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Cell Cycle Regulation of the p34^{cdc2} Inhibitory Kinases

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In cells of higher eukaryotic organisms the activity of the p34^{cdc2}/cyclin B complex is inhibited by phosphorylation of p34^{cdc2} at two sites within its amino-terminus (threonine 14 and tyrosine 15). In this study, the cell cycle regulation of the kinases responsible for phosphorylating p34^{cdc2} on Thr14 and Tyr15 was examined in extracts prepared from both HeLa cells and *Xenopus* eggs. Both Thr14- and Tyr15- specific kinase activities were regulated in a cell cycle-dependent manner. The kinase activities were high throughout interphase and diminished coincident with entry of cells into mitosis. In HeLa cells delayed in G2 by the DNA-binding dye Hoechst 33342, Thr14- and Tyr15- specific kinase activities remained high, suggesting that a decrease in Thr14- and Tyr15- kinase activities may be required for entry of cells into mitosis. Similar cell cycle regulation was observed for the Thr14/Tyr15 kinase(s) in *Xenopus* egg extracts. These results indicate that activation of CDC2 and entry of cells into mitosis is not triggered solely by activation of the Cdc25 phosphatase but by the balance between Thr14/Tyr15 kinase and phosphatase activities. Finally, we have detected two activities capable of phosphorylating p34^{cdc2} on Thr14 and/or Tyr15 in interphase extracts prepared from *Xenopus* eggs. An activity capable of phosphorylating Tyr15 remained soluble after ultracentrifugation of interphase extracts whereas a second activity capable of phosphorylating both Thr14 and Tyr15 pelleted. The pelleted fraction contained activities that were detergent extractable and that phosphorylated p34^{cdc2} on both Thr14 and Tyr15. The Thr14- and Tyr15-specific kinase activities co-purified through three successive chromatographic steps indicating the presence of a dual-specificity protein kinase capable of acting on p34^{cdc2}.

INTRODUCTION

In proliferating cells, the phosphorylation state of p34^{cdc2} changes in a cell cycle-dependent manner. p34^{cdc2} is progressively phosphorylated on tyrosine (Tyr15) and threonine (Thr14 and Thr161) residues beginning in S phase and peaking in late G2 phase (Draetta and Beach, 1988; Draetta *et al.*, 1988; Morla *et al.*, 1989; Krek and Nigg, 1991). Phosphorylation of p34^{cdc2} on tyrosine and threonine residues occurs in a cyclin-dependent manner (Solomon *et al.*, 1990; Meijer *et al.*, 1991; Parker *et al.*, 1991; Solomon *et al.*, 1992; Atherton-Fessler *et al.*, 1993). At the G2/M phase transition, p34^{cdc2} is dephosphorylated on tyrosine 15 and threonine 14, but not on threonine 161 (Draetta *et al.*, 1988; Dunphy and Newport, 1989; Gautier *et al.*, 1989; Morla *et al.*, 1989; Solomon *et al.*, 1990; Krek and Nigg, 1991; Norbury *et al.*, 1991). It is the abrupt dephosphorylation of Tyr15 and Thr14 that leads to activation of the p34^{cdc2} kinase and entry of cells into mitosis (Dunphy and Newport,

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1989; Gautier *et al.*, 1989; Morla *et al.*, 1989; Solomon *et al.*, 1990; Krek *et al.*, 1991; Norbury *et al.*, 1991). Following the degradation of cyclin B and exit of cells from mitosis, Thr161 is dephosphorylated during early G1 (Lorca, 1992). Thus it is clear that $p34^{cdc2}$ is phosphorylated and dephosphorylated at specific points in the cell cycle, but it is less clear what determines the timing of these phosphorylation and dephosphorylation events.

In *Schizosaccharomyces pombe*, the phosphorylation of Tyr15 is regulated by protein kinases encoded by *wee1*$^+$ and *mik1*$^+$ (Russell and Nurse, 1987; Lundgren *et al.*, 1991). *wee1*$^+$ encodes a 107 kDa protein kinase that directly phosphorylates $p34^{cdc2}$ on Tyr15 (Featherstone *et al.*, 1991; Parker *et al.*, 1991, 1992), while *mik1*$^+$ encodes a putative protein kinase that is also involved in maintaining $p34^{cdc2}$ in its tyrosine phosphorylated and inactive state (Lundgren *et al.*, 1991). Another mitotic control gene identified in *S. pombe*, *cdc25*$^+$, encodes a mitotic inducer and $p34^{cdc2}$ activator, which directly dephosphorylates $p34^{cdc2}$ on Tyr15 (Russell and Nurse, 1986; Millar *et al.*, 1991a). The protein-tyrosine phosphatase encoded by *pyp3*$^+$ can functionally substitute for *cdc25*$^+$ in the dephosphorylation of $p34^{cdc2}$ on Tyr15 (Millar *et al.*, 1992). Functional homologs of *cdc25*$^+$ have been identified in a variety of species and have been shown to encode phosphatases capable of dephosphorylating $p34^{cdc2}$ on Tyr15, as well as Thr14 (Dunphy *et al.*, 1991; Gautier *et al.*, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992; Sebastian *et al.*, 1993).

A human homolog of *wee1*$^+$ was cloned based on its ability to complement *wee1*$^+$ mutants of S. pombe (Igarashi *et al.*, 1991). Given that S. pombe *wee1*$^+$ encodes a protein kinase capable of phosphorylating serine, threonine, and tyrosine residues in vitro, the possibility arose that *wee1*$^+$ from higher eukaryotes might encode a true dual-specificity kinase that would phosphorylate $p34^{cdc2}$ on both Tyr15 and Thr14. However, the human homolog of *wee1*$^+$ was shown to encode a tyrosine-specific protein kinase that phosphorylates $p34^{cdc2}$ only on Tyr15 in vitro (Honda *et al.*, 1992; Parker and Piwnica-Worms, 1992; McGowen and Russell, 1993). Thus a second inhibitory kinase must exist in higher eukaryotes that is responsible for phosphorylating $p34^{cdc2}$ on Thr14. At this time, it is unknown whether this kinase is a serine/threonine-specific kinase that phosphorylates $p34^{cdc2}$ only on Thr14, or whether it might in fact be a dual-specificity kinase capable of phosphorylating both Thr14 and Tyr15.

The phosphorylation of $p34^{cdc2}$ on Thr14, Tyr15, and Thr161 during S and G2 parallels the synthesis and accumulation of cyclin B, a finding which reinforces the model that cyclin binding potentiates phosphorylation of $p34^{cdc2}$. Thus the timing of phosphorylation of $p34^{cdc2}$ is limited to some extent by the synthesis of its regulatory subunit, cyclin B. Regulation of the kinases (i.e., Wee1 and Mik1) and phosphatases (i.e., Cdc25), which act directly on $p34^{cdc2}$ represents another potential means by which the timing of critical phosphorylation/dephosphorylation events is determined.

In *S. pombe* and *Drosophila*, both the mRNA and protein levels of Cdc25 oscillate during the cell cycle, peaking at mitosis (Edgar and O'Farrell, 1989; Ducommun *et al.*, 1990; Edgar and O'Farrell, 1990; Moreno *et al.*, 1990). Thus in these organisms the synthesis of Cdc25 mRNA and protein determines the timing of dephosphorylation and activation of $p34^{cdc2}$. In *Xenopus* and humans, however, the abundance of Cdc25 remains constant throughout the cell cycle (Millar *et al.*, 1991b; Izumi *et al.*, 1992; Jessus and Beach, 1992; Kumagai and Dunphy, 1992). Cdc25 has been shown to physically associate with cyclin B in both *Xenopus* and human cells (Galaktionov and Beach, 1991; Jessus *et al.*, 1992), and this association may be required for the efficient dephosphorylation of $p34^{cdc2}$ (Zheng and Ruderman, 1993). Thus dephosphorylation of $p34^{cdc2}$ by Cdc25 may also be regulated by the formation of the $p34^{cdc2}$/cyclin B complex. In addition, Cdc25 is phosphorylated in a cell cycle dependent manner, with maximal phosphorylation occurring during M phase (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993). The phosphorylation of Cdc25 is associated with an increase in its phosphatase activity, which is reversible by dephosphorylation in vitro (Izumi *et al.*, 1992; Kumagai *et al.*, 1992; Hoffmann *et al.*, 1993).

Fission yeast $p107^{wee1}$ is negatively regulated by the cdr1/nim1 protein kinase, which phosphorylates $p107^{wee1}$ within its catalytic domain (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). Recent evidence from Tang *et al.* (1993) suggests that when fission yeast $p107^{wee1}$ (overproduced in insect cells) is mixed with M phase *Xenopus* egg extracts, its kinase activity is inhibited, and this inhibition is due to phosphorylation of its amino-terminal end (on sites distinct from those phosphorylated by the cdr1/nim1 kinase)(Tang *et al.*, 1993). These results corroborate an earlier report by Smythe and Newport (1992), which showed that M phase *Xenopus* egg extracts possessed diminished $p34^{cdc2}$-specific tyrosine kinase activity when compared with S phase extracts (Smythe and Newport, 1992). It has not been determined whether similar mechanisms of regulation hold true for mammalian cells. Further, these studies have been limited to the regulation of phosphorylation of $p34^{cdc2}$ at Tyr15. Virtually nothing is known about the regulation of the kinase responsible for the phosphorylation of $p34^{cdc2}$ on Thr14.

