Role of Phosphorylation in p34^{cdc2} Activation: Identification of an Activating Kinase

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Phosphorylation of p34^{cdc2} can both positively and negatively regulate its kinase activity. We have mapped two phosphorylation sites in *Xenopus* p34^{cdc2} to Thr-14 and Tyr-15 within the putative ATP-binding region of p34^{cdc2}. Mutation of these sites to Ala-14 and Phe-15 has no effect on the final histone H1 kinase activity of the cyclin/p34^{cdc2} complex. Phosphopeptide analysis shows that there is at least one more site of phosphorylation on p34^{cdc2}. When Thr-161 is changed to Ala, two phosphopeptide spots disappear and it is no longer possible to activate the H1 kinase activity of p34^{cdc2}. We suggest that Thr-161 is a third site of phosphorylation, which is required for kinase activity. All three phosphorylations are induced by cyclin. None of the phosphorylations appears to be required for binding to cyclin, as indicated by the ability of the triple mutant, Ala-14, Phe-15, Ala-161, to bind cyclin. The activating phosphorylation that requires Thr- or Ser-161 occurs even in a catalytically inactive K33R mutant of p34^{cdc2} and hence does not appear to be the result of intramolecular autophosphorylation. We have detected an activity in *Xenopus* extracts required for activation of p34^{cdc2} and present evidence that this is a p34^{cdc2} activating kinase which, in a cyclin-dependent manner, probably directly phosphorylates Thr-161.

INTRODUCTION

p34^{cdc2} is regulated by both association with cyclin and by multiple phosphorylations. Phosphorylation sites have been mapped in two organisms. In *Schizosaccharomyces pombe*, Tyr-15 is phosphorylated in G2 and is abruptly dephosphorylated on entry into mitosis (Gould and Nurse, 1989). Mutagenesis has indicated that this phosphorylation negatively regulates p34^{cdc2} activity, consistent with its location within the putative ATP-binding region of p34^{cdc2}. Phosphorylation sites have also been determined for chicken p34^{cdc2} (Krek and Nigg, 1991). Similar to *S. pombe*, chicken p34^{cdc2} is phosphorylated on Tyr-15, as well as on the adjacent Thr-14. Phosphorylation also occurs on Ser-277 during G1 and on a threonine that may correspond to Thr-161 during mitosis. The Thr-161 homologous residue is also believed to be phosphorylated in *S. pombe* p34^{cdc2} (cited in Krek and Nigg, 1991). It is generally believed that

homologs of the *S. pombe wee1$^+$* gene product phosphorylate Tyr-15 (and possibly Thr-14), although definitive proof is lacking (Featherstone and Russell, 1991; Parker *et al.*, 1991). There are currently no candidates for other kinases, although autophosphorylation of p34^{cdc2} on Thr-161 has been proposed because this site aligns with an autophosphorylation site found in a number of kinases (Krek and Nigg, 1991).

We recently examined the phosphorylation of p34^{cdc2} that occurred after the addition of purified cyclin protein to an interphase extract derived from *Xenopus* eggs (Solomon *et al.*, 1990). Monomeric p34^{cdc2} was unphosphorylated and inactive. Binding to cyclin induced the phosphorylation of p34^{cdc2} on at least three sites, two threonines and a tyrosine. (Cyclin-induced tyrosine phosphorylation of p34^{cdc2} has also been reported by Parker *et al.*, 1991 and by Meijer *et al.*, 1991.) Phosphorylation of the tyrosine and a nearby threonine was transient, serving to keep these newly formed complexes inactive until a threshold concentration of cyclin/p34^{cdc2} complexes was formed. Phosphorylation of the second threonine persisted into mitosis; dephosphorylation of this threonine in vitro led to

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inactivation of the complex (Lee et al., 1991). Although inhibitory phosphorylations of $p34^{cdc2}$ occurred immediately on cyclin binding, the timing of the activating phosphorylation and whether it is induced by cyclin binding, as opposed to being trapped in the presence of cyclin, were not explored.

To understand the nature and sequence of the phosphorylation reactions, it is necessary to map these phosphorylation sites. We have now determined the probable sites of phosphorylation of Xenopus $p34^{cdc2}$, using recombinant $p34^{cdc2}$ produced from the cloned gene (Milarski et al., 1991), purified cyclin protein, and interphase extracts derived from Xenopus eggs. After the identification of the sites we hope to identify the responsible kinases and ultimately to describe their regulation. Using various mutated forms of $p34^{cdc2}$, it is possible to distinguish autophosphorylation from phosphorylation driven by other kinases. From these experiments we have found that none of these phosphorylations is the result of autophosphorylation. The phosphorylation of the activating site on $p34^{cdc2}$ must therefore be due to an unidentified kinase activity. Using a specific assay for this activity, we have purified this kinase 100-fold. We have termed this the $p34^{cdc2}$ activating kinase (CAK). CAK activates $p34^{cdc2}$ by phosphorylation, probably on Thr-161, only in the presence of cyclin.

MATERIALS AND METHODS

Buffers

The following buffers were used extensively throughout this work: XB, 100 mM KCl, 50 mM sucrose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM K-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4, with 10 μg/ml each of leupeptin, chymostatin, and pepstatin; EB, 15 mM $MgCl_2$, 20 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM dithiothreitol (DTT), 80 mM K-β-glycerophosphate, pH 7.3, containing 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin; buffer A, 50 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM EDTA, 20 mM triethanolamine (pH 8.0 at 4°C) containing 10 μg/ml each of leupeptin, chymostatin, and pepstatin; and RIPA, 150 mM NaCl, 1.0% NP-40, 1.0% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM K-HEPES, pH 7.4 and containing 10 μg/ml each of leupeptin, chymostatin, and pepstatin.

Extracts and H1 Kinase Assays

Preparation of Xenopus interphase extracts (Solomon et al., 1990) and Xenopus oocyte extracts (Cyert and Kirschner, 1988) has been described. H1 kinase assays were performed exactly as described (Solomon et al., 1990).

Proteins and Other Reagents

Okadaic acid was purchased from Moana Bioproducts (Honolulu, Hawaii). $p13^{suc1}$-Sepharose, glutathione S-transferase cyclin B (GT-cyclin B) containing an N-terminal fusion of glutathione S-transferase to a sea urchin type B cyclin, and an anti-PSTAIR antiserum were prepared exactly as described previously (Solomon et al., 1990). A similar fusion to clam cyclin A (Solomon et al., 1991) was purified in the same manner. The 12CA5 monoclonal antibody to the peptide

YPYDVPDYA in the influenza hemagglutinin protein (Wilson et al., 1984; Field et al., 1988) was generously provided by Laura Davis (Whitehead Institute, Cambridge, MA).

Expression of Xenopus $p34^{cdc2}$ in reticulocyte lysates has been described (Gautier et al., 1991). ^{35}S-labeled $p34^{cdc2}$ was produced at $\sim$6 ng/μl at a specific activity of $\sim$3 $\times$ 10^5 cpm/μl. Extracts were supplemented with 0.4 mM L-Methionine for translation of unlabeled $p34^{cdc2}$ ($\sim$12 ng/μl). A clone of the Xenopus $p34^{cdc2}$ gene was generously provided by K. Milarski and J. Newport (UCSD). Single-stranded uracil-containing $p34^{cdc2}$ DNA was mutagenized (Zoller and Smith, 1984; Kunkel, 1985) using the following oligonucleotides

T14A: GATCGGAGAGGGCGCATATGGGGTTGTGTAC

Y15F: GATCGGAGAGGGCACATTTGGGGTTGTGTAC

T14A, Y15F: GATCGGAGAGGGCGCATTTGGGGTTGTGTAC

K33R: GTTGTTGCAATGCGCAAAATTCGATTG

K34R: GTTGCAATGAAGCGCATTCGATTGG

T161A: CGGGTTTACGCGCATGAGGTA

The triple mutant containing T14A, Y15F, and T161A was constructed by swapping Bsm I-BamHI fragments (position 458 to the polylinker beyond the 3' end of the gene).

Purification of H1 Kinase

H1 kinase was purified from a Xenopus extract to which GT-cyclin B was added, by virtue of the glutathione S-transferase tag on cyclin B. Four hundred microliters of interphase extract was incubated with 400 μl of GT-cyclin B in XB (125 nM final GT-cyclin B concentration) for 20 min at 23°C. This mixture was added to 800 μl of glutathione-agarose beads (S-linkage, Sigma, St. Louis, MO) that had been washed extensively with XB/0.5% NP-40/0.5 M NaCl followed by EB. After 20 min at 23°C, the beads were pelleted and washed in 10 ml once with EB, once with EB/0.2% NP-40, twice with EB/0.2% NP-40/0.5 M NaCl, and twice with EB. GT-cyclin B/$p34^{cdc2}$ complexes were eluted with three 0.8-ml volumes of EB/5 mM glutathione (reduced, freshly prepared)/0.1 mg/ml ovalbumin/0.05% NP-40/8% (vol/vol) glycerol/0.1 mM ATP. The pooled eluate was centrifuged briefly to pellet any remaining beads. The supernatant was concentrated in Centricon-30 cells (Amicon, Danvers, MA) to 230 μl, aliquoted, frozen in liquid nitrogen, and stored at -80°C.

Binding of ^{35}S-Labeled $p34^{cdc2}$ to Cyclin, $p13^{suc1}$, and the 12CA5 Monoclonal Antibody

For binding to cyclin and to $p13^{suc1}$, 1.0 μl of a ^{35}S-labeled reticulocyte lysate containing the Y15F form of $p34^{cdc2}$ was diluted with 20 μl of XB/0.1 mg/ml ovalbumin with or without 5 mM EDTA. For native immunoprecipitation, 1.0 μl of the reticulocyte lysate was diluted with 20 μl of XB/0.1 mg/ml ovalbumin/1% NP-40. This was added to 20 μl of beads containing GT-cyclin B, $p13^{suc1}$, or the 12CA5 antibody. For cyclin binding, glutathione agarose beads (S-linkage, Sigma) were washed twice with 150 mM NaCl, 0.5% NP-40, 5 mM DTT, 2 mM EDTA, 50 mM tris(hydroxymethyl) aminomethane (Tris), pH 8.0, containing 10 μg/ml each of leupeptin, chymostatin, and pepstatin, incubated with $\sim$250 nM GT-cyclin B for 20 min, washed twice more with 150 mM NaCl, 0.5% NP-40, 5 mM DTT, 2 mM EDTA, 50 mM Tris, pH 8.0, containing 10 μg/ml each of leupeptin, chymostatin, and pepstatin, and three times with XB. Control glutathione-agarose beads were washed similarly but were not incubated with GT-cyclin B. One sample was washed a final time with XB containing 5 mM EDTA. $p13^{suc1}$-Sepharose beads were washed once with XB/0.5 M NaCl/0.5% NP-40 and twice with XB. A final rinse with XB/5 mM EDTA was performed on appropriate samples. For immunoprecipitation, 20 μl of protein A-Sepharose beads was washed three times with XB/1% NP-40, incubated for 60 min with 2 μl ($\sim$4 μg) of 12CA5

antibody in a total volume of 100 μl of XB/1% NP-40, and washed three times with XB/1% NP-40. Control protein A beads were not incubated with antibody. After incubation with p34^{cdc2} for 20 min, beads were washed twice with XB/1% NP-40, once with XB, and resuspended in 40 μl of sample buffer. The entire sample was electrophoresed on 5–15% polyacrylamide gels. For binding of mutant p34^{cdc2} proteins to cyclin, 2 μl of ^{35}S-Met-labeled p34^{cdc2} was added to an interphase extract that was diluted fourfold into XB and containing ~500 nM GT-cyclin B. After 30 min at 23°C, the incubation was diluted with 500 μl of XB/1% NP-40 and added to 10 μl of glutathione-agarose beads. After 30 min at 23°C, the beads were pelleted, washed twice with XB/0.5 M NaCl, twice with XB, and specifically bound proteins were eluted with two 2-min rinses of 10 μl XB/50 mM glutathione.

