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Activation of Human Cyclin-Dependent Kinases In Vitro

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We have analyzed the activation of human cyclin-dependent kinases in a cell-free system. Human CDC2, cyclin-dependent kinase 2 (CDK2), cyclin A, and cyclin B1 were produced in insect cells by infection with recombinant baculoviruses. CDC2 or CDK2 monomers in lysates of infected cells could be activated by the addition of lysates containing cyclin A or B1. CDC2 activation by cyclin B1, as well as CDK2 activation by cyclins A and B1, was accompanied by the formation of high molecular weight complexes. In contrast, CDC2 did not bind effectively to cyclin A. CDC2 activation by cyclin B1 was studied in detail and was found to be accompanied by phosphorylation of CDC2 on Threonine 161. The binding of CDC2 to cyclin B1 also occurred under conditions where CDC2 phosphorylation was prevented, resulting in an inactive complex that could then be phosphorylated and activated on addition of cell extract. Highly purified CDC2 and cyclin B1 also formed inactive complexes that could be activated in an ATP-dependent fashion by unidentified components in crude cell extracts. These data suggest that the CDC2 activation process begins with cyclin binding, after which CDC2 phosphorylation, catalyzed by a separate enzyme, leads to activation.

INTRODUCTION

CDC2-like protein kinases are important regulators of the eukaryotic cell cycle. In yeast, CDC2 is required at two cell-cycle transitions: the commitment to DNA replication in G1 (Start) and the induction of mitosis at the G2/M boundary (Forsburg and Nurse, 1991). In higher eukaryotes, these transitions may be controlled by multiple CDC2-like kinases. The major vertebrate homologue, known as CDC2, has been implicated mainly in the control of M phase; it is most active during mitosis and is the catalytic subunit of M-phase promoting factor (MPF) (for reviews, see Murray and Kirschner, 1989; Draetta, 1990; Nurse, 1990; Morgan, 1992). A second CDC2-like kinase, known as cyclin-dependent kinase 2 (CDK2), recently has been identified in frogs and humans (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991; Rosenblatt *et al.*, 1992). CDK2 may play a role in earlier stages of the cell cycle than CDC2; in mammalian cells it is activated during the S and G2 phases (Rosenblatt *et al.*, 1992), and immunodepletion of CDK2 from *Xenopus* egg extracts results in an inhibition of DNA synthesis (Fang and Newport, 1991).

CDC2 and CDK2 are periodically activated during the cell cycle by various post-translational mechanisms. Both kinases are inactive as monomers, and activation requires association with regulatory subunits, known as cyclins, whose levels change during the cell cycle (Hunt, 1989). Several classes of human cyclins are known. These include cyclin B, whose levels peak during mitosis (Pines and Hunter, 1989), and cyclin A, whose levels peak between S phase and early mitosis (Pines and Hunter, 1990; Rosenblatt *et al.*, 1992). Cyclin B associates with CDC2 during mitosis to form the active MPF complex, whereas cyclin A associates mainly with CDK2 during the S and G2 phases to form an active complex that may be involved in the control of S phase (Girard *et al.*, 1991; Tsai *et al.*, 1991; Rosenblatt *et al.*, 1992).

The activity of CDC2 is also regulated by phosphorylation. Threonine 14 (T14) and tyrosine 15 (Y15), two residues adjacent to the ATP binding site in CDC2, are phosphorylated before mitosis (Gould and Nurse, 1989; Krek and Nigg, 1991a; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Phosphorylation at these sites is cyclin-dependent and is thought to suppress the kinase activity of the CDC2/cyclin B complex (Solomon *et al.*, 1990). Abrupt dephosphorylation of these residues at M phase leads to the activation of the CDC2/cyclin B complex. Thus, mutation of Y15 results in premature CDC2 activation and premature mitosis in fission yeast (Gould

and Nurse, 1989). Similarly, transient overexpression of vertebrate CDC2 mutated at T14 and Y15 results in premature mitotic events in HeLa cells (Krek and Nigg, 1991b). The importance of this phosphorylation remains unclear in budding yeast, where mutation of this tyrosine residue has no apparent effect on cell cycle control (Sorger and Murray, 1992).

CDC2 is also phosphorylated on an additional threonine residue that is probably threonine 161 (T161) (Gould *et al.*, 1991; Krek and Nigg, 1991a; Solomon *et al.*, 1992). Phosphorylation at this site is required for CDC2 activation because mutation of T161 results in the inactivation of CDC2 function and a loss of CDC2 kinase activity (Booher and Beach, 1986; Ducommun *et al.*, 1991; Gould *et al.*, 1991; Norbury *et al.*, 1991; Solomon *et al.*, 1992). The mechanism by which T161 phosphorylation leads to kinase activation is unclear. One possibility is that T161 phosphorylation promotes cyclin binding, because T161 mutation inhibits cyclin binding under some conditions (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Norbury *et al.*, 1991). This issue requires further exploration, however, because in some systems T161 mutation has no effect on cyclin binding (Solomon *et al.*, 1992).

To better understand the sequence of molecular events underlying CDK activation in human cells, we have developed a system in which the activation of these kinases can be reconstituted in vitro. Using human CDK and cyclin proteins produced with the baculovirus system, we have observed cyclin-dependent activation of CDK monomers. CDK activation by cyclins was accompanied by the formation of high molecular weight complexes and by CDK phosphorylation. The binding of CDC2 to cyclin B1 also occurred under conditions where CDC2 phosphorylation was prevented, indicating that phosphorylation is not required for cyclin binding. The inactive nonphosphorylated complex became phosphorylated and activated on addition of cell extract, suggesting that activation requires a separate enzyme whose function may be to catalyze CDC2 phosphorylation.

MATERIALS AND METHODS

Mutagenesis and Epitope Tagging

Oligonucleotide-mediated mutagenesis was performed with uracil-containing template DNA (Kunkel, 1985). The human CDC2 and CDK2 cDNAs (Lee and Nurse, 1987; Rosenblatt *et al.*, 1992) were cloned into pSM (Brodsky *et al.*, 1990) for transformation into *Escherichia coli* CJ236 (*dut- ung*-F'), and uracil-containing single stranded DNA was prepared by infecting cultures with M13 helper phage. Kinase-deficient versions of CDC2 and CDK2 were produced by replacing lysines in the ATP binding site region. In the CDC2(K−) mutant, lysines 33 and 34 are replaced with methionine and isoleucine (ATG.AAA.AAA.ATC changed to ATG.ATG.ATC.ATC). In CDK2(K−), lysines 33 and 34 are replaced with threonine and serine (CTT.AAG.AAA.ATC changed to CTT.ACT.AGT.ATC).

To obtain specific immunoprecipitates of CDC2 and CDK2 in some experiments, versions of these proteins were constructed with a short influenza hemagglutinin peptide fused to the C-terminus. This peptide epitope is recognized with high affinity by a monoclonal antibody (MAb) 12CA5, providing specific immunoblotting or immunoprecipitation of the tagged kinases (Field *et al.*, 1988). The epitope tag does not interfere with the function of these proteins because it does not affect the ability of CDK2 or CDC2 to complement *cdc28* mutations in *Saccharomyces cerevisiae*. In addition, the tag has no effect on the level of expression of the proteins or on the activation of these kinases in vitro. C-terminal fusion to the epitope tag was performed by introducing an *Nco* I site at the C-terminus of CDC2 and CDK2, allowing ligation to in-frame epitope tag sequences in the pSM vector. The C-terminus of CDC2 was changed from QIKKM to QIKTMAYPYDVPDYASLGPGL. The C-terminus of CDK2 was changed from LRL to LRLSMAYPYDVPDYASLGPGL. Residues recognized by MAb 12CA5 are underlined.

