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Effects of Phosphorylation by CAK on Cyclin Binding by CDC2 and CDK2

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The cyclin-dependent protein kinases (CDKs) are activated by association with cyclins and by phosphorylation at a conserved threonine residue by the CDK-activating kinase (CAK). We have studied the binding of various human CDK and cyclin subunits in vitro, using purified proteins derived from baculovirus-infected insect cells. We find that most CDK-cyclin complexes known to exist in human cells (CDC2-cyclin B, CDK2-cyclin A, and CDK2-cyclin E) form with high affinity in the absence of phosphorylation or other cellular components. One complex (CDC2-cyclin A) forms with high affinity only after CAK-mediated phosphorylation of CDC2 at the activating threonine residue. CDC2 does not bind with high affinity to cyclin E in vitro, even after phosphorylation of the CDC2 subunit. Thus, phosphorylation is of varying importance in the formation of high-affinity CDK-cyclin complexes.

The cyclin-dependent kinases (CDKs) are a family of enzymes involved in the control of eukaryotic cell cycle events (11, 18, 20, 22, 26). CDKs are small proteins (~34 kDa) containing little more than a protein kinase catalytic domain. They are inactive as monomers, and activation requires association with positive regulatory subunits known as cyclins. Cyclin binding is one of two major steps in CDK activation: complete activation also requires the phosphorylation of a conserved threonine residue (Thr-161 in human CDC2 and Thr-160 in CDK2) by a separate protein kinase known as the CDK-activating kinase (CAK) (16, 29). CAK is itself a CDK complex composed of a catalytic subunit, CDK7/MO15, and a regulatory subunit, cyclin H (9, 10, 25, 30).

In human cells, two CDKs are particularly well understood. CDC2 is activated primarily at the G_2/M transition and is involved in the induction of mitosis. Its major partner in the cell is cyclin B, whose levels peak at the G_2/M boundary (23). A minor fraction of CDC2 in the cell associates with cyclin A, whose levels rise early in S phase and decline just before mitosis (21, 24, 28). The other major human CDK, CDK2, is activated late in G_1 and remains active until the end of G_2; its major partners include cyclin E, whose levels peak around the G_1/S boundary, and cyclin A (7, 8, 14, 28, 32). CDK2-cyclin complexes appear to be involved in the control of G_1 events and DNA replication (2, 21, 33).

Previous studies have suggested that the affinity of the interaction between CDKs and cyclins may be affected by phosphorylation at the activating threonine. Several groups reported that cyclin binding by CDC2 is inhibited by mutation of Thr-161, suggesting that phosphorylation at this site stabilizes CDC2-cyclin complexes (6, 12, 19). In contrast, other groups have reported that CDC2 and cyclin bind with high affinity in the absence of phosphorylation (1, 5, 31). Thus, the precise effect of phosphorylation on cyclin binding remains unclear.

In the present studies, we set out to clarify the role of CDK phosphorylation in cyclin binding, using purified human CDK and cyclin proteins derived from baculovirus-infected insect cells. Our results suggest that certain CDK-cyclin complexes form readily in vitro in the complete absence of other proteins or phosphorylation, whereas other complexes form only when CDK phosphorylation is allowed to occur.

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MATERIALS AND METHODS

Protein expression. Human CDKs and cyclins were expressed at high levels in insect cells by infection with recombinant baculovirus vectors as described previously (5, 14). Human CDC2 and CDK2 proteins used in most experiments (all but that with CAK [see Fig. 3]) contained a C-terminal epitope tag from influenza virus hemagglutinin (HA) to allow immunoblotting and immunoprecipitation with monoclonal antibody (MAb) 12CA5 (5). Mutant CDC2 and CDK2 vectors were constructed by oligonucleotide-directed mutagenesis with uracil-containing template DNA (15). The templates used were the human CDK2 and CDC2 cDNAs, cloned into pSM as described previously (5, 13). The Thr-161 codon in CDC2 and the Thr-160 codon in CDK2 were changed to alanine codons to generate the CDC2T161A and CDK2T160A mutants, respectively.

