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Biochemistry

CDK2 encodes a 33-kDa cyclin A-associated protein kinase and is expressed before *CDC2* in the cell cycle

(cell cycle regulation/cyclins/threonine-serine protein kinase/phosphotyrosine)

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ABSTRACT **Critical cell cycle transitions are controlled by the coordinate actions of the p34^{cdc2} protein kinase and its regulatory subunits, cyclins. Recently we identified another human p34 homolog, cyclin-dependent kinase 2 (CDK2) by complementation of a *cdc28-4* mutation in *Saccharomyces cerevisiae* using a λYES human cDNA expression library. *CDK2* is 66% identical to *CDC2Hs* and 89% identical to the *Xenopus Eg1* gene, forming a distinct subfamily of CDC2-related protein kinases. We have found that *CDK2* encodes a 33-kDa cyclin A-associated protein kinase that contains phosphotyrosine, two characteristics it shares with *CDC2Hs*. However, we show that the subunit composition of these two protein kinase complexes can vary in different cell types, that they have different *in vitro* substrate preferences, and that CDK2 mRNA is observed much earlier than CDC2Hs mRNA when lymphocytes are stimulated to enter the cell cycle. We suggest that cells in different developmental or transformed states may have different mechanisms of cell cycle regulation.**

Critical transitions in the eukaryotic cell cycle are controlled by the coordinate action of a protein kinase(s), p34^{cdc2}, and the associated regulatory subunits, cyclins (1–3). Originally, cyclins were defined as proteins that varied in abundance with periodicity of the cell cycle (4–7); subsequently cyclins were shown to be a family of structurally related proteins that regulate the timing of activation of p34^{cdc2} (8–12). Evidence in budding yeast indicates that two distinct classes of cyclins, the G_1 cyclins (13, 14) and the mitotic B-type cyclins (15), interact with a single protein kinase, Cdc28 (the *Saccharomyces cerevisiae* Cdc2 homolog), to regulate the G_1-to-S and G_2-to-M transitions, respectively. The situation in higher eukaryotes is more complex. Lee and Nurse (16) identified *CDC2Hs*, a functional human homolog of the *Schizosaccharomyces pombe* gene *cdc2*. We recently identified another homolog of *CDC2Hs* (17), *CDK2*, by complementation of a *cdc28-4* mutation in *S. cerevisiae*, with a λYES human cDNA expression library (18). *CDK2* is 89% identical to the *Xenopus CDC2*-homolog *Eg1* (18, 19) but is only 66% identical to *CDC2Hs*, indicating that Cdk2 and Eg1 form a distinct subfamily of *CDC2*-related protein kinases. *In vitro* experiments with *Xenopus* egg extracts show that depletion of a Cdc2-like protein (20), which has recently been shown to be the *Xenopus* Cdk2 homolog (Cdc2b; Eg1), interferes with DNA synthesis (21). In contrast, depletion of Cdc2 alone does not affect DNA synthesis but blocks mitosis. This fact suggests that, in contrast to the situation in yeast, the G_1-to-S and G_2-to-M transition points may be controlled by different kinases in higher eukaryotes.

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A variety of mammalian cyclins have also been identified (5, 22–28), but except for the B-type cyclins their functions are largely unknown. The best-characterized cyclin complex is the mitotic cyclin B/p34^{cdc2} kinase, the active component of maturating promoting factor (5, 9–10). Cyclin A accumulates before cyclin B in the cell cycle (23) and may be involved in control of S phase (3, 23). In addition, cyclin A has been implicated in cellular transformation (27) and is found in complexes with E1a (29), transcription factors DRTF1 and E2F (30, 39), and the retinoblastoma protein (31). Although cyclin A associates with a Cdc2-like protein in HeLa cells, immunological data indicate this protein is not p34^{cdc2} (23).

Understanding how mammalian cells control cell cycle transitions will require extensive analysis of which cyclins associate with which kinases, the timing and localization of these associations, posttranslational modifications, and the target substrates *in vivo*. Toward these goals, we have initiated an analysis of the expression and subunit composition of the CDK2 protein kinase in human peripheral blood lymphocytes (PBLs) and HeLa cells. Here, we show that *CDK2* encodes a cyclin A-associated protein kinase, that *CDK2* contains phosphotyrosine, and that the *CDK2* mRNA is expressed earlier than *CDC2Hs* mRNA when cells are stimulated to enter the cell cycle.