Activation of p34^{cdc2} After Immunoprecipitation

p34^{cdc2} was immunoprecipitated from reticulocyte lysates as described above. Typically, 5 μl of beads containing the p34^{cdc2} from 2 μl of reticulocyte lysate was incubated with 5 μl of interphase extract containing 250 nM GT-cyclin B. After 30 min at 23°C, the beads were pelleted and washed twice with EB/0.2% NP-40, twice with EB/0.2% NP-40/0.5 M NaCl, twice with EB, and resuspended in 35 μl of EB for H1 kinase assay. Stringent washing with 0.8 M urea or with RIPA buffer containing 1.0 M LiCl did not reduce the H1 kinase activity on the beads.

Phosphopeptide Analysis

Interphase extracts (35–50 μl) were incubated with the indicated amounts of ^{32}P-orthophosphate (New England Nuclear, Boston, MA) for 10 min. In some experiments in vitro-translated p34^{cdc2} was added. In these cases, 50 μl of reticulocyte lysate containing the appropriate form of p34^{cdc2} was diluted eightfold with 150 mM NaCl, 20 mM HEPES (pH 7.4) containing 10 μg/ml each of leupeptin, chymostatin, and pepstatin and made 55% in ammonium sulfate. The extract was incubated for 15 min on ice, centrifuged at 10 000 $\times$ g for 10 min, rinsed once with 55% ammonium sulfate in the same buffer, resuspended in 25–30 μl of XB, and microdialyzed in two "dialysis boats" constructed out of small microfuge tube caps against XB for 60 min. The entire recovered volume was mixed with an equal volume of interphase extract. After incubation for 30 min with cyclin, p34^{cdc2} was retrieved from the extract either on glutathione agarose (by virtue of its association with GT-cyclin B) or by p13^{suc1} precipitation, as indicated in the figures. In either case, the p34^{cdc2} was subsequently immunoprecipitated, either with an anti-PSTAIR serum or with the 12CA5 antibody, as indicated in the figures. Double precipitations were necessary to remove contaminants from the crude Xenopus ex-

tracts. The use of GT-cyclin B or p13^{suc1} precipitation does not bias the recovery of p34^{cdc2} forms (Solomon et al., 1990). All precipitations (GT-cyclin B, p13^{suc1}, and immunoprecipitation) were as described (Solomon et al., 1990). Twelve and one-half microliters of 12CA5 antibody (~25 μg) and 25 μl of protein A-Sepharose beads were used per sample. Digestion of eluted p34^{cdc2} with trypsin, electrophoresis at pH 1.9 in acetic acid/formic acid/H$_2$O (195:65:1040), and chromatography in n-butanol/pyridine/acetic acid/H$_2$O (75:50:15:60) were as described (Solomon et al., 1990; Ward and Kirschner, 1990). Electrophoresis was from left (anode) to right (cathode) and chromatography was from bottom to top.

Assay of the p34^{cdc2} Activating Kinase

Two microliters of a sample to be assayed was mixed with 2 μl of a reticulocyte lysate containing Xenopus p34^{cdc2} (wild-type, ~12 ng/μl) and supplemented with 350 nM GT-cyclin B, 2 mM ATP, and 15 mM MgCl$_2$. After 30 min, 26 μl of EB containing 1 mg/ml ovalbumin was added and the samples were frozen in liquid nitrogen before assay of H1 kinase activity exactly as described (Solomon et al., 1990). When indicated, control reactions contained all components except the reticulocyte extract, which was replaced with EB.

Partial Purification of the p34^{cdc2} Activating Kinase

Two milliliters of an interphase extract (~140 mg protein) was diluted with 18 ml of buffer A and centrifuged at 40 000 $\times$ g for 30 min at 4°C in the SW50.1 rotor (Beckman, Fullerton, CA). The supernatant (~120 mg) was applied from below to an 8 ml DEAE-Sepharose column (1.0-cm diameter column) pre-equilibrated with buffer A. The column was run downward at 20 ml/h and 1-ml fractions were collected. The column was washed with 20 ml of buffer A after the sample was applied. A 100-ml linear gradient from buffer A to buffer A/400 mM NaCl was applied to the column. The protein concentrations of the fractions were determined using the Bradford assay (Bio-Rad, Richmond, CA) with bovine serum albumin as standard, and the NaCl concentrations were determined by conductivity measurements. Activity eluted with a peak at 115 mM total NaCl. Purification was sixfold with nearly complete recovery of activity. Activity in the pooled fractions was precipitated from 38% ammonium sulfate and resuspended in 4% of the input volume in buffer A for a further 5- to 10-fold purification with near quantitative recovery of activity. Gel filtration chromatography was performed on a Bio-Sil TSK-250 column (300 mm $\times$ 7.5 mm, Bio-Rad). The column was equilibrated in buffer A. The flow rate was 0.4 ml/min and 1-min fractions were collected. One hundred microliters of the resuspended ammonium sulfate precipitate was applied. This step resulted in removal of contaminating nucleic acids and a further protein purification of approximately
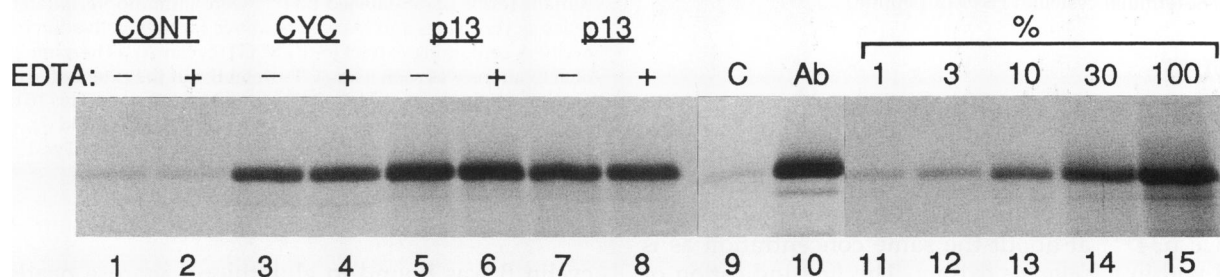

Figure 1. Binding of recombinant p34^{cdc2} to cyclin, p13^{suc1}, and a monoclonal antibody. ^{35}S-labeled p34^{cdc2} made in a reticulocyte lysate was diluted and incubated with glutathione agarose beads (lanes 1 and 2), GT-cyclin B prebound to glutathione agarose (CYC, lanes 3 and 4), p13^{suc1} coupled to Sepharose beads (p13, lanes 5–8), protein A-Sepharose (lane 9), or with monoclonal antibody 12CA5 against the hemagglutinin peptide sequence inserted at the C-terminus of p34^{cdc2} prebound to protein A-Sepharose beads (lane 10). Two preparations of p13^{suc1}-beads were used; the one in lanes 7 and 8 was known to deplete H1 kinase from an extract poorly. Binding was in the presence (+, lanes 2, 4, 6, and 8) or absence (−, lanes 1, 3, 5, and 7) of 5 mM EDTA. Lanes 11–15 contained 1%, 3%, 10%, 30%, and 100% of the input p34^{cdc2}, respectively, for quantitation. See MATERIALS AND METHODS for further details.

threefold. For the DEAE column profile 0.5 ml of interphase extract was diluted with buffer A, centrifuged for 30 min at 100 000 $\times$ g in the TL100.2 rotor, and the supernatant applied to a 2-ml column (1-cm diameter) and run under the same conditions as above.

Direct Phosphorylation of $p34^{cdc2}$ by the Activating Kinase

For native precipitation of $p34^{cdc2}$, 100 μl of protein A-Sepharose beads were rinsed three times in XB/1% NP-40, incubated with 20 μl of 12CA5 antibody ($\sim$40 μg), for 80 min at 4°C, washed four times in XB/1% NP-40/1 mg/ml ovalbumin, and aliquoted. For each 10 μl of beads in an aliquot, 2 μl of reticulocyte lysate containing either wild-type or mutant $p34^{cdc2}$ proteins ($\sim$12 ng/μl) and 60 μl of XB/1% NP-40/1 mg/ml ovalbumin were added. After 20 min on ice, the beads were pelleted, rinsed twice in XB/1% NP-40, twice in EB, once in EB/1 mg/ml ovalbumin, and further aliquoted. To these beads, 3 μl of GT-cyclin B (830 nM in EB/1 mg/ml ovalbumin) or EB/1 mg/ml ovalbumin alone, 3 μl of $p34^{cdc2}$ activating kinase (or EB/1 mg/ml ovalbumin alone), and 3 μl of an ATP mix made up of 30 μl of [γ-^{32}P]ATP (7.8 mCi/ml, 3000 Ci/mmol, ICN, Costa Mesa, CA), 2.7 μl of 1 mM ATP, and 7.3 μl of EB/1 mg/ml ovalbumin were added. The $p34^{cdc2}$ activating kinase fraction was purified through the ammonium sulfate fractionation and was at a concentration of $\sim$1.7 mg/ml. After 20 min at 23°C, the beads were pelleted and rinsed once in EB/0.2% NP-40, twice in RIPA buffer containing 0.25 M LiCl, once in RIPA buffer, once in 20 mM K-HEPES (pH 7.4), and resuspended in sample buffer for SDS-polyacrylamide gel electrophoresis on 5–15% gradient gels.

Treatment of $p34^{cdc2}$ Activating Kinase With $p13^{suc1}$-Sepharose Beads

Partially purified $p34^{cdc2}$ activating kinase (through the DEAE column, $\sim$1.4 mg/ml) was incubated with an equal volume of either protein A-Sepharose beads or $p13^{suc1}$-Sepharose beads. The beads were first rinsed twice with XB/0.5% NP-40/0.5 M NaCl and twice with buffer A. After 30 min on ice, the beads were pelleted. The supernatant was saved and the pellet was rinsed two times with buffer A and resuspended in sample buffer. The supernatants and the starting material were assayed for $p34^{cdc2}$ activating kinase activity as described above.