Purification of CDC2 and CDK2. Epitope-tagged CDC2 and CDK2 were purified from 300 to 500 ml of infected insect cell cultures by chromatographic procedures similar to those used previously to purify untagged CDK proteins (5, 27). HA-tagged CDK proteins (unlike the untagged proteins) were bound to and eluted by linear salt gradient from a DEAE-Sepharose column. Final preparations of CDC2 and CDK2 (~0.5 mg/ml; ~90 and ~95% homogeneous, respectively) were stored at −80°C in storage buffer (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.4], 250 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol). For the experiment with CAK (see Fig. 3), untagged CDC2 was purified as described previously (5).

Purification of cyclins. Human cyclins A and B1 were both purified by the mild chromatographic procedures described previously for the purification of cyclin B1 (5) except that the gel filtration step was omitted. The resulting proteins were ~90% pure and were stored at −80°C in storage buffer.

Human cyclin E is expressed at relatively low levels in infected insect cells (~1% of total cell protein, or about fivefold lower than the expression level of cyclin A or B). It was partially purified by the same S-Sepharose and Mono Q steps described previously for purification of cyclin B, resulting in a relatively impure preparation (~50% pure) that is nevertheless free of detectable CDKs, CAK activity, or histone H1 kinase activity.

Measurement of CDK-cyclin binding. Association between CDK and cyclin proteins was assessed by gel filtration and immunoblotting as described previously (5). Purified CDK and cyclin proteins were mixed at low concentrations (50 to 100 nM) in 250 μl of HBS (25 mM HEPES-NaOH [pH 7.4], 150 mM NaCl). After 20 min at 24°C, the mixtures were injected onto a Pharmacia Superose 12 column (HR 10/30, 0.4 ml/min) equilibrated with HBS plus 1 mM EDTA. Fractions (0.5 ml) were collected, and aliquots of each fraction were analyzed by immunoblotting with MAb 12CA5 prior to incubation with ^{125}I-labeled anti-mouse antibodies. Blots were quantitated with a PhosphorImager (Molecular Dynamics).

In some experiments, human HeLa cell cytoplasmic extract was added to CDK-cyclin mixtures. This extract was prepared by harvesting HeLa cells grown in suspension by centrifugation (800 × *g* for 10 min), swelling cells in hypotonic buffer (10 mM HEPES-NaOH [pH 7.4], 10 mM NaCl, 1 mM EDTA), centrifuging again, and then resuspending the pellet in 3 volumes of cold hypotonic lysis buffer (10 mM HEPES-NaOH [pH 7.4], 10 mM NaCl, 1 mM EDTA, 1 mM

phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml). Cells were ruptured in a Dounce homogenizer, and the resulting lysate was clarified by ultracentrifugation (100,000 × *g* for 60 min at 4°C). The supernatant (~10 to 20 mg/ml of total protein) was stored at −80°C.

Phosphorylation of CDC2-cyclin A by CDK-activating kinase (CAK). Reconstituted human CAK was prepared from baculovirus-derived, HA-tagged CDK7 and bacterially derived, histidine-tagged cyclin H, as described previously (10). Insect cell lysate containing ~2 μg of CDK7HA or CDK7(K41A)HA was mixed with 2 μg of purified cyclin H, incubated for 30 min at 24°C, and immunoprecipitated for 1 h at 4°C with 1 μg of MAb 12CA5 coupled to 25 μl of protein A-Sepharose. The beads were washed twice with HBST (HBS plus 0.1% Triton X-100) plus 10 mM EDTA and twice with HD (50 mM HEPES-NaOH [pH 7.4], 1 mM dithiothreitol). Immobilized, active CDK7-cyclin H complex was then incubated with a 100-μl mixture containing 12 μg of cyclin A (2.4 μM) and 6 μg of CDC2 (1.8 μM) in HBS plus 1 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, 1 mM ATP, and 10 mM $MgCl_2$. After 1 h at 24°C, the beads were pelleted and the supernatant was subjected to gel filtration as described above. Aliquots of fractions were analyzed by immunoblotting with affinity-purified rabbit anti-CDC2 antibodies (28) followed by incubation with ^{125}I-labeled anti-rabbit antibodies and analysis by PhosphorImager.

