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Human cyclin-dependent kinase 2 is activated during the S and G_2 phases of the cell cycle and associates with cyclin A

(CDC2 protein kinase)

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ABSTRACT **We have analyzed the cell cycle regulation of human cyclin-dependent kinase 2 (CDK2), a protein closely related to the cell cycle-regulatory protein kinase CDC2. We find that CDK2 activity, like that of CDC2, oscillates during the cell cycle in cultured mammalian fibroblasts. Unlike CDC2 activity (which peaks during mitosis), CDK2 activity rises in late G_1 or early S phase and declines during mitosis. Active S-phase CDK2 migrates in multiple large complexes on gel filtration, and CDK2 in one of these complexes is associated with cyclin A. These findings suggest that CDK2 and CDC2, in association with distinct cyclins, regulate separate functions in the mammalian cell cycle.**

The major events of the eukaryotic cell cycle are thought to be triggered by oscillations in the activity of a 34-kDa protein kinase originally identified as the product of the *CDC28* gene of *Saccharomyces cerevisiae* and the *cdc2+* gene of *Schizosaccharomyces pombe*. Genetic analysis in these organisms has demonstrated that the CDC2/CDC28 protein is required at two transition points in the cell cycle: the commitment to DNA replication in G_1 ("Start") and the preparation for mitosis at the G_2/M boundary (1). CDC2/CDC28 function at these transition points is regulated by various posttranslational mechanisms, including phosphorylation and association with regulatory subunits known as cyclins.

Many components of this regulatory system have been conserved in higher eukaryotes (2–4). A human CDC2 homologue has been identified that is closely related to the yeast proteins and complements both the G_1 and G_2 defects of *cdc2* mutants in *Sch. pombe* or *cdc28* mutants in *S. cerevisiae* (5, 6). A variety of cyclins have also been identified in human cells, including cyclin B, which activates CDC2 during mitosis (7), and cyclin A, which was originally identified as a mitotic cyclin and may also play a role in early cell cycle stages (8). Several human G_1 cyclin candidates have also been identified. These include cyclin D, which was found by three independent approaches: as a potential transforming gene in benign human parathyroid tumors (9), as a protein whose levels increase in late G_1 after growth factor treatment of quiescent mouse cells (10), and as a protein capable of rescuing a *cln1,2,3* triple deletion in *S. cerevisiae* (11, 12). This third approach has also led to the identification of cyclins C and E (11, 13).

The profusion of mammalian cyclins has been accompanied by increasing evidence that CDC2 is not the sole cyclin-activated kinase involved in mammalian cell cycle control. Cyclins A and D associate with 33- to 34-kDa proteins that are antigenically related but not identical to CDC2 (8, 10, 14, 15). In addition, despite an abundance of evidence implicating CDC2 in mitotic control, it has been difficult to unequivocally demonstrate a role for CDC2 alone at both Start and mitosis in mammalian cells (16, 17). Thus, we decided to search for additional human versions of the CDC2 protein. Using PCR to amplify CDC2-related sequences, we have cloned a cDNA for human cyclin-dependent kinase 2 (CDK2), which was recently isolated by others (15, 18, 19). We find that CDK2 is activated during the S and G_2 phases of the cell cycle and forms multiple high molecular weight complexes, one of which contains cyclin A.

MATERIALS AND METHODS

Cell Culture, Synchronization, and Lysis. Human HeLa S3 cells and mouse NIH 3T3 cells (clone A31) were grown as monolayers at 37°C in 5% CO_2, in DME-H16 medium containing 10% calf serum. Mitotic cells were isolated by a 14-hr treatment with nocodazole (0.04 μg/ml) (20), followed by shake-off and centrifugation. Synchronization of HeLa cells at the G_1/S boundary was achieved by a double block with thymidine and aphidicolin (9, 21). To prepare cell lysates, cells were washed, scraped from the dish, pelleted by centrifugation, and resuspended in 2 vol of lysis buffer [50 mM Hepes·NaOH, pH 7.4/0.1% Triton X-100/150 mM NaCl/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/50 mM NaF, 80 mM β-glycerophosphate/0.1 mM Na_3VO_4 with leupeptin (1 μg/ml) and aprotinin (20 units/μl)]. Lysates were clarified in a Beckman Airfuge (100,000 × *g*, 30 min). For gel filtration experiments, lysates were injected onto a Pharmacia Superose 12 column preequilibrated with column buffer (50 mM Hepes·NaOH, pH 7.4/0.1% Triton X-100/150 mM NaCl/5 mM EDTA/50 mM NaF/0.1 mM Na_3VO_4) and eluted at 0.4 ml/min (0.5-ml fractions).

