Phosphorylation Independent Activation of Human Cyclin-Dependent Kinase 2 by Cyclin A In Vitro

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p33^{Cdk2} is a serine-threonine protein kinase that associates with cyclins A, D, and E and has been implicated in the control of the G1/S transition in mammalian cells. Recent evidence indicates that cyclin-dependent kinase 2 (Cdk2), like its homolog Cdc2, requires cyclin binding and phosphorylation (of threonine-160) for activation in vivo. However, the extent to which mechanistic details of the activation process are conserved between Cdc2 and Cdk2 is unknown. We have developed bacterial expression and purification systems for Cdk2 and cyclin A that allow mechanistic studies of the activation process to be performed in the absence of cell extracts. Recombinant Cdk2 is essentially inactive as a histone H1 kinase ($<4 \times 10^{-5}$ pmol phosphate transferred $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$ Cdk2). However, in the presence of equimolar cyclin A, the specific activity is ~ 16 pmol $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$, 4×10^5 -fold higher than Cdk2 alone. Mutation of T160 in Cdk2 to either alanine or glutamic acid had little impact on the specific activity of the Cdk2/cyclin A complex: the activity of Cdk2^{T160E} was indistinguishable from Cdk2, whereas that of Cdk2^{T160A} was reduced by five-fold. To determine if the Cdk2/cyclin A complex could be activated further by phosphorylation of T160, complexes were treated with Cdc2 activating kinase (CAK), purified $\sim 12,000$ -fold from *Xenopus* eggs. This treatment resulted in an 80-fold increase in specific activity. This specific activity is comparable with that of the Cdc2/cyclin B complex after complete activation by CAK (~ 1600 pmol $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$). Neither Cdk2^{T160A}/cyclin A nor Cdk2^{T160E}/cyclin A complexes were activated further by treatment with CAK. In striking contrast with cyclin A, cyclin B did not directly activate Cdk2. However, both Cdk2/cyclin A and Cdk2/cyclin B complexes display similar activity after activation by CAK. For the Cdk2/cyclin A complex, both cyclin binding and phosphorylation contribute significantly to activation, although the energetic contribution of cyclin A binding is greater than that of T160 phosphorylation by ~ 5 kcal/mol. The potential significance of direct activation of Cdk2 by cyclins with respect to regulation of cell cycle progression is discussed.

INTRODUCTION

Critical transitions in the eukaryotic cell cycle are orchestrated, in part, by the coordinate action of a protein kinase(s), p34^{Cdc2}, and associated regulatory subunits called cyclins (Hunt, 1989; Murray and Kirschner, 1989; Gautier *et al.*, 1990; Nurse, 1990; Pines and Hunter, 1990). In yeast, a single p34 protein (Cdc28 in *Saccha-*

romyces cerevisiae and Cdc2 in *Schizosaccharomyces pombe*) interacts with two distinct classes of cyclins, the G1 cyclins (Cross, 1988; Hadwiger *et al.*, 1989; Richardson *et al.*, 1989; Ogas *et al.*, 1991) and the B-type mitotic cyclins (Booher and Beach, 1988; Hagan *et al.*, 1988; Wittenberg *et al.*, 1990; Surana *et al.*, 1991), to regulate the G1/S and G2/M transitions, respectively. In higher eukaryotes, the regulatory systems that govern

these two cell cycle checkpoints are more complex. Cell cycle progression involves not only p34^{cdc2} (Lee and Nurse, 1987), which is essential for the G2/M transition (Riabowol *et al.*, 1989; Hamaguchi *et al.*, 1992), but a related protein kinase, p33^{Cdk2}, as well. CDK2 was initially identified in *Xenopus* during a screen for cDNAs whose translational properties were altered upon fertilization (Paris *et al.*, 1991). Human CDK2 was independently identified based on its ability to carry out the essential G1/S functions of p34^{Cdc28} in *S. cerevisiae* (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991) and based on amino acid sequence conservation (66%) with CDC2 (Tsai *et al.*, 1991; Rosenblatt *et al.*, 1992). In mammalian cells, cyclin-dependent kinase 2 (Cdk2)¹ has been implicated in the regulation of early events in the cell cycle: it associates with cyclin E, a G1 cyclin (Koff *et al.*, 1992), with cyclin A during S and G2 phases (Tsai *et al.*, 1991; Elledge *et al.*, 1992; Rosenblatt *et al.*, 1992), and recently has been shown to interact with cyclin D1 as well (Xiong *et al.*, 1992). Cyclin A is required for DNA replication in both normal and transformed mammalian cell types (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992) and, in certain situations, can be oncogenic (reviewed in Hunter and Pines, 1991). In fact, the cyclin A gene in human cells was initially identified because it was the site of integration of the hepatitis B virus in a hepatocellular carcinoma (Wang *et al.*, 1990). Additionally, a complex containing Cdk2/cyclin A, p107, and E2F is a target of the adenovirus oncoprotein E1a (Cao *et al.*, 1992; Shirodkar *et al.*, 1992), suggesting that the Cdk2/cyclin A complex may play a role in oncogenic transformation by DNA tumor viruses (Hunter and Pines, 1991). Additional evidence that Cdk2 plays a role in the control of DNA replication comes from the *Xenopus* system where it has been shown that depletion of Cdk2 greatly reduces the rate of entry into S-phase in egg extracts (Fang and Newport, 1991), although in this case Cdk2 does not appear to be associated with an A-type cyclin.

Genetic and biochemical data have provided a picture of the multistep mechanism of Cdc2 activation (Solomon *et al.*, 1990, 1992; Gould *et al.*, 1991; Krek and Nigg, 1991; Norbury *et al.*, 1991; Desai *et al.*, 1992). H1 kinase activity has not been detected in Cdc2 generated by *in vitro* translation (Solomon *et al.*, 1992) or expressed in and purified from insect cells (Desai *et al.*, 1992). In

vitro, association of purified cyclin B with Cdc2 produces a complex that does not display significant kinase activity (Desai *et al.*, 1992; Solomon *et al.*, 1992), but this interaction is thought to be a prerequisite for the activating phosphorylation of T161 on Cdc2 by Cdc2 activating kinase (CAK) (Gould *et al.*, 1991; Desai *et al.*, 1992; Solomon *et al.*, 1992), an activity that has been purified from *Xenopus* egg extracts (Solomon *et al.*, 1992; Solomon, unpublished data). In both the *Xenopus* and *S. pombe* systems, cyclin binding is also a prerequisite for phosphorylation of T14 and Y15 near the ATP binding site by the wee1 kinase (Solomon *et al.*, 1990, 1992; Meijer *et al.*, 1991; Parker *et al.*, 1991). However, recent data with human wee1 suggest that Y15 phosphorylation *in vitro* is not absolutely dependent on cyclin binding (Parker and Piwnicka-Worms, 1992). Tyrosine-15 phosphorylation inhibits Cdc2 activity (Gould and Nurse, 1989; Parker *et al.*, 1991). Before mitosis, an inactive Cdc2/cyclin B complex accumulates containing phosphothreonine-14 and -161, as well as phosphotyrosine-15. Entry into mitosis is triggered by Cdc25, which dephosphorylates Y15 and presumably T14 (Morla *et al.*, 1989; Moreno *et al.*, 1990; Solomon *et al.*, 1990; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992).