CDC2 phosphorylation and histone H1 kinase activity. To measure the phosphorylation of CDC2 under various conditions, purified CDC2 (~200 ng, 100 nM) was incubated in 50 μl of HBS containing 10 mM $MgCl_2$, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP (3,000 mCi/mmol; Amersham). Purified cyclin (~50 nM) and/or HeLa cell extract (100 μg) was added to selected reaction mixtures. After 20 min at 24°C, the reaction was stopped by the addition of 500 μl of cold HBST plus 20 mM EDTA. CDC2 was then immunoprecipitated by addition of 1 μg of MAb 12CA5 plus 25 μl of protein A-Sepharose. After 2 h at 4°C, immunoprecipitates were washed three times at room temperature with HBST and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

To analyze the protein kinase activity of CDC2, purified CDC2 was incubated in reaction mixtures identical to those described above, except that labeled ATP was omitted. CDC2 was then immunoprecipitated with MAb 12CA5, washed with HBST, washed twice with HD, and mixed with a 30-μl reaction mix containing 10 mM $MgCl_2$, 50 μM ATP, 1 mM dithiothreitol, 5 μg of histone H1 (Boehringer Mannheim), and 2.5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham). After 5 min at 24°C, the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis.

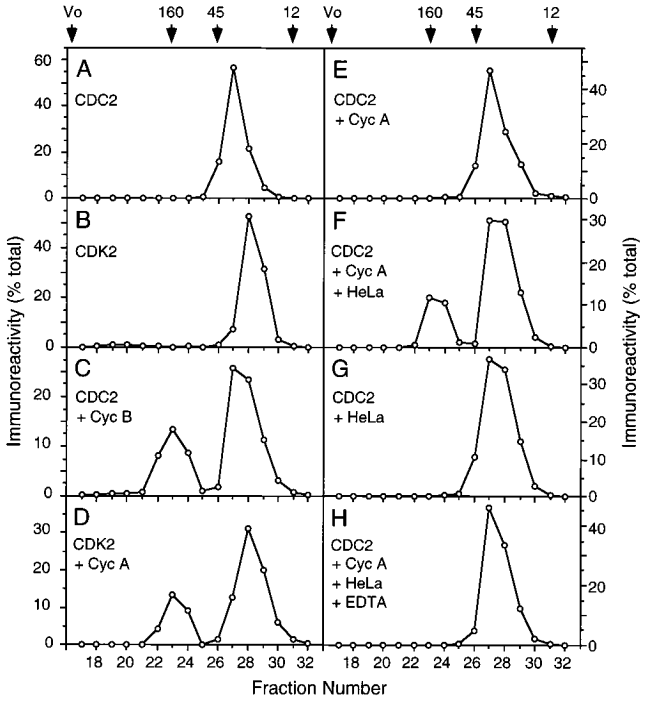

FIG. 1. Binding of purified CDK and cyclin subunits in vitro. Purified CDC2 or CDK2 (~100 nM) was incubated alone (A, B, and G) or in the presence of purified cyclin (~50 nM; C to F and H) in a total volume of 250 μl. Some reaction mixtures included 100 μg of HeLa cell extract plus 10 mM $MgCl_2$ and 50 μM ATP (F and G) or HeLa cell extract plus 20 mM EDTA (H). After 20 min of incubation at 24°C, the mixtures were injected onto a Pharmacia Superose 12 gel filtration column, and aliquots of fractions were subjected to immunoblotting with MAb 12CA5, which recognizes the HA epitope tag at the C terminus of the CDK proteins. Immunoblots were incubated with ^{125}I-labeled anti-mouse antibodies, followed by quantitation with a PhosphorImager. CDK immunoreactivity in each fraction is presented as a percentage of the total in all fractions. Molecular masses (in kilodaltons) of marker proteins, determined in parallel runs, are indicated by arrowheads (Vo, void volume; 160, immunoglobulin G; 45, ovalbumin; and 12, cytochrome *c*). Cyc A and Cyc B, cyclin A and cyclin B, respectively.