Baculovirus Construction, Infection, and Lysate Preparation

Wild-type or mutant cDNAs were cloned into baculovirus transfer vectors pVL941 or pVL1392 (Luckow and Summers, 1989). Transfer vectors were cotransfected with baculovirus genomic DNA into Sf9 insect cells, and recombinant baculoviruses were isolated and plaque purified as described (Summers and Smith, 1987; Morgan *et al.*, 1991). Monolayers of Sf9 cells (1×10^7 cells in 10-cm dishes) were infected at a multiplicity of infection of five plaque-forming units per cell, and 2 d after infection the cells were washed once with HBS (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-NaOH, pH 7.4, 150 mM NaCl), harvested by centrifugation, and lysed by resuspension in 400 μl cold hypotonic lysis buffer (10 mM HEPES-NaOH, pH 7.4, 10 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml leupeptin). NaCl was added (final concentration 150 mM), and the lysates were clarified by centrifugation in a microfuge (10 000 × *g*, 20 min) or Beckman (Fullerton, CA) airfuge (100 000 × *g*, 30 min). These lysates typically contained 5 mg/ml total protein, of which 2–7% was the foreign protein (several hundredfold higher than endogenous levels of these proteins; not shown). We estimated that these lysates contained the following concentrations of the foreign proteins: CDC2, 0.35 mg/ml, 10 μM; CDC2(K−), 0.07 mg/ml, 2 μM; CDK2, 0.2 mg/ml, 6 μM; cyclin A, 0.35 mg/ml, 7 μM; cyclin B1, 0.35 mg/ml, 7 μM. In many experiments, lysates were diluted to provide concentrations similar to those found in vivo. To estimate the CDC2 concentration in vivo, we used immunoblots to compare the amount of CDC2 in HeLa extracts with known amounts of purified CDC2 (not shown). We found that CDC2 represents ~0.025–0.05% of the protein in HeLa cell detergent extracts, in agreement with estimates by others (Draetta *et al.*, 1988). (CDK2 is present at ~5-fold lower levels, or ~0.005–0.01% of cell protein.) Based on this analysis, one HeLa cell contains an average of 0.5–1.0 × 10^6 CDC2 molecules, which is equivalent to a cellular concentration of roughly 0.2–0.4 μM (not taking into account the higher localized concentration in certain subcellular regions).

Various evidence indicates that CDC2 and CDK2 are not significantly phosphorylated when overexpressed in the insect cell. CDC2 and CDK2 were immunoprecipitated from lysates of cells labeled for the final 4 h of infection with ^{32}P-orthophosphate (not shown). When compared to $p60^{c\text{-}src}$, a protein known to be phosphorylated extensively when overexpressed in the insect cell, both CDC2 and CDK2 were very poorly labeled (as seen previously with CDC2) (Parker *et al.*, 1991). CDC2 and CDK2 were labeled on serine (~90%) and threonine (~10%); no phosphotyrosine was detected. Phosphorylation mainly occurred on several peptides that do not comigrate with previously identified phosphopeptides and are likely to reflect low levels of nonspecific phosphorylation. Phosphorylation of CDC2 and CDK2 was also assessed by analysis of their electrophoretic mobility, which changes on polyacrylamide gels when certain major sites are phosphorylated. In HeLa cells, CDK2 exhibits an increase in mobility when activated (Rosenblatt *et al.*, 1992); this shift correlates with T160 phosphorylation, and analysis of epitope-tagged CDK2 proteins ex-

pressed in COS and 3T3 cells reveals that changing T160 to alanine abolishes the shift (Gu and Morgan, unpublished data). T160 phosphorylation therefore increases CDK2 mobility. It is also known that phosphorylation of T14 and Y15 decreases CDC2 mobility (Draetta and Beach, 1988; Morla *et al.*, 1989). None of these shifts are exhibited by baculovirus-derived CDC2 and CDK2, which migrate as single bands with the mobility of nonphosphorylated proteins.

Activation, Phosphorylation, and Complex Formation in Crude Lysates

To obtain activation in vitro, 2 μl of insect cell lysate containing CDC2 or CDK2 was mixed with 2 μl lysate containing cyclin A or B1. In control experiments where one component was analyzed alone, 2 μl of lysate containing the desired protein was mixed with 2 μl of control lysate from cells infected with a recombinant baculovirus that does not encode a foreign protein. After 20 min incubation at 24°C, kinase activity in the mixture was assayed by addition of a 30 μl reaction mix containing 10 mM $MgCl_2$, 50 μM ATP, 1 mM dithiothreitol (DTT), 20 μg histone H1 (Boehringer Mannheim, Indianapolis, IN), and 2.5 μCi ^{32}P-γ-ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL). After 5 min at 24°C, reactions were boiled in sample buffer for analysis on polyacrylamide gels. Histone H1 bands were excised from gels, and Cerenkov counts were determined in a scintillation counter. In some cases, histone phosphorylation was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In some experiments, CDK proteins were isolated from lysates or column fractions by immunoprecipitation. Aliquots (25 μl) of Protein A-Sepharose beads were incubated 30 min at 4°C with 5 μg MAb 12CA5 (when epitope-tagged CDK proteins were used) or with 2 μg affinity-purified antibody against the C-terminus of CDC2 (Rosenblatt *et al.*, 1992). Beads were washed and then added to the desired lysate. After a 2-h incubation at 4°C, immunoprecipitates were washed three times at room temperature with HBST (HBS + 0.1% Triton X-100) and twice with 50 mM HEPES-NaOH, pH 7.4, 1 mM DTT. Histone H1 kinase activity was then measured in the precipitates as described above.

To measure phosphorylation of CDK proteins during activation, lysates were mixed and kinase reactions were performed as described above, except that histone H1 was omitted and higher amounts of ^{32}P-γ-ATP were used (10 μCi, 50 μM). Reactions were stopped by addition of 500 μl HBST containing 10 mM EDTA, and the epitope-tagged CDK was then immunoprecipitated from the mix with MAb 12CA5 for analysis on polyacrylamide gels. For tryptic phosphopeptide or phosphoamino acid analysis, proteins were electrophoretically transferred to Immobilon-P membranes. Phosphoamino acid analysis was performed as described (Morgan *et al.*, 1989; Kamps, 1991) using thin-layer electrophoresis at pH 3.5. Phosphopeptide analysis was performed by incubating excised Immobilon fragments in sequencing grade Trypsin (10 μg; Boehringer Mannheim) using described procedures (Boyle *et al.*, 1991; Luo *et al.*, 1991). Phosphopeptides were spotted onto thin layer cellulose (TLC) plates (Kodak Chromogram 13255, Rochester, NY) and subjected to electrophoresis at pH 1.9 (2.2% formic acid, 7.8% acetic acid), followed by chromatography in the second dimension (n-butanol:pyridine:acetic acid:H_2O, 75:50:15:60).

To obtain human CDC2 phosphorylated in vivo, HeLa cells were labeled for 2 h with ^{32}P-orthophosphate (0.8 mCi/ml) in phosphate-free medium supplemented with 10% dialyzed fetal calf serum. Cells were washed with HBS and lysed in HBST containing 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 50 mM NaF, 0.1 mM Na_3VO_4, and 80 mM β-glycerophosphate. Lysates were clarified by centrifugation (10 000 × *g*, 30 min, 4°C), and CDC2 was immunoprecipitated with affinity-purified rabbit antibodies against the C-terminal peptide of CDC2 (Rosenblatt *et al.*, 1992). Immunoprecipitates were run on polyacrylamide gels and transferred to Immobilon-P for tryptic phosphopeptide analysis as above.

Complex formation between CDK proteins and cyclins was measured by gel filtration analysis. Crude or purified CDK and cyclin were mixed for 20 min at 24°C in a total volume of 200 μl and then injected onto a Pharmacia (Piscataway, NJ) Superose 12 column (HR 10/30) pre-equilibrated with column buffer (50 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM EDTA) and eluted at 0.4 ml/min (0.5-ml fractions).

Immunoblotting

Proteins were subjected to electrophoresis on 10% polyacrylamide gels, followed by electrophoretic transfer to nitrocellulose. After blocking in 1% bovine serum albumin or 4% dry milk, blots were incubated overnight at 4°C in the desired primary antibodies: crude rabbit anti-PSTAIR serum (provided by C. Norbury and P. Nurse, University of Oxford, U.K.; 1:500) or crude rabbit anti-cyclin B or anti-cyclin A (provided by J. Pines and T. Hunter, The Salk Institute, La Jolla, CA; 1:1000). After an additional incubation with alkaline-phosphatase-conjugated second antibody (Promega, Madison, WI), antigens were visualized with alkaline phosphatase substrates from the Promega Protoblot Western Blot AP system. To quantitate the amount of antigen, the primary antibody was also detected by incubation for 1 h at room temperature with 1 μCi ^{125}I-labeled goat anti-rabbit antibodies (Amersham) plus 5% hemoglobin. Bands were quantified with a PhosphorImager.