Immunoblotting

Transfer and detection were as described before (Solomon *et al.*, 1990), except that the secondary antibody was horseradish peroxidase conjugated donkey anti-rabbit with development using the enhanced chemiluminescence method (Amersham, Arlington Heights, IL). The anti-PSTAIR serum was affinity purified on the 17 amino acid (including N-terminal cysteine) PSTAIR peptide.

RESULTS

Production of Functional $p34^{cdc2}$ In Vitro

The only expression system we have found suitable for production of activatable $p34^{cdc2}$ is in vitro translation in reticulocyte lysates (Gautier *et al.*, 1991), which can produce $p34^{cdc2}$ at about the same concentration as is found within a *Xenopus* extract. The first indication of the structural fidelity of this protein was its ability to bind to known $p34^{cdc2}$-binding proteins, cyclin and $p13^{suc1}$ (Figure 1). The cyclin was GT-cyclin B, a fusion of glutathione S-transferase to a sea urchin type B cyclin, expressed in *Escherichia coli* and affinity purified on glutathione-agarose beads (Solomon *et al.*, 1990). GT-
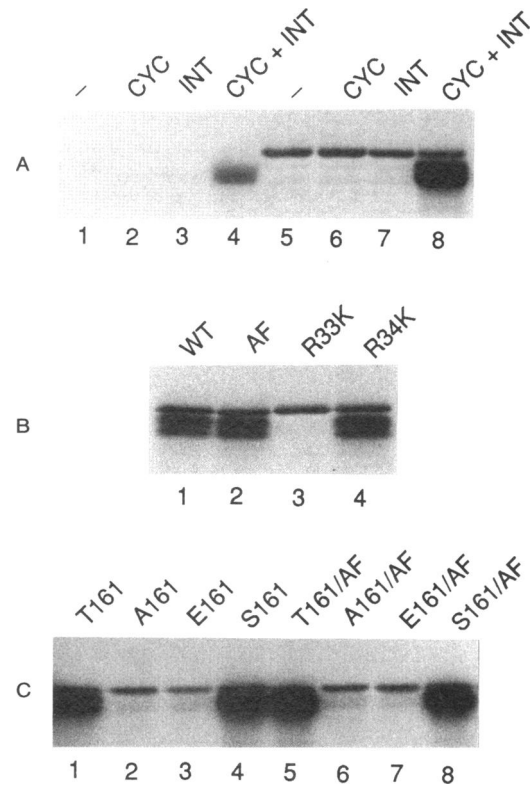

Figure 2. Activatability of wild-type and mutant $p34^{cdc2}$ protein. (A) Activation of recombinant $p34^{cdc2}$ requires GT-cyclin B and an extract factor. ^{35}S-labeled $p34^{cdc2}$ ("AF" mutant) was immunoprecipitated from a reticulocyte lysate (lanes 5–8) and incubated alone (lane 5), with $\sim$85 nM GT-cyclin B (lane 6), with an interphase extract (lane 7), or with both GT-cyclin B and the extract (lane 8). Equal volumes of the components were added. All reactions contained the same final volume; any deficit was made up with XB. The bead-bound $p34^{cdc2}$ was washed twice with EB after a 30-min incubation and assayed for H1 kinase activity. The uppermost band is the ^{35}S-labeled $p34^{cdc2}$ protein itself. Lanes 1–4 contained identically treated immunoprecipitates from an unprogrammed reticulocyte lysate. (B) Wild-type (lane 1), AF mutant (lane 2), K33R (lane 3), or K34R (lane 4) forms of ^{35}S-labeled $p34^{cdc2}$ were immunoprecipitated from reticulocyte lysates and incubated with a GT-cyclin B containing extract as above. After 30 min, the bead-bound $p34^{cdc2}$ was pelleted, washed thoroughly (see MATERIALS AND METHODS), and assayed for H1 kinase activity. (C) Mutant forms of ^{35}S-labeled $p34^{cdc2}$ were immunoprecipitated from reticulocyte lysates and assayed as above for their activation in a GT-cyclin A containing extract ($\sim$1 μM GT-cyclin A). (The same pattern of activations was seen using GT-cyclin B, but the extent of activation was somewhat lower.) The $p34^{cdc2}$ proteins contained Thr-161 (lanes 1 and 5), Ala-161 (lanes 2 and 6), Glu-161 (lanes 3 and 7), or Ser-161 (lanes 4 and 8) in combination with either Thr-14/Tyr-15 (lanes 1–4) or of Ala-14/Phe-15 (lanes 5–8).

cyclin B was bound to glutathione-agarose beads and $p13^{suc1}$ was covalently linked to Sepharose beads. Recombinant $p34^{cdc2}$ efficiently bound to cyclin (Figure 1, lanes 3 and 4) and to two preparations of $p13^{suc1}$ beads (Figure 1, lanes 5 and 6 and lanes 7 and 8; compare with loading standards in lanes 11–15). Induced phosphorylation of $p34^{cdc2}$ was not required for these inter-

Figure 3. Phosphorylation of p34[cdc2] on Thr-14 and Tyr-15. ^{35}S-labeled p34[cdc2] proteins produced in reticulocyte lysates were diluted 20-fold into an interphase extract in the presence of 250 nM GT-cyclin B in all cases and 2 mM vanadate in lanes 2, 4, 6, etc. Aliquots were taken after 30 min and analyzed by gel electrophoresis on 9% polyacrylamide gels (total acrylamide: bisacrylamide = 37.5). Aliquots of the same proteins before addition to the extract were also run (lanes 1, 3, 5, 7, 9, 11, and 13). The proteins were wild-type p34[cdc2] (TY, lanes 1 and 2), Ala-14/Phe-15 (AF, lanes 3 and 4), Ala-14 (AY, lanes 5 and 6), Phe-15 (TF, lanes 7 and 8), Ala-161 (A161, lanes 9 and 10), Ser-161 (S161, lanes 11 and 12), and Asp-161 (D161, lanes 13 and 14).
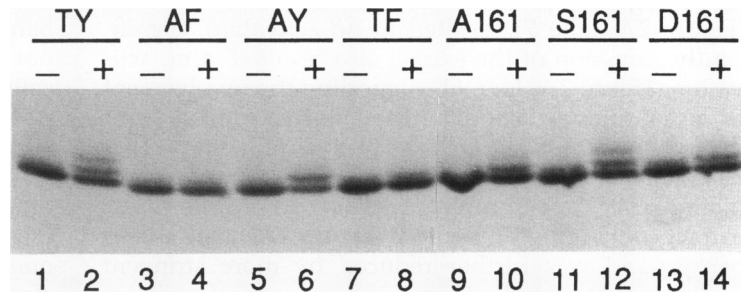
actions because binding occurred even in the presence of 5 mM EDTA (Figure 1, lanes 4, 6, and 8). The p34[cdc2] was produced with a C-terminal 14 amino acid extension derived from the influenza hemagglutinin protein, a peptide recognized by the 12CA5 monoclonal antibody (Wilson et al., 1984; Field et al., 1988). This antibody efficiently precipitated the recombinant p34[cdc2] (Figure 1, lane 10).

This recombinant p34[cdc2] could be activated as a kinase in a complete extract in the presence of cyclin. The p34[cdc2] immunoprecipitated from a reticulocyte lysate under nondenaturing conditions had no H1 kinase activity on its own (Figure 2A, lane 5). Addition of the immunoprecipitate to an interphase extract in the presence of GT-cyclin B activated the p34[cdc2] (Figure 2A, lane 8) to about the same extent (per ng of p34[cdc2]) as
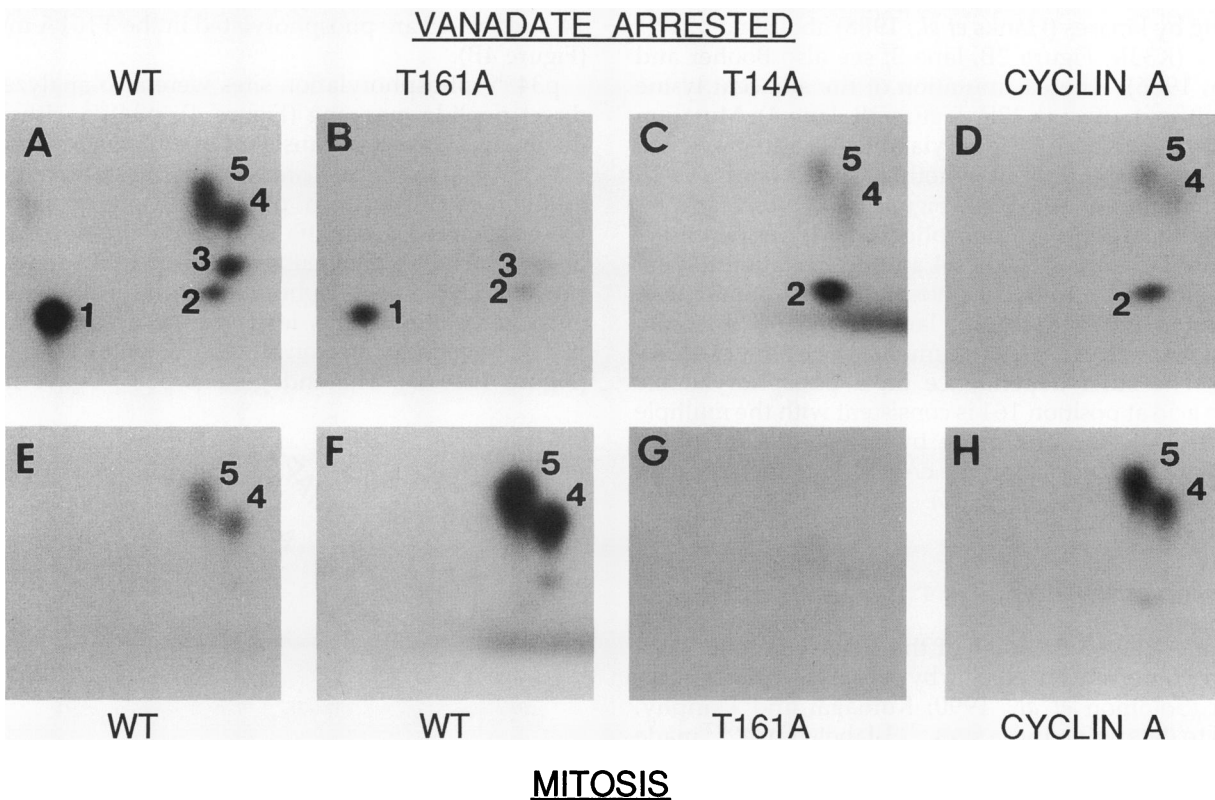

Figure 4. Phosphorylation of p34[cdc2] on Thr-161 and Thr-14 and phosphorylation induced by cyclin A. Endogenous wild-type p34[cdc2] (A, D, and H), wild-type p34[cdc2] expressed in a reticulocyte lysate (E and F), or mutant forms of p34[cdc2] expressed in a reticulocyte lysate (T161A, B and G; T14A, C) were ^{32}P-labeled in an interphase extract after addition of cyclin. A–D show extracts blocked from entering mitosis by 2 mM vanadate. E–H show extracts in mitosis. F is a longer exposure of E, to match the exposure of G. GT-cyclin B was added to 250 nM (A–C and E–G) and GT-cyclin A was added to 400 nM (D and H). p34[cdc2] was purified for phosphopeptide analysis from all extracts by p13[suc1] precipitation followed by immunoprecipitation (anti-PSTAIR for A, D, and H and 12CA5 for B, C, E, F, and G). D and H and B, C, E, F, and G are from single experiments. The following amounts of ^{32}P-orthophosphate and exposure times were used: A, 2.5 mCi/8 d; B, 3.8 mCi/8 d; C, 3.8 mCi/8 d; D, 3.9 mCi/5 d; E, 3.8 mCi/1 d; F, 3.8 mCi/4 d; G, 3.8 mCi/4 d; H, 3.9 mCi/1 d.

the endogenous p34^{cdc2}. As expected, omission of cyclin (Figure 2A, lane 7) resulted in no activation. Significantly, omission of the extract also resulted in no activation (Figure 2A, lane 6), suggesting the involvement of an additional factor that had not previously been demonstrated. Only low levels of contaminating H1 kinase activity were precipitated in the absence of recombinant p34^{cdc2}, even after incubation in an interphase extract containing GT-cyclin B (Figure 2A, lane 4). This background was further reduced by more stringent washing of the beads (Figure 2B).