RESULTS

In our previous studies, we found that purified human CDC2 and cyclin B bind with high affinity in the complete absence of phosphorylation or other cellular components (5). Complexes of other CDKs and cyclins were also studied, but only in crude cell extracts and not with purified components. In the present work, we extended these analyses by studying complex formation between purified CDKs and cyclins. Human CDC2, CDK2, and cyclins A and B were purified to near homogeneity by mild chromatographic procedures, diluted to physiological concentrations (50 to 100 nM), and mixed in various combinations. To measure the formation of CDK-cyclin complexes, proteins were subjected to gel filtration on a Superose 12 column, and the amount of CDK protein in each fraction was determined by immunoblotting. Using this method, we can readily estimate the molecular sizes of complexes and determine the relative amounts of bound and free CDK (5).

Purified CDC2 and CDK2 migrated as monomers on gel filtration (Fig. 1A and B), while cyclins A and B migrated anomalously at about 110 and 160 kDa, respectively (data not shown, but see reference 5). As seen previously, mixing of purified CDC2 with cyclin B resulted in the formation of high-molecular-weight CDC2 complexes (Fig. 1C), demonstrating the formation of CDC2-cyclin B complexes in the absence of other components. In these experiments, we estimate that CDC2 is present at about two- to threefold higher levels than cyclin, so that only a portion of the CDC2 shifts to the high-molecular-weight form (addition of more cyclin results in complete shifting of CDC2 to a high molecular weight [data not shown]). Purified CDK2 also formed high-molecular-weight complexes with purified cyclin A (Fig. 1D) and purified cyclin B (data not shown), indicating that these complexes also form with high affinity in the absence of phosphorylation or other components.

Interestingly, purified CDC2 and cyclin A did not form high-molecular-weight complexes (Fig. 1E), despite the fact that either protein was capable of forming stable complexes with other partners (Fig. 1C and D). Complex formation between CDC2 and cyclin A was also not observed when 10-fold-higher concentrations of the two proteins were mixed (1 μM [data not shown]). To determine if additional cellular components are required for CDC2-cyclin A binding, we tested the effect of adding a small amount of cytoplasmic extract of human HeLa cells, together with 10 mM $MgCl_2$ and 50 μM ATP. The addition of HeLa cell extract resulted in the formation of CDC2-cyclin A complexes (Fig. 1F). The shift in CDC2 size in the presence of HeLa cell extract required the presence of cyclin A (Fig. 1G). Complex formation was not seen when divalent cations were depleted by the addition of 20 mM EDTA (Fig. 1H).

As previous work from other laboratories had suggested that CDC2 phosphorylation at Thr-161 might stabilize cyclin binding (6), we analyzed the role of this phosphorylation in CDC2 binding to cyclin A. We prepared mutant CDC2 and CDK2 proteins in which the activating phosphorylation site was changed to alanine (CDC2T161A and CDK2T160A). Mutation of the activating threonine did not significantly affect the

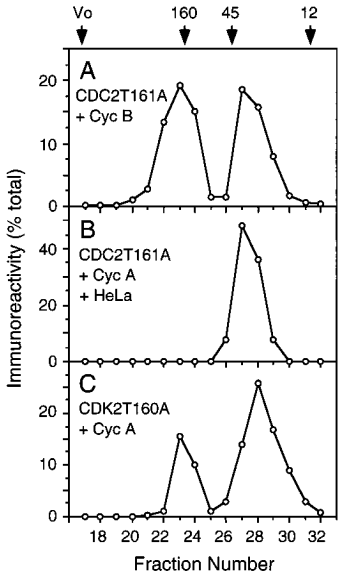

FIG. 2. Binding of cyclins to CDK subunits mutated at the Thr-160/Thr-161 phosphorylation site. Purified CDC2T161A (panels A and B) or CDK2T160A (panel C) was incubated with purified cyclin B (Cyc B; panel A) or cyclin A (Cyc A; panels B and C) under the same conditions as described in the legend for Fig. 1. The reaction mixture for panel B included 100 μg of HeLa cell extract plus 10 mM $MgCl_2$ and 50 μM ATP. The mixtures were analyzed as described for Fig. 1.

