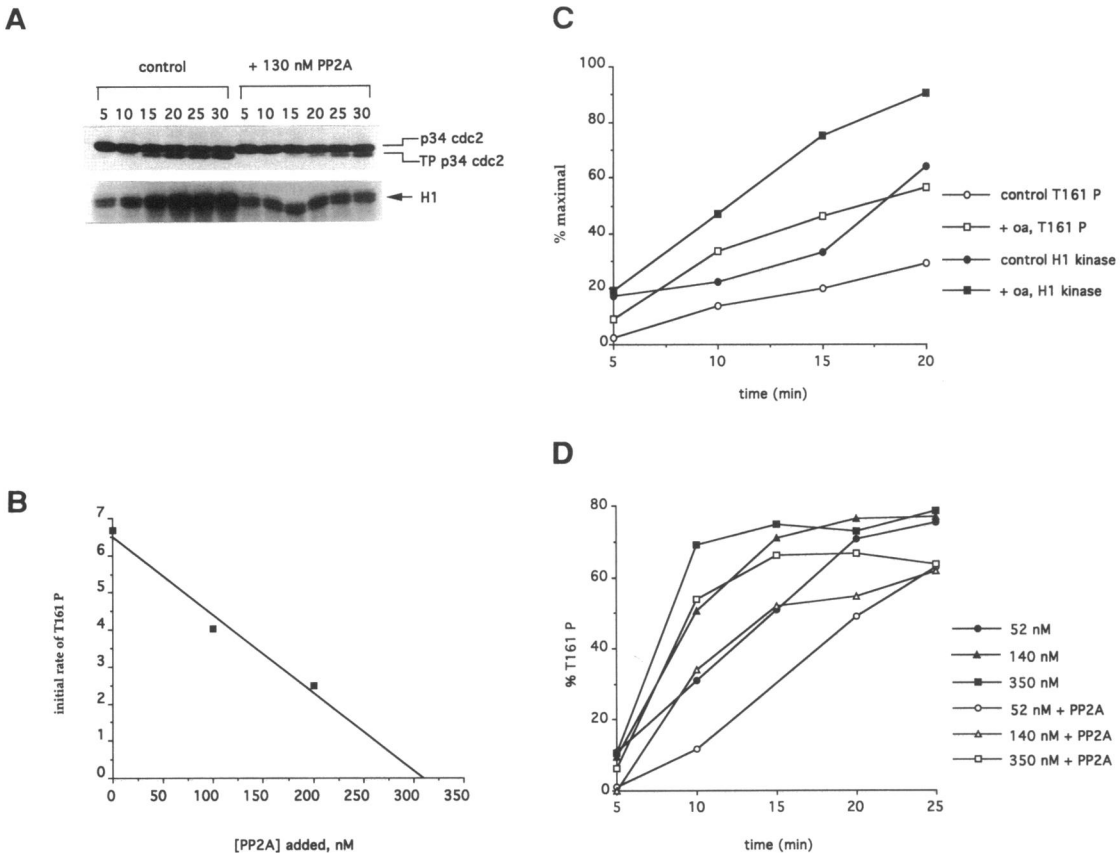

Figure 6. PP2A inhibits threonine 161 phosphorylation. (A) 0.4 μl of reticulocyte lysate that had been programmed with ^{35}S-methionine and the A14F15 cdc2 mRNA (Gautier *et al.*, 1992) and contained 10 ng/μl cdc2 protein was preincubated with 5 μl of interphase extract. After 10 min at room temperature, 1 μl of 0.5 μM GST-cyclin B was added in the presence of 4 μl of either 320 nM purified PP2A in XB or XB alone. At the indicated times, 0.5 μl of the reaction mixture was quenched with SDS sample buffer, and 1 μl of the reaction was diluted on ice for H1 kinase assay. The ^{35}S-labeled protein mixture was separated by SDS-PAGE (10%) to resolve the threonine 161 phosphorylated cdc2 from the unphosphorylated cdc2 and visualized by enhanced autoradiography as well as a phosphorimager system for quantitation. The upper panel depicts the accumulation of the shifted, phosphorylated ^{35}S-labeled cdc2, and the lower panel shows the H1 kinase activity in the reaction at the indicated times. (B) At a fixed concentration of cyclin, the initial rate of threonine 161 phosphorylation is determined by the level of PP2A in the extract, a graphical representation of an experiment such as described in A at three different concentrations of PP2A. The endogenous level of PP2A in the extract is estimated to be ~160 nM. The initial velocity (arbitrary units here) of T161 phosphorylation refers to the relative rate of the reaction during the initial 10 min. (C) Okadaic acid accelerates threonine 161 phosphorylation when it induces the premature activation of cdc2. cdc2-containing reticulocyte (0.5 μl of 10 ng/μl) lysate was preincubated in 6 μl of an interphase extract. After 10 min at room temperature, 2 μl of 0.4 μM GST-cyclin B + 12 μl of XB or XB containing 1.7 μM okadaic acid was added. At the indicated times, 2 μl of the reaction mixture was quenched with SDS sample buffer and 1 μl of the mix was diluted on ice for H1 kinase assay. The radioactivity in H1 was measured by directly counting the gel bands in a scintillation counter. (D) Increasing the cyclin level counteracts the inhibitory effect of PP2A on the initial rate of T161 phosphorylation. Interphase extract (2.5 μl) that had been preincubated with cdc2-containing reticulocyte lysate as above was incubated with 2.5 μl of 800 nM PP2A in XB or 2.5 μl of XB in the presence of 52, 140, or 350 nM GST-cyclin B (final concentration). At the indicated times, 0.5 μl of the reaction mixture was removed and quenched with SDS-sample buffer. The percentage of T161 phosphorylated form of cdc2 was resolved and quantitated as described in A.

bound p34^{cdc2} quantitated. As indicated in Figure 7, the rate of association (k ~ 0.07 min^{-1}) was not significantly affected by the presence of added PP2A (k ~ 0.06 min^{-1}). However, the rate of T161 phosphorylation in the same reaction was inhibited by 50% (k ~ 0.08 min^{-1} to k ~ 0.04 min^{-1} in the presence of added PP2A). Therefore, PP2A does not delay the rate of association of p34^{cdc2} and cyclin B but does inhibit the rate of T161 phosphorylation.