Like Cdc2, Cdk2 requires cyclin association for activation (Koff *et al.*, 1992; Rosenblatt *et al.*, 1992), and in addition, Cdk2 is phosphorylated on T14, Y15, and T160 *in vivo* (Gu *et al.*, 1992). However, unlike Cdc25 catalyzed activation of the mitotic Cdc2/cyclin B complex, Cdk2 activation appears to parallel closely the accumulation of cyclin regulatory subunits (Koff *et al.*, 1992; Rosenblatt *et al.*, 1992). This suggests that there may be differences in the mechanisms of activation of Cdc2 and Cdk2.

A detailed description of the mechanism of activation of Cdk2 will require both an elucidation of the individual chemical events that accompany activation as well as a determination of how these events alter reaction energetics and/or catalytic mechanism. To facilitate the analysis of Cdk2 activation, we have developed methods for isolation of soluble Cdk2 and cyclin A from bacteria. These purified proteins make it possible to examine how individual steps in the activation process alter the catalytic properties of Cdk2 in the absence of unknown modifications or protein associations that occur in crude extracts. Here we focus on the first step in the mechanism of activation of Cdk2 by cyclin A, cyclin binding. We find that cyclin A binding alone activates Cdk2 protein kinase activity by $\geq 4 \times 10^5$ -fold. However, the Cdk2/cyclin A complex is activated further (80-fold) after incubation with purified *Xenopus* CAK *in vitro*, and this activation is dependent on the presence of T160 in Cdk2. In contrast, activation of Cdk2 by cyclin B is completely CAK dependent.

¹ Abbreviations used: bp, base pair; CAK, Cdc2 activating kinase; cAMP, cyclic AMP; Cdk2, cyclin-dependent kinase 2; DTT, dithiothreitol; GST, glutathione S-transferase; HA, hemagglutinin A (for clarity, recombinant HA-Cdk2 is referred to as simply Cdk2); HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IPTG, isopropylthio- β -galactoside; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminoethane, DEAE, diethylaminoethane.

MATERIALS AND METHODS

Materials

T7 RNA polymerase-dependent expression plasmids for human Cdk2, cyclin A, and Ckshs1 were from a previous study (Elledge *et al.*, 1992), as was pET-HACdk2, which produces a version of Cdk2 containing an N-terminal 16 amino acid hemagglutinin A epitope tag (HA-Cdk2). A region within this peptide (MAYPYDVPDYASLGGH) is recognized by monoclonal antibody 12CA5. Affinity-purified polyclonal antibodies against Cdk2 and cyclin A were from a previous study (Elledge *et al.*, 1992). The following buffers were used extensively: buffer A, 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0, 2 mM EDTA, 2 mM dithiothreitol (DTT), 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml leupeptin; buffer B, 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 2 mM EDTA, 2 mM DTT, 1 mM PMSF; buffer C, 10 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM PMSF, 1 μ g/ml leupeptin; buffer D, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT; buffer E, 1 mM sodium phosphate, pH 6.5 or 7.0, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 mM PMSF; buffer F, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM EDTA, 2 mM DTT, 10% glycerol.

Mutagenesis of Cdk2

Replacement of threonine-160 in Cdk2 by glutamate was performed using the polymerase chain reaction (PCR) in conjunction with Vent polymerase (New England Biolabs, Beverly, MA). A 26-nucleotide primer overlapping the initiation codon and containing an *Nde* I site was used as the 5' primer (5'-CGGGCCATATGGAGAACTTCCAAAG), whereas a 41-nucleotide primer (5'-GCTCGGTACCACAGG-GTACCACCTCATGTTTCGTAAGTACG) overlapping both the T160 codon and an adjacent *Kpn* I site was used as the mutagenic 3' primer. After amplification, the 510-base pair (bp) product was digested with *Spl* I and *Kpn* I, and this 460-bp fragment was ligated into pET-HACdk2 previously digested with *Spl* I and *Kpn* I. Mutant expression plasmids were initially identified by direct sequencing of PCR-derived DNA fragments using flanking primers. Double-stranded sequencing was used to verify the entire sequence of the PCR-derived fragment. A similar strategy was used for Cdk2^{T160A}. M13 based mutagenesis was used to convert Asn-132 in Cdk2 to alanine. The oligonucleotide 5'-AACCTCAGGCTCTGCTT was used, and mutant phage was identified by DNA sequencing. An *Spl* I/*Kpn* I DNA fragment containing the mutation was introduced into pET-HACdk2 for expression, and the mutation in the expression plasmid was verified by double-strand DNA sequencing.

Other DNA Manipulations

An expression plasmid for the Cdk2 glutathione S-transferase (GST) fusion protein was generated by ligating a blunt-ended *Nde* I/*Bam*HI fragment containing the Cdk2 coding sequence (Elledge *et al.*, 1992) into pGEX2TK (Kaelin *et al.*, 1991) previously digested with *Sma* I. For expression in yeast, epitope-tagged Cdk2 coding sequences (as *Xho* I/*Bam*HI fragments) were cloned into the yeast expression plasmid pSE936 (Elledge and Spottswood, 1991) as described (Elledge *et al.*, 1992). The epitope tag has no significant effect on the ability of Cdk2 to complement a yeast strain carrying a *cdc28-4* temperature-sensitive mutation (Harper, unpublished data).

Purification of Cyclin A

Escherichia coli strain BL21(DE3)/pETcyclin A (2 l) was grown at 37°C until the OD₆₀₀ reached 1.0 and induced at 25°C with 0.4 mM isopropylthio- β -galactoside (IPTG) overnight. Sonicated extracts (in 70 ml buffer A/l culture containing 0.1 mg/ml lysozyme) were cleared by centrifugation (16 000 rpm, 20 min). After ammonium sulfate precipitation (80% wt/vol), proteins were dissolved in 20 ml buffer B and dialyzed against 1 l buffer B. The cleared supernatant was sub-

jected to chromatography on a 20-ml Toyopearl 650C DEAE column (Supelco, Bellefonte, PA) equilibrated in buffer C. After washing with 75 ml buffer C containing 100 mM NaCl, cyclin A was eluted with 75 ml buffer C containing 250 mM NaCl. Fractions containing cyclin A (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and immunoblotting) were then chromatographed on a 5-ml Pharmacia (Piscataway, NJ) Fast S Sepharose column equilibrated with buffer C and bound proteins eluted with a 100-ml gradient of NaCl (0.1–0.6 M NaCl; 0.8 ml/min). Peak fractions were individually diluted twofold into buffer D, filtered through a Centrux filter (Schleicher and Schuell, Keene, NH), and then applied to a Pharmacia MonoQ column equilibrated with buffer C. Proteins were eluted with a 0- to 500 mM NaCl gradient (40 min) at 0.8 ml/min, and aliquots were stored at -70°C. Peak fractions (~250 mM NaCl) were >95% homogeneous as assessed by SDS-PAGE. The yield of cyclin A was ~5 mg per 2 l culture.