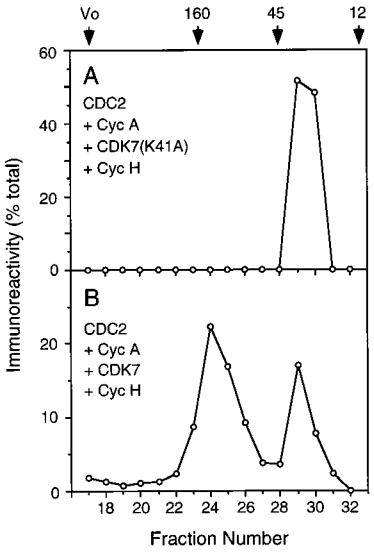

FIG. 3. Effect of phosphorylation by CAK on CDC2-cyclin A binding. Human CAK was prepared by mixing HA-tagged human CDK7 with purified cyclin H (Cyc H) (B), and kinase-deficient CAK was prepared with a mutant catalytic subunit, CDK7(K41A) (A). Mutant and wild-type CAK complexes were immunoprecipitated with MAb 12CA5, washed, and incubated with purified CDC2 and cyclin A (Cyc A) in the presence of 10 mM $MgCl_2$ and 1 mM ATP. CDC2-cyclin A mixtures were then subjected to gel filtration and anti-CDC2 immunoblotting analysis as described in Materials and Methods. Marker proteins are indicated as in Fig. 1.

binding of CDC2 to cyclin B or the binding of CDK2 to cyclin A (Fig. 2A and C), confirming that the formation of these complexes does not require phosphorylation and also indicating that the mutation does not grossly disrupt CDK conformation. However, CDC2 binding to cyclin A, even in the presence of HeLa cell extract, was prevented by mutation of Thr-161 (Fig. 2B). This observation suggests that phosphorylation at Thr-161 (by the CDK-activating kinase, CAK, in the HeLa cell extract) is required for high-affinity binding between CDC2 and cyclin A.

We explored the possibility that phosphorylation by CAK is sufficient to stabilize the CDC2-cyclin A complex. Human CAK was prepared by mixing baculovirus-derived CDK7 and bacterially produced cyclin H, as described previously (10). The active CAK complex was immunoadsorbed to protein A-Sepharose and incubated with CDC2 and cyclin A in the presence of $MgCl_2$ and ATP. Incubation with CAK promoted the formation of stable CDC2-cyclin A complexes (Fig. 3B). In a control experiment, we treated CDC2 and cyclin A with a kinase-deficient CAK mutant in which the CDK7 catalytic subunit carried a mutation in the conserved lysine of the ATP binding site (K41A). Incubation with mutant CAK did not facilitate CDC2-cyclin A binding (Fig. 3A). It therefore appears that phosphorylation of Thr-161 by CAK is necessary and sufficient to promote binding of CDC2 and cyclin A.

We next tested the abilities of CDC2 and CDK2 to bind human cyclin E. Whereas CDK2 readily formed complexes with cyclin E in vitro (Fig. 4A), CDC2 was unable to bind this cyclin, even in the presence of HeLa cell extract and Mg-ATP (Fig. 4B and C). Binding was also undetectable at 10-fold-higher concentrations of CDC2, cyclin E, and HeLa cell extract (data not shown).

The inefficient binding of CDC2 and cyclin E under these conditions could simply be due to a lack of CDC2 phosphorylation in the presence of cyclin E. To rule out this possibility, we analyzed CDC2 phosphorylation in various cyclin complexes in the presence and absence of HeLa cell extract (Fig. 5A). As expected, the addition of HeLa cell extract to CDC2-cyclin B and CDC2-cyclin A complexes resulted in extensive phosphorylation of CDC2 (Fig. 5A, lanes 4 and 7). This phosphorylation was diminished by mutation of Thr-161 (Fig. 5A, lanes 5 and 8), and tryptic phosphopeptide mapping (data not shown) confirmed that the majority of CDC2 phosphorylation