We have developed an assay system where the regulation of the kinases responsible for phosphorylating $p34^{cdc2}$ on Thr14 and Tyr15 can be studied in vitro. We report that the Thr14- and Tyr15-specific kinase activities are coordinately regulated in a cell cycle-dependent manner in both HeLa cells and in extracts prepared from *Xenopus* eggs. In addition, we have used this assay sys-

tem to begin to purify the Thr14- and Tyr15-specific kinases from interphase extracts prepared from *Xenopus* eggs.

MATERIALS AND METHODS

All procedures relating to Sf9 insect cell culture, generation of recombinant baculovirus, and propagation of recombinant baculovirus were performed as described elsewhere (Piwnica-Worms, 1990). Recombinant viruses encoding p34^{cdc2}(Arg 33) and p34^{cdc2}(Phe 15) have been described previously (Parker *et al.*, 1991; Atherton-Fessler *et al.*, 1993).

Mutagenesis of cdc2

Site-directed mutagenesis of the human cdc2 gene was performed as previously described (Parker *et al.*, 1991; Atherton-Fessler *et al.*, 1993). Single-stranded pSAF20 containing a 679-bp *Bam*HI-*Hinc*II N-terminal fragment of cdc2 served as the template for mutagenesis of threonine 14 to alanine (p34^{cdc2}[Ala 14]), as well as threonine 14 and tyrosine 15 to alanine and phenylalanine, respectively (p34^{cdc2}[Ala14/Phe15]). The oligonucleotides used in these cases were as follows:

5′-GGAGAAGGTGCCTATGGAGTTGTG-3′ (Thr14 to Ala14) and 5′-AGAAGGTGCCTTTGGAGTTGTGTA-3′ (Thr14/Tyr15 to Ala14/Phe15).

The wild-type cdc2 N-terminus of pSAF10 was then replaced with the mutant N-termini to generate full-length cdc2(Ala14) and cdc2(Ala14/Phe15). Mutations were confirmed by dideoxynucleotide sequencing and a 1.2 kb *Bam*HI to *Eco*RI fragment encoding each mutant was cloned into pVL1393 for the generation of recombinant baculoviruses as described (Piwnica-Worms, 1990).

Labeling of Sf9 Cells In Vivo

Insect cells infected with recombinant baculovirus were rinsed once with methionine-free Graces medium and then resuspended in methionine-free medium supplemented with 2 mM glutamine, 1.5% dialyzed calf serum, and 0.30-0.35 mCi/ml ^{35}S-Express protein label (NEN/DuPont); ~1.5×10^7 cells/ml. Cells were rocked at room temperature (25°C) for 3-4 h., then rinsed once in phosphate-buffered saline and stored as cell pellets at −80°C.

Preparation of Insect Cell Lysates

Insect cells were lysed in Nonidet P-40 (NP-40)/tris (hydroxymethyl) aminomethane (Tris) buffer [50 mM Tris (pH 7.4), 0.25 M NaCl, 50 mM NaF, 10 mM sodium pyrophosphate (NaPPi), 0.1% NP40, 10% glycerol] supplemented with 1 mM sodium orthovanadate, 0.15 U/ml of aprotinin, 20 μM pepstatin, and 20 μM leupeptin. Cell lysates were clarified at 10 000 × *g* for 15 min.

HeLa Cell Culture and Synchronization

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) with 10% calf serum (Sigma, St. Louis, MO). The double thymidine block release was performed essentially as described for the thymidine/aphidicolin double block release (Motokura *et al.*, 1991). Briefly, HeLa cells were grown to confluence, then incubated 16 h with 2 mM thymidine (Sigma). The cells were washed twice with Hank's balanced salt solution and trypsinized off the plates. Collected cells were then reseeded at ~4×10^6 cells per 100 mm tissue culture dish in DMEM/10% calf serum supplemented with 24 μM each of thymidine and deoxycytidine (Sigma) and incubated for 8 h. Cells were then incubated for a further 16 h in the presence of 2 mM thymidine. The plates were washed three times with prewarmed (37°C) DMEM and cultured in DMEM/10% calf serum for 0 to 14 h, as indicated in the appropriate figure legends (Figures 3 and 4). At the indicated times, plates were washed with ice cold phosphate-buffered saline (PBS), and stored at −80°C until use. Nocodazole arrested cells were obtained by incubating cells in the presence of 0.1 μg/ml nocodazole (Sigma) for 10 to 18 h. Mitotic cells were then collected by mitotic shake off. Cells were delayed in G2 by the addition of 7.5 μg/ml Hoechst 33342 (Calbiochem, San Diego, CA) 4 h after release from a double thymidine block (Crissman *et al.*, 1990). Plates were frozen at −80°C until needed. Immunoprecipitation assays (histone H1 kinase assays) or phosphorylation (shift) assays were performed as described below. For histone H1 kinase assays, HeLa cells were lysed in modified NETN buffer (20 mM Tris pH 8.0, 1 mM EDTA, 0.5% NP-40, 0.4 M NaCl, 10 mM NaF, 0.1 mM sodium vanadate, 2 mM dithiothreitol (DTT) and 5 μM microcystin (Gibco-BRL). For the Thr14/Tyr15 phosphorylation (shift) assays, HeLa cells were lysed in buffer consisting of 50 mM Tris pH 7.5, 5 mM EDTA, 2 mM DTT, 10 mM NaF, 0.1 mM sodium vanadate, 250 mM NaCl and 0.1% Brij-35.

Histone H1 Kinase Assays (HeLa Cells)

Approximately 4×10^6 HeLa cells (at different points in the cell cycle) were lysed in 1 ml of a modified NETN buffer (as described above). Anti-cyclin B serum (2.0 μl) (Atherton-Fessler *et al.*, 1993) was added to 50 μl of lysate, the reaction was brought up to 300 μl with NETN buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40] containing 1 mM sodium orthovanadate, 0.15 U/ml of aprotinin, 20 μM pepstatin, and 20 μM leupeptin. Reactions were incubated for 2 h at 4°C. Twenty microliters of 50% slurry of Sepharose CL-4B protein A beads were added, and the immunoprecipitation was continued for 1 h at 4°C. Immunoprecipitates were washed twice in NETN, twice in NETN containing 1 mM NaCl, and twice in incomplete kinase buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 5 mM DTT). Forty microliters of complete kinase buffer [50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.4 μg/ml histone H1, 10 μM ATP and 0.5 to 1.0 mCi/ml γ^{32}P-labeled ATP] was added to the washed immunoprecipitates and reactions were incubated at 30°C for 10 min. Reactions were terminated by the addition of sodium dodecyl sulfate (SDS)-sample buffer, and the reaction products were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Phosphorylation of histone H1 was detected by autoradiography and quantitated by Cherenkov counting in a liquid scintillation counter.

Thr14/Tyr15 Phosphorylation (Shift) Assays (HeLa Cells)

Lysates were prepared from insect cells infected with recombinant viruses encoding p34^{cdc2} (either wild-type or mutant forms) and labeled in vivo with ^{35}S-methionine. These (^{35}S)p34^{cdc2}-containing lysates were combined with insect cell lysates containing GST-cyclin B and incubated in the presence of 10 mM $MgCl_2$ and 1 mM ATP for 30 min at 25°C to allow for efficient formation of (^{35}S)p34^{cdc2}/GST-cyclin B complexes. (^{35}S)p34^{cdc2}/GST-cyclin B complexes were precipitated with glutathione agarose, and the precipitates were washed twice with NP-40/Tris buffer, and three times with Q buffer [QB: 20 mM triethanolamine-HCl, (pH 7.6) 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 5 mM sodium fluoride, 1 mM sodium pyrophosphate] containing 0.05% Brij-35 (SurfactAmps; Pierce, Rockford, IL) prior to use as a substrate for Thr14 and/or Tyr15 phosphorylation in vitro. Approximately 1 to 3 μg of p34^{cdc2} protein was present in each assay. HeLa cell lysates were added to (^{35}S)p34^{cdc2}/GST-cyclin B complexes, and phosphorylation reactions were initiated by the addition of 1/10 volume of reaction mixture consisting of QB(+0.05% Brij-35) supplemented with 100 mM $MgCl_2$, 10 mM sodium orthovanadate, and 20 mM ATP (final concentrations 10 mM $MgCl_2$, 1 mM sodium orthovanadate, and 2 mM ATP). HeLa cell lysates were normalized for total cell protein for Figure 3 (1.0 mg per condition). In Figure 4, several tissue culture dishes were seeded with ~4×10^6 S-phase arrested cells (after the double thymidine block), and one plate was harvested for each shift assay. Protein concentrations ranged from 1.0 to 2.0 mg throughout the course of the experiment. We typically measured a

15 to 20% increase in total cellular protein from cells harvested early in the time course (S and G2) as compared with those harvested later (M and early G1). Typical reaction volumes ranged from 0.5-1.0 ml depending on the protein concentration of HeLa lysates. Reactions were allowed to proceed at 25°C for 30-45 min., after which the (^{35}S)p34^{cdc2}/GST-cyclin B precipitates were washed twice with 0.5 M LiCl in 20 mM Tris pH 8.0. Precipitates were solubilized in SDS-sample buffer and boiled for 3-5 min. Proteins were resolved by SDS-PAGE (12% acrylamide gels) and visualized by Coomassie blue staining. (^{35}S)p34^{cdc2} was visualized and quantitated by autoradiography or PhosphorImage scanning.