Recombinant baculoviruses encoding CDC2 or CDK2 were constructed by established methods (22, 23) (D. Desai and D.O.M., unpublished work), and lysates of infected cells were prepared as described (23).

DNA synthesis was measured in duplicate wells of 12-well culture dishes by addition of [^{3}H]thymidine (5 μCi in 1 ml; 6.8 Ci/mmol; 1 Ci = 37 GBq) for 30 min, followed by washing, precipitation with 5% trichloroacetic acid, solubilization in 0.1 M NaOH, and liquid scintillation counting. Mitotic index was measured by staining methanol-fixed cells with Hoechst 33342 (1 μg/ml) before analysis with a fluorescence microscope.

Immunological Methods. Polyclonal antisera were raised against synthetic peptides corresponding to the C-terminal sequences of CDK2 (CDVTKPVPHLRL) and CDC2 (CLDNQIKKM). Peptides were coupled to keyhole limpet hemocyanin through their N-terminal cysteines with iodoacetic acid *N*-hydroxysuccinimide ester (24). Aliquots of coupled peptide (1 mg coupled to 5 mg of hemocyanin) were injected into rabbits (BAbCO, Richmond, CA), and anti-peptide an-

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Abbreviation: CDK2, cyclin-dependent kinase 2.

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tiserum was affinity-purified on columns of the peptide antigen (24). Immunoblotting was as described (25). For immunoprecipitations, aliquots (25 μl) of protein A-Sepharose beads were incubated 1 hr at 4°C with 1–5 μg of affinity-purified antibodies, washed, and then added to 500-μg aliquots of cell lysates for a 2-hr incubation at 4°C. Immunoprecipitates were washed at room temperature twice with 50 mM Hepes·NaOH, pH 7.4/150 mM NaCl/0.1% Triton X-100 and twice with 50 mM Hepes·NaOH, pH 7.4/1 mM dithiothreitol and then combined with a 30-μl reaction mix containing 40 μg of histone H1 (Sigma), 50 μM ATP, 10 mM $MgCl_2$, and 2.5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). After 15 min at room temperature, reaction mixtures were boiled in sample buffer for SDS/PAGE.

RESULTS

Isolation of a cDNA Encoding Human CDK2. We used PCR (26) to isolate cDNA sequences that were closely related to *CDC2*. *CDC2*-related sequences were amplified from a human placental cDNA library by using degenerate primers representing conserved regions of CDC2. Sequence analysis of several fragments revealed one sequence that was closely related, but not identical, to that of *CDC2*. A fragment containing this sequence was used to isolate a cDNA containing the entire coding region. The sequence of the predicted protein is identical to that of human CDK2 proteins recently described by others (15, 18, 19).

CDK2 Kinase Activity During the Cell Cycle. We analyzed CDK2 levels and activity in mammalian cells synchronized at various stages of the cell cycle. Rabbit antisera were developed against C-terminal peptides from CDK2 and CDC2 and affinity-purified on antigen columns. Immunoblotting of crude HeLa S3 cell extracts demonstrated that both antisera recognized single major bands at ≈34 kDa (Fig. 1*A*). The binding of both antibodies to these 34-kDa bands was completely inhibited by antigenic peptide (data not shown). To further confirm the specificity of the antibodies, we analyzed lysates of insect cells infected with baculoviruses encoding the two kinases. These lysates contain large amounts of the expressed kinase (2–5% of cell protein) and negligible amounts of endogenous CDC2 or CDK2. Immunoblotting of these lysates showed that the anti-CDK2 antibody recognized CDK2 but not CDC2 (Fig. 1*A*). Similarly, the anti-CDC2 antibody was completely specific for CDC2. As expected from their primary sequences, both proteins were recognized by antibodies against the highly conserved PSTAIR sequence (Fig. 1*A*). CDK2 was also recognized by $p13^{suc1}$ (data not shown), another reagent often used in the detection of CDC2 (27).