Purification of CDC2

A 700-ml culture of Sf9 cells (1.5×10^9 cells) was infected with CDC2 baculovirus by previously described procedures (Morgan *et al.*, 1991). After 2 d, the cells were harvested by centrifugation, washed gently in 100 ml HBS, and resuspended in 55 ml hypotonic lysis buffer (as above, except containing 25 mM NaCl). After Dounce homogenization, the lysate was clarified by centrifugation (100 000 × *g*, 1 h, 4°C). The supernatant was passed by gravity over a DEAE Sepharose Fast Flow column (2.5 × 3 cm; 15 ml) pre-equilibrated with lysis buffer. The flow-through was collected, brought to 50 mM NaCl, and passed by gravity over an S-Sepharose Fast Flow column (2.5 × 1.5 cm; 7.4 ml) pre-equilibrated with lysis buffer plus 50 mM NaCl. Ammonium sulfate was added to the flow-through, and the 45–70% ammonium sulfate pellet was dissolved in 10 ml 25 mM HEPES-NaOH, pH 7.4, 25 mM NaCl, 1 mM DTT, 1 mM EDTA and dialyzed against the same buffer at 4°C. The dialysate was centrifuged to remove particulates and then injected with a Pharmacia Superloop onto an ATP affinity column pre-equilibrated with the same buffer plus 10% glycerol. This column was composed of ATP coupled through ribose hydroxyls to 4% agarose (Sigma, St. Louis, MO; 1 × 2.5 cm; 2 ml). After washing, bound proteins were eluted with a 20-ml linear salt gradient (25–500 mM NaCl). Fractions containing CDC2 were pooled and concentrated (Centricon-30, Amicon, Beverly, MA) to 200 μl and injected on a Superose-12 column (HR10/30) pre-equilibrated with 25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10% glycerol. Fractions containing CDC2 (which was completely monomeric) were pooled, frozen in liquid nitrogen, and stored at −80°C.

Purification of Cyclin B

A 150-ml culture of Sf9 cells (2.5×10^8 cells) was infected with cyclin B1 virus and incubated 2 d. Cells were harvested by centrifugation, washed gently with 10 ml of HBS, and lysed by Dounce homogenization in 15 ml of hypotonic lysis buffer (as above). As cyclin B1 tended to aggregate under low salt concentrations, NaCl was immediately added to 150 mM. The lysate was centrifuged (100 000 × *g*, 1 h, 4°C) and the supernatant was injected onto an S-Sepharose fast flow column (1 × 6.4 cm; 5 ml) pre-equilibrated with HBS plus 1 mM EDTA. After washing, bound proteins were eluted with a 30-ml linear salt gradient (150–500 mM NaCl). Fractions containing cyclin B1 were pooled, diluted twofold, and injected onto a Pharmacia Mono Q HR 5/5 column pre-equilibrated with HBS plus 1 mM EDTA. Bound proteins were eluted with a 15-ml linear salt gradient (150–500 mM NaCl), and fractions containing cyclin B1 were pooled, concentrated

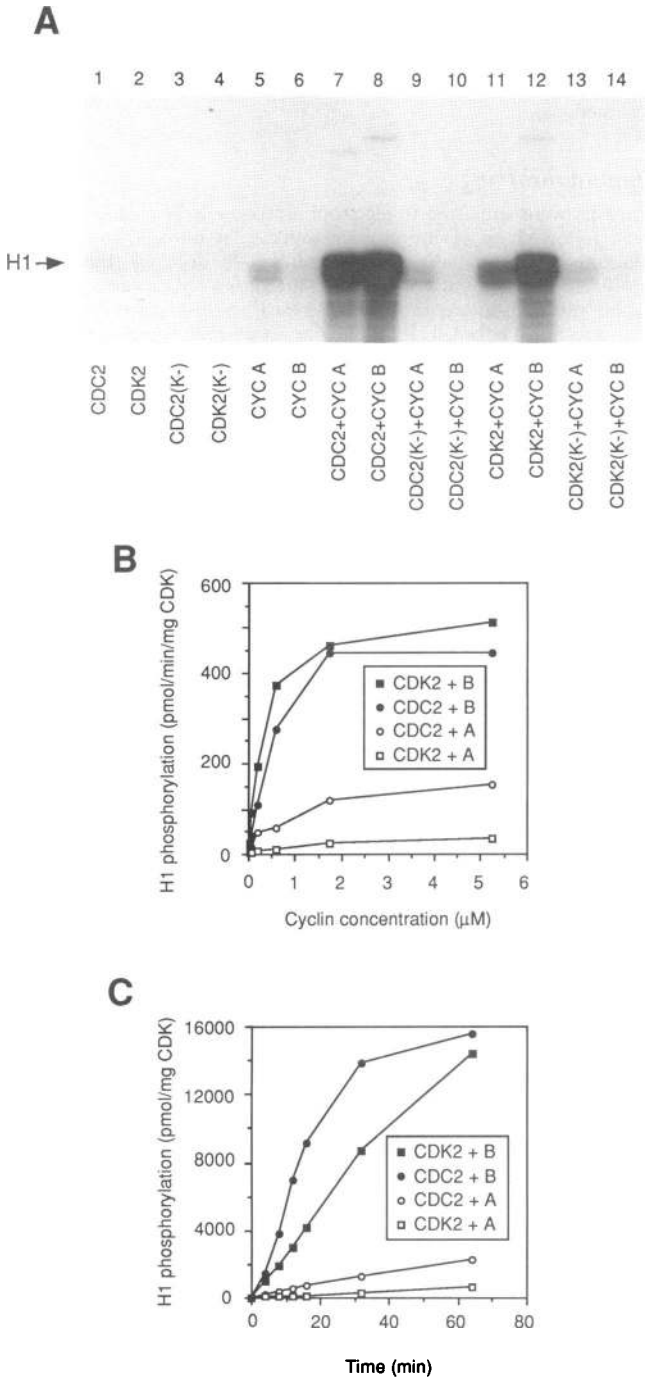


Figure 1. Activation of human cyclin-dependent kinases in vitro. (A) Lysates from baculovirus-infected cells expressing the indicated proteins were incubated alone (lanes 1–6) or in various combinations (lanes 7–14) for 20 min at 24°C, and then histone H1 kinase activity was measured (see MATERIALS AND METHODS). CDC2(K−) and CDK2(K−) are versions of these proteins with mutations in their ATP binding sites. (B) Fixed amounts of CDC2 (~2.5 μM; ○, ●) or CDK2 (~1.5 μM; □, ■) were mixed with the indicated concentrations of cyclin A (○, □) or cyclin B1 (●, ■). After a 20-min incubation at 24°C, histone H1 kinase activity was measured for an additional 5 min. Histone H1 bands were excised from gels and counted, and phosphate incorporation was normalized for the amount of CDK protein present. (C) Lysates containing CDC2 (2.8 μM; ○, ●) or CDK2 (2.8 μM; □, ■) were incubated with cyclin A (1.9 μM; ○, □) or B1 (1.9 μm; ●, ■). After incubation at 24°C for the indicated times, histone H1 kinase activity was measured in the mixtures for 1 min. Histone H1 bands were excised from gels and counted.

to 200 μl (Centricon-30), and injected on a Superose-12 column. Cyclin B1 eluted from the column in the 160-kDa range; fractions were pooled, frozen in liquid nitrogen, and stored at −80°C.

RESULTS

Activation of CDC2 and CDK2 by Cyclins A and B1 In Vitro

To analyze the biochemistry of human CDK activation, we used the baculovirus expression system to produce large quantities of human CDC2, CDK2, cyclin A, and cyclin B1. Recombinant baculoviruses encoding these proteins were constructed, and Sf9 insect cells infected with these viruses were found to express high levels of the proteins (generally 2–7% of extractable cell protein; see MATERIALS AND METHODS). In all cases, the baculovirus-derived proteins were completely soluble when cells were lysed under mild conditions. CDC2 and CDK2 were not significantly phosphorylated in the insect cell before lysis (see MATERIALS AND METHODS).

Lysates containing CDC2 or CDK2 were mixed with lysates containing cyclins A or B1, and after a brief incubation the histone H1 kinase activity in the mixture was measured (Figure 1A). Lysates containing CDC2 or CDK2 alone exhibited negligible amounts of kinase activity. On addition of lysates containing cyclins A or B1, the histone H1 kinase activities of both kinases were increased. We also analyzed kinase-deficient versions of CDC2 and CDK2, mutated at lysines in their ATP binding sites. (lysines 33 and 34 were changed to methionine and isoleucine in CDC2 and threonine and serine in CDK2.) These mutant proteins did not exhibit detectable kinase activity when cyclins were added.

When fixed amounts of CDC2 or CDK2 were mixed with increasing amounts of cyclin A or B1, activation of both kinases reached a plateau when the molar ratio of CDK to cyclin was approximately one (Figure 1B). Analysis of the time course of activation in these experiments (Figure 1C) revealed that activation occurred rapidly and without any apparent lag phase. Cyclin B1 generally activated both kinases to a greater extent than cyclin A.

CDK Proteins and Cyclins Form Complexes In Vitro

We analyzed the ability of CDKs to form high molecular weight complexes with cyclins in vitro. Mixtures of CDK and cyclin proteins, diluted to physiological concentrations, were subjected to gel filtration on Superose 12, and fractions were analyzed by immunoblotting and

histone H1 kinase assays. Using this method, we could readily estimate the molecular size of complexes and determine the relative amounts of bound and free CDK.