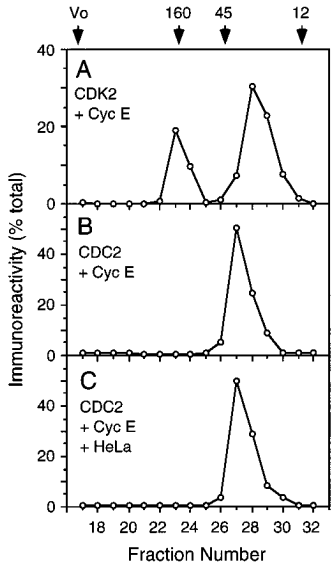

FIG. 4. Binding of CDC2 and CDK2 to cyclin E. Purified CDK2 (A) or CDC2 (B and C) was mixed with partially purified cyclin E (Cyc E) in the absence (A and B) or presence (C) of 100 μg of HeLa cell extract plus 10 mM $MgCl_2$ and 50 μM ATP. The mixtures were incubated and analyzed as described in the legend for Fig. 1.

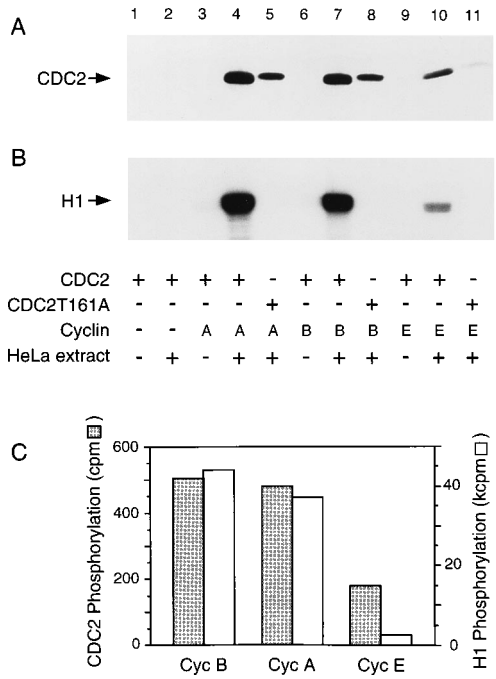

FIG. 5. CDC2 phosphorylation and activation in the presence of various cyclins. (A) Purified CDC2 (100 nM) (lanes 1 to 4, 6, 7, 9, and 10) or CDC2T161A (lanes 5, 8, and 11) was incubated without cyclin (−; lanes 1 and 2) or in the presence of 50 nM of the indicated cyclin (A, B, or E; lanes 3 to 11) in a 50-μl reaction volume containing 10 mM $MgCl_2$, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP (3,000 mCi/mmol). Certain reaction mixtures also contained 100 μg of HeLa cell extract (lanes 2, 4, 5, 7, 8, 10, and 11). After 20 min at 24°C, CDC2 was immunoprecipitated from the reaction mixtures with MAb 12CA5 and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. (B) Reaction mixtures identical to those described for panel A were prepared, except that ^{32}P-labeled ATP was omitted. After 20 min at 24°C, CDC2 was immunoprecipitated from the reaction mixture and tested for its ability to phosphorylate histone H1 (as described in Materials and Methods). (C) Labeled CDC2 and histone H1 bands were excised from lanes 4, 7, and 10 of the gels shown in panels A and B and subjected to Cerenkov counting. Background phosphorylation in the absence of cyclin was subtracted. The amounts of phosphate incorporated into CDC2 in the presence of HeLa cell extract and the indicated cyclin (from panel A) (shaded bars) and the levels of CDC2 activity toward histone H1 in the same complexes (from panel B) (open bars) are indicated.