The two antisera were capable of specifically immunoprecipitating CDK2 or CDC2 kinase activity from HeLa cell extracts. Immunoprecipitates of extracts from unsynchronized HeLa cells contained significant quantities of histone H1 kinase activity, and most of the activity was abolished when the immunoprecipitation was performed in the presence of excess antigenic peptide (Fig. 1*B*). The antibodies also specifically precipitated kinase activity from extracts of cells synchronized at two points in the cell cycle: at the beginning of S phase (by a thymidine/aphidicolin double block) and at metaphase (by nocodazole treatment and mitotic shake-off). As expected, CDC2 activity was highest in the metaphase-arrested cells (about 10-fold higher than in unsynchronized cells). CDK2 activity in these extracts did not vary so dramatically (Fig. 1*B*).

To investigate the activity of CDK2 during the cell cycle, HeLa cells were arrested at the G_1/S boundary by a thymidine/aphidicolin block and then released from the block to allow progress into S phase and beyond. According to analyses of DNA synthesis and mitotic index (Fig. 2*C*), these

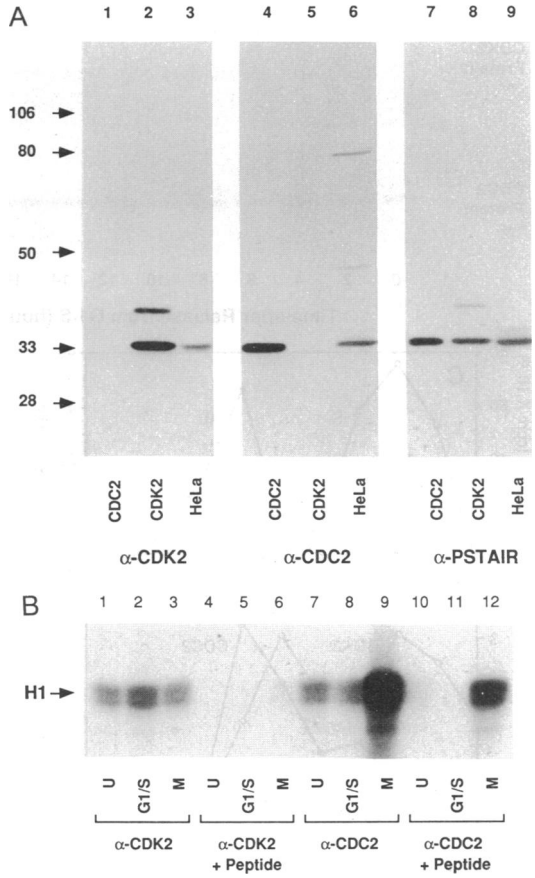

FIG. 1. Detection of CDK2 protein and kinase activity in HeLa cell lysates. (*A*) Lysates were prepared from unsynchronized HeLa cells (lanes 3, 6, and 9; 50 μg of total protein per lane) or from baculovirus-infected insect Sf9 cells overexpressing human CDC2 (lanes 1, 4, and 7; 50 ng of CDC2 per lane) or human CDK2 (lanes 2, 5, and 8; 50 ng of CDK2 per lane). Lysates were analyzed by immunoblotting with affinity-purified anti-CDK2 antibodies (lanes 1–3), affinity-purified anti-CDC2 antibodies (lanes 4–6), or crude antiserum against the PSTAIR peptide (lanes 7–9). Molecular masses (kDa) of prestained marker proteins (Bio-Rad) are indicated. The band above CDK2 in the insect cell lysates is an N-terminally extended version of the protein, resulting from a small amount of upstream translation initiation. (*B*) Lysates were prepared from unsynchronized HeLa cells (lanes 1, 4, 7, and 10), HeLa cells synchronized at the G_1/S boundary by thymidine/aphidicolin double block (lanes 2, 5, 8, and 11), or HeLa cells arrested in metaphase by nocodazole treatment and mitotic shake-off (lanes 3, 6, 9, and 12). Aliquots were immunoprecipitated with affinity-purified anti-CDK2 (lanes 1–6) or anti-CDC2 (lanes 7–12) in the absence (lanes 1–3 and 7–9) or presence (lanes 4–6 and 10–12) of an excess of the peptide antigen. Histone H1 kinase activity was measured in the immunoprecipitates, and the labeled histone H1 bands are shown.