When analyzed alone, CDC2 and CDK2 migrated as inactive monomers on this column (Figure 2, A–C), whereas cyclins A and B1 migrated as oligomers of ~110 and 160 kDa, respectively (Figure 2, D and E). When equal amounts of CDC2 lysate and cyclin B1 lysate were mixed and incubated briefly, gel filtration revealed that the CDC2 had shifted to high molecular weight fractions (Figure 2F). The same fractions contained histone H1 kinase activity, suggesting that the high molecular weight form of CDC2 represents an activated complex containing cyclin B1. The kinase-deficient mutant, CDC2(K−), was also capable of binding cyclin B1 (Figure 2G), indicating that kinase activity is not required for cyclin binding. In separate experiments, immunoprecipitation of CDC2 from mixed lysates resulted in the coprecipitation of cyclin B1 and histone H1 kinase activity, further demonstrating that CDC2 and cyclin B1 were physically associated (not shown).

CDK2 formed active complexes with both cyclins A and B1 (Figure 2, I and J). In the CDK2/cyclin A mixture (Figure 2J), an increased amount of unbound CDK2 was probably observed because the concentration of CDK2 (~0.3 μM) was greater than the concentration of cyclin A (~0.2 μM). In the experiments with cyclin B1 (Figure 2, F and I), the concentrations of CDK and cyclin were approximately equal (0.2 μM). In other studies (not shown), we have found that decreasing the relative amount of cyclin (or increasing the amount of CDK) results in a proportional increase in the amount of monomeric CDK. A simple explanation of these observations is that binding between these components is essentially complete and complexes contain equimolar amounts of the two subunits.

We did not observe efficient binding of CDC2 and cyclin A in these experiments, where the concentrations of cyclin A and CDC2 were roughly equal (0.2 μM) (Figure 2H). Complex formation was also insignificant when 10-fold higher concentrations of both components were mixed (not shown). As histone H1 kinase activity was found in high molecular weight fractions from these mixtures, some low level of complex formation was occurring.

The behavior of CDK/cyclin complexes formed in vitro was compared with that of active complexes isolated from HeLa cells. A mitotic HeLa cell extract, prepared from cells synchronized with nocodazole, was subjected to gel filtration. CDC2-associated kinase activity, measured in anti-CDC2 immunoprecipitates, migrated in heterogeneous complexes that were slightly larger than CDC2/cyclin B1 complexes formed in vitro (peak in fractions 20–21; not shown). The specific activity of mitotic HeLa cell CDC2 in these complexes was ~100-fold higher than the specific activity of CDC2 in complexes formed in vitro. Similarly, active CDK2/cyclin A complexes isolated from S-phase HeLa cells were slightly larger (peak in fractions 21–22) (Rosenblatt *et al.*, 1992) and several hundredfold more active than baculovirus-derived CDK2/cyclin A complexes. Thus, although these CDK and cyclin proteins bind effectively in vitro, some other aspect of the activation process is not complete.

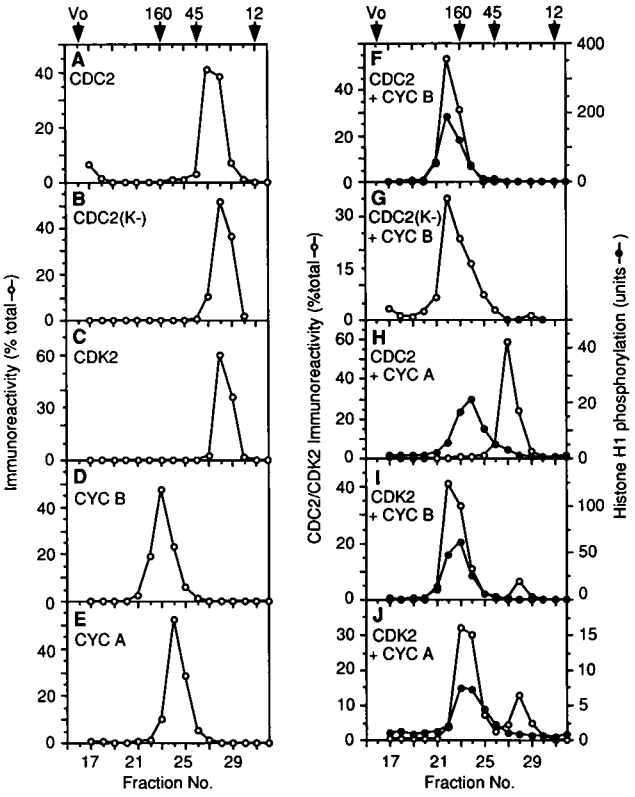
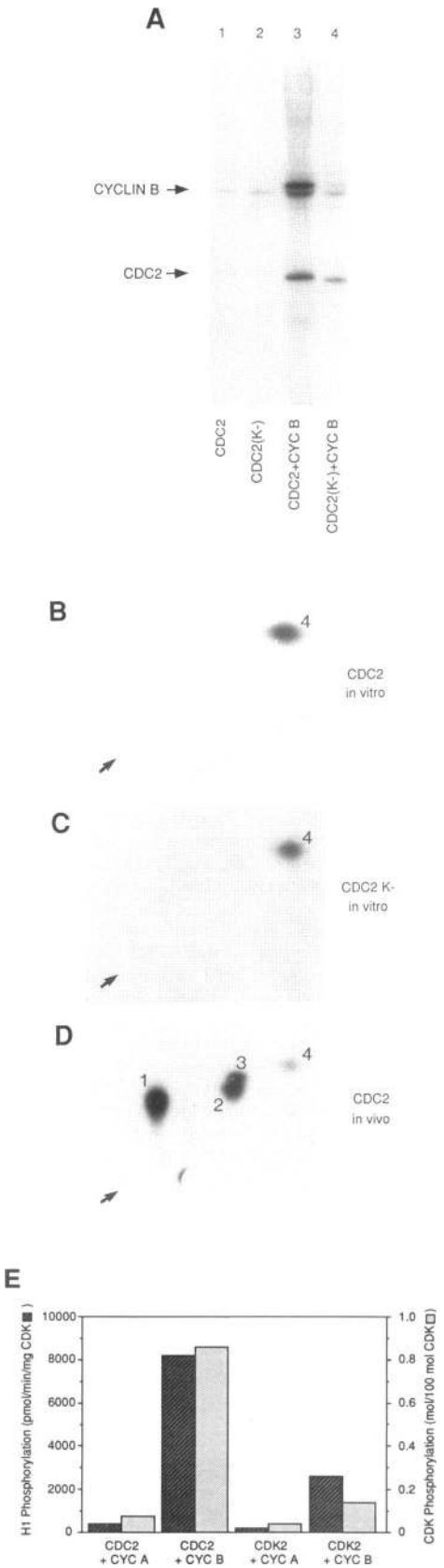


Figure 2. CDK and cyclin proteins form high molecular weight complexes in vitro. Diluted lysates containing the indicated proteins were incubated alone (A–E) or in various combinations (F–J) for 20 min at 24°C. CDK and cyclin concentrations in these mixtures were ~0.2 μM, except in G, where CDC2(K−) and cyclin B1 concentrations were 1 and 3 μM, respectively, and in J, where CDK2 concentration was ~0.3 μM. Mixtures were subjected to gel filtration on a Superose 12 column, and fractions were analyzed for the presence of CDC2 or CDK2 by immunoblotting with anti-PSTAIRE antibodies (A–C; F–J) or for the presence of cyclin A or B1 with anti-cyclin A or anti-cyclin B antibodies (D and E). Immunoblots were probed with ^{125}I-labeled secondary antibodies and quantified with a PhosphorImager (○). Histone H1 kinase activity was also measured in the fractions but is shown only for the four active combinations (F, H–J); activity in other panels was negligible. Kinase activity was quantitated by analysis with a PhosphorImager and is expressed in arbitrary units (●). Note the change in phosphorylation scales in the different panels. Molecular weights (in kDa) of marker proteins, determined in parallel runs, are indicated (Vo, Void volume; 160, IgG; 45, ovalbumin; 12, cytochrome C).