Xenopus Egg Extracts

The following buffers were used routinely in the preparation of extracts from *Xenopus* eggs: XB [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.7, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM sucrose], MMR (5 mM HEPES, pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1 mM EDTA). *Xenopus* egg extracts were prepared essentially as described (Murray and Kirschner, 1989), with minor modifications. Briefly, to prepare CSF-arrested (M-phase) extracts, *Xenopus* eggs were dejellied in 2% cysteine, pH 7.8, and transferred into XB containing 5 mM EGTA, 10 μg/ul of pepstatin A and chymostatin, and 100 μg/ul of cytochalasin B. Eggs were centrifuged for 3 min at 400 × *g* to remove excess buffer. Packed eggs were then crushed by centrifugation for 10 min at 10 000 × *g* in a swinging bucket rotor. The cytoplasmic layer was removed, placed on ice, and pepstatin A, chymostatin, and cytochalasin B were added to 10 μg/ul each. Extracts were then centrifuged for 15 min at 15 000 × *g* (4°C), followed by 15 min at 10 000 × *g* (4°C) to clarify. Interphase extracts were derived from CSF-arrested extracts by the addition of 100 μg/ml cycloheximide and 0.4 mM $CaCl_2$ and incubation for 40 min at 23°C. Alternatively, interphase extracts were obtained by activation of dejellied eggs with calcium ionophore. In this case, dejellied eggs were washed with 0.2X MMR, and then activated in a minimal volume of 0.2X MMR containing 5 μg/ml A23187 for 60 to 90 s. Eggs were washed extensively with 0.2X MMR and incubated for 40 min at 23°C to allow for complete entry into interphase. Extracts were then prepared as described above for CSF-arrested extracts. Finally, extracts were aliquoted, frozen, and stored at −80°C until needed. For time course experiments (Figure 6A), CSF-arrested extracts were activated with 0.4 mM $CaCl_2$ in the absence of added protease inhibitors or cycloheximide. Aliquots were removed at the times indicated after activation, quick frozen, and stored at −80°C. To monitor exit from M-phase into interphase, 3 μl of each extract were removed, diluted 10-fold in water, and were frozen at −80°C for histone H1 kinase assays. Histone H1 assays were performed for 10 min at 30°C in a final volume of 30 μl. Reactions consisted of 5 μl of diluted extract, 20 mM HEPES (pH 7.5), 5 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 200 μM ATP, 10 μg of histone H1, 10 μCi γ^{32}P ATP, and 0.2 μg of protein kinase A inhibitor. The interphase extracts used in figure 6, B and C were a gift from Dr. Peter Jackson (Dept. Cell Biology, Harvard Medical School) and were prepared as described by Murray (1991). To two 50 μl aliquots of interphase extract (~2 mg each) the following was added: 2.5 μl of a 20X energy mix (150 mM phosphocreatine, 20 mM ATP, 2 mM EGTA, 20 mM $MgCl_2$) and 5 μl of 10X protease inhibitor mix (0.1 mg/ml each pepstatin A, chymostatin, and leupeptin). The interphase extract was either incubated alone or in the presence of 2 μl (14 to 20 μg) of Δ90 cyclin (Glotzer *et al.*, 1991) at 23°C for 50 min to generate a stable mitotic extract. Three milliliters of each extract were removed for histone H1 kinase assays as described above. The remainder of each extract was diluted with 350 μl of QB containing 0.5% Brij-35, and 100 μl aliquots were used in shift assays by incubating with (^{35}S)p34^{cdc2}/GST-cyclin B precipitates for various periods of time as described below.

Thr14/Tyr15 Phosphorylation (Shift) Assays (Xenopus Egg Extracts)

(^{35}S)p34^{cdc2}/GST-cyclin B complexes were prepared as described above and washed with QB (+Brij-35). *Xenopus* egg extracts (1-2 mg total protein) or samples from selected column fractions were added to (^{35}S)p34^{cdc2}/GST-cyclin B complexes and phosphorylation reactions were initiated by the addition of 1/10 volume of reaction mixture consisting of QB(+Brij-35) supplemented with 100 mM $MgCl_2$, 10 mM sodium orthovanadate, and 20 mM ATP (final concentrations 10 mM $MgCl_2$, 1 mM sodium orthovanadate, and 2 mM ATP). For the shift assay shown in Figure 6B, reactions were adjusted to 1 μM microcystin. Typical reaction volumes ranged from 0.05 to 1.5 ml depending on the protein concentration of samples to be assayed. Reactions were allowed to proceed at 25°C for 30-45 min after which the (^{35}S)p34^{cdc2}/GST-cyclin B precipitates were washed twice with 0.5 M LiCl in 20 mM Tris pH 8.0. Precipitates were solubilized in SDS-sample buffer and boiled for 3-5 min. Proteins were resolved by SDS-PAGE (12% acrylamide gels) and visualized by Coomassie blue staining. (^{35}S)p34^{cdc2} was visualized by autoradiography or PhosphorImage scanning.

Separation of Two Activities in Xenopus Egg Extracts Capable of Phosphorylating p34^{cdc2} on Thr14/Tyr15

Eggs were collected and washed in 0.1 M NaCl. Interphase extract prepared as described above, was diluted two- to four-fold with buffer A (20 mM HEPES pH 7.6, 10% glycerol, 1 mM EDTA, 5 mM NaF, 1 mM sodium pyrophosphate) containing 50 mM NaCl, 1 mM DTT, 0.1 U/ml aprotinin, 10 μM pepstatin, and 10 μM leupeptin and was centrifuged at 150 000 × *g* for 20 min at 4°C. The supernatant was saved and referred to as 150S. The pellet (150P) was solubilized in buffer A containing 0.05% Brij-35 (Pierce) and placed on ice for 20 min prior to recentrifugation at 150 000 × *g*. The pellet from the second centrifugation was further extracted with buffer A containing 0.2 and 0.5% of Brij-35 as described above. Alternatively, the 150 000 × *g* pellet was extracted with buffer A containing 0.1 and 0.5% Triton X-100.

To purify Thr14 kinase activity from *Xenopus* extract, the 150 000 × *g* pellet was washed in buffer A containing 0.5 M NaCl and protease inhibitors, centrifuged at 150 000 × *g* for 20 min, and resolubilized in buffer A containing 0.5% Brij-35. The tube was placed on ice for 20 min and recentrifuged at 150 000 × *g*. The supernatant (150P0.5B) was saved and further fractionated by column chromatography (see below).

Phosphatase Assays

To characterize the nature of kinase(s) in both the 150 000 × *g* supernatant and the pellet, (^{35}S)-p34^{cdc2}/GST-Cyclin B was precipitated with glutathione agarose beads and incubated with either 150 000 × *g* supernatant or pellet solubilized with 0.5% Brij-35 under kinase conditions as described above. The reactions were stopped by washing the beads two times with gel shift buffer [50 mM Tris (pH 7.4), 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 5 mM sodium fluoride, 1 mM sodium pyrophosphate containing 0.05% Brij-35] and three times with phosphatase buffer (50 mM Tris pH 7.4, 2 mM EDTA, 2 mM DTT). The beads were then resuspended in 400 μl of phosphatase buffer and incubated in the presence or absence of 1 μg of yeast PTP1 phosphatase at 25°C for 30 min. The reactions were then washed with 0.5 M LiCl and analyzed on a 12% SDS-PAGE.

Fractionation of Xenopus Egg Extracts

The Thr14/Tyr15 kinase activities were solubilized from the 150 000 × *g* pellet by extraction with buffer A/0.5% Brij-35 as described above (~40-fold purification). A total of 36 ml of 150P0.5B fraction was loaded onto a 12 ml Q-Sepharose (Pharmacia, Piscataway, NJ) column equilibrated with buffer AB [20 mM HEPES (pH 7.6), 10% glycerol, 1 mM EDTA, 5 mM NaF, 1 mM sodium pyrophosphate, 0.05% Brij-35] containing 50 mM NaCl at a flow rate of 0.5 ml/min. Proteins bound to the column were eluted with a 100 ml NaCl gradient from 0.05 M to 1 M in buffer AB at 1 ml/min. Three milliliter fractions

were collected and assayed for the ability to retard the electrophoretic mobility of (^{35}S)-p34^{cdc2} in the phosphorylation assay described above (~3.6-fold purification). The active fractions (eluted at 220 to 450 mM NaCl) were pooled and desalted on a Sephadex G-25 column in buffer AB and then applied to a 7 ml S-Sepharose (Pharmacia) column at a flow rate of 0.5 ml/min. Bound proteins were eluted from the column with buffer AB containing 0.3 M NaCl (S0.3) (~6.5-fold purification). The S0.3 fraction was desalted in buffer AB to 24 ml in total volume, and 2 ml of desalted S0.3 were applied to a 1 ml Reactive Yellow 3 column (Sigma) equilibrated with buffer AB at 0.3 ml/min. Bound proteins were eluted with a 15 ml gradient of 0 to 1 M NaCl in buffer AB, and 1 ml fractions were collected and assayed for the ability to retard the electrophoretic mobility of (^{35}S)-p34^{cdc2} (~3-fold purification). The overall purification of the kinase activity was estimated to be ~2800-fold.

RESULTS

An In Vitro Assay for Thr14- and Tyr15-Specific Kinase Activities

Human p34^{cdc2} has been demonstrated to decrease in electrophoretic mobility upon phosphorylation of either Thr14 or Tyr15 residues, with a further decrease in mobility upon phosphorylation of both Thr14 and Tyr15 (Solomon *et al.*, 1992). Thus changes in the electrophoretic mobility of p34^{cdc2} can be used as a measure of changes in its state of phosphorylation at Thr14 and Tyr15. Phosphorylation of Thr161 does not result in a detectable change in the electrophoretic mobility of p34^{cdc2}. We took advantage of this fact to develop an assay that monitors the activities of Thr14- and Tyr15-specific kinases in HeLa cell lysates. To obtain a substrate for these kinases, insect cells overproducing p34^{cdc2} (either wild-type or mutant forms) were labeled with ^{35}S-methionine in vivo. Lysates prepared from these ^{35}S-labeled cells were then combined with lysates from insect cells producing cyclin B fused with the glutathione-S-transferase protein, GST-cyclin B, and the resulting p34^{cdc2}/GST-cyclin B complexes were isolated using glutathione agarose chromatography. By forming p34^{cdc2}/GST-cyclin B complexes in vitro, we could obtain large amounts of protein substrate in which only the p34^{cdc2} component was radioactively labeled. In addition, isolation of complexes by virtue of the GST-cyclin B fusion protein insures that 100% of the p34^{cdc2} isolated is associated with cyclin B and therefore is a preferred substrate for phosphorylation (Solomon *et al.*, 1990; Meijer *et al.*, 1991; Parker *et al.*, 1991, 1992).

The Thr14 and Tyr15 kinase activities of HeLa cells were assayed by mixing HeLa cell lysates with (^{35}S)p34^{cdc2}/GST-cyclin B complexes in the presence of MgATP. Inhibitors of both tyrosine- and serine/threonine-specific protein phosphatases were included in the reactions so that it was possible to specifically monitor the activities of the Thr14/Tyr15 kinases. Phosphorylation of Thr14 and Tyr15 was then assessed by monitoring the electrophoretic mobility of the ^{35}S-labeled p34^{cdc2}. As shown in Figure 1, upon incubation with HeLa cell lysate, p34^{cdc2} shifts from a single electrophoretic form at 34 kDa (lane 1) to three electrophoretic forms (lane 2) indicative of phosphorylation on either Thr14 and Tyr15. The middle band seen in lane 2 contains p34^{cdc2}, which is phosphorylated on Thr14 or Tyr15, while the topmost band contains p34^{cdc2} phosphorylated at both sites (see below). A kinase-deficient form of p34^{cdc2}, p34^{cdc2}(Arg33) (lanes 3 and 4), underwent a comparable shift in electrophoretic mobility after incubation with HeLa cell lysate (lane 4). To confirm that the observed shift in electrophoretic mobility corresponds to phosphorylation of p34^{cdc2} at Thr14 and Tyr15, the mobilities of nonphosphorylatable forms of p34^{cdc2} were examined after incubation alone or in the presence of HeLa cell lysate. When Thr14 is changed to Ala to generate p34^{cdc2}(Ala14) (lanes 5 and 6), p34^{cdc2} undergoes a single shift in electrophoretic mobility (lane 6) in the presence of HeLa cell lysate, indicative of phosphorylation at Tyr15. Likewise, when Tyr15 is changed to Phe, p34^{cdc2}(Phe15) (lanes 7 and 8), a single shift in mobility is observed (lane 8), indicative of phosphorylation at Thr14. If both Thr14 and Tyr15 are mutated, to Ala and Phe, respectively, the electrophoretic mobility of p34^{cdc2} is no longer affected by incubation with HeLa cell lysate (lanes 9 and 10). Thus phosphorylation of p34^{cdc2} at Thr14 and/or Tyr15 can be assessed by monitoring the electrophoretic mobility of (^{35}S)p34^{cdc2} (either wild-type or mutant forms). In addition, these results indicate that phosphorylation of Thr14 and Tyr15 can occur independent of one another.

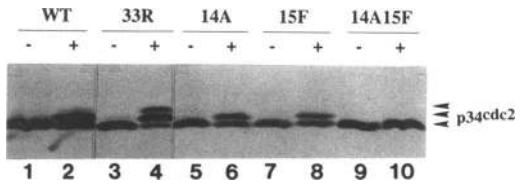

Figure 1. p34^{cdc2} is phosphorylated on Thr14 and Tyr15 in HeLa cell lysates. Insect cells producing wild-type p34^{cdc2} (WT, lanes 1 and 2), p34^{cdc2}(Arg33) (33R, lanes 3 and 4), p34^{cdc2}(Ala14) (14A, lanes 5 and 6), p34^{cdc2}(Phe15) (15F, lanes 7 and 8), or p34^{cdc2}(Ala14Phe15) (14A15F, lanes 9 and 10) were labeled in vivo with ^{35}S-methionine, lysed and combined with lysates from insect cells expressing GST-cyclin B. p34^{cdc2}/GST-cyclin B complexes were precipitated with glutathione agarose beads, and precipitates were incubated for 45 min at 25°C with buffer alone (−, lanes 1, 3, 5, 7, and 9) or with HeLa cell extract (+, lanes 2, 4, 6, 8, and 10). Proteins were resolved by SDS-PAGE and ^{35}S-labeled p34^{cdc2} was visualized by autoradiography.

Dependence of Thr14/Tyr15 Phosphorylation on HeLa Cell Protein Concentration

As shown in Figure 2 (top panel), with increasing amounts of HeLa cell protein, p34^{cdc2}(Arg33) shifts from a single electrophoretic form at 34 kDa to three electrophoretic forms indicative of phosphorylation at Thr14 and Tyr15. With the addition of 0.5 mg of HeLa cell protein, ~30% of p34^{cdc2}(Arg33) was phosphorylated on one or both sites (as indicated by a decrease in elec-

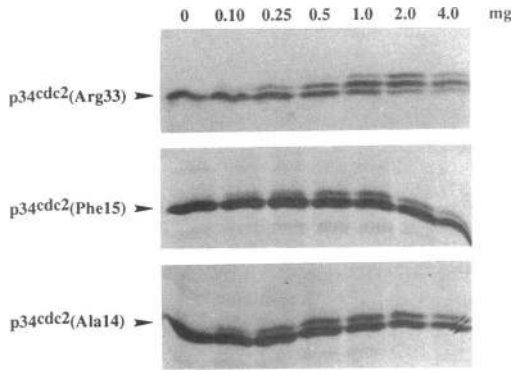

Figure 2. Dependence of Thr14- and Tyr15-specific kinase activities on concentration of HeLa cell lysates. Protein precipitates containing $p34^{cdc2}$(Arg33) (top), $p34^{cdc2}$(Phe15) (middle), or $p34^{cdc2}$(Ala14) (bottom) in complex with GST-cyclin B were prepared as described in Figure 1. $p34^{cdc2}$/GST-cyclin B complexes were incubated for 30 min at 25°C with varying amounts of HeLa cell lysate (0–4 mg total protein). Proteins were resolved by SDS-PAGE and ^{35}S-labeled $p34^{cdc2}$ was visualized by autoradiography.

trophoretic mobility), and this fraction increased to >80% with the addition of 2 mg of HeLa protein. Phosphorylation of $p34^{cdc2}$(Arg33) followed saturation kinetics up to 2 mg of HeLa cell protein. At the highest concentration of HeLa cell protein (4 mg in this experiment), the $p34^{cdc2}$(Arg33)/GST-cyclin B substrate began to elute from the glutathione agarose, leading to a slight decrease in detected kinase activity.