T161 Phosphorylation Stabilizes the p34^{cdc2}/cyclin B Complex

In the process of studying the affect of PP2A on p34^{cdc2}/cyclin complex formation, we found that although the rate of association between p34^{cdc2} and cyclin B is not affected by T161 phosphorylation, T161 phosphorylation lowers the rate of dissociation of the complex. As shown in Figure 8, a comparison of the stability of the complex formed between cyclin B and p34^{cdc2} phos-

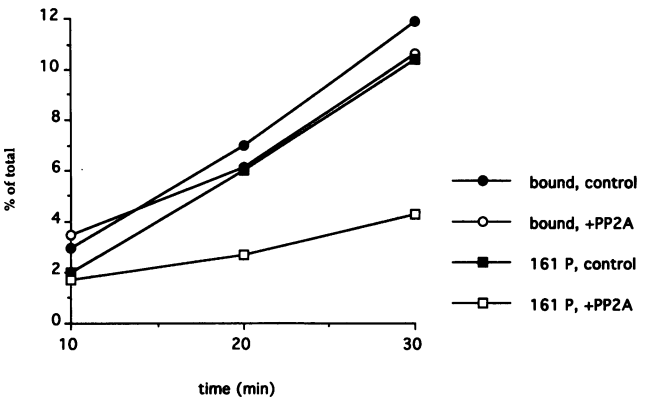

Figure 7. Although it inhibits threonine 161 phosphorylation, PP2A does not inhibit the association of cdc2 and cyclin. One hundred nanagrams of GST-cyclin B was prebound to each 8-μl aliquot of glutathione agarose beads (60 min on ice). Six microliters of interphase extract that had been preincubated for 10 min at room temperature with 0.5 μl of reticulocyte lysate containing 10 ng/μl ^{35}S-labeled A14F15 cdc2 protein was added to each aliquot of beads in the presence of 10 μl of either 500 nM purified PP2A in XB or XB alone. At the indicated times, the beads were rinsed once with 200 μl XB and mixed with SDS sample buffer. Of the total cyclin-bound cdc2, the threonine 161 phosphorylated and unphosphorylated forms of cdc2 were resolved by SDS-PAGE (10%) and quantitated on a phosphorimager system.

phorylated on T161 with the complex formed between cyclin B and p34^{cdc2} that cannot be phosphorylated on T161 (provided by the alanine 161 p34^{cdc2} protein) showed that the phosphorylated p34^{cdc2} was completely stable, whereas the complex containing the unphosphorylatable p34^{cdc2} dissociated at a significant rate with successive 20-fold dilutions. Therefore, the phosphorylation of p34^{cdc2} on T161 or some other consequence of T161 phosphorylation stabilizes the interaction between p34^{cdc2} and cyclin B. This result may explain the discrepancies among different investigators with respect to the ability of the unphosphorylated form of p34^{cdc2} to bind cyclin (Ducommun *et al.*, 1991; Desai *et al.*, 1992; Solomon *et al.*, 1992). The result would depend on the stringency of the binding assay, although the significance in vivo may be minimal because the concentrations of p34^{cdc2} and cyclin in the cell are apt to be higher than the estimated dissociation constant of ~1 nM.

AF p34^{cdc2} Is Sensitive to Inhibition by PP2A

The results presented so far demonstrate that PP2A affects only one known reaction during the lag, that of T161 phosphorylation. This would indicate that PP2A exerts its inhibitory effect on the activation of p34^{cdc2} by modulating the T161 phosphorylation reaction. If, in fact, PP2A were acting solely through the regulation of T161 phosphorylation, we would predict that the A14F15 p34^{cdc2} protein, which cannot be inhibited by tyrosine phosphorylation (Gould and Nurse, 1989), would retain sensitivity to inhibition by PP2A. To test this hypothesis, wild-type and A14F15 p34^{cdc2} containing the HA epitope tag (Field *et al.*, 1988) at the C-terminus were translated in reticulocyte lysates (as in Gautier *et al.*, 1991) and added to the interphase extract such that the exogenous AF p34^{cdc2} comprised ~50% of the total p34^{cdc2}. Thirty minutes after cyclin B addition, the exogenous p34^{cdc2} was specifically removed by immunoprecipitation with a monoclonal antibody against the HA epitope and its H1 kinase activity assessed. Figure 9A compares the H1 kinase activity achieved by the wild-type protein by 30 min in the presence of added PP2A (compare lanes 3 and 4 to lane 2) to that achieved by the A14F15 protein in the presence of the same levels of PP2A (compare lanes 6 and 7 to lane 5). At these concentrations of PP2A, the A14F15 p34^{cdc2} is, in fact, sensitive to PP2A. This result is consistent with our finding that PP2A can regulate p34^{cdc2} through inhibiting the phosphorylation of T161 and seems to leave no role for cdc25 in the activation process. Yet the A14F15 mutant shows precocious activation (Krek and Nigg, 1991; Norbury *et al.*, 1991), suggesting that the inhibitory sites can be rate limiting. We therefore carefully examined the sensitivity of the A14F15 and wild-type protein to PP2A. Figure 9B shows a dose response of both forms of p34^{cdc2} to a range of PP2A concentrations. The results indicate that although the activation of the A14F15 protein shows some sensitivity to PP2A, it is much less sensitive than the wild-type protein. Whereas the activation of the normal p34^{cdc2} is completely blocked by the addition of 100 nM

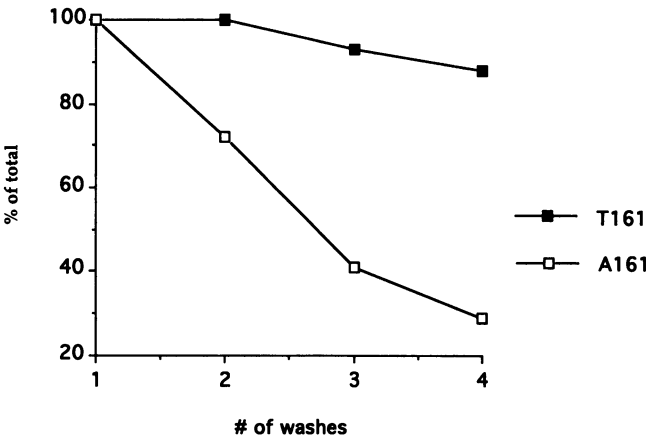

Figure 8. Threonine 161 phosphorylation stabilizes the cdc2/cyclin complex. One hundred nanograms of GST-cyclin B was prebound to 8-μl aliquots of glutathione agarose beads. Six microliters of interphase extract, which had been preincubated with 0.5 μl of reticulocyte lysate containing 10 ng/μl of ^{35}S-labeled wild-type or A161 (that cannot be phosphorylated on threonine 161) cdc2 protein, and 10 μl of XB was added to each aliquot of the cyclin-bound beads. After 30 min at room temperature to allow mitotic entry and thus threonine 161 phosphorylation of the wild-type cdc2, each aliquot of beads was washed 1, 2, 3, or 4 times with 200 μl of XB. The proteins remaining on the beads were then mixed with SDS sample buffer and resolved on SDS-PAGE. The cyclin-bound cdc2 was visualized and quantitated using a phosphorimager system.