CDK Proteins are Phosphorylated During Activation

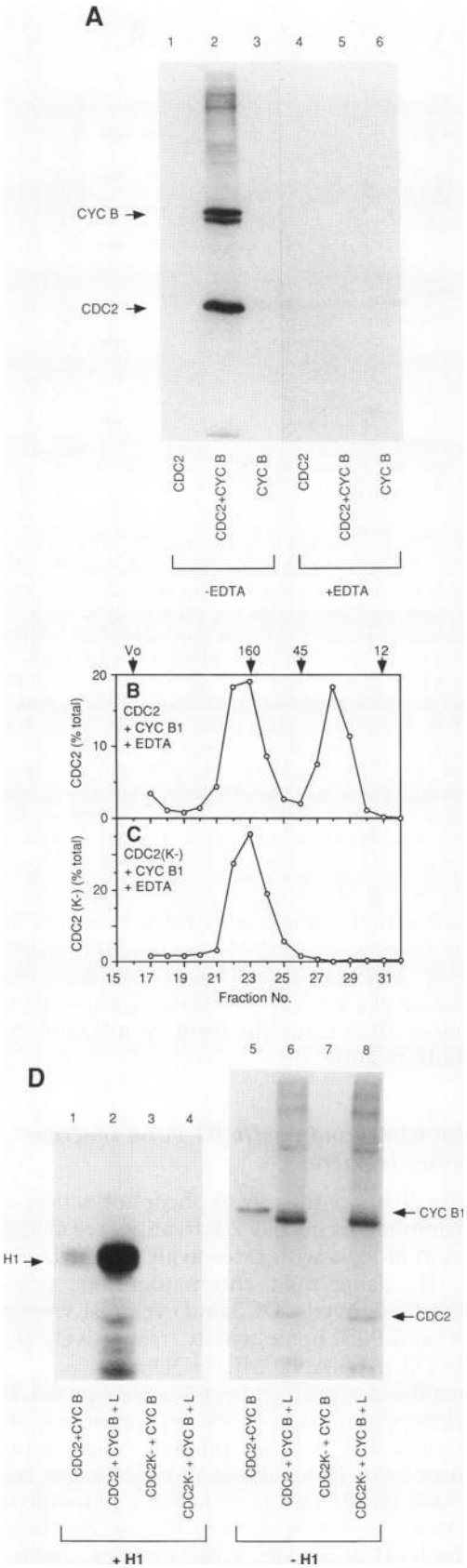
To determine if CDC2 phosphorylation was occurring during activation in insect cell lysates, lysates containing



epitope-tagged CDC2 and cyclin B1 were mixed and kinase reactions were performed in the absence of Histone H1. CDC2 was then immunoprecipitated from the reaction and analyzed on polyacrylamide gels (Figure 3A). CDC2 and cyclin were both phosphorylated in these reactions. CDC2 phosphorylation was greatly stimulated by the addition of cyclin B1 (generally 20 to 100-fold). Phosphoamino acid analysis indicated that CDC2 was being phosphorylated on threonine, whereas cyclin was phosphorylated on serine (not shown). Quantification of phosphate incorporation revealed that ~1 mol phosphate was incorporated per 100 mol CDC2 during a 20-min reaction.

The site of phosphorylation on CDC2 was further analyzed by tryptic phosphopeptide mapping (Figure 3, B–D). CDC2 phosphorylated in vitro was compared with CDC2 immunoprecipitated from HeLa cells labeled with ^{32}P-orthophosphate. When phosphorylated in vitro, CDC2 contained a single major phosphopeptide that comigrated with peptide 4, one of the four major phosphopeptides from CDC2 labeled in vivo. We have identified the phosphorylation site in peptide 4 by analyzing epitope-tagged CDC2 mutants expressed in COS and 3T3 cells (Gu and Morgan, unpublished data). Peptide 4 labeling in CDC2 is abolished by mutation

Figure 3. Phosphorylation of CDC2 and cyclin B1 during activation. (A) Lysates containing 2 μM CDC2 (lanes 1 and 3) or 2 μM CDC2(K−) (lanes 2 and 4) were mixed with equal volumes of control lysates (lanes 1 and 2) or lysates containing 3 μM cyclin B1 (lanes 3 and 4). Mixtures were immediately combined for 20 min at 24°C with ^{32}P-γ-ATP (50 μM) and $MgCl_2$ (see MATERIALS AND METHODS). CDC2 was then immunoprecipitated from the reaction and analyzed on a 10% polyacrylamide gel. The mobilities of cyclin B1 and CDC2 are indicated; CDC2(K−) migrates slightly faster than the wild-type protein. CDC2 bands were excised and counted, and results from several experiments (e.g., Figures 4A and 6D) indicate that the addition of cyclin B1 to CDC2 increased CDC2 phosphorylation 20- to 100-fold. CDC2(K−) was consistently phosphorylated at two- to threefold lower levels than the wild-type protein. (B and C) CDC2 bands from lanes 3 and 4 in A were excised and digested with trypsin. Peptides were spotted onto thin-layer cellulose plates (origin indicated by the arrow) and subjected to electrophoresis at pH 1.9 in the horizontal dimension (anode at left) and chromatography in the vertical dimension (see MATERIALS AND METHODS). (D) Human CDC2 was immunoprecipitated from HeLa cells labeled with ^{32}P-orthophosphate and subjected to the same tryptic phosphopeptide analysis shown in B and C. Numbers indicate the four major phosphopeptides, which contain the following phosphoamino acids: peptide 1, phosphotyrosine and phosphothreonine; peptide 2, phosphotyrosine; peptide 3, phosphothreonine; and peptide 4, phosphothreonine. Analysis of mixed samples indicated that the peptide phosphorylated in vitro (B) comigrated with peptide 4 from CDC2 labeled in vivo (not shown). (E) The indicated combinations of epitope-tagged CDK and cyclin proteins, at the same concentrations used in Figure 1C, were incubated for 15 min at 24°C with ^{32}P-γ-ATP and $MgCl_2$. CDK proteins were then immunoprecipitated from the reaction and subjected to gel electrophoresis. CDK bands were excised and counted in a scintillation counter, and background CDK phosphorylation in the absence of cyclin was subtracted (light bars). Histone H1 kinase activity was measured in parallel immunoprecipitates of the same complexes (dark bars).



of T161 to alanine. In addition, changing T160 in CDK2 to serine results in phosphoserine labeling on peptide 4. We therefore conclude that labeling of CDC2 on peptide 4 in vitro represents phosphorylation of T161.

We also analyzed the phosphorylation of the kinase-deficient mutant, CDC2(K−). This protein was also phosphorylated on threonine in peptide 4 (Figure 3, A and C), although the level of phosphorylation was decreased two- to threefold. Cyclin phosphorylation was essentially abolished. Thus it appears that cyclin phosphorylation, but probably not CDC2 phosphorylation, requires an active CDC2 kinase subunit.

We did not detect any phosphorylation of CDC2 on tyrosine residues in these experiments. In addition, baculovirus-derived CDC2 is not phosphorylated on tyrosine in the insect cell before lysis (see MATERIALS AND METHODS) (Parker *et al.*, 1991). These observations, combined with our observation that activation occurs without any lag phase (Figure 1C), suggest that CDC2 activation in this system does not involve or require any changes in the phosphorylation state of the inhibitory site at Y15.

The CDK subunit was also phosphorylated when other CDK/cyclin mixtures were analyzed (Figure 3E),

Figure 4. CDC2 forms an inactive complex with cyclin B1 when phosphorylation is prevented. (A) Infected insect cell lysates were prepared in standard lysis buffer (lanes 1–3) or in the same buffer plus 20 mM EDTA (lanes 4–6). CDC2 and/or cyclin B1 lysates were incubated in kinase reactions as in Figure 3, and CDC2 was immunoprecipitated from the reactions and analyzed on a 10% polyacrylamide gel. (B) Lysates containing CDC2 and cyclin B1 were prepared in the presence of 20 mM EDTA and desalted by passage over Sephadex G-25 columns pre-equilibrated with lysis buffer plus 20 mM EDTA. Mixtures of desalted lysates containing CDC2 (2.7 μM) and cyclin B1 (1.9 μM) were incubated 20 min at 24°C and subjected to gel filtration on Superose 12 in the usual column buffer plus 10 mM EDTA. Fractions were analyzed for the presence of CDC2 by immunoblotting. (C) Desalted lysates containing CDC2(K−) (0.5 μM) and cyclin B1 (1.9 μM) were mixed and analyzed as in B. (D) CDC2/cyclin B1 complexes were immunoprecipitated from high molecular weight fractions from the columns shown in B and C. These immunoprecipitates were incubated for 20 min at 24°C in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a crude insect cell lysate and then washed and analyzed for the presence of histone H1 kinase activity (lanes 1–4). In lanes 5–8, immunoprecipitates containing EDTA-treated CDC2/cyclin B1 complexes (lanes 5 and 6) or CDC2(K−)/cyclin B1 complexes (lanes 7 and 8) were incubated with ^{32}P-γ-ATP (10 μCi, 50 μM) and $MgCl_2$ (10 mM), either in the absence (lanes 5 and 7) or presence (lanes 6 and 8) of a crude insect cell lysate. Equal amounts of CDC2 and CDC2(K−) complexes were immunoprecipitated, as judged by immunoblotting of parallel immunoprecipitates. After a 20-min incubation at 24°C, immunoprecipitates were washed and analyzed on a 10% polyacrylamide gel. The heavily labeled band just below cyclin is the heavy chain of the 12CA5 immunoglobulin, which is phosphorylated by the crude insect cell lysate (this is less apparent in other experiments because the antibody is usually added after the kinase reaction has been stopped; see MATERIALS AND METHODS). Autoradiograph exposure time was 5 h (−80°C) in lanes 1–4 and 48 h (−80°C) in lanes 5–8.

although the level of CDK phosphorylation varied considerably. Analysis of histone H1 kinase activity in CDK immunoprecipitates of the same mixtures revealed a striking correlation between the level of CDK phosphorylation and the histone H1 kinase activity in the complex (Figure 3E).