Purification of Cdk2 and GST-Cdk2 Proteins

Strain BL21(DE3)/pET-HACdk2 (or corresponding strains for Cdk2 mutants) was grown to an OD₆₀₀ of approximately one at 37°C and induced with 0.4 mM IPTG at 25°C overnight. Cells from 0.5 l were suspended in 35 ml of 20 mM Tris, pH 7.5, 10% sucrose, 1 mM PMSF, 1 μ g/ml leupeptin, and 0.1 mg/ml lysozyme and incubated 45 min on ice. After sonication (5 $\times$ 15 s on ice), the insoluble material was removed by centrifugation (14 000 rpm, 20 min), and the soluble extract was dialyzed against 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM PMSF, 1 μ g/ml leupeptin, 1 mM EDTA, 1 mM DTT (1 l, 4°C). The cleared extract was then loaded on a 15-ml Trisacryl SP column (IBF Biotechnics, Villeneuve-la-Garenne, France) equilibrated in the same buffer. The column flowthrough (plus one column volume of column wash) was immediately loaded on a TSK-DEAE column (15 ml) in the same buffer, and the flowthrough and first column volume were collected. At this stage, Cdk2 constituted 50–70% of the total protein. Final purification was achieved using a 1- to 2-ml ATP-agarose column (type IV, Sigma, St. Louis, MO) equilibrated in buffer E. After application of extract representing ~150 ml of culture, the column was washed with four column volumes of buffer E and Cdk2 eluted with buffer E containing 0.2 M NaCl. Pooled fractions were either dialyzed against buffer F or supplemented with one-fifth volume of 5 $\times$ buffer F before storage at either -70 or 4°C. Proteins were >95% homogeneous as assessed by SDS-PAGE. Yields of Cdk2 ranged from 300 to 800 μ g per 0.15 l culture.

For GST-Cdk2, 1 l of strain BL21(DE3)/pGST-Cdk2 was grown to OD₆₀₀ ~ 1 at 37°C and induced with 0.1 mM IPTG at 25°C overnight. Cells were collected and resuspended in 70 ml NETN (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40) containing 1 mM PMSF and 5 μ g/ml leupeptin. Cells were disrupted by sonication, and the cleared lysate was incubated with 1.4-ml glutathione sepharose (1:1 in NETN + 0.5% powdered milk) for 30 min. After washing three times with NETN, proteins were eluted with 1 ml of 20 mM glutathione in 50 mM Tris-HCl, pH 9.6, 120 mM NaCl per 0.2 ml beads (10 min at 4°C), and then dialyzed against 20 mM Tris-HCl, pH 8, 10 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol.

For ATP-agarose chromatography, GST-Cdk2 was dialyzed against buffer E before application to a 1 ml ATP-agarose (Sigma type IV) column. The column was washed with 10 ml buffer E containing 0.1 M NaCl, and elution was accomplished with buffer E containing 0.4 M NaCl. Peak fractions were dialyzed as described above for Cdk2 before storage at -70°C. GST-Cdk2 was >90% homogeneous by SDS-PAGE. Samples were quantitated by the method of Bradford (1976). The yield of GST-Cdk2 before ATP-agarose chromatography was ~1 mg/l culture. Recovery from the ATP column was typically ~50%.

Purification of Ckshs-1

Strain BL21(DE3)/pLysS/pET-Ckshs-1 was induced and harvested as described previously (Richardson *et al.*, 1990), and soluble proteins

were precipitated with ammonium sulfate before chromatography on a TSK-DEAE column. Peak fractions (>95% homogeneous) were further purified on a C4 reverse-phase high-performance liquid chromatography column using 0.1% trifluoroacetic acid and acetonitrile gradients. The protein was coupled to affigel-10 at a concentration of 5 mg/ml of resin (Elledge *et al.*, 1992). To examine the interaction of Ckshs-1 with Cdk2 and Cdk2 mutant proteins, 25 μ l of Ckshs-1 beads or control affigel beads were incubated with 2 μ g of purified Cdk2 in a total volume of 50 μ l (15 mM HEPES, 1 mM EDTA, 2 mM DTT, 5% glycerol, 0.1% NP-40) for 30 min at 4°C. The supernatant was diluted into an equal volume of 2 $\times$ sample buffer, and the beads were washed three times with 1 mL of 25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.1% NP-40. Bound proteins were released from the beads using 2 $\times$ sample buffer. Samples were electrophoresed on a 12% SDS gel and proteins detected by Coomassie blue staining.

Kinase Assays

The concentrations of cyclin A and Cdk2 proteins used were determined by amino acid analysis or by Bradford analysis using standard curves that were based on Cdk2 and cyclin A concentrations determined by amino acid analysis. Assays were typically performed in 25 μ l at 37°C in 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 10 mM MgCl₂, 30 μ M [γ -³²P]ATP (0.3 nCi/pmol), 1.3 μ M histone H1 (Boehringer Mannheim, Indianapolis, IN), and <0.1% glycerol using the indicated concentrations of cyclin A, GST-cyclin B, and kinase subunits, unless noted otherwise. Reactions were quenched at the indicated time by addition of 2 $\times$ sample buffer and boiled for 3 min before analysis of 20 or 25 μ l of the sample by SDS-PAGE and autoradiography. Histone H1 associated radioactivity in each lane was quantitated using a Betagen scanner (Betagen, Mountain View, CA) (corrected for 20% counting efficiency), or in some cases histone H1 bands were excised from gels and radioactivity quantitated by liquid scintillation. Thin-layer chromatography was used to assess the purity of stock γ -³²P-ATP solutions. Less than 10% of the ATP had hydrolyzed during storage. Preliminary experiments indicated that cyclin A-dependent Cdk2 protein kinase activity was somewhat sensitive to repeated freeze-thawing. Therefore, stock solutions of both cyclin A and Cdk2 were aliquoted before storage at -70°C, and all experiments shown in Figures 2–4 were performed using samples that had been thawed only once, unless otherwise noted. Additional control experiments indicated that Cdk2 protein kinase activity was stable for $\geq$ 3 mo when stored at 4°C. For reactions involving long incubation times (Figure 3A), ovalbumin (1 mg/ml) was included as carrier. Control reactions indicated that ovalbumin has no effect on the specific activity of Cdk2.

In Vitro Activation by CAK

CAK was purified ~12 000-fold from *Xenopus* egg extracts. Extract preparation, DEAE chromatography, and ammonium sulfate fractionation were as described (Solomon *et al.*, 1992) and were followed by sequential chromatography on hydroxyapatite, MonoQ, and heparin. The final preparation contained ~10% CAK by weight; the vast bulk of the non-CAK protein was in a single contaminating band whose distribution across column fractions did not follow CAK activity. CAK activation of Cdk2/cyclin A complexes was performed as described in the legend to Figure 5A.