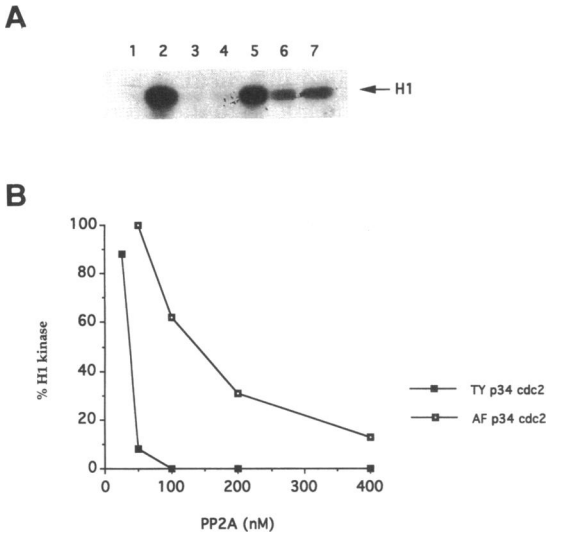

Figure 9. (A) Activation of the A14F15 cdc2 protein is inhibited by PP2A. HA epitope-tagged wild-type (lanes 2–4), A14F15 (lanes 5–7), and A161 (lane 1 as a negative control) cdc2 were translated in reticulocyte lysates to ~10 ng/μl. Two microliters of reticulocyte lysate was mixed with 3 μl of interphase extract and 1 μl of 0.5 μM GST-cyclin B in the presence of 5 μl of 800 nM purified PP2A in XB (lanes 3 and 6), 400 nM PP2A (lanes 4 and 7), or XB alone (lanes 1, 2, and 5). After a 30-min incubation at room temperature, each sample was mixed with 10 μl of anti-HA antibody-bound protein A sepharose beads and incubated on ice for 30 min. The beads were then washed with 3 × 1 ml (XB + 0.5 M NaCl + 1% NP40 + 1 μM okadaic acid) × 1 h at 4°C with rotation, followed by 2 × 1 ml brief washes in XB. The immunoprecipitates were then assayed for H1 kinase activity. (B) Activation of the A14F15 cdc2 protein is less sensitive to PP2A. Two microliters of reticulocyte lysate containing the wild-type or A14F15 cdc2 protein were mixed with 3 μl of interphase extract and 1 μl of 0.5 μM GST-cyclin B in the presence of 5 μl of 0, 100, 200, 400, or 800 nM purified PP2A in XB. After 30 min, the HA epitope-tagged cdc2 was immunoprecipitated from each reaction as in A and assayed for H1 kinase activity. One hundred percent refers to the amount of activity achieved by 30 min in the absence of added PP2A.

PP2A, the A14F15 protein is able to partially activate even in the presence of 400 nM PP2A. We conclude, from these results, that although the rate of T161 phosphorylation might normally be an important determinant of the kinetics of cdc2 activation, it is not the only determinant and that PP2A also regulates $p34^{cdc2}$ through the inhibitory phosphorylation sites.

There is an apparent paradox. PP2A seems to have no effect on the rate of tyrosine phosphorylation or dephosphorylation during the lag, yet the kinetics of activation seem to be strongly influenced by the availability of the inhibitory phosphorylation sites (Krek and Nigg, 1991; Norbury *et al.*, 1991) or by the addition of excess cdc25 (see Gautier *et al.*, 1991). One way of reconciling these results is that PP2A may regulate the tyrosine kinase and phosphatase activities indirectly, through the regulation of T161 phosphorylation. By delaying T161 phosphorylation during the lag, PP2A might delay indirectly the activation of the tyrosine phosphatase and the inactivation of the tyrosine kinase. However, in the absence of the inhibitory phosphorylation sites (provided by the A14F15 protein), the rate of T161 phosphorylation is the only limiting reaction.

Active cdc2 Is Not Sufficient to Turn on Its Own Activation

These results suggest that the activation process may involve several intermediates. For example, Solomon *et al.* (1990) initially proposed that the accumulation of a critical threshold level of active $p34^{cdc2}$, phosphorylated on T161 but not on T14Y15, might be a prequisite to activating a positive feedback loop that would subsequently activate the bulk of the $p34^{cdc2}$ by tyrosine dephosphorylation. The data presented here might suggest that PP2A, by slowing the rate of T161 phosphorylation, would delay the accumulation of the critical threshold level of active $p34^{cdc2}$ molecules and thus prevent the subsequent activation of the bulk of the $p34^{cdc2}$. According to this proposal, the rate of T161 phosphorylation and consequently the rate of accumulation of active $p34^{cdc2}$ during the lag would comprise the rate-determining step or switch to activated cdc2. A simple test of such a model would be to add a superthreshold level of active $p34^{cdc2}$/cyclin B complex to an interphase extract. The added kinase should bypass the initial requirement and result in the activation of the positive feedback loop, resulting in a stable mitotic state. (The use of a nondegradable cyclin, provided by the glutathione S-transferase portion of GST-cyclin B, could prevent the subsequent cyclin degradation). We therefore added a superthreshold level of active $p34^{cdc2}$/cyclin B complex to an interphase extract (Figure 10A, lane 1). Surprisingly, most of the H1 kinase activity of the added complex was quickly lost (lane 2). Inactivation occurred even when the level of the added complex exceeded the amount that would have been generated in the recipient extract by the addition of an equivalent amount of free cyclin (see below). The activated $p34^{cdc2}$/cyclin complex was thus insufficient to turn on the enzymes required to maintain cdc2 in the active state. The inactivation of the H1 kinase could be subsequently reversed by the addition of bacterially expressed and purified cdc25 to the inactivated and reisolated complex (Figure 10A, lane 4), demonstrating that the inactivation was reversible, not as if it were caused by proteolysis but rather through phosphorylation on T14Y15.

We compared the effect of cyclin bound to active cdc2 with that of free cyclin to ensure that the level of active complex we were adding exceeded the threshold levels of cyclin that would normally be sufficient to activate stable levels of cdc2 activity. GST-cyclin B, prebound to glutathione beads, was incubated in interphase extracts for different lengths of time. At the indicated times, we isolated the cyclin on the glutathione beads and either assayed for the H1 kinase activity on the beads (Figure 10B, lanes 1–4) or incubated the beads in a second interphase extract for 20 or 40 min (lanes 5–