cells progressed synchronously through S phase in about 8 hr and then moved through mitosis with reasonable synchrony after 12–14 hr. The ensuing G_1 phase then continued for an additional 6–8 hr, after which an asynchronous entry into the next S phase began. The behavior of CDC2 in these cells followed the expected pattern. Immunoblotting with anti-CDC2 (Fig. 2*B*) indicated that the level of the protein remained the same throughout the cycle, and previously observed bands of shifted mobility were found to increase during S and G_2 and disappear at the time of mitosis (28, 29). CDC2 kinase activity increased during S and G_2 and peaked at 12 hr, coincident with the peak mitotic index (Fig. 2*D*).

The pattern of CDK2 activation appeared quite distinct from that of CDC2 activation (Fig. 2*D*). CDK2 activity increased to a peak 4 hr after release from the G_1/S block, at the same time as the peak of DNA synthesis. It fell slightly

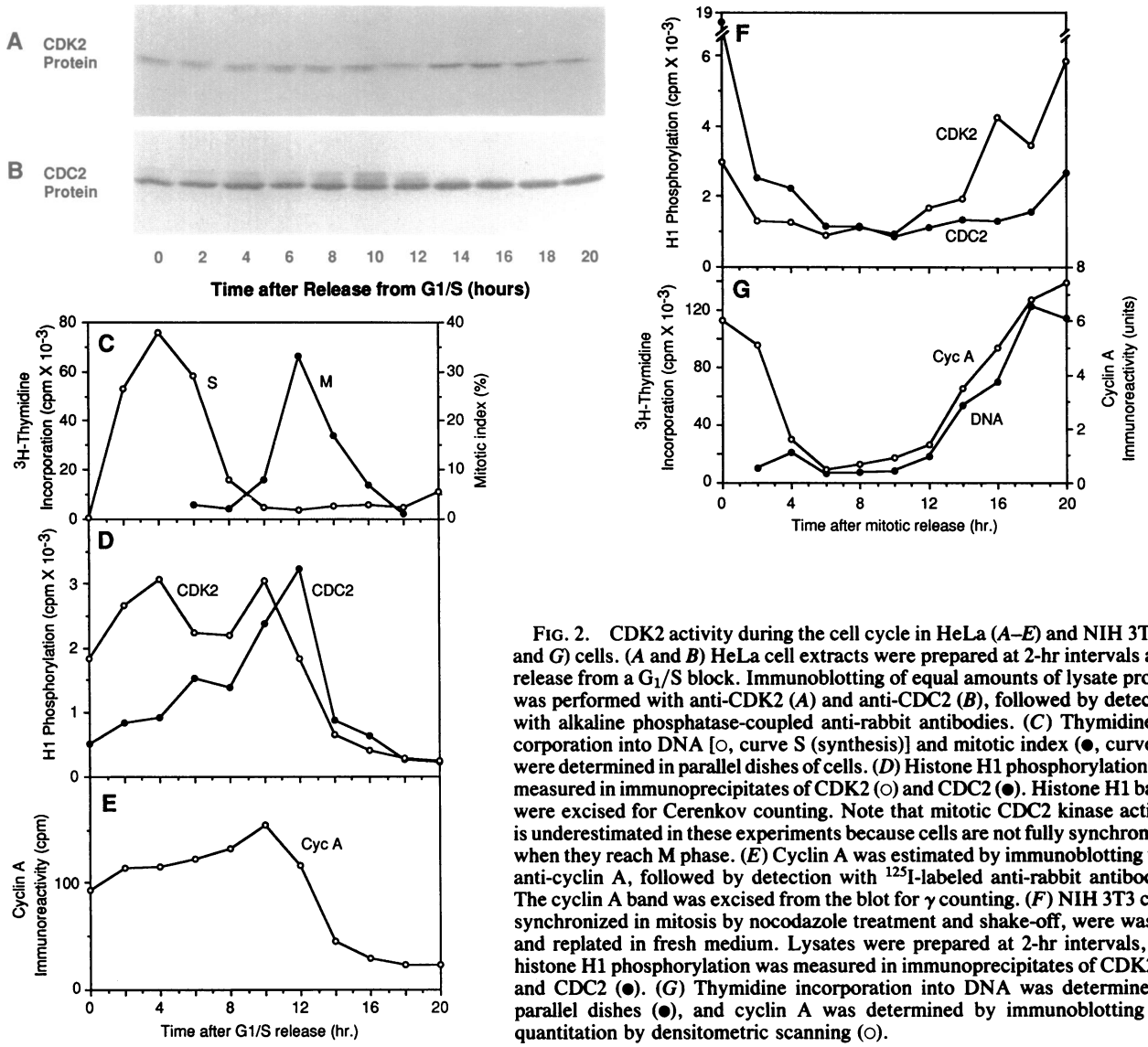

FIG. 2. CDK2 activity during the cell cycle in HeLa (*A–E*) and NIH 3T3 (*F* and *G*) cells. (*A* and *B*) HeLa cell extracts were prepared at 2-hr intervals after release from a G_1/S block. Immunoblotting of equal amounts of lysate protein was performed with anti-CDK2 (*A*) and anti-CDC2 (*B*), followed by detection with alkaline phosphatase-coupled anti-rabbit antibodies. (*C*) Thymidine incorporation into DNA [○, curve S (synthesis)] and mitotic index (●, curve M) were determined in parallel dishes of cells. (*D*) Histone H1 phosphorylation was measured in immunoprecipitates of CDK2 (○) and CDC2 (●). Histone H1 bands were excised for Cerenkov counting. Note that mitotic CDC2 kinase activity is underestimated in these experiments because cells are not fully synchronized when they reach M phase. (*E*) Cyclin A was estimated by immunoblotting with anti-cyclin A, followed by detection with ^{125}I-labeled anti-rabbit antibodies. The cyclin A band was excised from the blot for γ counting. (*F*) NIH 3T3 cells, synchronized in mitosis by nocodazole treatment and shake-off, were washed and replated in fresh medium. Lysates were prepared at 2-hr intervals, and histone H1 phosphorylation was measured in immunoprecipitates of CDK2 (○) and CDC2 (●). (*G*) Thymidine incorporation into DNA was determined in parallel dishes (●), and cyclin A was determined by immunoblotting and quantitation by densitometric scanning (○).

thereafter and then peaked again 10 hr after release, 2 hr before the peak of mitotic CDC2 activity. It then decreased during mitosis, in agreement with our measurement of CDK2 activity in cells more highly synchronized in mitosis by nocodazole arrest (Fig. 1*B*). The twin peaks of CDK2 activity were reproducible: a similar pattern of activity was observed in three separate experiments.

Immunoblotting showed that the level of CDK2 remained unchanged during progress through the cell cycle (Fig. 2*A*). During the first 12 hr after release from the block, CDK2 appeared as two equally intense bands: one at the usual position on the gel (at the same position as baculovirus-expressed protein; data not shown) and another at a lower position. The lower band was not seen in the presence of excess antigenic peptide (data not shown). This mobility shift may reflect a modification in CDK2 that accompanies activation (see *Discussion*).

To better assess the onset of CDK2 activation, mitotic NIH 3T3 fibroblasts were isolated by nocodazole treatment and then washed free of the drug to allow progress through G_1. DNA synthesis in these cells (Fig. 2*G*) indicated that S phase began 8–10 hr after release from the block. CDK2 activity in these cells decreased after mitosis and rose gradually in late G_1 or early S phase, roughly in parallel with DNA synthesis (Fig. 2*F*). Immunoblotting showed that CDK2 levels did not change during these experiments, and CDK2 activation was accompanied by an increase in electrophoretic mobility (data not shown).