The comparison of CAK activation of cyclin A and cyclin B/Cdk complexes (Figure 5C) was carried out according to Solomon *et al.* (1992) with some modifications. Cdc2 was expressed in reticulocyte lysates at ~12 ng/ μ l. Sea urchin cyclin B was expressed in *E. coli* as a fusion protein to GST and purified as described (Solomon *et al.*, 1990). The indicated amounts of Cdc2 and Cdk2 were incubated in EB (15 mM MgCl₂, 20 mM EGTA, 10 mM DTT, 80 mM K- β -glycerophosphate, pH 7.3, containing 25 μ g/ml aprotinin, 25 μ g/ml leupeptin, 1 mM benzamidin, 0.5 mM PMSF, 10 μ g/ml pepstatin) with 1 mg/ml ovalbumin and 0.5 mM ATP in the presence of 25 nM cyclin A or 20 nM cyclin B, when appropriate, and partially purified CAK. The

mixture was incubated at 23°C for 30 min, diluted with four volumes of EB containing 1 mg/ml ovalbumin, and assayed for H1 kinase activity as described (Solomon *et al.*, 1990).

Expression of Cdk2 in Yeast

To examine the effects of mutation of Cdk2 in yeast, coding sequences were placed under control of the galactose-inducible GAL1 promoter using the plasmid pSE936 (Elledge and Spottswood, 1991). Plasmids were transformed into yeast strain Y61[ura3-52, trp1, cdc28-4] (Elledge and Spottswood, 1991) that contains a temperature-sensitive p34^{Cdc28} protein. This strain is viable at 30°C but arrests primarily at G1 upon shift to 34°C. Individual transformants were screened for complementation at the restrictive temperature on minimal media plates containing either glucose or galactose but lacking uracil. To examine expression of Cdk2 in yeast, 2-ml cultures of the appropriate strains were grown to OD₆₀₀ ~ 1 in minimal media lacking uracil, the cells were washed with water, and then grown in 4 ml yeast peptone containing 2% galactose overnight. Cells from 1.7-ml of culture were collected and resuspended in 0.35 ml of 2 $\times$ SDS sample buffer containing 1 mM benzamidin, 1 μ g/ml leupeptin, 1 μ g/ml antipain, 1 μ g/ml soybean trypsin inhibitor, 60 μ g/ml aprotinin, and 1 mM PMSF before cell disruption with glass beads. Equal quantities of proteins from each strain (determined by Coomassie staining of SDS gels) were immunoblotted, and the blots were probed with affinity-purified anti-Cdk2 antibodies (Elledge *et al.*, 1992). Detection was accomplished using chemiluminescence (ECL, Amersham, Arlington Heights, IL).

RESULTS

Cdk2 and Cyclin A Purification

Examination of the mechanism of Cdk2 activation required the availability of enzyme preparations that were devoid of contaminating kinase activity that can greatly influence the detection limit for histone H1 phosphorylation. This was achieved using a bacterial T7 RNA polymerase expression system (Studier *et al.*, 1990) (Figure 1A) that produces a version of Cdk2 containing a 16 amino acid N-terminal epitope tag (Elledge *et al.*, 1992). When expression was performed at 37°C, >90% of the Cdk2 present in whole cell extracts was insoluble. As attempts to refold guanidine-denatured Cdk2 were unsuccessful, alternative methods for production of soluble Cdk2 were developed. Ultimately, we found that when expression was induced at 25°C, a substantial portion (50–70%) of Cdk2 was soluble and could be readily purified (see MATERIALS AND METHODS).

The final purification step involved affinity chromatography on ATP-agarose, a method that has been exploited successfully in the purification of Cdc2 expressed in insect cells (Desai *et al.*, 1992). Sequence conservation within the protein kinase family suggests that Cdk2 is likely to adopt the “bi-lobal” structure characteristic of cyclic AMP (cAMP)-dependent protein kinase (A-kinase). Based on the A-kinase crystal structure, ATP binds at the interface between the two domains (Knighton *et al.*, 1991a,b). Because the architecture of the ATP-binding site is dependent on proper folding of both domains, ATP affinity chromatography is expected to select for the population of properly folded Cdk2 molecules

Figure 1. (A) Schematic diagram of pET HA-Cdk2 showing restriction sites used for cloning and mutagenesis. Stippled box represents the T7 promoter, heavy bars represent sequences encoding the HA epitope tag, and light bars represent the T7 terminator. (B) SDS-PAGE of purified Cdk2 and cyclin A proteins. Samples from the peak fractions of the last purification step in each case (see MATERIALS AND METHODS) were subject to electrophoresis on a 12% gel and stained with Coomassie brilliant blue. (C) Immunoblot of Cdk2 proteins shown in B. The proteins (125 ng) were immunoblotted with α -Cdk2 polyclonal affinity-purified antibodies (1:2000) and detected using chem-

Figure 2. Activation of Cdk2 by cyclin A. Kinase assays were performed by incubating the indicated concentrations of Cdk2, cyclin A, and histone H1 alone or in various combinations in the presence of [γ - 32 P]ATP for 1 h at 37°C (see MATERIALS AND METHODS). The reactions were quenched, and half of the sample was subjected to SDS-PAGE and autoradiography. Exposure time was 4 h with an intensifying screen.

present in the crude preparation. Typically, 30–60% of Cdk2 present in partially purified preparations bound ATP-agarose and was eluted with 200 mM NaCl. For comparison, Cdc2 elutes with $\sim$ 250 mM NaCl under slightly different conditions (Desai *et al.*, 1992). Overall yields of purified protein ranged from 2 to 5 mg/l of culture, and proteins were >95% homogeneous as assessed by SDS-PAGE (Figure 1B). The identity of the purified protein was verified by immunoblotting (Figure 1C) using affinity-purified polyclonal Cdk2 antibodies (Elledge *et al.*, 1992). Previously, we demonstrated that both monomeric and cyclin A-associated Cdk2 from HeLa cells binds tightly to Cks1 (Elledge *et al.*, 1992), the human homolog of the *S. pombe* protein p13^{suc+} (Richardson *et al.*, 1990). Recombinant Cdk2 protein also readily associated with immobilized Cks1, providing additional evidence for proper folding (Figure 1D).

Cyclin A (Figure 1B) was purified from the soluble fraction of an *E. coli* lysate induced at 25°C with the hope of enriching for properly folded protein (see Solomon *et al.*, 1990). As with Cdk2, we found that low temperature induction greatly increased the proportion of soluble cyclin A, facilitating its purification.