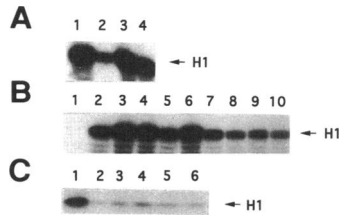

Figure 10. The active cdc2/cyclin complex cannot convert an interphase extract to the mitotic state in the absence of excess free cyclin. (A) The active complex becomes inactivated in an interphase extract. Twenty microliters of interphase extract was mixed with 20 μl of 100 nM GST-cyclin B in XB at room temperature for 30 min to allow activation of cdc2. After 30 min, 10 μl aliquots were removed and each bound to 10 μl of glutathione agarose beads for 15 min at room temperature. Each aliquot of beads was rinsed with 0.5 ml of XB and incubated with 5 μl of interphase extract + 5 μl of XB (lanes 2 and 4), with 5 μl of interphase extract + 5 μl of XB containing 2 μM okadaic acid (lane 3), or with 10 μl of XB alone (lane 1). After 15 min at room temperature, each sample was washed once in XB^{++} and once in XB. The sample in lane 4 was further treated with 10 μl of 3 μM bacterially expressed *Drosophila* cdc25 for 30 min at room temperature and then washed as described. Finally, each aliquot was assayed for H1 kinase activity. (B) The inability of the active cdc2/cyclin complex to drive the interphase to mitotic transition is not a threshold effect. One hundred fifty nanograms of GST-cyclin B were bound to each of 10–10 μl aliquots of glutathione agarose beads on ice for 60 min. The beads were briefly rinsed in 200 μl XB, then incubated with 10 μl of a 1:1 diluted interphase extract in XB for 0 (lanes 1, 5, and 6), 20 (lanes 2, 7, and 8), 40 (lanes 3, 9, and 10), or 80 (lane 4) min at room temperature. At the respective times, the beads were rinsed with 200 μl of XB and left untreated (lanes 1–4) or incubated with 10 μl of a fresh, 1:1 diluted interphase extract in XB for 20 (lanes 5, 7, and 9) or 40 (lanes 6, 8, and 10) min at room temperature. At the respective times, each (both treated and untreated) aliquot of beads was washed in 0.5 ml XB and assayed for H1 kinase activity. (C) A mitotic extract that has been depleted of the cdc2/cyclin complex is rapidly converted to an interphase extract. Thirty microliters of interphase extract was activated with 30 μl of 100 nM GST-cyclin B. After 30 min at room temperature, each 10-μl aliquot was mixed with 10 μl of glutathione agarose beads and incubated at room temperature for 15 min to allow binding of the active complex to the beads. The unbound mitotic extract in each aliquot was separated from its bead bound active complex for 2 (lane 3), 5 (lane 4), 10 (lane 5), or 15 min (lane 6) at room temperature, then added back to the beads. After 15 min, the beads were washed as described in A and assayed for H1 kinase activity. Lane 1 shows the H1 kinase activity of the untreated active complex, and lane 2 shows the H1 kinase activity of the active complex after isolation and incubation with a fresh interphase extract (diluted 1:1 with XB) for 15 min.

10). We then reisolated the complex and assayed for H1 kinase activity. Lanes 1–4 indicate the time course of H1 kinase activation in the first incubation, and lanes 5–10 indicate the H1 kinase activity remaining on the beads at each time point after incubation in a second interphase extract in the absence of any additional cyclin. As shown in lanes 1–4, H1 kinase activity begins to increase during the first incubation at 20 min, reaches a maximum level by 40 min, and remains stable to 80 min. As shown in lanes 5 and 6, at a time when cyclin has no associated H1 kinase activity in the first extract (lane 1), it is fully capable of activating a second extract. However, by 20 min in the first extract (lane 2), it is no longer capable of activating a second interphase extract (lanes 7 and 8). Instead, most of the H1 kinase activity associated with cyclin during the incubation in the first extract (lane 3) is rapidly lost upon addition to the second extract (lanes 9 and 10). These findings demonstrate that, although the presence of a critical threshold level of active cdc2 may be rate-limiting, it is not sufficient to activate the enzymes that maintain its own activity. In fact, as cyclin binds to $p34^{cdc2}$ and turns on the kinase activity of cdc2, it appears to lose the capacity to induce a stable mitotic state. As shown in Figure 10A, lane 3, the H1 kinase activity of the $p34^{cdc2}$/cyclin complex is not lost when added to a second interphase extract containing 1 μM okadaic acid. Thus PP2A appears to act at a step distinct from the initial accumulation of the active cdc2/cyclin complex.

The addition of a nondegradable cyclin to an interphase extract leads to the activation and stable maintenance of cdc2/cyclin in an active state (Figure 10B, lane 4) (Murray *et al.*, 1989; Solomon *et al.*, 1990). Because the ability to drive an interphase extract into M phase does not reside in the fully activated cdc2/cyclin complex, we asked whether a mitotic extract that has been depleted of the active cdc2/cyclin complex retains the ability to stabilize the active complex. For this, we used glutathione agarose beads to deplete a stable mitotic extract of the cdc2/cyclin complex (Figure 10C, lanes 3–6). To probe the state of the depleted extract, we added back the active complex (lane 1) after incubating the depleted extract for various times. As shown in Figure 10C, the ability to stabilize the active cdc2/cyclin complex is lost rapidly (within 2 min, lanes 3–6) from the mitotic extract upon depletion of the active complex, suggesting that although the cdc2/cyclin complex is not sufficient by itself to initiate all of the events required for its stabilization, it is necessary for its own stabilization. Furthermore, because the activity required for the stabilization of the cdc2/cyclin active complex is lost upon separation of the active complex from a mitotic extract, we conclude that the continued interaction of the active complex with some unidentified factor(s) is required for the maintenance, and probably also the generation, of the mitotic state.

DISCUSSION

The accumulation of a critical threshold level of cyclin protein triggers the interphase to mitotic transition during the first *Xenopus* embryonic cell cycle (Murray and Kirschner, 1989). The cyclin threshold is determined, at least in part, by the level of PP2A (Solomon *et al.*, 1990); therefore, PP2A must limit the action of cyclin such that at subthreshold concentrations, the extract remains in interphase, whereas at above threshold concentrations, the extract rapidly enters mitosis. This antagonistic relationship between cyclin and PP2A is essential for the switch-like response to a continously increasing pool of cyclin, i.e., in the absence of PP2A activity, cyclin activates cdc2 proportionately and with-

out a significant lag (Felix *et al.*, 1990; Solomon *et al.*, 1990). Central to understanding the all or none mechanism for entering mitosis is the identification of the link between cyclin and PP2A.

We have used purified PP2A from *Xenopus* eggs and *Xenopus* interphase extracts to explore the mechanism by which PP2A and cyclin oppose one another. The mitotic state is produced by the activation of the cdc2/cyclin protein kinase complex. Because cyclin is an essential component of this key regulator, the link between cyclin and PP2A should lie somewhere along the pathway to cdc2 activation. Before this study, we knew in a broad sense the sequence of events after cyclin addition. Cyclin associated with cdc2 to form a complex that was inactive because it lacked an essential phosphorylation on the activating site, T161 (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Krek and Nigg, 1992; Solomon *et al.*, 1992). Cyclin binding allowed phosphorylation on T161 (Solomon *et al.*, 1992), but it also simultaneously induced phosphorylation on two inhibitory residues Y15 and T14 (Solomon *et al.*, 1990; Meijer *et al.*, 1991; Parker *et al.*, 1991). Below the threshold concentration of cyclin, the complex remained in the triply phosphorylated form and was inhibited (Solomon *et al.*, 1990). Above this threshold, cyclin catalyzed a series of events, which after a lag, led to a switch in the balance between the tyrosine kinase and phosphatase activities such that the complex was abruptly dephosphorylated and activated (Solomon *et al.*, 1990). Thus the activities that dephosphorylated the tyrosine 15 residue and that phosphorylated the tyrosine 15 residue were markedly different before and after the mitotic transition (Solomon *et al.*, 1990; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Smythe and Newport, 1992). There was no obvious modulation of the activating phosphorylation on T161 throughout the transition (Solomon *et al.*, 1992). To identify, among these phosphorylation and dephosphorylation reactions on cdc2, the triggering event, we compared each reaction pathway under two conditions, one in which cyclin exceeded the threshold and one in which cyclin was below threshold. These conditions could be achieved by varying slightly (2- to 3-fold) the ratio of cyclin to PP2A.