The abilities of forms of p34^{cdc2} mutated in putative catalytic and phosphorylation sites to activate were assessed. We will show below that Thr-14 and Tyr-15 are the inhibitory sites of p34^{cdc2} phosphorylation. (The complete sequence of the *Xenopus* p34^{cdc2} gene has recently been determined [Milarski *et al.*, 1991].) Mutation of these residues to Ala-14 and Phe-15 had no effect on the activatability of p34^{cdc2} ("AF" mutant; Figure 2B, compare lanes 1 and 2), indicating that p34^{cdc2} need not pass through an inactivated intermediate form as an obligate step on the activation pathway. On the other hand, mutation of a conserved lysine involved in ATP-binding by kinases (Hanks *et al.*, 1988) abolished p34^{cdc2} activity (K33R, Figure 2B, lane 3; see also Booher and Beach, 1986), whereas mutation of the adjacent lysine was without effect (K32R, Figure 2B, lane 4). Mutation of Thr-161 to a nonphosphorylatable Ala also prevented activation, regardless of whether Thr-14 and Tyr-15 were mutated to Ala/Phe (Figure 2C, lanes 2 and 6), although mutation to a phosphorylatable Ser permitted activation (Figure 2C, lanes 4 and 8). Mutation to Glu, in an attempt to mimic a phosphorylated amino acid, blocked activity (Figure 2C, lanes 3 and 7). Similarly, we found that the Asp-161 mutation also blocked activity. The strict dependence on a phosphorylatable amino acid at position 161 is consistent with the multiple structural interactions made by the equivalent phosphorylated amino acid in the cAMP-dependent protein kinase (Knighton *et al.*, 1991).

Phosphorylation Sites on p34^{cdc2}

We have taken advantage of the electrophoretic retardation conferred on p34^{cdc2} by inhibitory phosphorylation (Solomon *et al.*, 1990; Kumagai and Dunphy, 1991) to determine these sites. ^{35}S-labeled p34^{cdc2} made in reticulocyte lysates was incubated with GT-cyclin B in an interphase extract in the presence of 2 mM vanadate to prevent entry into mitosis by blocking tyrosine dephosphorylation of p34^{cdc2} (Morla *et al.*, 1989; Solomon *et al.*, 1990). This treatment traps wild-type p34^{cdc2} in a triply phosphorylated intermediate state that is phosphorylated on a tyrosine, a nearby threonine, and a second threonine (Solomon *et al.*, 1990). All reticulocyte lysate-produced p34^{cdc2} proteins migrated as a

closely spaced doublet, with a relatively faint upper band (see, e.g., Figure 3, lane 1). This upper band does not comigrate with either of the strong electrophoretically retarded bands corresponding to phosphorylated p34^{cdc2}. Phosphorylation of wild-type p34^{cdc2} in a vanadate arrested extract led to the appearance of two slower migrating rungs of a ladder. These new forms did not appear in the "AF" double mutant, T14A/Y15F (Figure 3, lane 4). The single mutants had a maximum one-rung retardation (Figure 3, lanes 6 and 8); this was clearer for the T14A mutant because vanadate treatment leads to higher stoichiometry phosphorylation of Tyr-15 than of Thr-14. Thus, each unit of retardation reflects an incremental phosphorylation of p34^{cdc2}; the phosphorylated sites are Thr-14 and Tyr-15. Phosphorylation of Thr-14 and Tyr-15 occurred even in the context of Thr-161 mutations (Figure 3, lanes 9–14). There appeared to be less phosphorylation of these sites in the inactive forms (Figure 3, lanes 10 and 14) than in the Ser-161 mutant (Figure 3, lane 12). We do not know if this perceived reduction is genuine or if the second incremental retardation requires Thr-161 phosphorylation. As shown below by direct ^{32}P-labeling, both Thr-14 and Tyr-15 are phosphorylated in the T161A mutant (Figure 4B).

p34^{cdc2} phosphorylation sites were also analyzed by direct peptide mapping (Figure 4). p34^{cdc2}, either endogenous or in vitro translated in a reticulocyte lysate, was ^{32}P-labeled in a *Xenopus* interphase extract after addition of GT-cyclin B. p34^{cdc2} phosphorylation sites were examined in mitotic extracts (Figure 4, E–H) and in extracts that were incubated with 2 mM vanadate to prevent p34^{cdc2} dephosphorylation on tyrosine to block entry into mitosis; this leads to the accumulation of p34^{cdc2} phosphorylated on all sites (Solomon *et al.*, 1990) (Figure 4, A–D). The endogenous p34^{cdc2} gave rise to
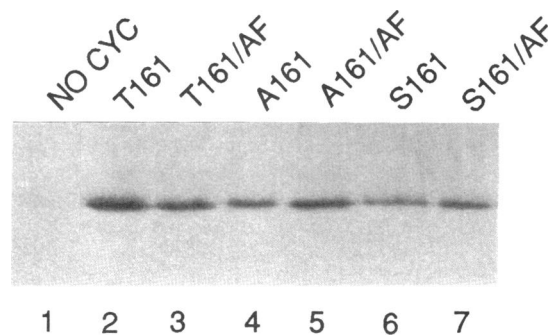

Figure 5. Phosphorylation of Thr-161 is not required for association of p34^{cdc2} with GT-cyclin B. Equal amounts of ^{35}S-labeled p34^{cdc2} from reticulocyte lysates were added to interphase extracts in the presence of GT-cyclin B. GT-cyclin B-bound p34^{cdc2} was precipitated on glutathione-agarose beads after 30 min. Lane 1 contained wild-type p34^{cdc2} but no GT-cyclin B. The other forms of p34^{cdc2} were Thr-161 (lanes 2 and 3), Ala-161 (lanes 4 and 5), and Ser-161 (lanes 6 and 7) in the context of Thr-14/Tyr-15 (lanes 2, 4, and 6) or of Ala-14/Phe-15 (lanes 3, 5, and 7). Equal counts were input to each binding reaction.
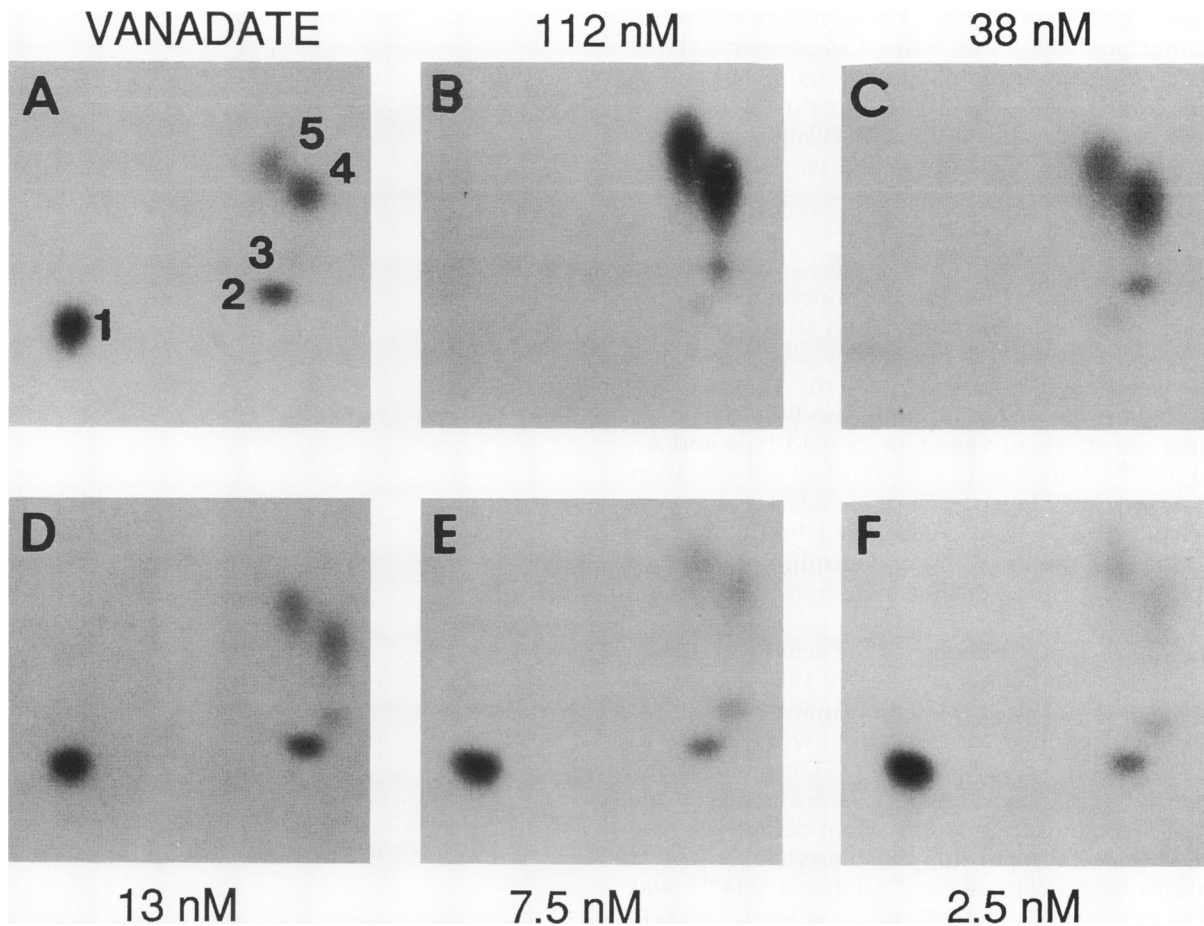
Figure 6. Phosphorylation of p34^{cdc2} at subthreshold concentrations of cyclin. Different concentrations of GT-cyclin B were prebound to glutathione-agarose beads and added to interphase extracts containing ^{32}P-orthophosphate. The final GT-cyclin B concentrations are indicated (A, 250 nM). Vanadate 2 mM was added to the sample shown in A. The glutathione-agarose beads were pelleted after 20 min and the p34^{cdc2} further purified by denaturing immunoprecipitation with an anti-PSTAIR antiserum before phosphopeptide analysis. The following amounts of ^{32}P and exposure times were used: A, 2.5 mCi/5 d; B, 2.5 mCi/5 d; C, 5 mCi/5 d; D, 10 mCi/5 d; E, 10 mCi/8 d; F, 30 mCi/8 d.