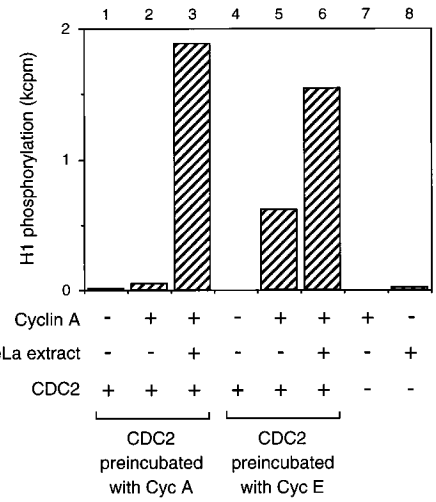

FIG. 6. Activation of phosphorylated CDC2 monomer by purified cyclin A. In reactions 4 to 6 (reaction numbers are indicated above the bars), monomeric CDC2 was immunoprecipitated with MAb 12CA5 from a low-molecular-weight fraction (fraction 27) from the gel filtration experiment whose results are shown in Fig. 4C, in which CDC2 had been incubated with cyclin E (Cyc E) and HeLa cell extract. In reactions 1 to 3, similar amounts of CDC2 monomer were immunoprecipitated from a low-molecular-weight fraction from the experiment whose results are shown in Fig. 1E, in which CDC2 had been incubated with cyclin A (Cyc A). CDC2 immunoprecipitates were washed and then incubated alone (reactions 1 and 4) or in the presence of 200 ng of purified cyclin A (reactions 2, 3, 5, and 6); reaction mixtures 3 and 6 also included 100 μg of HeLa cell extract. Control immunoprecipitates lacking CDC2 were also analyzed (reactions 7 and 8). All reaction mixtures included 10 mM $MgCl_2$ and 50 μM ATP. After 20 min at 24°C, the immunoprecipitates were washed and tested for their ability to phosphorylate histone H1 as described in Materials and Methods. Histone H1 bands were excised from a dried gel and quantitated by Cerenkov counting.

was found on the phosphopeptide containing Thr-161 (peptide 4 in previous work [13]). Some phosphorylation was also seen at other sites. All phosphorylation was dependent on both cyclin and the HeLa cell extract.

CDC2 was also phosphorylated at Thr-161 in the presence of cyclin E, although the level of phosphorylation was reduced two- to threefold (Fig. 5A, lanes 10 and 11). Despite this phosphorylation, the histone H1 kinase activity of CDC2 in the presence of cyclin E was 15-fold lower than activity in the presence of cyclin B or A (Fig. 5B and C). These results, combined with our binding results (Fig. 4C), are consistent with a low-affinity interaction between CDC2 and cyclin E that is sufficient to promote considerable CDC2 phosphorylation at Thr-161. However, although phosphorylated CDC2 accumulates in the reaction mixture, its low affinity for cyclin E impairs the activation of its histone H1 kinase activity. In addition, it is possible that cyclin E binding does not induce effective CDC2 activation.

Thus, incubation of CDC2 and cyclin E with HeLa cell extracts appears to result in the accumulation of a Thr-161-phosphorylated CDC2 monomer. This phosphorylated monomer should be present in the population of CDC2 that migrates at 34 kDa upon gel filtration of a reaction mixture containing CDC2, cyclin E, and HeLa cell extract (as shown in Fig. 4C). To investigate this possibility, monomeric CDC2 was immunoprecipitated from these low-molecular-weight fractions. Although the CDC2 monomer from these fractions exhibited very low levels of kinase activity in the absence of cyclin, the addition of purified cyclin A resulted in a major stimulation of activity (Fig. 6, reaction 5). The addition of crude cell extract further activated CDC2, as expected if the monomeric CDC2 was only partially phosphorylated (Fig. 6, reaction 6). In contrast, purified cyclin A did not significantly activate unphosphorylated CDC2 monomer (Fig. 6, reaction 2). These results are consistent with the notion that exposure of CDC2 to cyclin E and HeLa cell extract results in the formation of a monomeric CDC2 species that is phosphorylated at Thr-161 but does not bind cyclin E with high affinity. The phosphorylated monomer can be activated by purified cyclin in the absence of CAK.