In past studies, the problem of identifying the trigger, i.e., that event that was sensitively regulated by cyclin and PP2A, was complicated by two major issues. The first complication arises in distinguishing those events that are a direct target of PP2A and are potentially rate limiting for the initiation of the transition from those events that occur after the transition, downstream of the trigger. By blocking the initiation of the transition, PP2A ultimately affects all of the events that would accompany the transition. The second complication arises from the difficulty in assessing the physiological relevance of partial reactions that are catalyzed among purified components, particularly for an enzyme (like PP2A) that exhibits a broad substrate specificity in vitro (Cohen, 1989) and little selectivity at high concentrations. The present study is an attempt to deal with both of these issues.

The problem of cause and effect is illustrated by the relationship between PP2A and cdc25. The addition of okadaic acid to an interphase extract and cyclin results in the premature conversion of cdc25 to the highly phosphorylated and active, mitotic form (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). Similarly, the addition of purified PP2A to an interphase extract with cyclin blocks the activation of cdc25 (Clarke *et al.*, 1993). These results imply that PP2A negatively regulates the activity of cdc25. But whether cdc25 could be part of the trigger for the transition depends on whether PP2A directly inhibits cdc25 or whether PP2A inhibits cdc25 by preventing the transition from occurring. To separate the primary effects of PP2A from those effects that occur as a consequence of blocking the initiating event, we examined the effect of PP2A on cdc25 activity during the lag, between the time of cyclin addition and the time of the transition. The lag period is the relevant period for a trigger because this is the time when either the sufficiency or insufficiency of the cyclin level is being evaluated by the kinetics of the reaction pathways leading to the activation of cdc2. Our results suggest that although PP2A may inhibit cdc25 after the transition, the level of PP2A does not modulate cdc25 activity before the transition. It appears, therefore, that the inhibitory effect of PP2A on cdc25 is an indirect consequence of the inhibitory effect of PP2A on the trigger for mitosis. During the transition itself, changes in the activity of cdc25 may be important in adding an autocatalytic acceleration to the acquisition of the mitotic state, as has been suggested previously (Dorée *et al.*, 1989; Hunt, 1989; Solomon *et al.*, 1990).

Similarly, we can rule out a role for changes in tyrosine phosphorylation in the trigger for mitosis by studying the effect of PP2A on the tyrosine kinase activity during the lag. Previous studies showed that the addition of okadaic acid to an extract arrested in interphase, with high tyrosine kinase activity, led to a 10-fold reduction in the tyrosine kinase activity (Smythe and Newport, 1992), suggesting that PP2A regulated the tyrosine kinase. We find no significant difference in the tyrosine kinase activity, during the lag, in the presence of elevated PP2A activity. Therefore the effect of PP2A on the tyrosine kinase must also be downstream of some other regulator after the transition. Because we did not look directly at T14 phosphorylation, it remains a possibility that the T14 kinase is modulated by PP2A, independently of Y15 phosphorylation. However, Norbury *et al.* (1991) have shown that abolishing either one of the inhibitory T14 and Y15 residues for phosphorylation had no discernible effect on the kinetics of cdc2 activation, suggesting that either T14 or Y15 phosphorylation is sufficient for conferring negative regulation on cdc2. In agreement with their report, we find that both A14Y15 and T14F15 cdc2 molecules are as

sensitive to inhibition by PP2A as is the wild-type protein (unpublished data). In light of this observation, it is unlikely that downregulation of the T14 kinase, even if modulated independently of the Y15 kinase, would comprise a rate-limiting step, because dephosphorylation of T14 in the absence of Y15 dephosphorylation would not activate cdc2. In summary, it appears that a change in the balance of the kinase(s) and phosphatase(s) that control the inhibitory sites on cdc2 is not part of the trigger, but rather a consequence of the trigger.

The second complication described above, that of determining which reactions are physiologically relevant, became clear when we initially examined the effect of PP2A on T161 dephosphorylation. We had previously demonstrated the ability of purified PP2A to directly dephosphorylate T161 on cdc2 and to inactivate it (Gould *et al.*, 1991; Lee *et al.*, 1991). On the basis of this result, we suggested that perhaps PP2A regulated cdc2 by dephosphorylating cdc2 on T161. We noted, however, that micromolar concentrations of PP2A were required to see this effect, whereas nanomolar concentrations of PP2A were sufficient to block the initial activation of cdc2.

The importance of the T161 dephosphorylation reaction is best evaluated in a complex extract under more physiological conditions. In these conditions T161 dephosphorylation does not occur at a measurable rate. This is not to suggest that T161 dephosphorylation is not important at some step of mitosis. Lorca *ei al.* (1992) have demonstrated a requirement for T161 dephosphorylation on cdc2, subsequent to cyclin degradation, for the inactivation of cdc2. Thus dephosphorylation of T161 could potentially play a regulatory role in the exit from M phase, although a situation in which it is rate limiting has yet to be observed.

Despite its broad substrate specificity in vitro (Cohen, 1989), when PP2A is added to an interphase extract at levels that just prevent the cyclin-triggered transition, it has no discernible effect on most of the reactions governing cdc2 activity. Neither cyclin binding to cdc2, nor the phosphorylation or dephosphorylation reactions on Y15 are affected by PP2A. But at the same time, the T161 phosphorylation reaction is sensitively modulated by PP2A. Therefore, under more physiological conditions in limiting amounts, PP2A exhibits a degree of substrate specificity. The addition of PP2A to the extract lowers the initial rate of T161 phosphorylation in a dose-dependent manner. Furthermore, this inhibitory effect can be counteracted by increasing the level of cyclin added to the reaction, arguing in favor of the T161 phosphorylation reaction being part of the link between cyclin and PP2A. Whether the regulation of T161 phosphorylation occurs at the level of the kinase that phosphorylates T161 is not known. Solomon *et al.* (1992) have shown in fact that the activity of the T161 kinase (CAK) is not changed between interphase and mitosis. The regulation by PP2A may set a baseline value for CAK activity that is independent of cell-cycle states. Alternatively, CAK activity may be regulated indirectly, and the effect of PP2A may be lost upon dilution of the extract for the CAK assay. As expected, inhibition of PP2A activity in the extract with okadaic acid accelerates T161 phosphorylation. Formally, this could be an indirect consequence of the acceleration of the transition; however, the observation that T161 phosphorylation can be modulated in the absence of the transition suggests that the acceleration by okadaic acid probably reflects a more direct effect of PP2A on the T161 phosphorylation reaction.