Active CDK2 Is a Component of High Molecular Weight Complexes. We next addressed the possibility that the activation of CDK2, like that of CDC2, requires association with cyclins in high molecular weight complexes. We analyzed the molecular size of native CDK2 at a time in the cell cycle (S phase) when its activity is high. Lysates were prepared from HeLa cells 4 hr after release from a G_1/S block and fractionated by gel filtration on Superose 12. Fractions were analyzed by immunoblotting and immunoprecipitation with anti-CDK2. CDK2 immunoreactivity migrated at two major positions (Fig. 3*A*). First, a major fraction of the protein migrated at 30–40 kDa, as expected for monomeric CDK2. A second broad peak of CDK2 immunoreactivity was present in high molecular weight fractions. The kinase activity of CDK2 migrated on the column at the same position as the high molecular weight forms of CDK2 seen on the immunoblot, suggesting that complexed CDK2, and not the monomeric form, was active as a histone H1 kinase (Fig. 3*B*). The peak of kinase activity, like the peak immunoreactivity, migrated in the 300-kDa range, suggesting a heterogeneous complex of multiple subunits. There was also a small but significant shoulder of activity migrating at ≈100 kDa. To be certain that this second peak was not an artifact caused by the Triton X-100 used in lysis and column buffers, the experiment was repeated by extracting S-phase HeLa cells by homogeniza-

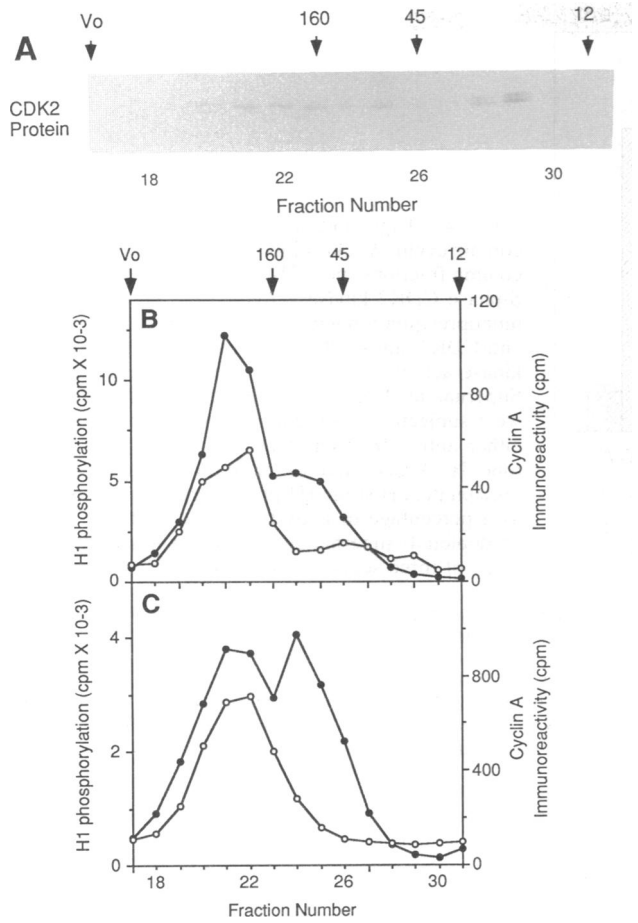

FIG. 3. Active CDK2 is found in multiple high molecular weight complexes. A Triton X-100 extract prepared from HeLa cells 4 hr after release from a G_1/S block was fractionated on a Superose 12 gel filtration column. (*A*) Fractions were analyzed by immunoblotting with anti-CDK2 antibodies. (*B*) Histone H1 kinase activity was measured in anti-CDK2 immunoprecipitates of each fraction (●), and the amount of cyclin A was determined by immunoblotting followed by detection with ^{125}I-labeled anti-rabbit antibodies (○). (*C*) CDK2 kinase activity (●) and cyclin A levels (○) were determined in a similar experiment, except that the Hela extract was prepared by Dounce homogenization in hypotonic lysis buffer instead of by Triton X-100 solubilization. The molecular masses (kDa) of marker proteins, determined in parallel runs, are indicated (Vo, void volume; 160, IgG; 45, ovalbumin; 12, cytochrome *c*).

tion in a detergent-free hypotonic buffer. This extract was run over the column in the absence of detergent, and analysis of the fractions indicated that the smaller peak of CDK2 activity was even more prominent (Fig. 3*C*).