five labeled peptides (Figure 4A). Peptide 1 contained both phosphotyrosine and phosphothreonine, and we suggested that it was a doubly phosphorylated version of peptide 2, which contained only phosphotyrosine (Solomon et al., 1990). Peptides 4 and 5, which persisted into mitosis (Figure 4, E and F), contained only phosphothreonine (Solomon et al., 1990). Peptide 3 was not observed previously. Mutation of Thr-14 to Ala (T14A) in the recombinant p34^{cdc2} eliminated peptides 1 and 3 and dramatically increased the intensity of peptide 2 (Figure 4C). This result confirms the identification of Thr-14 as the second phosphorylation site in peptide 1 and indicates that peptides 2 and 3 are the singly phosphorylated forms of peptide 1. Mutation of Thr-161 to Ala (T161A) eliminated peptides 4 and 5 from both the vanadate arrested extract (Figure 4B) and from the mitotic extract (Figure 4G). Traces of residual phosphorylation of Thr-14 and of Tyr-15 were visible (Figure 4, F and G). (Note that the labeling and exposure condi-

tions in F and G were identical for the wild-type and T161A mutant of the recombinant p34^{cdc2}.) We interpret these results to indicate that peptides 4 and 5 contain the same phosphorylation site and that incomplete digestion by trypsin gives rise to two phosphorylated peptides. We cannot exclude the possibility that a second phosphorylation occurs that is absolutely dependent on the first. As the simplest interpretation, we will refer to this phosphorylation as occurring on a single site, Thr-161. Further experiments are in progress to clarify these points.

We determined the phosphorylation sites of p34^{cdc2} induced by a type A cyclin to see whether these could account for the different activation kinetics and behaviors of p34^{cdc2} in response to type A and B cyclins (Minshull et al., 1990; Solomon et al., 1991). We previously found by following electrophoretic mobility that p34^{cdc2} became phosphorylated on both Thr-14 and Tyr-15 after binding a clam type A cyclin fused to glutathione

S-transferase (Solomon *et al.*, 1991). Figure 4 demonstrates that peptides 4 and 5 are also phosphorylated in both a vanadate-arrested extract (Figure 4D) and in a mitotic extract (Figure 4H). This experiment also confirms the cyclin A-induced phosphorylation of Tyr-15 (Figure 4D); Thr-14 phosphorylation was not detected in this experiment.

The Roles of p34^{cdc2} Phosphorylation

We wanted to know whether phosphorylation of Thr-14 and Tyr-15, like phosphorylation of Tyr-15 in *S. pombe*, exerted negative control over p34^{cdc2} activity. Because vanadate treatment leads to the accumulation of p34^{cdc2} phosphorylated on both Thr-14 and Tyr-15, we tested the effects of vanadate on wild-type and AF mutant p34^{cdc2}. Vanadate (2 mM) blocked the activation of wild-type p34^{cdc2} in a p13^{suc1}-depleted interphase extract (reduced to 36% of control) but had almost no effect on the AF mutant (89% of control). We have not yet explored the effects of the single phosphorylations on p34^{cdc2} activity.

Mutations of Thr-161 abolished the activity of p34^{cdc2} (Figure 2C; see also Booher and Beach, 1986, for results in *S. pombe*); the trivial explanation that p34^{cdc2} phosphorylation is required for association with cyclin was excluded. The binding of p34^{cdc2} to GT-cyclin B in the presence of 5 mM EDTA (Figure 1, lane 4) tends to rule out a required phosphorylation. More definitively, we examined the binding of nonphosphorylatable p34^{cdc2} mutants to cyclin (Figure 5). ^{35}S-labeled p34^{cdc2} was added to an interphase extract and complexes with GT-cyclin B were retrieved on glutathione-agarose beads. Association with GT-cyclin B was independent of the amino acid at position 161, whether activatable (Thr or Ser; Figure 5, lanes 2, 3, 6, and 7) or not (Ala; Figure 5, lanes 4 and 5), and was independent of the presence of nonphosphorylatable amino acids at residues 14 and 15.

Putative T161 Phosphorylation does not Require Activation of the Mitotic State but Requires Cyclin Binding

It is important to understand whether the phosphorylation that seems to occur on Thr-161 is a consequence of mitosis or a prerequisite for mitosis. A test of this is to determine whether this phosphorylation occurs at subthreshold concentrations of cyclin. Our previous analysis (Solomon *et al.*, 1990) showed that this site was phosphorylated in mitotic and vanadate-arrested extracts containing high concentrations of GT-cyclin B but did not indicate whether this activating phosphorylation also occurred at low concentrations of cyclin, such as are present during interphase, before the decision to enter mitosis. To answer this question, we examined the phosphorylations of p34^{cdc2} induced by different concentrations of GT-cyclin B, down to very low
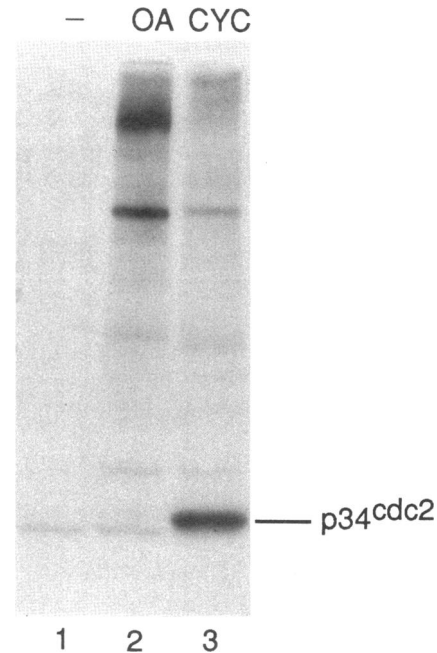

Figure 7. Phosphorylation of Thr-161 does not occur in the absence of cyclin. An interphase extract containing 2.5 mCi ^{32}P-orthophosphate was mixed with an equal volume of XB alone (lane 1), XB containing 2.5 μM okadaic acid (OA, lane 2), or XB containing 500 nM GT-cyclin B (CYC, lane 3). After a 30-min incubation, p34^{cdc2} was recovered by p13^{suc1} precipitation followed by immunoprecipitation with an anti-PSTAIR antiserum. Labeled p34^{cdc2} was released with sample buffer and analyzed by electrophoresis on a 5–15% polyacrylamide gel.

concentrations of GT-cyclin B (Figure 6). In this experiment, the threshold concentration for p34^{cdc2} activation was 38 nM (Figure 6C). The amount of ^{32}P-orthophosphate used and the exposure times of the autoradiograms were adjusted to produce equal sensitivities in the presence of varying cyclin concentrations (see Figure 6 legend). Relative to phosphorylation of Tyr-15, peptides 4 and 5 were equally phosphorylated in a vanadate-arrested extract (Figure 6A) and at subthreshold GT-cyclin B concentrations (Figure 6, D–F), indicating that the degree of phosphorylation of this site is likewise proportional to the amount of cyclin present.

This result does not distinguish between a cyclin-induced phosphorylation of p34^{cdc2} on peptides 4 and 5 and a stabilization by cyclin binding of the phosphorylated form in rapid equilibrium with the unphosphorylated state. (A similar hypothesis for the cyclin-induced phosphorylation of Tyr-15 was previously excluded because such phosphorylation did not occur after vanadate treatment alone [Solomon *et al.*, 1990].) This constitutive phosphorylation would have to be extremely low because it has never been observed, even at very high specific activity labelings, and because the extent of phosphorylation is proportional to even the lowest concentrations of cyclin (Figure 6F). A further experiment also suggested that Thr-161 is not phosphorylated in

the absence of cyclin. Okadaic acid inhibits INH, a type 2A phosphatase that can dephosphorylate p34^{cdc2}, presumably on Thr-161 (Lee *et al.*, 1991). However, no phosphorylation of p34^{cdc2} was trapped even in the presence of a high concentration of okadaic acid, sufficient to also inhibit type 1 phosphatases (Figure 7, lane 2). In contrast, p34^{cdc2} was strongly phosphorylated by addition of GT-cyclin B (Figure 7, lane 3). We cannot yet distinguish between phosphorylation after cyclin binding and phosphorylation coupled to binding.

Is Thr-161 Phosphorylation an Intramolecular Event?

The proximity of Thr-161 to the autophosphorylation sites found in a number of kinases (Hanks *et al.*, 1988) has led to the suggestion that p34^{cdc2} might also autophosphorylate (Krek and Nigg, 1991). This possibility was tested by determining the phosphorylation capability of an inactive p34^{cdc2} mutant. Lys-33 of p34^{cdc2} aligns with a lysine found in all kinases that is essential for binding ATP (Hanks *et al.*, 1988; Knighton *et al.*, 1991). Mutation of Lys-33 to Arg abolished p34^{cdc2} activity whereas the adjacent K34R mutation had no effect (Figure 2B, lanes 3 and 4). Reticulocyte lysate-produced p34^{cdc2} and to the endogenous p34^{cdc2} (Figure 4). Thus sites of phosphorylation determined. The labeling of these mutants was identical to an added wild-type p34^{cdc2} and to the endogenous p34^{cdc2} (Figure 8). Thus intramolecular autophosphorylation by p34^{cdc2}, if it occurs at all, is not the only route to phosphorylation of any of these sites.

A p34^{cdc2} Activating Kinase

The phosphorylation of this catalytically inactive form of p34^{cdc2} within an extract demonstrates the existence of a kinase capable of phosphorylating p34^{cdc2} on the activating site. This kinase is especially important be-

cause the resulting phosphorylation is essential for p34^{cdc2} activation. We screened different cell cycle extracts as a preliminary to purification of this activity. Reticulocyte lysate-produced p34^{cdc2} was incubated with diluted *Xenopus* interphase and mitotic extracts and GT-cyclin B and then assayed for the H1 kinase activity of the activated p34^{cdc2}. The extracts were diluted to reduce the effect of activation of the endogenous p34^{cdc2} and to provide a roughly quantitative measure of relative activities. An interphase extract could be diluted 27-fold and still maximally activate the added p34^{cdc2}; partial activation occurred after dilution by 81-fold (Figure 9A, lanes 9 and 10). The H1 kinase activity in the absence of added p34^{cdc2} (Figure 9A, lanes 1–3) was due to activation of the endogenous p34^{cdc2}. Similarly, a mitotic extract partially activated the added p34^{cdc2} at a dilution of 81-fold (Figure 9B, lane 10). Little or no H1 kinase activity was detectable at comparable dilutions in the absence of added p34^{cdc2} (Figure 9B, lanes 1–5). A slightly more dilute extract prepared from prophase arrested oocytes had comparable activity (Figure 9C). This activity has been termed the "p34^{cdc2} activating kinase" (or CAK) on the basis of data presented below (Figure 11).