DISCUSSION

We have measured the abilities of various human CDK and cyclin subunits to form high-affinity complexes in vitro. Our gel filtration method measures the formation of complexes that form at low concentrations (50 to 100 nM) and do not dissociate when subjected to substantial dilution during the 1-h column run. Although this method does not provide accurate measurement of binding affinities, it does provide a reasonable assessment of complex formation at CDK and cyclin concentrations that roughly approximate concentrations in the cell. Our results indicate that several complexes (CDC2-cyclin B

and CDK2-cyclin A, B, or E) form readily in the absence of phosphorylation or other proteins. However, CDC2 and cyclin A do not bind with high affinity unless phosphorylation occurs at Thr-161. Finally, one complex (CDC2-cyclin E) does not bind with high affinity even if Thr-161 phosphorylation has occurred. In general, the affinities of various complexes in vitro are consistent with their relative abundances in vivo: the primary partner for CDC2 in the cell is cyclin B (23), while CDK2 is the major partner for cyclins A and E (7, 8, 14, 28, 32). CDC2-cyclin A complexes are less abundant (24, 28). Interestingly, CDK2-cyclin B complexes are generally not found in the cell, despite their high-affinity interaction in vitro.

Thr-161 phosphorylation clearly enhances the affinity of at least one complex (CDC2-cyclin A). It is possible that it also increases the affinity of high-affinity complexes (e.g., CDK2-cyclin A) (3), although our method would not detect this increase. The effect of phosphorylation on CDK2-cyclin A affinity may be difficult to assess; we have found that even in the absence of phosphorylation this complex does not dissociate measurably over extended periods, even in the presence of strong chaotropic agents (28a). More sophisticated methods will be required to measure the precise affinities of these complexes in the presence and absence of phosphorylation and also to measure the low affinities that we suspect are exhibited by unphosphorylated CDC2-cyclin A and CDC2-cyclin E complexes.

The stabilization of CDC2-cyclin A binding by Thr-161 phosphorylation may result from a direct interaction between the phosphorylated residue and cyclin; this possibility is supported by crystal structure analysis of the CDK2 monomer, which indicates that the activating threonine is on a flexible loop close to the putative cyclin binding site (4, 17). It is also possible that Thr-161 phosphorylation enhances cyclin binding indirectly by inducing a conformational change that both activates the kinase and alters the cyclin binding site. These issues will be resolved only by structural analysis of a phosphorylated CDK-cyclin complex.

Previous work from a number of groups has produced conflicting results regarding the effect of CDK phosphorylation on cyclin binding. Whereas some previous work has indicated that CDK phosphorylation is important for high-affinity cyclin binding (6, 12, 19), other work has suggested that phosphorylation is not required (1, 3, 5, 31). Our results suggest that both possibilities may be correct, depending on the particular CDK and cyclin subunits under study. For example, support for the importance of phosphorylation comes mainly from the studies of Ducommun et al. (6), who used coprecipitation methods to show that mutation of Thr-161 in human CDC2 resulted in decreased binding to human cyclin A. This is consistent with our results with the human CDC2-cyclin A complex. On the other hand, much of the evidence that phosphorylation is not required for cyclin binding comes from studies of CDC2-cyclin B or CDK2-cyclin A (1, 3, 5, 31) and is also consistent with our results. Some results remain unexplained, including the findings that mutation of Thr-161 (or its equivalent) inhibits binding of mouse CDC2 and frog cyclin B (19) or fission yeast CDC2 and the B-type cyclin Cdc13 (12). These discrepancies may be explained by species-specific differences in the requirements for stable CDK-cyclin complex formation.

Our studies have also addressed the related question of whether Thr-161 phosphorylation alone can activate CDC2. We find that after incubation of CDC2 and cyclin E in a HeLa cell extract, gel filtration can be used to isolate CDC2 monomers that are phosphorylated at Thr-161. The phosphorylated CDC2 monomer exhibits minimal kinase activity, indicating that Thr-161 phosphorylation alone cannot fully activate CDC2 (although we cannot rule out low levels of activation). This is consistent with our recent results indicating that phosphorylation of monomeric CDK2 by purified CAK does not result in activation (10). Thus, the activation of these CDKs requires cyclin binding as well as phosphorylation.

ACKNOWLEDGMENTS

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