Activation of Cdk2 by Cyclin A

Recombinant Cdk2 was essentially inactive as a histone H1 kinase: incubation of 700 nM Cdk2 with histone H1 and ATP (1 h, 37°C) resulted in no detectable phos-

iluminescence. (D) Association of purified Cdk2 with Cks1. Experiments were performed as described under MATERIALS AND METHODS using a Cdk2 sample that was stored at 4°C and bound proteins detected by Coomassie Blue staining. As a control for protein levels, Cdk2 (2 μ g) was run on the same gel as indicated. S, supernatant fraction; P, bead-associated fraction.

phorylation of histone H1 (Figure 2). In contrast, addition of 100 nM cyclin A to reaction mixtures containing 70 nM Cdk2 resulted in readily detectable H1 kinase activity (~ 16 pmol ^{32}P transferred $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$). The concentration of Cdk2 used in this experiment is within the range estimated for that of Cdk2 in HeLa cells (40–80 nM) (Desai *et al.*, 1992). Control incubations with cyclin A and histone H1 alone demonstrated that H1 phosphorylation was not due to contaminants in the cyclin A preparation (Figure 2). Cyclin A, at ~ 58 kDa, was also readily phosphorylated by Cdk2 in these reactions, presumably at the single Cdc2/Cdk2 consensus sequence (E-S-P-H, residues 153–156).

Although these results indicated that cyclin A binding alone can activate Cdk2, additional experiments were required to determine the extent of activation. Using relatively high Cdk2 concentrations (1.3 μM) and extended reaction times (6 h), we attempted to estimate the maximal catalytic activity attributable to recombinant Cdk2 alone (Figure 3A). Control experiments indicated that incubation of Cdk2 at 37°C resulted in a time-dependent decrease in specific activity, measured after addition of excess cyclin A. Inactivation appeared to have two components: the initial rapid phase, which resulted in loss of 20% activity over 2 h, was followed by a slower phase in which a further 6% loss of activity was observed over the next 4 h. After 16 h, $<10\%$ activity remained. On the basis of replicate measurements corrected for both trace levels of kinase activity in the histone H1 preparation and the 26% loss of activity observed over 6 h in control reactions, the specific activity of Cdk2 alone was estimated to be $<4 \times 10^{-5}$ pmol $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$. This represents an upper limit because trace bacterial kinases in the Cdk2 preparation could contribute to the total histone phosphorylation under these conditions. Indeed, the absolute phosphorylation of histone H1 was indistinguishable in the presence or absence of Cdk2.

To establish the conditions for maximal cyclin A activation, reactions were performed using 70 nM Cdk2 and a range of cyclin A concentrations (10–1000 nM). Maximum activity (16 pmol $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$) was achieved between 50 and 100 nM cyclin A, approximately equimolar with Cdk2 (Figure 3B). These data indicated that cyclin A binding alone increased the catalytic activity of Cdk2 toward histone H1 by $\geq 4 \times 10^5$ -fold.

Apparent k_{cat} and K_{m} values were determined for histone H1 under conditions where cyclin activation was maximal (Figure 3C). Nonlinear least-squares fit of the kinetic data (Figure 3D) gave a K_{m} value of 1.4 μM , near the H1 concentration used in the standard assay, 1.3 μM . This value is similar to that determined for purified Cdc2/cyclin B from *Xenopus* eggs (1.9 μM) (Erikson and Maller, 1989). k_{cat} for this substrate is 0.4/min. Although we have used ATP-agarose to select for properly folded Cdk2, we do not know whether all of the molecules

contain properly folded cyclin and substrate binding sites. Thus, the specific activity and k_{cat} values given represent lower limits. In addition, histone H1 contains a number of potential phosphorylation sites and therefore kinetic constants represent the average of all phosphorylation sites.

To verify that the cyclin-dependent kinase activity was in fact due to Cdk2, two additional experiments were performed. First, GST-Cdk2 was purified using a procedure decidedly different from that used for Cdk2 (see MATERIALS AND METHODS). As shown in Figure 3E, this fusion protein displayed undetectable activity toward histone H1, although the fusion protein was detectably labeled in this reaction, presumably by a contaminating bacterial kinase. Addition of cyclin A, however, resulted in substantial activation of GST-Cdk2, comparable with that found with Cdk2 itself. In addition to histone H1 and cyclin A phosphorylation, phosphorylation of GST-Cdk2 was also greatly enhanced by cyclin A. This modification presumably occurs at the single Ser-Pro dipeptide located at the junction between the GST and Cdk2 domains. This sequence is not present in Cdk2.

Second, the requirement for the Cdk2 active site in catalysis was established by replacement of Asn-132 by alanine. This residue is invariant in the catalytic loop of serine-threonine protein kinases and forms a hydrogen bond with the catalytic base in cAMP-dependent protein kinase (Knighton *et al.*, 1991a,b). Cdk2^{N132A} was purified by a procedure identical to that used for the wild-type enzyme (see MATERIALS AND METHODS). Although this protein bound to ATP-agarose and Ckshs-1-affigel (see MATERIALS AND METHODS), histone H1 kinase activity was not detected in the presence of cyclin A even after long incubation periods (24 h) with relatively high concentrations of mutant Cdk2 protein (Figure 3F). The specific activity of Cdk2^{N132A} in the presence of cyclin A is $\geq 10^4$ lower than Cdk2 bound to cyclin A. Thus, the ability of Cdk2 to be activated by cyclin A is dependent on the integrity of its active site.

Mutation of T160 in Cdk2

Given the importance of T161 in Cdc2 activation (Gould *et al.*, 1991; Desai *et al.*, 1992; Solomon *et al.*, 1992) and the recent finding that the corresponding residue in Cdk2, T160, is phosphorylated (Gu *et al.*, 1992), we examined the effects of mutation of T160 to either glutamic acid or alanine. The former replacement potentially mimics phosphorylation, whereas the latter change removes the phosphorylation site. As shown in Figure 4A, the T160A mutant (85 nM) was also activated by equimolar cyclin A, but the specific activity was $\sim 15\%$ that of the unmodified Cdk2 protein kinase (2.5 pmol $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$). Interestingly, high concentrations

Figure 3. (A) Activity of Cdk2 in the absence of cyclin A. Kinase assays were performed using Cdk2 (1.3 μM) alone or in the presence of H1 (1 μM). Incubations were for 6 h at 37°C using a Cdk2 sample that was stored at 4°C. Exposure time was 4 h with an intensifying screen. Specific activities were determined by liquid scintillation of histone H1 bands excised from gels. (B) Activation of Cdk2 by cyclin A. Assays were performed using 70 nM Cdk2, 1.3 μM histone H1, and the indicated cyclin A concentrations (0–1000 nM). Reaction mixtures were incubated for 1 h at 37°C before SDS-PAGE and autoradiography. Exposure time was 4 h with an intensifying screen. (C) Concentration dependence for histone H1 phosphorylation. Assays were performed with 70 nM Cdk2, 350 nM cyclin A, and the indicated concentration of histone H1 for 1 h at 37°C (exposure time 4 h). Histone phosphorylation was determined using the Betagen scanner. (D) Nonlinear least-squares fit of the kinetic data obtained from C. (E) Activation of GST-Cdk2 by cyclin A. GST-Cdk2 (170 nM) was incubated with 1.3 μM histone H1 in the presence or absence of the indicated concentration of cyclin A for 1 h at 37°C as described under MATERIALS AND METHODS. Half of each reaction mixture was

of cyclin A (0.5–1 μM) appeared to be a better substrate for Cdk2^{T160A} than histone H1 at a similar concentration (1.3 μM). These relative activities were consistently obtained with the T160A mutant in several experiments. In contrast, wild-type Cdk2 preferred histone H1 over cyclin A at comparable substrate concentrations (Figure 3B, lane 6). The finding that Cdk2^{T160A} displays substantial kinase activity in the presence of cyclin A indicates that phosphorylation of this residue is not essential for activation by cyclin A and rules out the possibility that trace phosphorylation of T160 in Cdk2, either by a contaminating bacterial kinase or by auto-phosphorylation, is responsible for the cyclin A-dependent activation observed.