CDK2 species of two electrophoretic mobilities were observed in immunoblots of the column fractions (Fig. 3*A*). Monomeric CDK2 was composed of both forms, while CDK2 in high molecular weight complexes was entirely composed of the faster migrating form. Thus, as seen in the experiments above (Fig. 2*A*), the lower band accompanied the active form of the CDK2 kinase. However, the presence of the lower band in the inactive monomer fraction indicates that the mobility shift, if indeed it is required for activation, is not sufficient.

Association of CDK2 with Cyclin A. The observation that active CDK2 was found in high molecular weight complexes suggested that active CDK2, like active CDC2, is complexed with cyclin molecules. Our observation that the cell cycle timing of CDK2 activation was similar to previously observed cell cycle changes in cyclin A levels (8) suggested that CDK2 might be bound to cyclin A. We used specific polyclonal antibodies (8) to analyze the levels of cyclin A in our crude and fractionated extracts of synchronized HeLa and NIH 3T3 cells. Immunoblotting confirmed that the timing of cyclin A expression correlated reasonably well with CDK2 activity in the cell (Fig. 2 *E* and *G*). In addition, analysis of fractionated S-phase extracts showed that cyclin A migrated with the high molecular weight peak of CDK2 activity (Fig. 3 *B* and *C*) but not with the low molecular weight peak of CDK2 activity.

These correlations suggested that a subpopulation of CDK2 might be associating with cyclin A. More-direct evidence for an association was obtained by serial immunoprecipitation experiments. Aliquots of column fractions containing the high molecular weight CDK2 complex were depleted of CDK2, CDC2, or cyclin A by immunoprecipitation (Fig. 4*A*). The supernatants from these immunoprecipitations were then reprecipitated with the various antibodies, and histone H1 kinase activity was measured in the pellets. The first immunoprecipitation step removed the majority of the antigen, since only a small fraction of the original activity was detected when supernatants were reprecipitated with the same antibody used in the first precipitation (Fig. 4*B*, bars 1, 5, and 9). Depletion of cyclin A resulted in the loss of most CDK2 from the supernatant (bar 7), suggesting that the majority of CDK2 in these fractions was associating with cyclin A. Reprecipitation of a CDK2-depleted fraction with anti-cyclin A revealed that the majority of the cyclin A had coprecipitated with CDK2 (bar 3). Immunoblot analysis of these supernatants and pellets, although less sensitive than kinase assays, yielded similar results. These results suggest that most of the CDK2 in these fractions associates with most of the cyclin A, but that there may be additional subpopulations of both CDK2 and cyclin A that form active kinase complexes with other partners.

DISCUSSION

We have identified and characterized human CDK2, a member of the CDC2-like family of cyclin-dependent protein kinases. Like CDC2, CDK2 exhibits histone H1 kinase activity that oscillates during the cell cycle. However, while CDC2 activity reaches a peak during mitosis, CDK2 activity exhibits a more complex pattern of activation that includes peaks of activation coinciding with the S and G_2 phases. This complex pattern of CDK2 activation is consistent with a role in multiple cell cycle processes ranging from S-phase initiation or maintenance to the preparation for mitosis.

The kinase activity of CDK2, like that of CDC2, appears to be regulated by association with cyclins. Both kinases are activated in COS cells when coexpressed with high levels of human cyclin A or B (unpublished work), and both kinases are activated by cyclins *in vitro*, as shown by using proteins overexpressed with the baculovirus system (D. Desai and D.O.M., unpublished work). In addition, the periodic oscillations of CDK2 activity during the cell cycle are consistent with an activation mechanism involving an association with cyclin regulatory subunits. Finally, gel filtration of HeLa cell extracts shows that the active form of the CDK2 kinase (like active CDC2) exists in high molecular weight complexes that contain cyclins; monomeric CDK2 appears inactive. Active CDK2 exhibits an increased mobility in polyacrylamide gels, and [^{32}P]phosphate labeling of HeLa cells indicates that this shift is probably due to phosphorylation (Y.G., unpublished work). Thus we speculate that CDK2 activation, like CDC2 activation, involves changes in its phosphorylation state as well as cyclin association.