We have partially purified the p34^{cdc2} activating kinase 100-fold on the basis of its ability to activate exogenous p34^{cdc2}. The starting material was an interphase extract because it is simple to prepare and because CAK activity is no higher in other extracts. In brief, the extract was diluted and centrifuged to remove a large mass of yolk proteins and other aggregates. The supernatant was applied to a DEAE-Sepharose column and eluted with a NaCl gradient. The pooled peak of activity was fractionated by ammonium sulfate precipitation for a two-step enrichment of CAK activity of 30- to 60-fold. A further approximately threefold purification was achieved by gel filtration chromatography on a TSK-250 column, which also provided a size estimate of
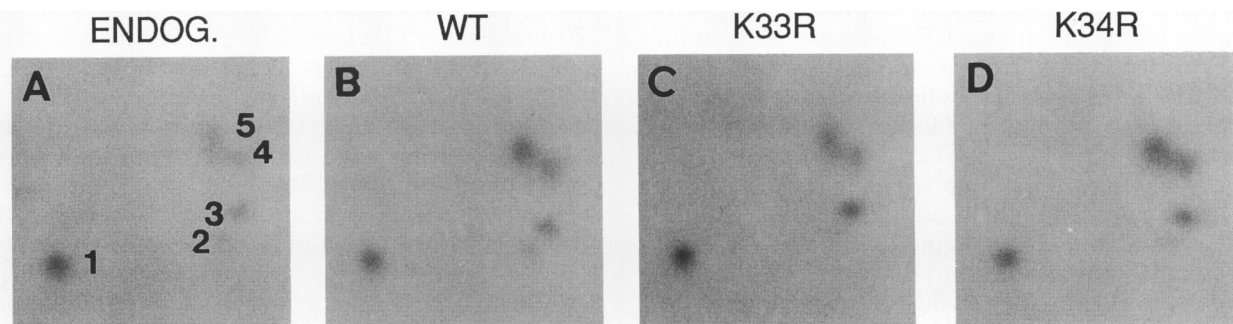

Figure 8. Intramolecular autophosphorylation is not an obligate pathway for p34^{cdc2} phosphorylation. GT-cyclin B was added to interphase extracts containing ^{32}P-orthophosphate and only endogenous p34^{cdc2} (A) or also p34^{cdc2} made in a reticulocyte lysate (wild-type, B; K33R mutant, C; K34R mutant, D). The final GT-cyclin B concentration was 250 nM. The K33R mutant is catalytically inactive presumably due to its inability to bind ATP. After 30 min, the p34^{cdc2} was recovered for phosphopeptide analysis by p13^{suc1} precipitation followed by immunoprecipitation with an anti-PSTAIR antiserum (A) or monoclonal antibody 12CA5 (B–D). All extracts contained 2.5 mCi ^{32}P-orthophosphate. Exposure times were 8 d.
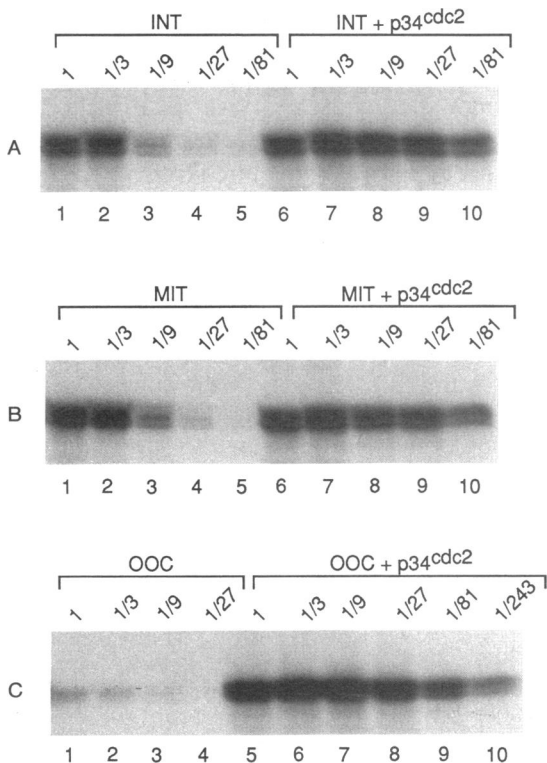
Figure 9. Activity of the p34^{cdc2} activating kinase in interphase, mitotic, and oocyte extracts. An interphase extract (A, ~70 mg/ml) was serially diluted threefold into EB (lanes 1–5 and lanes 6–10). These samples were incubated with reticulocyte lysates containing p34^{cdc2} and GT-cyclin B (lanes 6–10) or just with GT-cyclin B (lanes 1–5) and assayed for H1 kinase activity. Lanes 1 and 6 contained the undiluted extract and lanes 5 and 10 contained extract diluted 81-fold. A mitotic extract produced by incubating an interphase extract with 450 nM GT-cyclin B for 30 min was similarly diluted and assayed (B). An oocyte extract (Cyert and Kirschner, 1988) (~14 mg/ml) was similarly diluted and assayed with GT-cyclin B alone (lanes 1–4) or with a reticulocyte lysate containing p34^{cdc2} in addition (lanes 5–10). See MATERIALS AND METHODS for further details.

~200 kDa. Even the most highly enriched fractions still contained a complex mixture of proteins. Figure 10 shows the fractionation on a DEAE-Sepharose column. All the applied CAK activity bound to the column (IN), although half the total protein flowed through. Activity eluted fairly sharply during a linear salt gradient, with a peak at the fairly low NaCl concentration of ~115 mM. There was near quantitative recovery of CAK activity and a purification of about sixfold.

Partially purified CAK (purified through the ammonium sulfate step) was used to determine its substrate requirements (Figure 11). Wild-type p34^{cdc2} was immunoprecipitated from reticulocyte lysates and incubated with [γ-^{32}P]ATP, GT-cyclin B, and CAK (Figure 11, lane 2). p34^{cdc2} was phosphorylated as was a protein tentatively identified as GT-cyclin B. In the absence of GT-cyclin B there was only a low background phosphorylation of p34^{cdc2} (Figure 11, lane 3), presumably

on misfolded molecules. CAK is thus a cyclin-dependent p34^{cdc2} kinase, as predicted from the extract experiments. The T161A mutant of p34^{cdc2} was not significantly phosphorylated (Figure 11, lane 4), confirming that the correct site was utilized. Omission of either p34^{cdc2} (Figure 11, lane 6) or CAK (Figure 11, lane 7) eliminated the observed phosphorylation. Importantly, the catalytically inactive K33R mutant of p34^{cdc2} was also phosphorylated (Figure 11, lane 5), confirming that intramolecular autophosphorylation is not part of this reaction sequence. The same results were seen in the AF mutant background of p34^{cdc2}: phosphorylation of p34^{cdc2} and of GT-cyclin B (Figure 11, lane 8) but no phosphorylation of either in the absence of GT-cyclin B or with Ala-161 (Figure 11, lanes 9 and 10). We tested whether GT-cyclin B was phosphorylated by CAK or whether its phosphorylation depended on the activity of p34^{cdc2}. In all cases, GT-cyclin B only became phosphorylated when p34^{cdc2} was both phosphorylated and capable of activation; simple phosphorylation of an inactivatable form of p34^{cdc2} was insufficient to cause GT-cyclin B phosphorylation (Figure 11, lane 5). We conclude that the vast bulk of GT-cyclin B phosphorylation is due to activated p34^{cdc2} rather than to a component in the CAK preparation.

CAK Activity is not due to a p34^{cdc2} Protein Kinase

We examined the possibility that p34^{cdc2} could autophosphorylate intermolecularly, i.e., that CAK activity is due to p34^{cdc2}. A number of experiments ruled this out. First, purified H1 kinase had no CAK activity (Figure 12A). Dilutions of purified H1 kinase were added to a control buffer (Figure 12A, lanes 1–5), to an unprogrammed reticulocyte lysate (Figure 12A, lanes 6–10), or to a reticulocyte lysate containing wild-type Xenopus p34^{cdc2} (Figure 12A, lanes 11–15). No activation of p34^{cdc2} was detectable; the H1 kinase activity seen was that present in the initial H1 kinase sample itself (Figure 12A, lanes 1–5). Second, partially purified CAK contained no H1 kinase activity (Figure 12B). Although p34^{cdc2} activation occurred after CAK dilution of 10- to 270-fold (Figure 12B, lanes 3–6), 10- and 30-fold diluted CAK displayed no detectable H1 kinase activity (Figure 12B, lanes 1 and 2). Third, incubation of partially purified CAK with p13^{suc1}-Sepharose beads depleted p34^{cdc2}-like proteins but had no effect on CAK activity (Figure 12, C and D). p13^{suc1} binds to p34^{cdc2} and very close family members from all species examined (Brizuela et al., 1987; Dunphy et al., 1988; Solomon et al., 1990). Dilutions of CAK were assayed alone (Figure 12C, lanes 1–4), or after incubation with control protein A-Sepharose beads (Figure 12C, lanes 5–8), or after incubation with p13^{suc1}-Sepharose beads (Figure 12C, lanes 9–12). There was no detectable difference in activity other than the small dilution of both samples that had been incubated with beads (compare Figure 12C, lanes 8 and 12 with lane 4).
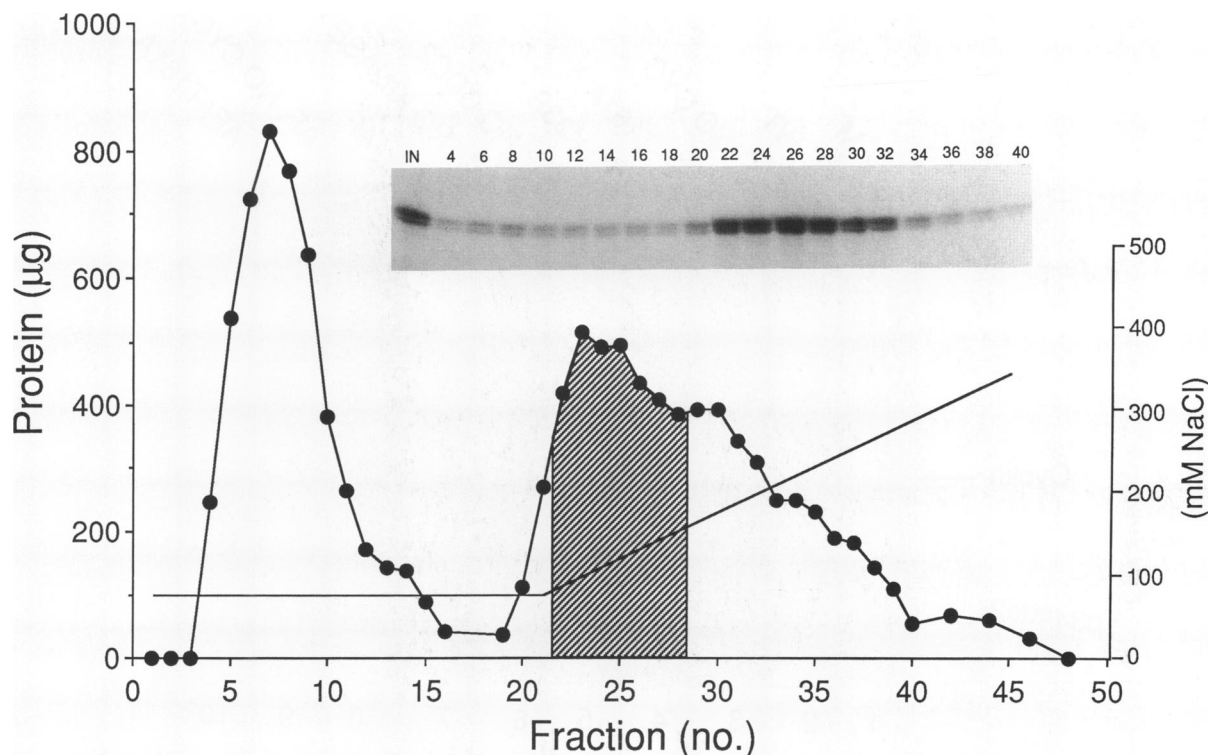
Figure 10. DEAE-Sepharose chromatography of the p34^{cdc2} activating kinase. A clarified interphase extract was applied to a DEAE-Sepharose column as described in MATERIALS AND METHODS and eluted with a linear NaCl gradient from 50 mM in fraction 20 to 345 mM in fraction 45. Fractions were assayed for CAK activity (gel insert) and for total protein (graph; see MATERIALS AND METHODS). Fractions 23–28 were pooled (hatched area) following reassay of diluted fractions to more accurately determine the peak of activity.