Like Cdk2, Cdk2^{T160E} was inactive as an H1 kinase (specific activity $< 4 \times 10^{-5}$ pmol·min⁻¹·μg⁻¹) but was readily activated on addition of cyclin A (Figure 4B). Interestingly, the specific activity of this mutant at the optimal cyclin A concentration was indistinguishable from that of the wild-type enzyme (~ 18 pmol·min⁻¹·μg⁻¹).

Activation of Cdk2/Cyclin A Complexes by *Xenopus* CAK

Although cyclin A binding alone activates Cdk2 by $\geq 4 \times 10^5$ -fold, available data indicate that additional modifications are required for maximal activation. The activity of Cdk2^{T160A} produced in mammalian cells and purified by immunoprecipitation is ≥ 40 -fold lower than that obtained with the wild-type protein (Gu *et al.*, 1992). In addition, anti-cyclin A immune complexes contain almost exclusively the T160 phosphorylated form of Cdk2 (Elledge *et al.*, 1992; Gu *et al.*, 1992; Rosenblatt *et al.*, 1992), indicating that cyclin A regulation is accompanied by phosphorylation of T160. Finally, the apparent specific activity of Cdk2/cyclin complexes obtained by immunoprecipitation from mammalian cells (Elledge *et al.*, 1992) is substantially higher (two to three orders of magnitude) than that of the recombinant complex with cyclin A formed in vitro.

To examine whether the Cdk2/cyclin A complex could be further activated by phosphorylation of T160, complexes were incubated with *Xenopus* CAK. The CAK preparation used was purified ~ 12 000-fold (see MATERIALS AND METHODS) and contains only trace levels of contaminating histone H1 activity. Incubations were also performed using mutant Cdk2 proteins in

separated by SDS-PAGE before autoradiography. (F) Cdk2^{N132A} is not activated by cyclin A. Cdk2^{N132A} (350 nM) was incubated with histone H1 (1.3 μM), the indicated concentration of cyclin A for 24 h at 37°C, and reactions processed as described above. As a control, 70 nM Cdk2 was incubated for 1 h at 37°C with the indicated concentration of cyclin A and histone H1 (left). Exposure was 4 h with an intensifying screen.

Figure 4. (A) Activation of Cdk2^{T160A} by cyclin A. Assays were performed using 85 nM Cdk2^{T160A}, 1.3 μ M histone H1, and the indicated concentrations of cyclin A. (B) Activation of Cdk2^{T160E} by cyclin A. Assays were performed using 70 nM Cdk2^{T160E}, 1.3 μ M histone H1, and the indicated concentrations of cyclin A. All reactions were incubated for 1 h at 37°C and processed in conjunction with the reactions shown in Figure 3B. The last lane of A, showing the reaction with 1000 nM cyclin A, was underloaded as assessed by Coomassie staining of histone H1 protein.

which T160 was replaced by either alanine or glutamic acid. As shown in Figure 5A, treatment with CAK resulted in a further 80-fold activation of the Cdk2/cyclin A complex, whereas no effect on activity was observed with either the Cdk2^{T160A}/cyclin A complex (Figure 5A) or the Cdk2^{T160E}/cyclin A complex (Solomon and Harper, unpublished data). As with Cdc2 (Solomon *et al.*, 1992), CAK activation was dependent on the presence of cyclin A. The extent of activation induced by CAK in these experiments is a lower limit because CAK activation and H1 phosphorylation occurred simultaneously during the 60-min incubation (Figure 5A). However, under the conditions used, Cdc2 activation in the presence of cyclin B is essentially complete within 5–10 min. An accurate assessment of CAK-dependent activation of Cdk2 necessitated the use of low concentrations of the kinase subunit (~ 1 nM) such that histone phosphorylation was in the linear range. This made it difficult to accurately determine the specific activity of unphosphorylated Cdk2/cyclin A complexes under the same experimental conditions (see Figure 2). A value of $4.5 \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$ was obtained for the unphosphorylated complex in this experiment, compared with $360 \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$ in the presence of CAK. This

Figure 5. (A) Activation of the Cdk2/cyclin A complex by CAK. Standard histone H1 kinase reactions (see MATERIALS AND METHODS) were performed using the following concentrations of the indicated components: 1 nM Cdk2 and Cdk2^{T160A}, 50 nM cyclin A, and ~ 1 nM CAK. All components were added at the beginning of the 60-min incubation. Data were quantitated by scanning the dried gel with a PhosphorImager, normalized to the specific activity of the CAK + cyclin A + Cdk2 sample determined by Cerenkov counting of the excised portion of the gel. Where appropriate, values were corrected for the background phosphorylation of H1 in the presence of CAK alone or with cyclin A (1–4% of the activity of CAK + cyclin A + Cdk2 sample). Deviations in background activities measured under the conditions of these experiments are not considered significant because the absolute reaction rates are very low and approach the detection limit. The specific activity of the CAK + cyclin A + Cdk2 sample was $360 \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$ Cdk2. (B) Cdk2 is not activated directly by cyclin B. The indicated mixtures of Cdk2 and GST-cyclin B, Cdk2 and cyclin A, or individual protein components were incubated with histone H1 and [γ -³²P]ATP for 60 min at 37°C, and reaction products were analyzed as described under MATERIALS AND METHODS. Activity toward histone H1 is shown. Relative activities of the various complexes (see RESULTS) were determined by scintillation counting of histone H1 bands excised from the gel. (C) Activation of Cdc2 and Cdk2-cyclin complexes by CAK. Activities were measured as described under MATERIALS AND METHODS using 18 nM *in vitro* translated Cdc2 or 41 nM Cdk2 in combination with 25 nM cyclin A or 20 nM cyclin B. Activity toward histone H1 is shown. The specific activities of Cdk2 complexes in this experiment are reduced by about ninefold compared with those in A. This is likely due to freeze-thaw instability.

3.5-fold reduction in specific activity for the unphosphorylated complex measured here, relative to the specific activity measured at much higher Cdk2 concentrations (see Figure 3, A and B), could be attributable to the multiple freeze-thawings that these particular Cdk2 and cyclin A samples underwent during the course of these experiments (see MATERIALS AND METHODS). However, the fold-activation observed in the presence of CAK is unaltered by the precise specific activity of the Cdk2 used.