To monitor the Thr14 and Tyr15 kinase activities independently, either $p34^{cdc2}$(Phe15) or $p34^{cdc2}$(Ala14) were assayed with varying amounts of HeLa cell protein. As shown in Figure 2 (middle), when $p34^{cdc2}$(Phe15) is incubated with increasing amounts of HeLa cell protein, it becomes progressively phosphorylated on Thr14, as indicated by the appearance of a slower electrophoretic form. Similarly, $p34^{cdc2}$(Ala14) becomes progressively phosphorylated on Tyr15 with increasing amounts of HeLa cell protein (Figure 2, bottom). These results indicate that the protein concentration dependence of both activities are comparable, with maximal phosphorylation occurring at 1 to 2 mg HeLa protein. Thus, for experiments designed to detect potential differences in the Thr14 and/or Tyr15 kinase activities, an assay of 0.5 to 1 mg of HeLa lysate was judged to be within the linear range of both kinase activities.

Cell Cycle Regulation of Thr14- and Tyr15-Specific Kinase Activities in HeLa Cells

During a normal cell division cycle, $p34^{cdc2}$/cyclin B complexes begin to accumulate during late S phase, and these complexes are maintained in an inactive state by phosphorylation at Thr14 and Tyr15 (Draetta and Beach, 1988; Draetta *et al.*, 1988; Dunphy and Newport, 1989; Gautier *et al.*, 1989; Morla *et al.*, 1989; Solomon *et al.*, 1990; Krek *et al.*, 1991; Norbury *et al.*, 1991). At the transition from G2 to M phase, Thr14 and Tyr15 are rapidly dephosphorylated and $p34^{cdc2}$ is activated as a protein kinase (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Morla *et al.*, 1989; Solomon *et al.*, 1990; Krek *et al.*, 1991; Norbury *et al.*, 1991). We wished to determine whether the Thr14- and Tyr15-specific kinase activities present in HeLa cells are regulated in a cell cycle dependent manner. In Figure 3, HeLa cells were tested at various stages of the cell cycle for their ability to phosphorylate $p34^{cdc2}$ at Thr14 and/or Tyr15 using the assay described above. HeLa cells were synchronized in S phase by a double thymidine block and were then released and allowed to proceed through G2 into mitosis (M) or were delayed in G2 with the DNA binding dye, Hoechst 33342. Lysates prepared from aynchronous HeLa cells were capable of phosphorylating $p34^{cdc2}$(Arg33) on both Thr14 and Tyr15, resulting in the appearance of two retarded electrophoretic forms (Figure 3, top panel). Interestingly, as shown in Figure 3 (top), lysates prepared from both S phase and G2 phase cells phosphorylated Thr14 and Tyr15 to the same extent as those prepared from asynchronous cells [~70-80% of $p34^{cdc2}$(Arg33) phosphorylated on one or both sites]. However, mitotic cells obtained after release from a double thymidine block possessed significantly less Thr14- and/or Tyr15-specific kinase activity, as judged by a decrease in the proportion of $p34^{cdc2}$(Arg33) migrating with a retarded electrophoretic mobility (~35% phosphorylated; Figure 3, top). If mitotic cells were obtained after a nocodazole arrest, these results were even more striking, in that $p34^{cdc2}$(Arg33) was not detectably phosphorylated on either Thr14 or Tyr15 (Figure 3, top) when compared with controls.

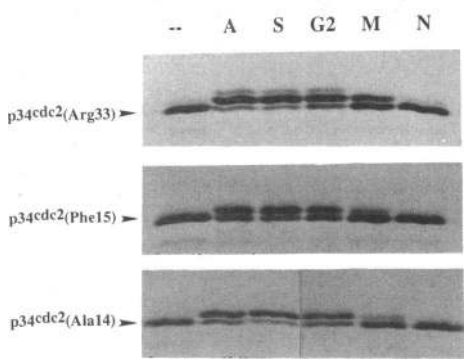

Figure 3. Phosphorylation of $p34^{cdc2}$ on Thr14 and Tyr15 is diminished in mitotic HeLa cell lysates. Protein precipitates containing $p34^{cdc2}$(Arg33) (top), $p34^{cdc2}$(Phe15) (middle), or $p34^{cdc2}$(Ala14) (bottom) in complex with GST-cyclin B were prepared as described in Figure 1. $p34^{cdc2}$/GST-cyclin B complexes were incubated with lysates prepared from asynchronous HeLa cells (A); HeLa cells synchronized in S phase (S; double thymidine block); G2 phase (G2; release from double thymidine into H33342); or mitosis (M; mitotic shake-off following release from double thymidine block); or nocodazole arrested mitotic HeLa cells (N). Proteins were resolved by SDS-PAGE, and ^{35}S-labeled $p34^{cdc2}$ was visualized by autoradiography and quantitated by PhosphorImager.

Thr14- and Tyr15-specific kinase activities were distinguished by using p34cdc2(Phe15) and p34cdc2(Ala14) as substrates for phosphorylation by HeLa cells synchronized in S phase, G2 phase, or mitosis. As in the case of p34cdc2(Arg33), S- phase and G2-phase cells readily phosphorylated both p34cdc2(Phe15) (Figure 3, middle) and p34cdc2(Ala14) (Figure 3, bottom) to the same extent as asynchronous cells. The phosphorylation of both p34cdc2(Phe15) and p34cdc2(Ala14) by mitotic cells decreased by ~65%, suggesting that both Thr14- and Tyr15-specific kinase activities are negatively regulated at the G2 to M phase transition. Again, these results were enhanced in nocodazole arrested HeLa cells, with no detectable phosphorylation of either p34cdc2(Phe15) or p34cdc2(Ala14) when compared with controls. These results indicate that the kinase(s) responsible for phosphorylating p34cdc2 on Thr14 and Tyr15 are regulated in a cell cycle dependent manner, such that both activities are reduced during mitosis.

To more closely examine the cell cycle regulation of the Thr14 and Tyr15 kinase activities, particularly at the G2/M phase transition, HeLa cell lysates were assayed for their ability to phosphorylate p34cdc2(Arg33) after release from an S-phase block in the presence or the absence of the DNA binding dye, Hoechst 33342 (Figure 4A). Entry into mitosis was monitored by assaying cell lysates at selected time points for histone H1 kinase activity associated with anti-cyclin B immunoprecipitates (Figure 4B). Cells progressed into mitosis 10 h after release from the double thymidine block, and exit from mitosis was complete by 13 h after the release. Incubation of cells with Hoechst 33342 caused a significant G2 delay. In Figure 4A, p34cdc2(Arg33) was readily phosphorylated on Thr14 and Tyr15 throughout S phase and G2 phase, with >95% of p34cdc2(Arg33) migrating with a reduced electrophoretic mobility. Concomitant with entry of cells into mitosis, phosphorylation of p34cdc2(Arg33) at Thr14 and Tyr15 decreased to ~50% of S and G2 phase levels. Finally, as cells exited mitosis into G1 of the next cell cycle, phosphorylation of Thr14 and Tyr15 increased, reflecting increased activity of the Thr14- and Tyr15-specific kinase(s). In the presence of H33342, entry into mitosis was delayed (Figure 4B) and the Thr14- and Tyr15-kinase activities remained elevated (Figure 4A).

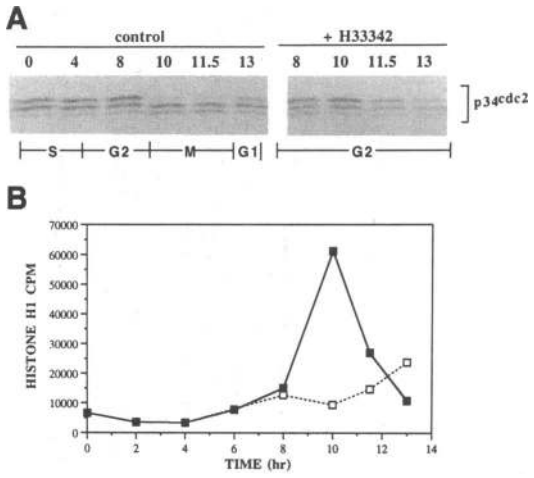

Figure 4. Cell cycle regulation of Thr14- and Tyr15-specific kinase activities in HeLa cells. HeLa cells were synchronized in S phase by a double thymidine block (time 0) and then allowed to progress through G2 and mitosis as described in MATERIALS AND METHODS. (A) p34cdc2(Arg33)/GST-cyclin B complexes (where p34cdc2 was labeled with ^{35}S-methionine) were incubated with lysates prepared from HeLa cells that had been collected at various times after release from an S phase block in the absence (control lanes) or in the presence of Hoechst 33342 (+H33342 lanes). Proteins were resolved by SDS-PAGE, and ^{35}S-labeled p34cdc2(Arg33) was visualized by autoradiography and quantitated by PhosphorImager. (B) HeLa cell lysates prepared as described above were immunoprecipitated with anti-cyclin B antiserum and precipitates were assayed for histone H1 kinase activity. Shown is a plot of radioactivity incorporated into histone H1 for control cells (■) and cells delayed in G2 (+H33342, □).