CDC2 Binds Cyclin B1 in the Absence of Phosphorylation

We next performed detailed studies of CDC2 activation by cyclin B1 to explore the role of CDC2 phosphorylation in the activation process. We wished to determine if phosphorylation was required for the binding of CDC2 and cyclin B1 or was involved in a later step in activation. Some of our observations had already suggested that phosphorylation was not required for CDC2/cyclin binding. In particular, it was clear that the binding of CDC2 and cyclin B1 was complete (Figure 2F) under conditions where the stoichiometry of CDC2 phosphorylation was low (1%) (Figure 3E). In addition, the phosphorylation of CDC2 was cyclin dependent (Figure 3A), implying that cyclin binding preceded the increase in CDC2 phosphorylation. To explore this issue further, we assessed the binding of CDC2 and cyclin B1 under conditions where phosphorylation was essentially absent. Lysates containing CDC2 and cyclin B1 were prepared in buffer containing 20 mM EDTA to chelate divalent cations. When EDTA-treated CDC2 and cyclin B1 lysates were mixed and incubated with 32P-γ-ATP, no phosphorylation was detected (Figure 4A). However, gel filtration analysis revealed that CDC2 and cyclin B1 still formed high molecular weight complexes even when the lysates were further depleted of ionic components by passage over Sephadex G-25 columns (Figure 4B). Similar results were obtained with lower concentrations of CDC2 and cyclin B1 (0.3 and 0.2 μM; not shown). The kinase-deficient mutant, CDC2(K−), was also capable of binding cyclin B1 in the absence of phosphorylation (Figure 4C).

Nonphosphorylated CDC2/cyclin B1 complexes were isolated from high molecular weight fractions by immunoprecipitation, followed by washing to remove EDTA. These complexes exhibited very low levels of histone H1 kinase activity, which could be greatly activated by a brief incubation in a crude insect cell lysate (Figure 4D, lanes 1 and 2). CDC2 in the inactive complex was not phosphorylated during the kinase reactions but became phosphorylated on addition of crude cell extract (Figure 4D, lanes 5 and 6). Interestingly, the cyclin B1 subunit in the inactive complex was phosphorylated to some extent, despite the low histone H1 kinase activity in the complex.

We also measured CDC2 and cyclin phosphorylation in complexes containing CDC2(K−) and cyclin B1 (Figure 4D, lanes 7 and 8). In the absence of cell lysate, cyclin in these complexes was not phosphorylated, suggesting that the cyclin phosphorylation seen in the wild-type complex is the result of phosphorylation by CDC2. However, addition of cell lysate did cause the phosphorylation of CDC2(K−), suggesting as before that this phosphorylation is not the result of intramolecular autophosphorylation.

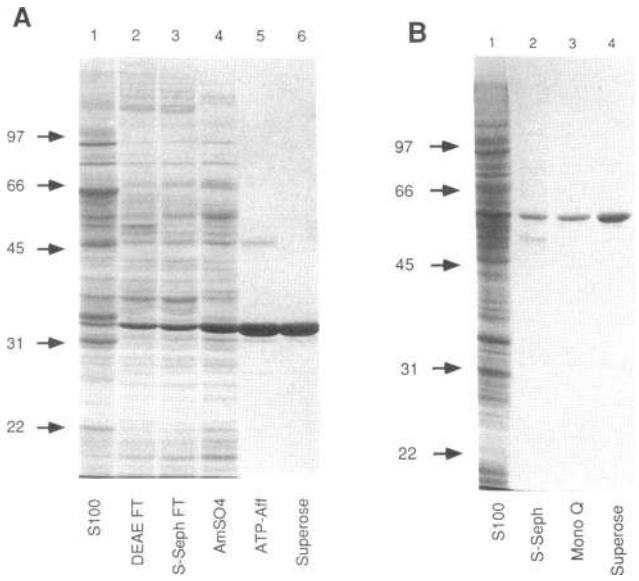
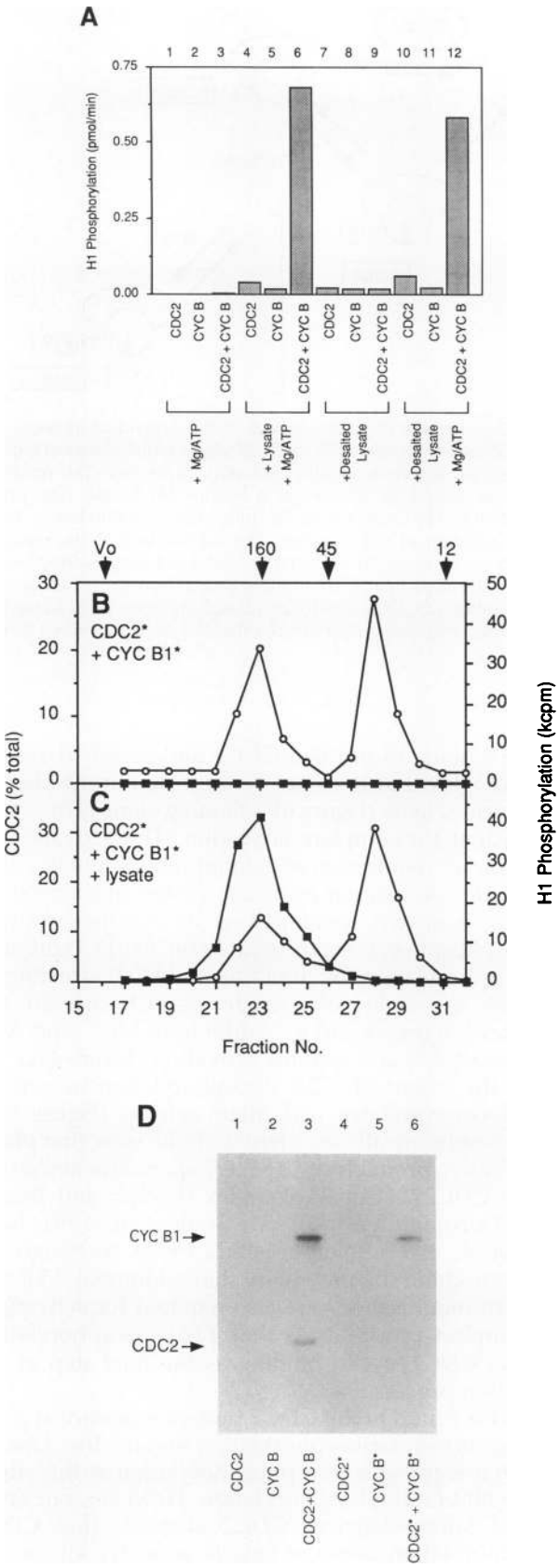


Figure 5. Purification of human CDC2 and cyclin B1. (A) Human CDC2 was purified as described in MATERIALS AND METHODS. Samples from each purification step were analyzed on a 10% polyacrylamide gel, and the Coomassie-Blue stained gel is shown. Lane 1, starting lysate; lane 2, DEAE Sepharose flow-through; lane 3, S-Sepharose flow-through; lane 4, 45–70% ammonium sulfate precipitate; lane 5, ATP affinity column eluate; and lane 6, peak Superose 12 fraction. Molecular weights (kDa) of marker proteins (Bio-Rad, Richmond, CA) are indicated. (B) Human cyclin B1 was purified as described in MATERIALS AND METHODS, and samples from each step were analyzed as in A. Lane 1, starting lysate; lane 2, S-Sepharose eluate; lane 3, Mono Q eluate; and lane 4, peak Superose 12 fraction. Molecular weights (kDa) of marker proteins (Bio-Rad) are indicated.

Purified CDC2 and Cyclin B1 Form Inactive Complexes In Vitro

To further explore the role of phosphorylation and separate components in CDC2 activation, we analyzed the activation process with extensively purified CDC2 and cyclin B1. Using mild chromatographic procedures, baculovirus-derived CDC2 and cyclin B1 were purified to ~98 and 90% homogeneity, respectively (Figure 5; see MATERIALS AND METHODS).

When the two purified proteins were mixed, they did not exhibit significant histone H1 kinase activity, even if Mg^{++} and ATP were included in the reaction (Figure 6A, lanes 1–3). The addition of crude insect cell lysate to the two purified proteins led to a dramatic increase in Histone H1 kinase activity (Figure 6A, lanes 4–6), and the level of activity achieved was similar to that



seen with crude proteins mixed under the same conditions (not shown).