Cyclin B Activates Cdk2 only in Combination with CAK

It was of interest to determine whether cyclin B could also activate Cdk2 in the absence of T160 phosphorylation. Although cyclin B has never been shown to interact with Cdk2 in vivo, previous studies have demonstrated that Cdk2 can be activated by cyclin B in the presence of crude insect cell lysates containing CAK activity (Desai *et al.*, 1992). In addition, among the known cyclins, cyclins A and B display the highest degree of sequence identity, so it seemed plausible that cyclin B would retain the ability to activate Cdk2 directly.

As shown in Figure 5B, mixtures containing pure Cdk2 (100 nM) and excess purified cyclin B (in the form of a GST fusion protein) display an activity toward histone H1 indistinguishable from that of Cdk2 or cyclin B alone. Although histone H1 was not detectably phosphorylated in these experiments, GST-cyclin B and GST breakdown products (migrating slightly faster than histone H1) were trace labeled. The activity of the Cdk2/cyclin A complex was >1000-fold greater than that of the Cdk2/cyclin B complex determined in the same experiment (Figure 5B, lanes 8 and 9).

To ensure that our Cdk2 preparation was capable of being activated by cyclin B, assays were performed using CAK and various combinations of Cdk2, cyclin A, and cyclin B (Figure 5C). Additional incubations were performed with Cdc2 and cyclins as a control. Cdk2 was activated to a similar extent by both cyclins A and B in the presence of CAK, indicating that the inability of cyclin B to directly activate Cdk2 (Figure 5B) was not simply due to the inability of bacterial Cdk2 to associate with cyclin B. These data suggest that the ability of cyclin A to activate Cdk2 directly is due to some unique aspect of its primary structure and not just the presence of a cyclin box.

Expression of Cdk2 Mutants in *S. cerevisiae*

Previous studies with Cdc2 in *S. pombe* (Gould *et al.*, 1991) indicate that phosphorylation of T167 (equivalent to T161 in human Cdc2) is required for in vivo function. Mutation to either alanine or glutamate produces a nonfunctional Cdc2 protein. Because Cdk2 can func-

tionally replace both the G1 and M-phase functions of Cdc28 in *S. cerevisiae* (Elledge and Spottswood, 1991), it was of interest to determine whether the nonphosphorylatable Cdk2 T160A and T160E mutants retained sufficient activity for in vivo function. Mutant and wild-type proteins were expressed under control of the galactose-inducible GAL1 promoter in yeast strain Y61 (Elledge and Spottswood, 1991). This strain contains a temperature-sensitive Cdc28 protein (*cdc28-4* allele) and arrests in G1 at the nonpermissive temperature of 34°C. In galactose at 34°C, Cdk2 readily replaced Cdc28 function (Figure 6), presumably by interacting with one or more G1 cyclins. In contrast, neither of the mutant proteins complemented *cdc28^{ts}* (Figure 6). Although the mutant proteins were present at approximately fivefold lower levels than wild-type Cdk2 expressed from the same promoter, other mutant proteins (e.g., T14A) expressed at this same low level fully complemented *cdc28-4* in this strain (see MATERIALS AND METHODS) (Connell-Crowley and Harper, unpublished data). Expression of Cdk2^{T160A} and Cdk2^{T160E} proteins at the permissive temperature slows cell growth substantially; small colonies were visible after 4–5 days at 30°C (Figure 6). This result suggests that these proteins can compete with the temperature-sensitive Cdc28 for limiting proteins (e.g., cyclins and/or substrates). A similar result was found with the corresponding Cdc2 mutants in *S. pombe* (Gould *et al.*, 1991). However, expression of Cdk2^{T160A} or Cdk2^{T160E} had no apparent effect on cell viability when expressed in a strain (CRY2) containing a wild-type Cdc28 protein (Connell-Crowley and Harper, unpublished data), suggesting that the semidominant effect was dependent on a compromised Cdc28 protein.

Although these results are consistent with a requirement for phosphorylation of Cdk2 for function, as suggested previously for Cdc2 (Gould *et al.*, 1991), they should be interpreted with caution because we do not know the identity of the *S. cerevisiae* cyclins with which Cdk2 interacts or whether these cyclins activate Cdk2 to the extent that cyclin A does. In addition, we have not ruled out the possibility that ~80-fold overproduction of a cyclin/Cdk2^{T160A} complex would be capable of complementing Cdc28^{ts}.

DISCUSSION

A variety of mechanisms have evolved to allow the activities of particular enzymes to be varied over several orders of magnitude. One particularly extreme example of a highly regulated enzyme system is that of the cyclin-dependent protein kinases typified by Cdc2 and Cdk2. These enzymes are regulated both by protein–protein interactions (with cyclins) and by phosphorylation of multiple residues with both positive and negative influences on catalysis. The dynamic range over which the

Figure 6. Cdk2^{T160A} and Cdk2^{T160E} fail to complement a temperature-sensitive mutation of Cdc28 in *S. cerevisiae*. Plasmids expressing the indicated Cdk2 proteins (containing an N-terminal epitope tag) or a control plasmid lacking an insert were transformed into strain Y61 as described under MATERIALS AND METHODS. Single colonies (in duplicate for T160 mutant strains) were replica streaked on plates lacking uracil but containing glucose or galactose (to induce Cdk2 expression) and incubated at the indicated temperature for 4 d.

activity of Cdk2 is regulated is very large ($\sim 10^7$) due to the fact that the catalytic subunit alone is a very poor catalyst. The low intrinsic activity of cyclin-dependent kinases is crucial because the total concentration of these enzymes is essentially constant over the cell cycle; substantial activity outside of the normal window of activation could lead to disruption of normal cell cycle control (Murray *et al.*, 1989; Ghiara *et al.*, 1991; Roy *et al.*, 1991).

Although the actual chemical and physical steps involved in Cdk2 and Cdc2 activation are complex, the overall result of the activation process is to simply lower the energy barrier to catalysis. Any particular activating event could reduce the energy barrier to catalysis in several ways: 1) increasing the energetic contribution of residues that make similar interactions with substrate in both the ground and transition states, 2) increasing the contribution of active site residues involved only in transition state stabilization, or 3) altering the catalytic mechanism in a manner that changes the nature of the

rate-determining step for phosphate transfer. Although the same general activation pathways appear to be used by Cdc2 and Cdk2, the sequence differences displayed among both the kinases and the cyclins with which they predominantly associate implies that the molecular details of how the energy barrier to catalysis is reduced need not be identical for the two enzymes. Understanding subtle features of the activation pathways for these enzymes could provide insight into why multiple kinase and cyclin subunits have evolved to regulate different cell cycle transitions in higher eukaryotes. In the present article, we have examined the first two steps in Cdk2 activation, cyclin binding, and phosphorylation by CAK.