Xenopus Egg Extracts also Possess Thr14- and Tyr15-Specific Kinase Activities

Using the assay described above for HeLa cells, the characteristics of the Thr14- and Tyr15-specific kinase activities found in *Xenopus* egg extracts were examined. Activated *Xenopus* egg extracts were arrested in interphase by the addition of cycloheximide and assayed for the ability to phosphorylate several mutant forms of p34cdc2 in complex with GST-cyclin B (Figure 5A). Again, phosphorylation of Thr14 and/or Tyr15 was monitored by a shift in electrophoretic mobility of p34cdc2. When

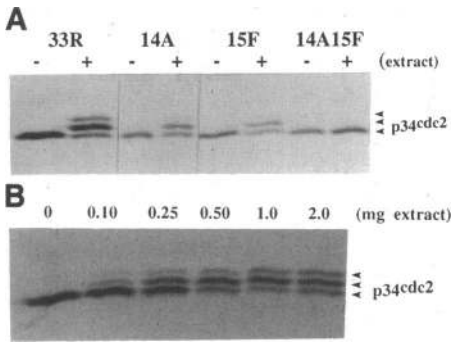

Figure 5. Characterization of Thr14- and Tyr15-specific kinase activities in *Xenopus* egg extracts. (A) Protein precipitates containing p34cdc2(Arg33) (33R), p34cdc2(Ala14) (14A), p34cdc2(Phe15) (15F), p34cdc2(Ala14Phe15) (14A15F) in complex with GST-cyclin B were prepared as described in Figure 1. p34cdc2/GST-cyclin B complexes were incubated in the absence (−) or in the presence (+) of interphase egg extracts (~1 mg total protein). Proteins were resolved by SDS-PAGE, and ^{35}S-labeled p34cdc2 was visualized by autoradiography. (B) (^{35}S)-p34cdc2(Arg33)/GST-cyclin B complexes were isolated as described in Figure 1. Precipitates were then incubated with varying amounts of interphase egg extract (0–2 mg total protein). Proteins were resolved by SDS-PAGE, and ^{35}S-labeled p34cdc2 was visualized by autoradiography.

p34^{cdc2}(Arg33) was incubated with interphase extract in the presence of MgATP and vanadate, it was readily phosphorylated on both Thr14 and Tyr15, as indicated by the appearance of two slower electrophoretic forms of p34^{cdc2}(Arg33) (Figure 5A; 33R). Similarly, p34^{cdc2}(Ala14) and p34^{cdc2}(Phe15) were phosphorylated on Tyr15 and Thr14, respectively, when incubated with interphase extract, indicated by a single shift in electrophoretic mobility (Figure 5A; 14A and 15F). The altered mobility observed after incubation of p34^{cdc2} with interphase extract was due to phosphorylation of Thr14 and/or Tyr15, as addition of interphase extract did not alter the electrophoretic mobility of p34^{cdc2}(Ala14Phe15) (Figure 5A; 14A and 15F).

To assess the activity of the *Xenopus* Thr14- and Tyr15-kinases, phosphorylation of Thr14 and Tyr15 was assayed with varying amounts of *Xenopus* egg extract. As shown in Figure 5B, the extent of phosphorylation of p34^{cdc2}(Arg33) increased with increasing amounts of interphase extract, as measured by a decrease in electrophoretic mobility. Phosphorylation of both Thr14 and Tyr15 was detected with as little as 100 μg extract, and maximal levels of phosphorylation were achieved with 0.5 to 1.0 mg extract. Thus, as measured in this assay, *Xenopus* egg extracts contain approximately twice the level of Thr14 and Tyr15 kinase activities found in HeLa cell lysates. However, it is unclear from these experiments whether this difference reflects a difference in the abundance of the Thr14/Tyr15 kinase(s), the activity of the kinase(s), or simply the method by which the extracts were prepared.

Cell Cycle Regulation of Thr14- and Tyr15-Specific Kinase Activities in Xenopus Egg Extracts

Earlier studies of the phosphorylation state of p34^{cdc2} in *Xenopus* egg extracts suggested that the rate of phosphorylation of p34^{cdc2} on Tyr15 is diminished in mitotic versus interphase extracts (Solomon *et al.*, 1990). Smythe and Newport (1992) reported that *Xenopus* egg extracts naturally arrested in M phase possessed less p34^{cdc2}-specific tyrosine kinase activity than extracts arrested in S phase by the presence of aphidicolin (Smythe and Newport, 1992). To assess both the Thr14- and Tyr15-specific kinase activities at different stages of the cell cycle in *Xenopus* egg extracts, two approaches were taken. In one case, CSF-arrested extracts (arrested in M phase) were induced to enter interphase by the addition of 0.4 mM $CaCl_2$. Extract samples were removed at selected time points after activation and incubated with (^{35}S)p34^{cdc2}(Arg33)/GST-cyclin B complexes, MgATP, and vanadate (Figure 6A). The Thr14- and Tyr15-kinase activities were low in M phase extracts (Figure 6A, 0'). Interestingly, both activities increased as early as 5 min after exit from M phase and remained relatively constant as the extract progressed fully into interphase (Figure 6A).

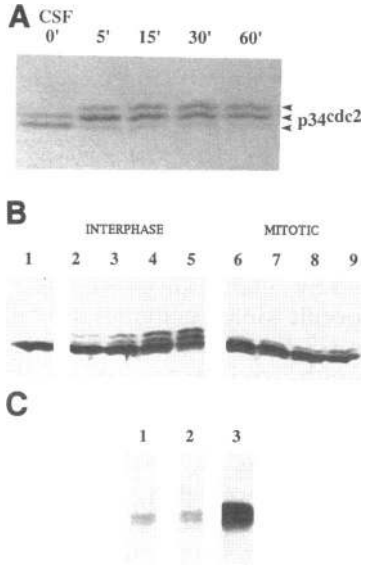

Figure 6. Cell cycle regulation of Thr14- and Tyr15-specific kinase activities in *Xenopus* egg extracts. (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B precipitates were prepared as described in Figure 1. (A) CSF-arrested egg extracts were activated with 0.4 mM $CaCl_2$ and incubated at 23°C. Aliquots of extract were removed at 5, 15, 30, and 60 min after activation and then incubated for 45 min at 25°C with (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B precipitates. (B) Interphase extracts were prepared from *Xenopus* eggs as described in the MATERIALS AND METHODS. Mitotic extracts were prepared by the addition of Δ90 cyclin (a nondegradable form of sea urchin cyclin B) to the interphase extracts. (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B precipitates were incubated in the interphase (lanes 2 to 5) and mitotic (lanes 6 to 9) extracts for 5, 10, 15, and 30 min at room temperature. Lane 1: (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B precipitates incubated in buffer alone for 30 min at room temperature. (C) Interphase and mitotic extracts were assayed for their ability to phosphorylate histone H1. Lane 1: interphase extract; lane 2: interphase extract incubated at 23°C for 50 min; lane 3: interphase extracts incubated at 23°C for 50 min in the presence of Δ90 sea urchin cyclin B. Proteins were resolved by SDS-PAGE, and ^{35}S-labeled p34^{cdc2}(Arg33) and ^{32}P-labeled histone H1 were visualized by autoradiography.

As a second approach, interphase extracts were prepared and were induced to enter M-phase by the addition of a nondegradable form of sea urchin cyclin B (Glotzer *et al.*, 1991) (Figure 6, B and C). The extracts were then assayed for levels of histone H1 kinase activity (Figure 6C) and were incubated with (^{35}S)p34^{cdc2}(Arg33)/GST-cyclin B complexes for various periods of time as described above (Figure 6B). As seen in Figure 6B (lanes 2-5), interphase extracts contained Thr14- and Tyr15-kinase activities. However, when these extracts were induced to enter a stable M-phase state, a loss in both activities was observed (lanes 6 to 9). These results suggest that the Thr14- and Tyr15-specific kinase activities are both activated immediately upon exit of cells from mitosis, remain high throughout interphase, and then decrease as cells progress from G2

into mitosis. Thus the results obtained with *Xenopus* egg extracts parallel those obtained for HeLa cells.