To determine the step in activation at which crude lysate was required, we analyzed the binding of purified CDC2 and cyclin B1 in vitro. According to gel filtration analysis, mixtures of purified CDC2 and cyclin B1 formed complete complexes in vitro (Figure 6B). The CDC2 concentration in these experiments was in the physiological range (~0.3 μM; see MATERIALS AND METHODS) and was higher than that of cyclin B1 (~0.2 μM); as a result there was considerable unbound CDC2. Thus we conclude that binding can occur in the absence of other components. The resulting complex has negligible kinase activity, unless a crude lysate is added to the purified proteins before gel filtration (Figure 6C).

Mixing purified CDC2 and cyclin B1 did not result in CDC2 phosphorylation (Figure 6D). Because complex formation does occur in these mixtures (Figure 6B), these results confirm our earlier conclusion that CDC2 can bind cyclin B1 when CDC2 phosphorylation is not detectable. They also indicate that CDC2 autophosphorylation is not significant in pure inactive complexes. As seen in the inactive complexes isolated from desalted lysates (Figure 4D), some autophosphorylation on the cyclin subunit was observed in the purified complexes (about 4 mol phosphate incorporated per 100 mol cyclin B1).

Crude lysates therefore contain some component that is required not for binding but for some postbinding step in activation. It seems likely that this postbinding step is CDC2 phosphorylation. Our EDTA treatment experiments, which suggest that the postbinding activation step requires divalent cations, are consistent with this possibility (Figure 4). To fur-

Figure 6. Purified CDC2 and cyclin B1 form inactive complexes that can be activated in an ATP-dependent fashion by crude cell extracts. (A) Purified CDC2 (0.02 μM; 30 ng/reaction) and purified cyclin B1 (0.02 μM; 50 ng/reaction) were incubated alone or together in the presence of the following components: lanes 1–3, 20 mM $MgCl_2$ and 1 mM ATP; lanes 4–6, $MgCl_2$, ATP, and a hypotonic extract of insect Sf9 cells (150 μg protein); lanes 7–9, the same amount of hypotonic extract, desalted by passage over a Sephadex G-25 column; and lanes 10–12, desalted extract plus $MgCl_2$ and ATP. After 20 min at 24°C, CDC2 was immunoprecipitated from the mixture with affinity-purified anti-peptide antibodies and histone H1 kinase activity was measured in the immunoprecipitate. (B and C) Purified CDC2 (0.3 μM) and cyclin B1 (0.2 μM) were incubated for 20 min at 24°C either alone (B) or after addition of 100 μl crude Sf9 insect cell lysate (C). Mixtures were subjected to gel filtration on Superose 12, and fractions were analyzed for the presence of CDC2 by immunoblotting (○) or for histone H1 kinase activity (■). (D) Crude or purified CDC2 and cyclin B1, alone or in combination, were incubated in standard kinase reactions as in Figure 3 and analyzed on a 10% polyacrylamide gel. CDC2 in crude lysates (lanes 1–3) was immunoprecipitated from the reaction before analysis. Purified components (lanes 4–6, indicated by asterisks) were analyzed directly.

ther pursue this possibility, we determined the ATP requirement of activation (Figure 6A). The purified CDC2/cyclin B1 complex was incubated with a crude lysate that had been desalted by passage over a G-25 column (Figure 6A, lanes 7–9). As before, activation was prevented. However, the addition of Mg^{++} and ATP to this desalted lysate completely restored its ability to activate the pure complex (Figure 6A, lanes 10–12).

DISCUSSION

We have developed a cell-free system in which we can dissect the activation of human cyclin-dependent kinases. Using proteins overexpressed with the baculovirus system, we have reconstituted the activation of human CDC2 and CDK2 by human cyclins A and B1. Using highly purified CDC2 and cyclin B1, it is possible in this system to divide the activation process into discrete steps (Figure 7). The first step is the binding of CDC2 and cyclin B1, which can occur in the absence of other proteins and in the absence of phosphorylation. The resulting complex has negligible histone H1 kinase activity. The activation of this complex requires the addition of crude cell lysates, is ATP-dependent, and is accompanied by CDC2 phosphorylation.

The binding of purified CDC2 to purified cyclin B1 was effective at concentrations (0.2–0.3 μM) that approximate the concentration of CDC2 in the intact HeLa cell. Although detailed kinetic analysis will be required to clearly assess the affinity of this interaction, it appears from our results that the affinity is sufficient to allow effective binding at physiological concentrations in the absence of other proteins or CDC2 phosphorylation.

Others recently have reported that mutation of T161 inhibits the binding of CDC2 and cyclin (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Norbury *et al.*, 1991). Binding was demonstrated by measuring the coprecipitation of one protein when the other was immunoprecipitated or otherwise immobilized on Sepharose beads. These beads were washed extensively with detergent-containing buffers. Perhaps under these relatively stringent conditions T161 phosphorylation has an effect on binding, although we have found that our CDC2/cyclin B1 complexes remain intact after gel filtration in buffers containing Triton X-100 (not shown). We have also observed that an epitope-tagged CDC2 protein bearing a T161 mutation binds cyclin normally when stably expressed in 3T3 cells (Gu, unpublished data). Solomon *et al.* (1992) have also reported that T161 mutation has no effect on cyclin binding in their *Xenopus* extracts. Nevertheless, the possibility remains that T161 phosphorylation, although not strictly required for binding under physiological conditions, increases the affinity of the interaction. To test this possibility directly, we are attempting to develop conditions under which complete phosphorylation can be obtained in vitro.

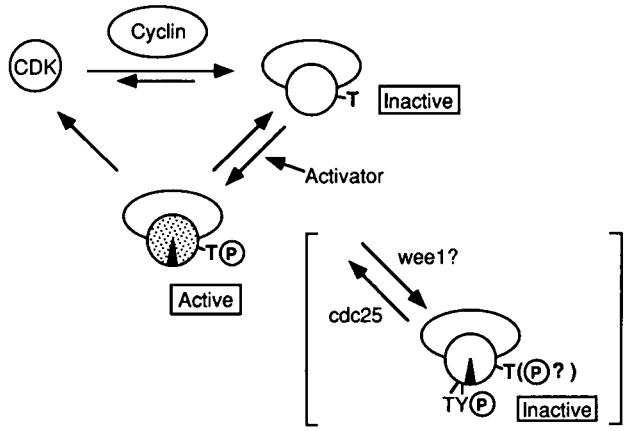


Figure 7. Steps in the activation of cyclin-dependent kinases. Activation begins with reversible cyclin binding, which does not require phosphorylation events or other proteins in the cell. The resulting complex is essentially inactive as a histone H1 kinase. The phosphorylation of T161, catalyzed by an unidentified protein kinase, leads to the activation of the complex. The inactivation of the complex probably involves cyclin degradation and T161 dephosphorylation, although the sequence in which these processes occur is not known. The inhibition of CDC2 activity by phosphorylation of T14 and Y15 is an added regulatory step in vivo that we do not observe in our system.

The binding of purified CDC2 and cyclin B1 results in a complex that does not possess significant histone H1 kinase activity (Figure 6B). Binding alone is therefore insufficient for complete activation. The activation of the complex requires an additional component in crude cell lysates. Activation by crude lysates, like activation in vivo, is always accompanied by cyclin-dependent CDC2 phosphorylation (probably on T161). Inhibition of phosphorylation (by desalting and EDTA treatment) prevents the ability of a crude lysate to activate the complex (Figures 4 and 6). Addition of Mg^{++} and ATP to a desalted lysate restores activation (Figure 6A). Finally, the extent of CDK phosphorylation in various complexes correlates with their activity (Figure 3E). These results are all consistent with the view that phosphorylation, probably at T161, is required for activation of the CDC2/cyclin B1 complex (Booher and Beach, 1986; Ducommun *et al.*, 1991; Gould *et al.*, 1991; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Although we cannot exclude the possibility that additional ATP-dependent modifications are also required for activation, the simplest possibility is that T161 phosphorylation follows CDC2/cyclin binding as the final step in the activation process.

T161 is found in the CDC2 protein sequence at a site analogous to autophosphorylation sites in other kinases and so it is possible that phosphorylation at this site is the result of autophosphorylation. However, our analysis of kinase-deficient CDC2 suggests that CDC2 phosphorylation does not require an active kinase do-

main and is probably not due to intramolecular autophosphorylation. Similarly, Solomon *et al.* (1992) recently reported that a kinase-deficient mutant of CDC2 is phosphorylated at T161 in *Xenopus* extracts. It therefore seems likely that T161 phosphorylation is catalyzed by a separate protein kinase (although it is not possible to exclude the possibility that these mutant CDC2 proteins retain sufficient activity for autophosphorylation). Studies of the identity and regulation of this protein kinase will undoubtedly provide new insight into the regulation of CDC2 activity and cell-cycle control.