We have partially characterized the components of high molecular weight complexes containing active CDK2. During S phase, two distinct CDK2-containing complexes are apparent on gel filtration analysis. The larger complex (≈300 kDa) comigrates with cyclin A on the gel filtration column,

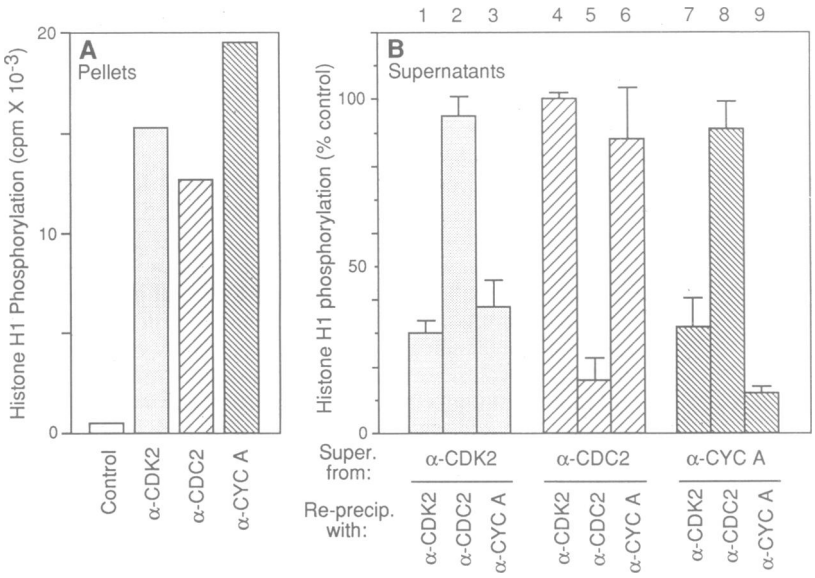

FIG. 4. High molecular weight complexes of CDK2 contain cyclin A. (*A*) Aliquots of high molecular weight column fractions (nos. 21–22 from Fig. 3*B*) containing S-phase CDK2 kinase activity were subjected to immunoprecipitation with normal rabbit serum (control), anti-CDK2, anti-CDC2, and anti-cyclin A. Histone H1 kinase activity was measured in the precipitates. (*B*) Supernatants from the four immunoprecipitations in *A* were subjected to a second immunoprecipitation with either anti-CDK2, anti-CDC2, or anti-cyclin A. Histone H1 kinase activity was then measured in the precipitates. Histone H1 phosphorylation is presented as a percentage of activity in precipitates of control (undepleted) supernatants. Results shown are the average of three separate experiments (± SEM).

and immunodepletion studies suggest that the majority of CDK2 in this complex is associated with cyclin A. Our results therefore agree with the recent work of Tsai *et al.* (15), who demonstrated by peptide mapping that CDK2 associates with cyclin A. Although our analysis is restricted to S-phase CDK2 complexes, we suspect that the p33 protein associated with cyclin A during G_2 (14) is also CDK2.

The nature of the smaller CDK2 complex, which does not comigrate with cyclin A, remains unclear. Although this form of CDK2 may simply represent an activated multimer of CDK2, it seems likely that this complex represents CDK2 bound to a different cyclin. Potential candidates include cyclin E, which can interact with CDK2 when expressed in yeast (13), and cyclin D, which associates with a protein that is related but not identical to CDC2 (10).

The identification of a second human CDC2-like kinase suggests that higher eukaryotes have evolved multiple forms of CDC2 to fulfill the function of a single form in yeast. It remains to be seen how the Start and mitotic functions of CDC2 in yeast are divided among multiple proteins in higher eukaryotes. Since previous evidence supports a role for CDC2 in mitosis and does not clearly indicate a role in Start (16, 17), it is tempting to speculate that human CDK2 and CDC2 are responsible for nonoverlapping functions at Start and mitosis, respectively. However, the complex timing of CDK2 activation and its association with cyclin A are consistent with a role for CDK2 in both S phase and mitosis. Similarly, several lines of evidence implicate CDC2 in the control of early stages of the cell cycle (30, 31). Thus, it seems likely that CDK2 and CDC2 are involved in the control of overlapping cell cycle events.

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