Western blotting with an anti-PSTAIR antiserum that detects p34^{cdc2} and closely related proteins showed that the bulk of p34^{cdc2} purified away from CAK activity (Figure 12D). Most flowed through the DEAE-Sepharose column (Figure 12D, compare lane 3 [flow through] with lane 2 [input]). A small amount of p34^{cdc2} remained in the pooled peak of CAK activity (Figure 12D, lane 4) along with more of a protein with greater electrophoretic mobility, presumably Eg1 (CDK2) (Solomon et al., 1990; Paris et al., 1991), and some minor proteins with mobilities similar to p34^{cdc2}. These proteins were enriched by ammonium sulfate precipitation of CAK activity (Figure 12D, lane 5). The pooled DEAE eluate that was incubated with protein A- and p13^{suc1}-Sepharose beads (Figure 12C) was analyzed by Western blotting for p34^{cdc2}-related proteins (Figure 12D, lanes 6–9 and 10–13). No p34^{cdc2}-related proteins bound to the protein A-Sepharose beads (Figure 12D, lanes 7 and 11) whereas all of the Eg1 (CDK2) and some of the minor proteins bound quantitatively to p13^{suc1} (Figure 12D, lanes 9 and 13). We assume that the minor proteins that do not bind to p13^{suc1} (Figure 12D, lanes 8 and 12) do not represent forms of p34^{cdc2} because both p34^{cdc2} and Eg1 bind tightly to p13^{suc1} (Brizuela et al., 1987; Dunphy et al., 1988; Solomon et al., 1990) and because Eg1 has been quantitatively depleted by p13^{suc1} in this experi-

ment. These proteins may be more distantly related members of the p34^{cdc2} family of protein kinases that contain recognizable "PSTAIR" sequences. It will be interesting to follow these proteins during further purification.

DISCUSSION

p34^{cdc2} Phosphorylation Sites

The synthesis of cyclin is not sufficient to generate the mitotic state. Several phosphorylation events are interspersed between cyclin binding and p34^{cdc2} activation. These phosphorylation reactions govern the entry into mitosis and provide important targets for feedback regulation. The identification of the enzymes responsible for regulating these phosphorylation events has been difficult. The wee1 and/or mik1 (Lundgren et al., 1991) proteins may phosphorylate the inactivating sites (Featherstone and Russell, 1991; Parker et al., 1991), although direct evidence is lacking. cdc25 is now thought to be the protein tyrosine phosphatase that acts on the same sites (Gautier et al., 1991; Strausfeld et al., 1991). The kinase(s) and phosphatase(s) acting on the activating site are unknown, although INH, a type 2A protein phosphatase, can inactivate p34^{cdc2} and remove the activating phosphorylation (Lee et al., 1991). To
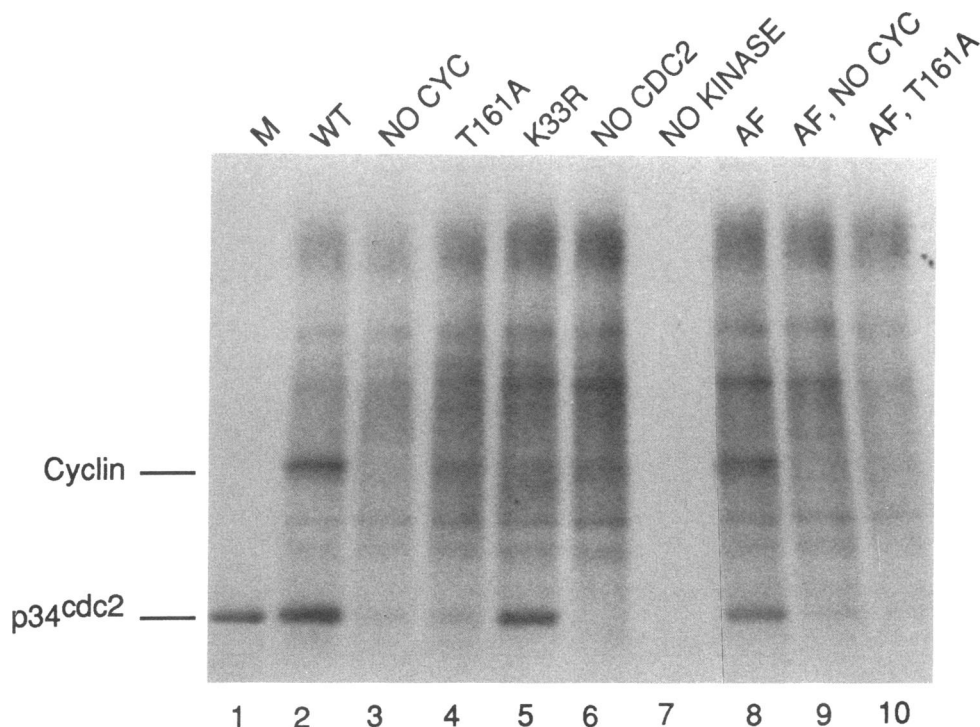
Figure 11. Direct cyclin-dependent phosphorylation of p34^{cdc2} by the p34^{cdc2} activating kinase. Reticulocyte lysate produced forms of p34^{cdc2} were immunoprecipitated and incubated with [γ-^{32}P]ATP in the presence or absence of GT-cyclin B and/or the activating kinase. Lane 1, ^{35}S-labeled p34^{cdc2} from reticulocyte lysate (0.1 μl, ~30 000 cpm in p34^{cdc2}). This is the only ^{35}S-labeled sample shown. Lane 2, Incubation with the activating kinase and GT-cyclin B (125 nM). Lanes 3–5, Same as lane 2 but without GT-cyclin B (lane 3) or using the T161A mutant of p34^{cdc2} (lane 4) or the K33R mutant (lane 5). Lane 6, p34^{cdc2} was omitted. Lane 7, no activating kinase added. Lanes 8–10, The p34^{cdc2} contained the mutations T14A and Y15F. Lane 8, Complete incubation. Lane 9, No cyclin. Lane 10, p34^{cdc2} also contained the T161A mutation.

study these reactions we need to identify the phosphorylation sites, mutate these sites to identify their functions, and identify the kinases and phosphatases responsible. This analysis is complicated by the fact that the object of this regulation, p34^{cdc2}, is itself a kinase that could, a priori, phosphorylate some of these sites by an intramolecular or intermolecular route.

We have identified sites of phosphorylation of *Xenopus* p34^{cdc2} on Thr-14, Tyr-15. An additional, activating, phosphorylation probably occurs on Thr-161. Phosphorylations of Thr-14 and Tyr-15 were readily detectable by the electrophoretic retardation they conferred (Figure 3). The sites of phosphorylation were already known to reside on the same tryptic peptide (Solomon *et al.*, 1990), which, as it turns out, contains a single threonine and two tyrosines (Milarski *et al.*, 1991). Added wild-type p34^{cdc2} ("TY") gave rise to two electrophoretically retarded forms, a single mutant ("AY") gave rise to a single electrophoretically retarded form, and the double "AF" mutant could not be phosphorylated and gave rise to no new forms. Direct ^{32}P-labeling of the T14A mutant confirmed the assignments and clearly demonstrated the phosphorylation of this site. Thr-161 was shown to be a likely site of phosphorylation by direct ^{32}P-labeling of mutant p34^{cdc2} proteins:

phosphorylation of this site in both a vanadate-arrested extract and in a mitotic extract was eliminated by a T161A mutation. We cannot yet exclude the remote possibility that Thr-161 is not phosphorylated and that mutation of Thr-161 to nonphosphorylatable amino acids actually prevents phosphorylation at another threonine in the p34^{cdc2} molecule. We assume for now that Thr-161 is indeed phosphorylated. Experiments are in progress to resolve this issue. Evidence presented here and elsewhere (Solomon *et al.*, 1991) indicates that a type A cyclin protein induces phosphorylation of the same sites and acts through the same pathway. We have suggested that differences in subcellular localization, rather than biochemistry, determine the different activation pathways followed by cyclin A- and cyclin B-p34^{cdc2} complexes (Solomon *et al.*, 1991).