On the basis of these results, we might expect that cdc2 mutants insensitive to the inactivating phosphorylations (the A14F15 mutant) should show some sensitivity to regulation of the T161 phosphorylation rate. In fact, we find the activation of the A14F15 protein to be sensitive (although not as strongly as the wild-type protein) to the level of PP2A in the extract. However, the decreased sensitivity of the A14F15 protein suggests that somehow PP2A regulates the level of tyrosine phosphorylation, even though it seems to have no direct effect on those reactions. We are left with the conclusion that PP2A exerts an inhibitory effect on the pathway controlling Y15 phosphorylation at least in part through its effect on T161 phosphorylation, which suggests to us that T161 phosphorylation might be upstream to the switch in tyrosine kinase and phosphatase activities that in turn participate in the transition. Thus T161 phosphorylation, which appears to be modulated by cyclin and PP2A, is potentially a rate-limiting step in the initiation of mitosis. However, definitive proof of the importance of T161 phosphorylation in limiting the trigger clearly awaits the ability to specifically modulate the rate of this reaction independently of PP2A.

The kinetic data on cdc2 activation fits nicely with a model in which PP2A sets the threshold through its regulation of T161 phosphorylation. Such a model was initially proposed by Solomon *et al.* (1990) and later by Hoffman *et al.* (1993), and we call it the positive feedback loop model. This model postulates that cyclin addition leads to the proportionate accumulation of a low but critical threshold level of active cdc2/cyclin complexes. This active fraction of cdc2 then triggers a switch in the balance between tyrosine kinase and tyrosine phosphatase activities, which subsequently leads to the activation of the bulk of the cdc2/cyclin complexes initially held inactive by tyrosine phosphorylation. By delaying the onset of T161 phosphorylation, PP2A could delay the accumulation of a critical threshold level of active cdc2. As a consequence, tyrosine phosphorylation would outcompete T161 phosphorylation, not allowing the active complexes (the trigger) to form. This model places T161 phosphorylation central to the trigger as the rate-limiting step for M phase initiation.

A prediction of the positive feedback loop model is that the addition of the activated cdc2/cyclin complex to an interphase extract would bypass the requirement

for the initial trigger and directly induce the mitotic state. To our surprise, the simple test of the model failed. We found that adding the fully activated cdc2/cyclin complex to an interphase extract, rather than accelerating the conversion of the interphase extract into a mitotic extract, resulted in the inactivation of the complex, suggesting that the active cdc2/cyclin complex failed to activate cdc25 and/or inactivate the tyrosine kinase, a prerequisite for a stable mitotic state. In agreement with these results, M. Gonzales-Kuyvenhoven, C.L. Ashorn, J.E. Penkala, and J. Kuang (unpublished data) have demonstrated the inability of the cdc2/cyclin complex to mitotically phosphorylate cdc25 when added to an interphase extract. Whether or not the tyrosine kinase is similarly maintained in the interphase state remains to be determined, but the rapid kinetics of inactivation would suggest that the tyrosine kinase is active. The failure of the active cdc2/cyclin complex to drive an interphase extract into a stable mitotic state (unless PP2A is first inactivated by the addition of okadaic acid) raises several important points. First, a positive feedback loop in which cdc2 turns on its own activators cannot by itself explain mitotic initiation. The result of Hoffmann *et al.* (1993), demonstrating the ability of the cdc2/cyclin B complex to directly phosphorylate recombinant cdc25, is potentially interesting but does not in itself establish a feedback loop. Second, although T161 phosphorylation might dictate when entry into M phase occurs under certain conditions, it cannot be the only rate-limiting step. Third, there must exist an additional target of PP2A. The simplest interpretation summarizing these points is that PP2A controls more than one rate-limiting component of the mitotic trigger. A component resides in the pathway regulating T161 phosphorylation and therefore determining the initial rate of accumulation of active cdc2/cyclin complexes, as well as at least one other, as yet unidentified pathway. Activation of both pathways may be required for the initiation of the mitotic transition. The rate-limiting events may be required either to overcome a constant level of PP2A, or to downregulate PP2A activity during the transition. The existing information argues, in general, against the cell cycle regulation of cytosolic PP2A activity (Kinoshita *et al.*, 1990; Ruediger *et al.*, 1991; Nakamura *et al.*, 1992), and we also see no variation in the activity of PP2A (using β-casein as a substrate) throughout the G2/M transition in cyclin-activated interphase extracts (unpublished data). However, we cannot rule out the possibility that subpopulations of PP2A are differentially regulated (Nakamura *et al.*, 1992) and that PP2A activity is cell cycle regulated in other situations or other systems, nor can we exclude the possibility that PP2A activity towards specific substrates is regulated during the transition into M phase.

Although INH/PP2A was originally characterized as an inhibitor of the G2/M transition in oocytes, we have studied here the role of INH/PP2A in the mitotic cell cycle. The finding, that the pathway regulating T161 phosphorylation may be a relevant target of PP2A during the mitotic cell cycle, appears initially to be at odds with the information available from studies in the oocyte, suggesting that T161 phosphorylation is not rate limiting for entry into M phase (Dunphy and Newport, 1991; Gautier *et al.*, 1991). However, our findings reveal the existence of at least one additional target of PP2A that is closely involved in the initiation of the G2/M transition. It is conceivable that the rate-limiting step(s) for M phase initiation differs in different situations, as has been revealed by studies in the early *Drosophila* embryo (Edgar and O'Farrell, 1990). The question concerning the rate-limiting event in oocyte maturation is an important one but is not the subject of this paper and thus awaits future studies.

Although cyclin added to the interphase extract can initiate all of the events required for mitotic entry, cyclin bound to cdc2 and fully activated as a protein kinase complex cannot. Because the only known end product of cyclin addition is its fully activated complex with cdc2, the finding that the cdc2 complex is unstable is unexpected and appears to undermine the importance of the active cdc2/cyclin complex in triggering mitotic initiation. However, we show that removal of active cdc2 from a mitotic extract causes the rapid conversion of the mitotic extract to an interphase extract, strongly suggesting that the cdc2/cyclin complex plays an active role in the maintenance of the mitotic state (as shown by Dunphy *et al.*, 1988). Because the enzymes stabilizing the mitotic forms of the tyrosine phosphatase and the tyrosine kinase are inactivated upon separation of the active cdc2 complex from the mitotic extract and yet cdc2 itself does not retain the ability to convert the interphase forms to their mitotic counterparts, the active cdc2/cyclin complex must act in concert with other unidentified factors to initiate the conversion of cdc25 and the tyrosine kinase to the mitotic state. Thus cyclin addition may trigger the initiation of mitosis by activating a multicomponent system (as has been suggested for MPF itself, Kuang *et al.*, 1991). We do not know what form of cyclin is responsible for activating the partner to the cdc2/cyclin complex in the trigger. A candidate might be a complex between the unphosphorylated form of cyclin and cdc2 that is present in the early phases of activation, to be replaced by the phosphorylated form of cyclin in the mitotic state. However, it could also be free cyclin, or cyclin bound to a kinase other than cdc2.