Characterization and Partial Purification of the Thr14/Tyr15 Kinase(s) from Xenopus Extracts

We used the shift assay described above to begin purifying the Thr14 and Tyr15 kinases from interphase extracts prepared from *Xenopus* eggs (Figure 7 and 8). The assay requires the use of the p34^{cdc2}/cyclin B complex because this is the preferred substrate for phosphorylation. Interphase extract was tested for its ability to shift the electrophoretic mobility of (^{35}S)-p34^{cdc2} as described previously (Figure 7A, lane 1). Alternatively, interphase extract was centrifuged at 150 000 × *g*, and the supernatant fraction (Figure 7A, lane 2) or the pellet fraction [extracted with varying concentrations of either Brij-35 (lanes 3, 4, 5), or Triton X (lanes 6 and 7)] were assayed for their ability to shift the electrophoretic mobility of (^{35}S)-p34^{cdc2}. The 150 000 × *g* supernatant fraction contained an activity capable of causing a single shift in the electrophoretic mobility of p34^{cdc2} (this represents p34^{cdc2} singly phosphorylated on either Thr14 or Tyr15). In contrast the 150 000 × *g* pellet contained activities (that were extractable with either Brij-35 or Triton X) that caused a double shift in the electrophoretic mobility of p34^{cdc2}. To further characterize these activities, phosphatase experiments were performed using PTP1, a tyrosine-specific protein phosphatase from *S. cerevisiae* (Figure 7B) (Hannig *et al.*, 1993). p34^{cdc2}/cyclin B was incubated with interphase extracts (lanes 2 and 3), with the 150 000 × *g* supernatant (lanes 4 and 5) or the 150 000 × *g* pellet that had been solubilized in 0.5% Brij-35. p34^{cdc2}/cyclin B was then incubated in phosphase buffer in the absence or the presence of PTP1. In these experiments, Tyr15 was dephosphorylated by PTP1, whereas phosphorylated Thr14 remained intact. These experiments demonstrated that the supernatant fraction contained an activity that exclusively phosphorylated p34^{cdc2} on Tyr15 (lanes 4 and 5), whereas the pellet fraction contained activities capable of phosphorylating both Thr14 and Tyr15 (lanes 6 and 7).

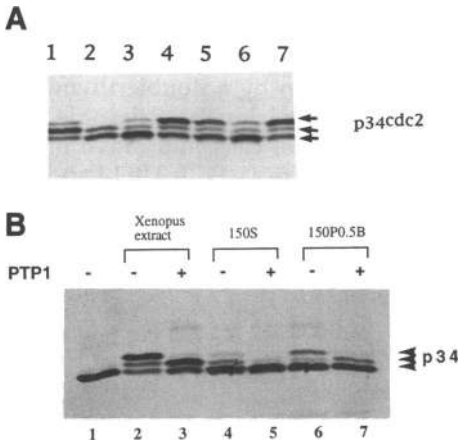

Figure 7. Identification of two activities in *Xenopus* interphase extracts capable of phosphorylating p34^{cdc2}. (A) (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B purified on glutathione beads was incubated with interphase extract prepared from *Xenopus* eggs (lane 1). Interphase extract was centrifuged at 150 000 × *g*. The 150 000 × *g* supernatant fraction (lane 2) and the 150 000 × *g* pellet fraction [extracted with varying concentrations of either Brij-35 (lanes 3–5) or Triton X (lanes 6 and 7)] were assayed for their ability to shift the electrophoretic mobility of (^{35}S)-p34^{cdc2}. (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B was resolved by SDS-PAGE on a 12% gel. ^{35}S-labeled p34^{cdc2} was visualized by autoradiography. Lane 1: interphase extract; lane 2: 150 000 × *g* supernatant; lane 3: the 150 000 × *g* pellet was solubilized in 0.05% Brij-35, recentrifuged at 150 000 × *g*, and the supernatant was assayed; lane 4: pellet from 3 was solubilized in 0.2% Brij-35, was recentrifuged at 150 000 × *g* and the supernatant was assayed; lane 5: pellet from 4 was solubilized in 0.5% Brij-35, was recentrifuged at 150 000 × *g* and the supernatant was assayed; lane 6: 150 000 × *g* pellet was solubilized in 0.1% Triton X, was recentrifuged at 150 000 × *g*, and the supernatant was assayed; lane 7: pellet from 6 was solubilized in 0.5% Triton X, was recentrifuged at 150 000 × *g*, and the supernatant was assayed. (B) (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B purified on glutathione beads was incubated with interphase extract, with the 150 000 × *g* supernatant fraction (150S) or with the 150 000 × *g* pellet fraction solubilized in 0.5% Brij-35 (150P0.5B). Reactions were stopped by washing with phosphatase buffer and further incubated in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of PTP1. Lane 1: substrate only. (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B was resolved by SDS-PAGE on a 12% gel. ^{35}S-labeled p34^{cdc2} was visualized by autoradiography.

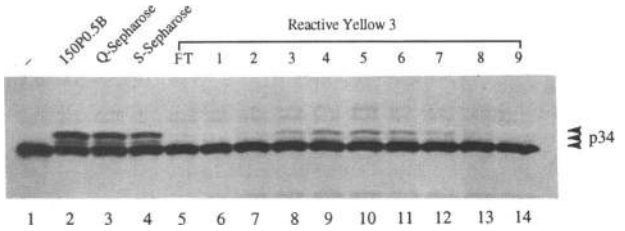

Figure 8. Partial purification of the Thr14/Tyr15 kinase(s) from *Xenopus* egg extract. Proteins solubilized from the 150 000 × *g* pellet were fractionated through Q-Sepharose, S-Sepharose, and reactive yellow 3 columns. Various column fractions were assayed for the ability to phosphorylate (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B complex. Proteins were resolved by SDS-PAGE, and ^{35}S-labeled p34^{cdc2} was visualized by autoradiography. (^{35}S)-p34^{cdc2}(Arg33)/GST-cyclin B was incubated with buffer (lane 1), the 150 000 × *g* pellet solubilized with 0.5% Brij-35 (lane 2), the Q-Sepharose pool (lane 3), the S-Sepharose pool (lane 4), the reactive yellow 3 flowthrough (lane 5), and the fractions of reactive yellow 3 gradient (lanes 6–14).

To further characterize the detergent extractable activity in the pellet fraction, we used the assay conditions described above to partially purify the activities (Figure 8). The Thr14/Tyr15 kinase activities were solubilized from the 150 000 × *g* pellet by extraction with 0.5% Brij-35 and tested for their ability to shift the electrophoretic mobility of p34^{cdc2} (lane 2). Extracts were applied to a Q-Sepharose anion exchange column. Bound proteins were eluted with a gradient of NaCl (0.05 to 1 M), and fractions were assayed for their ability to shift the electrophoretic mobility of p34^{cdc2}. A single peak of

activity (220 to 450 mM NaCl) was detected that catalyzed the phosphorylation of both Thr14 and Tyr15. Peak fractions were pooled (lane 3), desalted on a Sephadex G-25 column, and were then loaded onto an S-Sepharose column. The Thr14/Tyr15 kinase activities were eluted from the column with 0.3 M NaCl. The S-Sepharose fraction (lane 4) was desalted and further applied to a reactive yellow 3 column. Proteins bound to the yellow 3 column were eluted with a 0-1 M NaCl gradient. Flow through (lane 5) and eluted fractions (lanes 6-14) were tested in the shift assay described above. Peak fractions of activity eluted between 0.15 and 0.4 M NaCl. Both Thr14- and Tyr15-kinase activities co-purified through the three column steps.

DISCUSSION

This study describes the cell cycle regulation of the kinase(s) responsible for phosphorylation of $p34^{cdc2}$ on both Thr14 and Tyr15. Phosphorylation of Thr14 and/or Tyr15 inhibits $p34^{cdc2}$ kinase activity, and it is the abrupt dephosphorylation of these residues that triggers the activation of $p34^{cdc2}$ and entry of cells into mitosis (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Morla *et al.*, 1989; Solomon *et al.*, 1990; Krek and Nigg, 1991; Norbury *et al.*, 1991). To monitor the phosphorylation of Thr14 and Tyr15, an assay was developed that relies on the fact that phosphorylation of $p34^{cdc2}$ on either residue results in a decrease in its electrophoretic mobility (Solomon *et al.*, 1992). Phosphorylation of $p34^{cdc2}$ on both residues leads to a further retardation in its mobility (Solomon *et al.*, 1992). Thus the state of phosphorylation of $p34^{cdc2}$ (at Thr14 and Tyr15) can be monitored by changes in its electrophoretic mobility. Based on this, ^{35}S-labeled $p34^{cdc2}$ (in complex with GST-cyclin B) was used as a substrate for phosphorylation by various cell lysates. By using ^{35}S-labeled $p34^{cdc2}$ as a substrate, changes in electrophoretic mobility (due to phosphorylation) were easily detected by autoradiography. Furthermore, the Thr14- and Tyr15-specific kinase activities could be examined individually by monitoring changes in the electrophoretic mobility of mutant forms of $p34^{cdc2}$ in which either Thr14 or Tyr15 was changed to a nonphosphorylatable residue. $p34^{cdc2}$(Ala14) and $p34^{cdc2}$(Phe15) each undergo a single shift in electrophoretic mobility, indicative of phosphorylation of Tyr15 and Thr14, respectively.