Three factors were necessary for our ability to determine these phosphorylation sites: recombinant p34^{cdc2} mutants, high stoichiometry labeling resulting from high concentrations of added cyclin, and the defined arrest points resulting from vanadate treatment ("late G2") and a nondegradable cyclin (mitosis). Similar phosphorylation sites have been identified in p34^{cdc2}s from other species: Tyr-15 in *S. pombe* p34^{cdc2} and both Thr-14 and Tyr-15 in chicken p34^{cdc2} (Gould and Nurse,
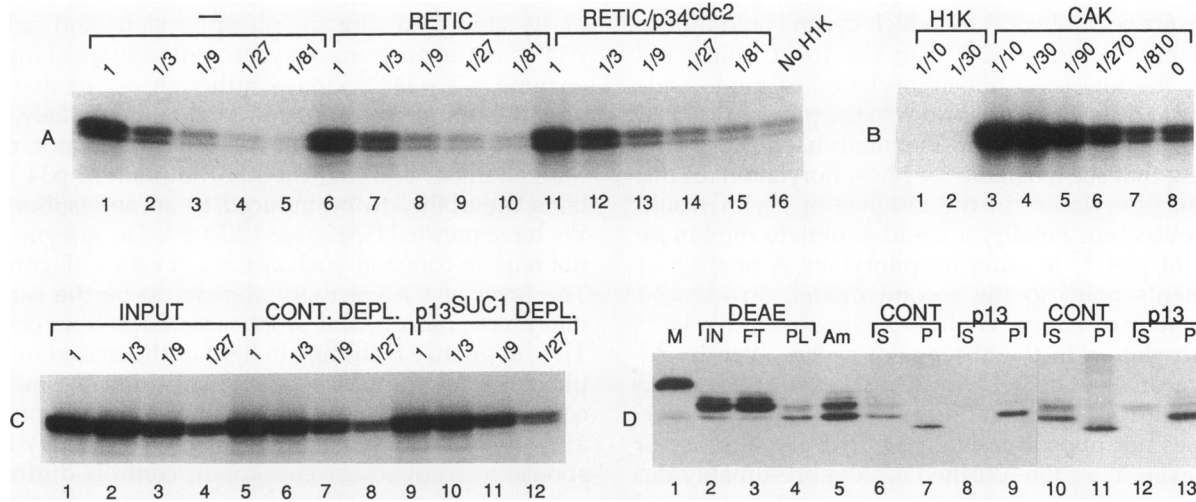
Figure 12. p34^{cdc2} activating kinase activity is not due to intermolecular autophosphorylation. (A) Purified H1 kinase (see MATERIALS AND METHODS) was diluted 5-fold (lanes 1, 6, and 11), 15-fold (lanes 2, 7, and 12), 45-fold (lanes 3, 8, and 13), 135-fold (lanes 4, 9, and 14), and 405-fold (lanes 5, 10, and 15) in EB/1 mg/ml ovalbumin and assayed for its p34^{cdc2} activating kinase activity. Lanes 1–5 were control reactions (see MATERIALS AND METHODS) and show the resulting H1 kinase activity of the diluted enzyme itself. Lanes 6–10 contained in addition unprogrammed reticulocyte lysate and lanes 11–16 contained reticulocyte lysate that had synthesized wild-type Xenopus p34^{cdc2}. No H1 kinase was added to lane 16. (B) The p34^{cdc2} activating kinase has no detectable H1 kinase activity. p34^{cdc2} activating kinase (~3.5 mg/ml) partially purified through ammonium sulfate fractionation was diluted 10-fold (lanes 1 and 3), 30-fold (lanes 2 and 4), 90-fold (lane 5), 270-fold (lane 6), or 810-fold (lane 7) and assayed for its ability to activate p34^{cdc2} in a reticulocyte lysate (lanes 3–7) or, following a control incubation, assayed for its own H1 kinase activity (lanes 1 and 2). (C) Treatment of p34^{cdc2} activating kinase with p13^{suc1}-Sepharose beads. Partially purified p34^{cdc2} activating kinase (through DEAE chromatography) was left untreated (lanes 1–4) or incubated with control protein A-Sepharose beads (lanes 5–8) or with p13^{suc1}-Sepharose beads (lanes 9–12). Supernatants were left undiluted (lanes 1, 5, and 9) or diluted 3-fold (lanes 2, 6, and 10), 9-fold (lanes 3, 7, and 11), or 27-fold (lanes 4, 8, and 12) into EB/1 mg/ml ovalbumin and assayed for their ability to activate p34^{cdc2} in reticulocyte lysates. Lanes 1–4 contained about twice as much protein as the corresponding lanes 5–8 and 9–13. (D) Immunoblot of p34^{cdc2}-related proteins during purification of the p34^{cdc2} activating kinase. Lane 1, 2 ng of reticulocyte lysate produced p34^{cdc2}. Lane 2, 10 µg of the input to the DEAE column. Lane 3, Flow-through from the DEAE column. Lane 4, 10 µg of pooled peak fractions from the DEAE column. Lane 5, 10 µg of 0–38% ammonium sulfate fraction of the DEAE peak. Lanes 6–9, supernatant (lanes 6 and 8) and pellet (lanes 7 and 9) fraction after incubation of 10 µg of the pooled DEAE peak with protein A-Sepharose (lanes 6 and 7) or with p13^{suc1}-Sepharose (lanes 8 and 9). Lanes 10–13 show a longer exposure of lanes 6–9.

1989; Krek and Nigg, 1991). Thr-161 may be a site of phosphorylation in chicken p34^{cdc2} (Krek and Nigg, 1991) and the homologous Thr-167 is probably used in S. pombe p34^{cdc2} (cited in Krek and Nigg, 1991). We have never seen evidence for phosphorylation of Ser-277 as occurs in chicken p34^{cdc2} (Krek and Nigg, 1991). A possible explanation is that the early embryonic Xenopus cell cycle lacks a G1 phase, the time at which phosphorylation of this site is highest (Krek and Nigg, 1991).

The Roles of Phosphorylation in p34^{cdc2} Activation

Phosphorylation of Thr-14 and Tyr-15 negatively regulate p34^{cdc2} activity (see RESULTS), whereas phosphorylation of the activating site, assumed to be Thr-161, is absolutely required for its kinase activity (see Figure 2). Mutagenesis of S. pombe Tyr-15 also indicates that this phosphorylation plays a negative role (Gould and Nurse, 1989). Conversely, Thr-167 (equivalent to Thr-161 of Xenopus p34^{cdc2}) is required for the function of S. pombe p34^{cdc2}, although the reason for this dependence was not explored (Booher and Beach, 1986).

An activating phosphorylation of p34^{cdc2}, probably on Thr-161, is inherently required for p34^{cdc2} activity; it does not function indirectly, either to stimulate dephosphorylation of Thr-14 and/or Tyr-15 or to promote association with cyclin. The triple mutant of p34^{cdc2}, containing Ala-14, Phe-15, and Ala-161 is still not activatable (Figure 2C, lane 6) and binds GT-cyclin B as well as the wild-type protein. Because GT-cyclin B bound efficiently to p34^{cdc2} in the presence of EDTA (Figure 1) and to forms of p34^{cdc2} mutated at Thr-161 (Figure 5), phosphorylation of this site is not required for this association. Although a strong case can be made that any cyclin-induced phosphorylation of p34^{cdc2} must increase the affinity of p34^{cdc2} for cyclin, it appears that the interaction between Xenopus p34^{cdc2} and GT-cyclin B is sufficiently strong to occur in the absence of phosphorylation (see Figure 5). Binding of cyclin B to p34^{cdc2} is, however, insufficient for kinase activity (see Figure 2A). We have not tested whether this interaction is strengthened by any of the p34^{cdc2} phosphorylations or if the interaction between other p34^{cdc2}s and cyclins requires this incremental binding energy.

There are several ways by which cyclin could induce the phosphorylation of p34^{cdc2} on Thr-161. Cyclin could stabilize the low level phosphorylation of monomeric p34^{cdc2} and drive the equilibrium to the phosphorylated form. It could induce a conformational change in p34^{cdc2}, rendering that site available for phosphorylation. In this case phosphorylation in the absence of cyclin would not be observed. Finally, it could stimulate the kinase activity of p34^{cdc2} to autophosphorylate. A number of experiments point to the second model, an induced conformational change. We have never observed p34^{cdc2} phosphorylation in the absence of cyclin, even in the presence of okadaic acid to trap the phosphorylated form (Figure 7). Furthermore, the p34^{cdc2} activating kinase does not phosphorylate p34^{cdc2} in the absence of cyclin, even in a more purified system presumably deficient in phosphatase activities (Figure 11). Induced autophosphorylation also does not seem to be operative. We have no evidence that autophosphorylation ever occurs (Figures 8, 11, and 12). Moreover, the p34^{cdc2} activating kinase exists and is capable of phosphorylating the correct site in a catalytically inactive form of p34^{cdc2} in the presence of GT-cyclin B (Figure 11).

The sequence of phosphorylations is not yet clear but their dependencies are. The activating phosphorylation depends on cyclin binding and does not require activation of the mitotic state. All three phosphorylations are thus dependent on cyclin binding (Solomon *et al.*, 1990) and may reflect a common induced conformational change that makes these sites accessible to the appropriate kinases. The activating phosphorylation also does not depend on Thr-14 and/or Tyr-15 phosphorylation (Figures 2 and 11). Similarly, Thr-14 and Tyr-15 phosphorylation do not depend on the activating phosphorylation (Figures 3 and 4). The extent of the activating phosphorylation does not appear to be regulated by entry into mitosis to produce an autoactivating process. In contrast, entry into mitosis does regulate Thr-14 and Tyr-15 phosphorylation through both the kinase and the phosphatase reactions (Solomon *et al.*, 1990). All of this suggests independent regulation of the activating and inhibitory phosphorylations and leaves unclear the role of cyclin phosphorylation (see also Izumi and Maller, 1991).

A "p34^{cdc2} Kinase"

A new and central component in the regulatory network is the kinase that phosphorylates p34^{cdc2} on its activating site, probably Thr-161. We found that this phosphorylation does not occur by either intramolecular or intermolecular autophosphorylation; instead, a cellular activity is required. There are no obvious genetic candidates for this enzyme, which could suggest that it has multiple roles in cell physiology. We have termed this enzyme the p34^{cdc2} activating kinase (CAK) on the basis of its ability to directly phosphorylate and activate p34^{cdc2} in the presence of cyclin. Properly speaking, this enzyme is a p34^{cdc2} kinase, although use of that term would likely cause confusion. Although unlikely, it remains formally possible that CAK is an activator, rather than a kinase, and that the K33R mutant of p34^{cdc2} retains the ability to be induced to autophosphorylate. We have purified CAK over 100-fold. The enzyme does not require cofactors such as Ca^{2+} or cAMP (Figure 11). The level of CAK activity appears to be the same in interphase, mitosis, and prophase extracts from oocytes. This invariance is striking in light of the crucial role this phosphorylation plays in p34^{cdc2} activation. Despite this, control of the extent of Thr-161 phosphorylation is an attractive route for regulation of p34^{cdc2} activity in response to feedback or checkpoint controls during the cell cycle. Further efforts to purify and identify the p34^{cdc2} activating kinase and to study its regulation under other cell cycle conditions should yield insights into the coordination of p34^{cdc2} activity with cellular signals.

ACKNOWLEDGMENTS

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