In summary, we began this study with the intent to identify the link between cyclin and PP2A in hopes of understanding the mechanism of the trigger for mitosis. Our results suggest that T161 phosphorylation and the accumulation of active cdc2 during the lag may be part of the trigger for mitotic initiation and that the switch in tyrosine kinase and tyrosine phosphatase activities reside downstream of the initiating event. However, our results also suggest that the accumulation of active cdc2/cyclin complexes during the lag is only one of the

events required for triggering entry into mitosis. The identification of the other reaction pathway(s) initiated by cyclin, and opposed by PP2A, will lead to a more complete understanding of the trigger for mitosis.

ACKNOWLEDGMENTS

We thank Mark Solomon for his generous contribution of purified CAK and DEAE eluates from which INH was purified. We also thank Marc Mumby for his helpful suggestions and generosity in sharing antibodies against PP2A, Doug Kellog for sharing his GST-*Xenopus* cyclin B1 construct, and Yong Gu for providing us with cdc25 protein. Discussions with Jian Kuang and members of the Kirchner laboratory played an important role in this work. We also thank Peter Jackson, Jian Kuang, and Andrew Murray for careful reading of the manuscript. This work was supported by the National Institute of General Medical Sciences and the Program of Excellence in Molecular Biology of the National Heart, Lung, and Blood Institute.

REFERENCES

Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988). cdc2 is a component of the M phase specific histone H1 kinase: evidence for identity with MPF. Cell *55*, 371–378.

Bialojan, C., and Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Biochem. J. *256*, 283–290.

Booher, R.N., Alfa, C.E., Hyams, J.S., and Beach, D. (1989). The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. Cell *58*, 485–497.

Booher, R., and Beach, D. (1986). Site-specific mutagenesis of $cdc2^{+}$, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. Mol. Cell. Biol. *6*, 3523–3530.

Booher, R.N., Deshaies, R.J., and Kirschner, M. (1993). Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of $p34^{cdc28}$ in response to G_1 and G_2 cyclins. EMBO J. *12*, 3417–3426.

Clarke, P., Hoffman, I., Draetta, G., and Karsenti, E. (1993). Dephosphorylation of cdc25-C by a type 2A protein phosphatase: specific regulation during the cell cycle in *Xenopus* egg extracts. EMBO J. *12*, 53–63.

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. *58*, 485–497.

Cyert, M.S., and Kirschner, M.W. (1988). Regulation of MPF activity in vitro. Cell *53*, 185–195.

Desai, D., Gu, Y., and Morgan, D.O. (1992). Activation of human cyclin-dependent kinases in vitro. Mol. Biol. Cell *3*, 571–582.

Dorée, M., Labbe, J.C., and Picard, A. (1989). M phase promoting factor: its identification as the M phase-specific H1 histone kinase and its activation by dephosphorylation. J. Cell Sci. Suppl. *12*, 39–51.

Draetta, G., and Beach, D. (1988). Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. Cell *54*, 17–26.

Ducommon, B., Brambilla, P., Felix, M.A., Franza, B.R.J., Karsenti, E., and Draetta, G. (1991). cdc2 phosphorylation is required for its interaction with cyclin. EMBO J. *10*, 3311–3319.

Dunphy, W.G., Brizuela, L., Beach, D., and Newport, J. (1988). The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell *54*, 423–431.

Dunphy, W.G., and Kumagai, A. (1991). The cdc25 protein contains an intrinsic phosphatase activity. Cell *68*, 787–797.

Dunphy, W.G., and Newport, J.W. (1989). Fission yeast p13 blocks mitotic activation and tyrosine dephosphorylation of the *Xenopus* cdc2 protein kinase. Cell *58*, 181–191.

Edgar, B.A., and O'Farrell, P.H. (1990). The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G2 by *string*. Cell *62*, 469–480.

Evans, T., Rosenthal, E.T., Youngbloom, J., Distel, D., and Hunt, T. (1983). Cyclin: a protein specificied by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell *33*, 389–396.

Featherstone, C., and Russell, P. (1991). Fission yeast p107 wee1 mitotic inhibitor is a protein tyrosine/serine kinase. Nature *349*, 808–811.

Felix, M.A., Cohen, P., and Karsenti, E. (1990). cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cell cycle: evidence from the effects of okadaic acid. EMBO J. *9*, 675–683.

Fesquet, D., Labbe, J.C., Derancourt, J., Capony, J.P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M., and Cavadore, J.C. (1993). The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr 161 and its homologues. EMBO J. *12*(8), 3111–3121.

Field, J., Nikawa, J.I., Broek, D., McDonald, B., Rodgers, L., Wilson, I.A., Lerner, R., and Wigler, M. (1988). Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159–2165.

Gautier, J., Matsukawa, T., Nurse, P., and Maller, J. (1989). Dephosphorylation and activation of *Xenopus* p34 cdc2 protein kinase during the cell cycle. Nature *339*, 626–629.

Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J. (1990). Cyclin is a component of maturation-promoting factor from *Xenopus*. Cell *60*, 487–494.

Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988). Purified maturation promoting factor contains the product of a *Xenopus* homologue of the fission yeast cell cycle control gene cdc2+. Cell *54*, 433–439.

Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F., and Kirschner, M.W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34 cdc2. Cell *67*, 197–211.

Gerhart, J., Wu, M., and Kirschner, M. (1984). Cell cycle dynamics of an M-phase specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. J. Cell Biol. *98*, 1247–1255.

Goris, J., Hermann, J., Hendrix, P., Ozon, R., and Merlevede, W. (1989). Okadaic acid, a specific protein phosphatase inhibitor, induces maturation and MPF formation in *Xenopus laevis* oocytes. FEBS Lett. *245*, 91–94.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S., and Nurse, P. (1991). Phosphorylation at T167 is required for *Schizosaccharomyces pombe* p34 cdc2 function. EMBO J. *10*, 3297–3309.

Gould, K.I., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature *342*, 39–45.

Hoffman, I., Clarke, P.R., Marcote, M.J., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. *12*, 53–63.

Hunt, T. (1989). Embryology. Under arrest in the cell cycle. Nature *342*, 483–484.