Cyclin phosphorylation also occurs during the activation process and is probably carried out by the CDC2 subunit in the complex. This is consistent with previous observations of cyclin phosphorylation by CDC2 in complexes isolated from mammalian cells or *Xenopus* extracts (Draetta and Beach, 1988; Draetta *et al.*, 1988; Izumi and Maller, 1991). Cyclin autophosphorylation is also detected in purified relatively inactive CDC2/cyclin complexes (Figures 4D and 6D). However, the rate of phosphate incorporation into cyclin in these reactions is very low and consistent with the low level of histone H1 kinase activity. This cyclin phosphorylation is not sufficient to fully activate the complex, because Histone H1 kinase activity remains low in purified complexes in which cyclin is phosphorylated. Thus, the function of this phosphorylation is unclear, as suggested by previous work showing that mutagenesis of cyclin phosphorylation sites has no apparent effect on cyclin function in *Xenopus* extracts (Izumi and Maller, 1991).

Activation of Different CDK/Cyclin Complexes

We observed considerable differences in the activity of different CDK/cyclin complexes in our system. This may be due in part to differences in the affinity of various CDK/cyclin interactions. In particular, CDC2 does not bind effectively to cyclin A. We have also expressed human cyclin E in this system (Desai, unpublished observations) and have found that cyclin E, like cyclin A, binds completely to CDK2 and poorly to CDC2. Thus, CDC2, cyclin A, and cyclin E have a limited binding specificity when compared with CDK2 and cyclin B1.

However, it is unlikely that the activity of different CDK/cyclin complexes in our system is determined strictly by binding affinities. In three cases (CDC2/cyclin B1, CDK2/cyclin B1, and CDK2/cyclin A), complex formation was essentially complete and kinase activity still varied among the different complexes. Thus, a postbinding step in activation (probably CDK phosphorylation) is rate-limiting in these experiments and determines the relative activity of the various complexes. This possibility is supported by the correlation between CDK phosphorylation and kinase activity (Figure 3E). The low stoichiometry of CDK phosphorylation in all complexes (<1%) also suggests that a limiting amount of the activating kinase (or kinases) is present in these reactions. The specificity of the insect cell-derived activating kinase may also be a limiting factor. For example, although CDK2/cyclin A complexes are poorly activated in insect cell lysates, we have found that HeLa cell extracts activate these complexes as effectively as CDC2/cyclin B1 complexes (Desai, unpublished observations).

Our observations suggest that the catalytic and regulatory subunits of this family are all capable of interacting with each other in vitro, even though these kinases probably do not interact with all cyclins in a normal cell cycle. Two of the four complexes we observe (CDC2/cyclin B and CDK2/cyclin A) clearly exist in the HeLa cell (Pines and Hunter, 1989; Tsai *et al.*, 1991; Rosenblatt *et al.*, 1992). The CDC2/cyclin A complex also exists to some extent in human cells, but cyclin A seems to interact mainly with CDK2 (Pines and Hunter, 1989, 1990; Rosenblatt *et al.*, 1992). Our studies suggest that formation of the CDC2/cyclin A complex may be limited in human cells by the relatively low affinity of the interaction. Finally, we have observed effective binding of CDK2 to cyclin B1. This complex has not been reported to exist in vivo and seems an unlikely combination because CDK2 activity declines at a time in the cell cycle (mitosis) when cyclin B1 levels are maximal (Rosenblatt *et al.*, 1992). In this case, mechanisms must exist inside the cell that prevent complex formation between these proteins despite their high binding affinity.

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REFERENCES

Booher, R., and Beach, D. (1986). Site-specific mutagenesis of *cdc*2+, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. Mol. Cell. Biol. *6*, 3523–3530.

Boyle, W.J., van der Geer, P., and Hunter, T. (1991). Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. *201*, 110–149.

Brodsky, M.H., Warton, M., Myers, R.M., and Littman, D.R. (1990). Analysis of the site in CD4 that binds to the HIV envelope glycoprotein. J. Immunol. *144*, 3078–3086.

Draetta, G. (1990). Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. TIBS *15*, 378–383.

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

Draetta, G., Piwnica-Worms, H., Morrison, D., Druker, B., Roberts, T., and Beach, D. (1988). Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate. Nature *336*, 738–744.

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

Elledge, S.J., and Spottswood, M.R. (1991). A new human p34 protein kinase, CDK2, identified by complementation of a cdc28 mutation in Saccharomyces cerevisiae, is a homolog of Xenopus Eg1. EMBO J. *10*, 2653–2659.

Fang, F., and Newport, J.W. (1991). Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. Cell *66*, 731–742.

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

Forsburg, S.L., and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Annu. Rev. Cell. Biol. *7*, 227–256.

Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N.J.C. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell *67*, 1169–1179.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S., and Nurse, P. (1991). Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* $p34^{cdc2}$ function. EMBO J. *10*, 3297–3309.

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

Hunt, T. (1989). Maturation promoting factor, cyclin and the control of M-phase. Curr. Opin. Cell Biol. *1*, 286–274.

Izumi, T., and Maller, J.L. (1991). Phosphorylation of Xenopus Cyclins B1 and B2 is not required for cell cycle transitions. Mol. Cell. Biol. *11*, 3860–3867.

Kamps, M.P. (1991). Determination of phosphoamino acid composition by acid hydrolysis of protein blotted to immobilon. Methods Enzymol. *201*, 21–27.

Krek, W., and Nigg, E.A. (1991a). Differential phosphorylation of vertebrate $p34^{cdc2}$ kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO J. *10*, 305–316.

Krek, W., and Nigg, E.A. (1991b). Mutations of $p34^{cdc2}$ phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to $p34^{cdc2}$ kinase activation in vertebrates. EMBO J. *10*, 3331–3341.

Kunkel, T.A. (1985). Rapid and efficient site-directed mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. *82*, 488–492.

Lee, M.G., and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. Nature *327*, 31–35.

Luckow, V.A., and Summers, M.D. (1989). High level expression of non-fused foreign genes with Autographa californica nuclear polyhedrosis virus expression vector. Virology *170*, 31–39.

Luo, K., Hurley, T.R., and Sefton, B.M. (1991). Cyanogen bromide cleavage and proteolytic peptide mapping of proteins immobilized to membranes. Methods Enzymol. *201*, 149–152.

Morgan, D.O. (1992). Cell cycle control in normal and neoplastic cells. Curr. Opin. Genetics Develop. *2*, 33–37.

Morgan, D.O., Kaplan, J.M., Bishop, J.M., and Varmus, H.E. (1989). Mitosis-specific phosphorylation of $p60^{c\text{-}src}$ by $p34^{cdc2}$-associated protein kinase. Cell *57*, 775–786.

Morgan, D.O., Kaplan, J.M., Bishop, J.M., and Varmus, H.E. (1991). Production of $p60^{c\text{-}src}$ by baculovirus expression and immunoaffinity purification. Methods Enzymol. *200*, 645–660.

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

Murray, A.W., and Kirschner, M.W. (1989). Dominoes and clocks: the union of two views of the cell cycle. Science *246*, 614–621.

Ninomiya-Tsuji, J., Nomoto, S., Yasuda, H., Reed, S.I., and Matsumoto, K. (1991). Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast *cdc28* mutation. Proc. Natl. Acad. Sci. *88*, 9006–9010.

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

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature *344*, 503–508.

Paris, J., Le Guellec, R., Couturier, A., Le Guellec, K., Omilli, F., Camonis, J., MacNeill, S., and Phillipe, M. (1991). Cloning by differential screening of a Xenopus cDNA coding for a protein highly homologous to cdc2. Proc. Natl. Acad. Sci. *88*, 1039–1043.

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

Pines, J., and Hunter, T. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell *58*, 833–846.

Pines, J., and Hunter, T. (1990). Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. Nature *346*, 760–763.

Rosenblatt, J., Gu, Y., and Morgan, D.O. (1992). Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. Proc. Natl. Acad. Sci. *89*, 2824–2828.

Solomon, M., Glotzer, M., Lee, T.H., Phillipe, M., and Kirschner, M. (1990). Cyclin activation of $p34^{cdc2}$. Cell *63*, 1013–1024.

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

Sorger, P.K., and Murray, A.W. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of $p34^{cdc28}$. Nature *355*, 365–368.

Summers, M.D., and Smith, G.E. (1987). *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*. College Station, Texas, Texas Agricultural Station.

Tsai, L.-H., Harlow, E., and Meyerson, M. (1991). Isolation of the human cdk2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. Nature *353*, 174–177.