HeLa cells possess readily detectable Thr14 and Tyr15 kinase activities, as evidenced by the phosphorylation of $p34^{cdc2}$(Arg33), as well as $p34^{cdc2}$(Ala14) and $p34^{cdc2}$(Phe15) in vitro (Figures 1-3). To examine the Thr14 and Tyr15 kinase activities across the cell cycle, HeLa cells were synchronized by a double thymidine block and then allowed to progress through G2, mitosis, and into early G1. When synchronized cells were analyzed at various stages of the cell cycle, it was observed that both the Thr14 and Tyr15 kinase activities were coordinately regulated in a cell cycle-dependent manner, with both activities high until the G2/M transition (Figures 3 and 4). Upon entry of cells into mitosis, the Thr14- and Tyr15-specific kinase activities were reduced. This inactivation was reversed as cells exited mitosis and progressed into G1. Interestingly, when HeLa cells were arrested in mitosis by the addition of nocodazole, the Thr14 and Tyr15 kinase activities were undetectable (Figure 3). This exaggerated inhibition of the Thr14/Tyr15 kinase activities observed in nocodazole treated cells might be the result of an extended arrest in mitosis, leading to a cumulative inhibition or turn-over of the Thr14/Tyr15 kinases.

The Thr14 and Tyr15 kinase activities were also examined in HeLa cells that were delayed in their progression through G2 by the DNA binding dye, Hoechst 33342. Hoechst 33342 binds to DNA, and interferes with the proofreading mechanisms, which occur during G2, thus delaying the initiation of mitosis (Crissman *et al.*, 1990). When Hoechst 33342 is added to cells following synchronization by a double thymidine block, activation of histone H1 kinase activity is delayed by $\geq$4 h relative to control cells, indicative of a delay in entry into mitosis (Figure 4). Both Thr14 and Tyr15 specific kinase activities remained high throughout this extended G2 phase, thus maintaining $p34^{cdc2}$ in its phosphorylated and inactive state. It is noteworthy that the activity of the Thr14/Tyr15 kinase(s) did not increase during the G2-induced delay, suggesting that normal interphase levels of Thr14 and Tyr15 kinase activities are sufficient to counteract any Cdc25 phosphatase activity that may be present. In *Xenopus*, G2 delays induced by the presence of unreplicated DNA have been shown to stimulate the activity of Tyr15 kinase (Smythe and Newport, 1992) and more recently the Thr14 kinase (Kornbluth *et al.*, 1994). Differences between the human and *Xenopus* results may be explained if unreplicated DNA and Hoechst binding to DNA operate to activate distinct feedback control pathways. Addressing these questions will be greatly facilitated once cDNAs encoding the *Xenopus* and human Thr14 and Tyr15 kinases are available.

It has been proposed that the abrupt activation of $p34^{cdc2}$/cyclin B at the G2/M transition is triggered by the Cdc25 phosphatase, and that the activation of Cdc25 and $p34^{cdc2}$ are intertwined in an autocatalytic loop (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Clarke *et al.*, 1993; Hoffmann *et al.*, 1993). According to this model, Cdc25 dephosphorylates and activates $p34^{cdc2}$, which then phosphorylates and further activates Cdc25 to dephosphorylate $p34^{cdc2}$, and so on. In fact, in *Xenopus* and humans, Cdc25 is activated by phosphorylation (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Clarke *et al.*, 1993; Hoffmann *et al.*, 1993), and $p34^{cdc2}$ is capable of phosphorylating and activating Cdc25 in vitro (Hoffmann *et al.*, 1993). Based on the results obtained in this study as well as those reported by others

(Smythe and Newport, 1992; Tang *et al.*, 1993), it is clear that the inactivation of Thr14 and Tyr15 kinase activities also plays a role in regulating the timing of activation of p34^{cdc2} at G2/M. Accordingly, the state of phosphorylation of p34^{cdc2} at Thr14 and Tyr15 is likely determined by the balance of Thr14 and Tyr15 kinase activities and Cdc25 phosphatase activities during late G2. Several possible mechanisms can be envisioned in which the down-regulation of the Thr14/Tyr15 kinase(s) might combine with the activation of Cdc25 and contribute to the rapid dephosphorylation and activation of p34^{cdc2}. One model might be that Cdc25 and the Thr14/Tyr15 kinase(s) are regulated by independent mechanisms which converge during late G2 to precisely time the activation of p34^{cdc2}/cyclin B. Alternatively, Cdc25 and the Thr14/Tyr15 kinase(s) might share a common regulator that is responsible for both activating Cdc25 and inactivating the Thr14 and Tyr15 kinase activities at the G2/M transition. Interestingly, Cdc25 (from *Xenopus* and humans) has been shown to be activated by phosphorylation (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Clarke *et al.*, 1993; Hoffmann *et al.*, 1993), while p107^{wee1} (from fission yeast) is inactivated upon phosphorylation by the Cdr1/Nim1 kinase (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). Furthermore, Tang *et al* (1993) demonstrated that fission yeast p107^{wee1} is also phosphorylated and inhibited when incubated with mitotic *Xenopus* egg extracts, suggesting that higher eukaryotes possess kinase activity (distinct from the Cdr1/Nim1 kinase), which is capable of down-regulating p107^{wee1} during M phase. The present evidence indicates that the Thr14 and Tyr15 kinase activities of HeLa cells are inhibited in mitosis, and one might predict by analogy that this inhibition is due to phosphorylation of the Thr14/Tyr15 kinase(s). In that case, a common regulatory kinase and/or phosphatase could simultaneously activate the Cdc25 phosphatase and inactivate the Thr14/Tyr15 kinase(s), thus leading to an abrupt change in the phosphorylation state of p34^{cdc2} at the G2/M transition. Most likely, a combination of regulatory mechanisms is involved in the precise control of the state of phosphorylation of p34^{cdc2} by Cdc25 and the Thr14/Tyr15 kinase(s).

The regulation of Thr14 and Tyr15 kinase activities was examined in *Xenopus* egg extracts in addition to HeLa cells. Like HeLa cells, *Xenopus* egg extracts contained kinase activities capable of phosphorylating p34^{cdc2} on both Thr14 and Tyr15. The *Xenopus* Thr14/Tyr15 kinase(s) also displayed a cell cycle dependent regulation with low activity in M phase. In *Xenopus* egg extracts, Thr14 and Tyr15 kinase activities appeared immediately upon exit from M phase and entry into interphase. In addition, the activities were inhibited when interphase extracts were driven into M-phase by the addition of a nondegradable form of cyclin B. These findings corroborate studies reported by Solomon *et al.* (1990) and Smythe and Newport (1992), in which the p34^{cdc2}-specific tyrosine kinase activity of *Xenopus* egg extracts was shown to decrease during mitosis. Of interest in the present study is the fact that Thr14-specific kinase activity is also regulated in a cell cycle dependent manner, and the regulation of its activity parallels that of the Tyr15-specific kinase activity. Similar results have recently been reported by Kornbluth *et al.* (1994). These results suggest that Thr14 and Tyr15 kinase activities are regulated in a similar manner throughout the cell cycle. Given that Thr14 and Tyr15 specific kinase activities are diminished during mitosis in both *Xenopus* and HeLa cells, and that fission yeast p107^{wee1} is also inhibited in the context of an M phase extract (Tang *et al.*, 1993), down-regulation of the kinase(s) responsible for the inhibitory phosphorylation of p34^{cdc2} may represent a conserved mechanism by which activation of p34^{cdc2} and entry of cells into mitosis is regulated. *Xenopus* egg extracts should be readily amenable to dissecting the cell cycle regulation of the Thr14 and Tyr15 kinases at the biochemical level.

To date, only a single human kinase capable of phosphorylating p34^{cdc2} on Tyr15 and inhibiting the p34^{cdc2}/cyclin B complex has been identified (Honda *et al.*, 1992; Parker *et al.*, 1992; McGowen and Russell, 1993). The gene encoding this kinase (denoted WEE1Hu) was identified in a genetic screen looking for functional homologs of *wee1+* (Igarashi *et al.*, 1991). The kinase responsible for Thr14 phosphorylation has not been identified. We have detected two activities capable of phosphorylating p34^{cdc2} on Thr14 and Tyr15 in interphase extracts prepared from *Xenopus* eggs. An activity capable of phosphorylating Tyr15 remains soluble after centrifugation of interphase extracts at 150 000 $\times$ *g*. A second activity capable of phosphorylating both Thr14 and Tyr15 pellets during the 150 000 $\times$ *g* spin but is detergent extractable. Similar findings have recently been reported by Kornbluth *et al.* (1994). We have begun to biochemically purify the detergent extractable enzyme. The assay requires the use of the p34^{cdc2}/cyclin B complex as this is the preferred substrate for phosphorylation. The kinases are detected in various fractions based on their ability to cause a shift in the electrophoretic mobility of p34^{cdc2} on SDS-gels. The kinases co-purify through three successive chromatographic steps. Further steps will be required to purify the enzyme(s) to homogeneity for future study, but the intriguing possibility exists that a dual-specificity kinase capable of phosphorylating p34^{cdc2} on both Thr14 and Tyr15 is present in higher eukaryotic cells.

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