Izumi, T., Walker, D.H., and Maller, J.L. (1992). Periodic changes in phosphorylation of the *Xenopus* cdc25 phosphatase regulate its activity. Mol. Biol. Cell *3*, 927–939.

Kinoshita, N., Ohkura, H., and Yanagida, M. (1990). Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. Cell *63*, 405–415.

Krek, W., and Nigg, E.A. (1991). Mutations of p34 cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34 cdc2 kinase activation. EMBO J. *10*, 305–316.

Krek, W., and Nigg, E.A. (1992). Cell cycle regulation of vertebrate p34 cdc2 activity: identification of Thr 161 as an essential in vivo phosphorylation site. New Biol. *4*, 323–329.

Kuang, J., Penkala, J.E., Ashorn, C.L., Wright, D.A., Saunders, G.F., and Rao, P.N. (1991). Multiple forms of maturation promoting factor in unfertilized *Xenopus* eggs. Proc. Natl. Acad. Sci. USA, *88*(24), 11530–11534.

Kumagai, A., and Dunphy, W.G. (1992). Regulation of the Cdc25 protein during the cell cycle in *Xenopus* extracts. Cell *70*, 139–151.

Labbé, J.C., Lee, M.G., Nurse, P., Picard, A., and Doree, M. (1988). Activation at M phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene cdc2+. Nature *355*, 251–254.

Labbé, J.C., Picard, A., Peaucellier, G., Cavadore, J.C., Nurse, P., and Doree, M. (1989). Purification of MPF from starfish: identification as the H1 histone kinase p34 cdc2 and a possible mechanism for its periodic activation. Cell *57*, 253–263.

Lee, T.H., Solomon, M.J., Mumby, M.C., and Kirschner, M.W. (1991). INH, a negative regulator of MPF, is a form of protein phosphatase 2A. Cell *64*, 415–423.

Lohka, M.J., Hayes, M.K., and Maller, J.L. (1988). Purification of maturation promoting factor, an intracellular regulator of early mitotic events. Proc. Natl. Acad. Sci. USA *85*, 3009–3013.

Lorca, T., Labbe, J.C., Devault, A., Fesquet, D., Capony, J.P., Cavadore, J.C., Le Bouffant, F., and Doree, M. (1992). Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. EMBO J. *11*, 2381–2390.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M.W., and Beach, D. (1991). mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell *64*, 1111–1122.

Masui, Y., and Markert, C.L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool. *177*, 129–146.

Mayer, R.E., Hendrix, P., Cron, P., Matthies, R., Stone, S.R., Goris, J., Merlevede, W., Hofsteenge, J., and Hemmings, B.A. (1991). Structure of the 55-kDa regulatory subunit of protein phosphatase 2A: evidence for a neuronal-specific isoform. Biochemistry *30*, 3589–3596.

McGowan, C.H., and Russell, P. (1993). Human wee1 kinase inhibits cell division by phosphorylating p34 cdc2 exclusively on Tyr 15. EMBO J. *12*, 75–85.

Meijer, L., Azzi, L., and Wang, J.Y. (1991). Cyclin B targets p34 cdc2 for tyrosine phosphorylation. EMBO J. *10*, 1545–1554.

Miake-Lye, R., Newport, J., and Kirschner, M. (1983). Maturation-promoting factor induces nuclear envelope breakdown in cycloheximide-arrested embryos of *Xenopus laevis*. J. Cell Biol. *97*, 81–91.

Millar, J.B.A., and Russell, P. (1992). The cdc25 M-phase inducer: an unconventional protein phosphatase. Cell *68*, 407–410.

Minshull, J., Blow, J.J., and Hunt, T. (1989). Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. Cell *56*, 947–956.

Morla, A.O., Draetta, G., Beach, D., and Wang, J.Y.J. (1989). Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. Cell *58*, 193–203.

Murray, A.W., and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. Nature *339*, 275–280.

Murray, A.W., Solomon, M.J., and Kirschner, M.W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature *339*, 280–286.

Nakamura, K., Koda, T., Nakinuma, M., Matsuzawa, S., Kitamura, K., Mizuno, Y., and Kikuchi, K. (1992). Cell cycle dependent gene expressions and activities of protein phosphatases PP1 and PP2A in mouse NIH 3T3 fibroblasts. Biochem. Biophys. Res. Comm. *187*, 507–514.

Norbury, C., Blow, J., and Nurse, P. (1991). Regulatory phosphorylation of the p34 cdc2 protein kinase in vertebrates. EMBO J. *10*, 3321–3329.

Parker, L.L., Atherton-Fessler, S., Lee, M.S., Ogg, S., Falk, J.L., Swenson, K.J., and Piwnica-Worms, H. (1991). Cyclin promotes the tyrosine phosphorylation of p34 cdc2 in a wee1$^+$ dependent manner. EMBO J. *10*, 1255–1263.

Parker, L.L., and Piwnica-Worms, H. (1992). Inactivation of the p34 cdc2 cyclin B complex by the human wee1 tyrosine kinase. Science *257*, 1955–1957.

Poon, R.Y., Yamashita, K., Adamczewski, J.P., Hunt, T., and Suttleworth, J. (1993). The cdc2-related protein p40MO15 is the catalytic subunit of a protein kinase that can activate p33 cdk2 and p34 cdc2. EMBO J. *12*(8), 3123–3132.

Ruediger, R., Van Wart Hood, J.E., Mumby, M., and Walter, G. (1991). Constant expression and activity of protein phosphatase 2A in synchronized cells. Mol. Cell. Biol. *11*, 4282–4285.

Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homologue. Cell *49*, 559–567.

Smith, L.D., and Ecker, R.E. (1971). The interaction of steroids with *Rana pipiens* oocytes in the induction of maturation. Dev. Biol. *25*, 232–247.

Smythe, C., and Newport, J.W. (1992). Coupling of mitosis to the completion of S phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates p34 cdc2. Cell *68*, 787–797.

Solomon, M.J., Glotzer, M., Lee, T.H., Philippe, M., and Kirschner, M.W. (1990). Cyclin activation of p34 cdc2. Cell *63*, 1013–1024.

Solomon, M.J., Harper, J.W., and Shuttleworth, J. (1993). CAK, the p34^{cdc2} activating kinase, contains a protein identical or closely related to p40MO15. EMBO J. *12*(8), 3133–3142.

Solomon, M.J., Lee, T., and Kirschner, M.W. (1992). Role of phosphorylation in p34^{cdc2} activation: identification of an activating kinase. Mol. Biol. Cell *3*, 13–27.

Strausfeld, U., Labbe, J.C., Fesquet, D., Cavadore, J.C., Picard, A., Sadhu, K., Russell, P., and Doree, M. (1991). Dephosphorylation and activation of a p34 cdc2/cyclin B complex in vitro by human cdc25 protein. Nature *351*, 242–248.

Wasserman, W., and Masui, Y. (1975). Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in *Xenopus oocytes*. Exp. Cell Res. *91*, 381–392.