In vitro activity measurements performed using relatively high concentrations of free recombinant Cdk2 and extended incubation times indicate that the specific activity is $< 4 \times 10^{-5} \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$ Cdk2. This represents an upper limit because trace bacterial kinases could contribute significantly to histone H1 phosphorylation under these conditions. In the presence of

equivalent concentrations of cyclin A, the specific activity of Cdk2 is increased by 4×10^5 -fold (Figures 2, 3, and 7A). The extent of cyclin-dependent activation of Cdk2 was not substantially affected by replacement of T160 with alanine or glutamic acid (Figure 4). However, maximal activity ($\sim 10^7$ -fold activation compared with Cdk2 alone) was achieved only after incubation with purified *Xenopus* CAK that presumably phosphorylated T160 in Cdk2. In support of this residue as the site of CAK action, the Cdk2^{T160A}/cyclin A complex was not activated further by treatment with CAK (Figures 5 and 7A). As our estimates of the specific activity of free Cdk2 represent an upper limit, the actual extent of activation by cyclin could be higher. In addition, assays were performed at histone H1 concentrations near the K_m . Thus, maximal activities are approximately twofold higher. Interestingly, the specific activity of Cdk2/cyclin A complexes after CAK treatment is similar (within fourfold) to that of maximally activated *Xenopus* Cdc2/cyclin B (Figure 7A).

Our results indicate that activation of Cdk2 occurs in two steps, each contributing significantly to catalysis (Figure 7B). Cyclin A binding reduces the energy barrier for the reaction by ~ 8 kcal/mol, whereas phosphorylation by CAK contributes an additional 2.8 kcal/mol of free energy to the reaction. Studies of Cdc2 have focused on the second step in the activation pathway (Desai *et al.*, 1992; Solomon *et al.*, 1992) in which 50- to 100-fold activation was measured above background levels of kinase activity. At present, the absolute catalytic activity of Cdc2 is unknown, and it is not yet clear whether cyclin A binding alone can partially activate Cdc2. Our attempts to produce Cdc2 in bacteria using a procedure analogous to that used for Cdk2 have been unsuccessful (Harper, unpublished data). It has, therefore, not been possible to examine whether cyclin A activation of Cdc2 parallels that of Cdk2, and other investigators who have pure Cdc2 produced in insect cells (Desai *et al.*, 1992) have not addressed this aspect of cyclin A-dependent activation of Cdc2 in detail. Interestingly, cyclin B did not activate Cdk2 directly, although the complex was readily activated by CAK (Figure 5, B and C). This finding parallels that of Cdc2, where activation by cyclin B is observed only in the presence of CAK (Desai *et al.*, 1992; Solomon *et al.*, 1992). Further experiments are required to enumerate the mechanistic similarities and differences in Cdc2 and Cdk2 activation by the various cyclins with which they associate, including cyclin E (Koff *et al.*, 1992), and to identify the critical structural features in cyclin A responsible for direct Cdk2 activation.

As with Cdc2, it is impossible to distinguish whether the structural effects of phosphorylation of T160 in Cdk2 alter reaction energetics directly or are propagated to the Cdk2 active site via cyclin A. Rate and equilibrium constants for the individual substrate binding and cat-

Figure 7. (A) Specific activity of Cdk2/cyclin A and Cdc2/cyclin B complexes with and without CAK treatment. Values for Cdk2 were obtained as described under RESULTS, whereas the values for *Xenopus* Cdc2/cyclin B were deduced from previous data (Solomon *et al.*, 1992; Solomon, unpublished data). (B) Diagram depicting the mechanism of activation of Cdk2 by cyclin A. ΔG_f is the reduction in the free energy barrier for catalysis, compared with the unactivated enzyme, for each of the intermediates. Values of ΔG_f for Cdk2 are based on the specific activity determined in vitro (see RESULTS).

alytic steps for Cdk2 have not yet been determined. In addition, the extremely low activity of free Cdk2 makes it difficult to determine kinetically whether it can bind peptide substrates, although ground-state ATP-binding does not appear to be defective. Thus, it is not possible at present to define the catalytic step(s) on which cyclin A or T160 phosphorylation acts. However, it is plausible that at least some of the observed rate enhancement is due to alterations in the orientation of key catalytic residues that selectively enhance transition state interactions.

Our data indicate that T160 phosphorylation of Cdk2 is not required for cyclin A binding (also see Parker *et al.*, 1991; Desai *et al.*, 1992; Solomon *et al.*, 1992). Although the true dissociation constant for the Cdk2/cyclin A complex is unknown, K_d appears to be substantially lower than 10 nM. It is plausible, based on the results of previous immunoprecipitation studies with human and *S. pombe* Cdc2 (Ducommun *et al.*, 1991; Gould *et al.*, 1991), that phosphorylation of T161 increases the affinity of Cdc2 for cyclin. Alternatively, replacement of T161 in Cdc2 by alanine (or other amino acids) could lead to structural changes elsewhere in the molecule that reduce cyclin affinity. Clearly, under the conditions of our kinetic assay, mutation of T160 in Cdk2 did not have a significant effect on cyclin A binding because maximal activity was observed at ~1:1 molar ratios of the two proteins. However, if the binding constant for Cdk2/cyclin A is subnanomolar, then a significant (>10-fold) reduction in affinity would not have been observed here.

Previous work with cAMP-dependent protein kinase demonstrated that glutamic and aspartic acid could partially mimic phosphothreonine, a modification that is crucial for the interaction of the catalytic subunit with the regulatory subunit (Levin and Zoller, 1990). As mentioned above, replacement of T160 by glutamate had no effect on the specific activity of the cyclin complex in vitro, a result that indicates that glutamate is a poor mimic of phosphothreonine in this case, at least with respect to its activating function. Thus, the mere presence of a negative charge at this position is not sufficient to induce the catalytically crucial structural changes that accompany T160 phosphorylation. However, it is still possible that glutamate could increase the affinity of Cdk2 for cyclin, as compared with unphosphorylated threonine.

Available in vivo data in *S. pombe* (Ducommun *et al.*, 1991; Gould *et al.*, 1991), as well as our limited experiments with Cdk2 in *S. cerevisiae* (see RESULTS), are consistent with the idea that CAK activation, and therefore maximal Cdc2 activity, is required for normal cell cycle progression. Although the relevance of direct cyclin activation to Cdk2 enzymology is obvious, the question of whether the unphosphorylated complex plays a biological role remains unanswered. In vivo rate constants for Cdk2/cyclin complex formation and T160 phosphorylation by CAK are unknown, and it is therefore impossible to know whether unphosphorylated complexes exist transiently or whether there is any compartmentalization effects on CAK-dependent activation. In addition, turnover numbers for CAK may vary with the identity of the cyclin subunit or its modification. Thus, it is plausible that the relatively low activity of an unphosphorylated Cdk2/cyclin A complex could serve to sense the concentration of accumulating complexes during particular cell cycle transitions. A further

understanding of the regulatory circuits controlling Cdk2 activation during the G1/S and G0/G1 transitions could provide insight into the biochemical pathways that control cell proliferation.

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