MATERIALS AND METHODS

Recombinant DNA Manipulations. Bacterial expression plasmids for Cdk2, Cdc2Hs, cyclin A, cyclin B, cyclin D, and Ckshs-1 were obtained by subcloning PCR-derived fragments into pET-T7 expression vectors (32). Plasmid DNAs for Cdc2Hs and Cdk2 (17) or human cDNA libraries (18) were used as PCR template. To generate the hemagglutinin A (HA) epitope-tag (33) expression vector (pXHA), oligonucleotides containing 5′ *Nco* I and 3′ *Bam*HI restriction sites and encoding sequence MAYPYDVPDYASLGGHMRPRI were subcloned into a modified version of pET8c digested with *Nco* I and *Bam*HI. Coding sequences for Cdk2 and Cdc2Hs (as *Nde* I–*Bam*HI fragments) were subcloned into pXHA vector in-frame at Met-17, and the resulting HA-fusion cassettes (as *Xho* I–*Bam*HI fragments) were transferred to plasmid pSE936 (18) for expression in *S. cerevesiae*. These epitope-tagged kinases are referred to as HA–Cdk2 and HA–Cdc2Hs. Corresponding plasmids for Cdk2 and Cdc2Hs were from a previous study (17).

Abbreviations: PBL, peripheral blood lymphocyte; CDK2, cyclin-dependent kinase 2; HA, hemagglutinin A; α-Cdk2^{CT}, C-terminal-specific Cdk2 antibodies; α-cyclin A^{NT}, N-terminal-specific cyclin A antibodies; α-cyclin A^{CHLA-1}, C-terminal-specific cyclin A antibodies; RF-A, replication factor A; PHA, phytohemagglutinin-P.

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Antibodies. Proteins for antibody production were produced in *Escherichia coli* strain BL21(DE3) (32), isolated from inclusion bodies by preparative SDS/PAGE, and injected into rabbits (34). For affinity purification, α-Cdk2 antiserum was first depleted of antibodies that react with Cdc2Hs by passage through a Cdc2Hs-Affi-Gel column (≈1 mg of Cdc2Hs per ml of resin). Nonabsorbed antibodies were then passed through a Cdk2-Affi-Gel column, and the adsorbed antibodies were released with 0.2 M glycine (pH 3). N-Terminal-specific cyclin A antibodies (α-cyclin A^{NT}) were affinity-purified by using cyclin A(1–114)-Affi-Gel. Antibodies recognizing the C-terminal Cdk2 sequence TKPVPHLRL, α-$Cdk2^{CT}$, were generated in rabbits and affinity-purified by using the peptide linked to Affi-Gel-10. α-Cdc2Hs IgG, recognizing the C terminus of Cdc2Hs (DNQIKKN), and antibodies recognizing the C-terminal region of cyclin A (α-cyclin A^{CHLA-1}) were from a previous study (35). α-Phosphotyrosine was provided by Tony Pawson, Mount Sinai Hospital, Toronto. Coupling of antibodies to protein A-Sepharose beads used standard procedures (34). Although the beads were washed extensively with 0.1 M glycine (pH 3) to remove most of the noncovalently bound antibodies before use, a small amount of IgG was released from some batches of beads with SDS sample buffer.

Cell Culture, Immunoprecipitations, and Protein Purification. Freshly isolated PBLs (2×10^6 cells per ml) in RPMI 1640/10% fetal calf serum were stimulated with phytohemagglutinin-P (PHA, 1 μg/ml, Burroughs Wellcome).

Suspension HeLa S3 cells (ATCC CCL2.2) were grown in RPMI medium/7% fetal calf serum and harvested at midlogarithmic phase. Adherent HeLa cells (derived from ATCC CCL 2) were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum and harvested at 50–70% confluence. Washed cells were lysed by using 0.1% Nonidet P-40 buffers (9). For immunoprecipitations, extracts (2 mg of protein) were incubated with either ≈5 μg of α-cyclin A^{NT}, 10 μg of preimmune serum, 40 μl of Ckshs-1 beads, 0.5–10 μg of α-Cdc2Hs IgG, 20 μl of α-cyclin A^{NT} beads, 50 μl of α-$Cdk2^{CT}$ beads, or 20 μl of preimmune beads for 1 hr (4°C), and immunoprecipitates were collected on protein A-Sepharose (40 μl) where necessary. Beads were washed three times in extraction buffer, and bound proteins were released by using 2× SDS sample buffer containing 100 mM *N*-ethylmaleimide unless otherwise noted, before immunoblotting. Under these conditions most antibodies released from protein A by SDS migrated at >100 kDa. For kinase assays, 10% of Cdk2 and Cdc2Hs immune complexes from suspension HeLa cell extracts (2 mg of protein) were incubated with 1 μg of histone H1 and 25 μM [γ-^{32}P]ATP (25 μl, total volume) for 15 min at 37°C, and the relative activities were determined by using phosphocellulose filter binding. Aliquots corresponding to equal quantities of histone H1 activity for the Cdk2 and Cdc2Hs immune complexes and an equal volume of preimmune control beads were then incubated with either 1 μg of human replication factor A (RF-A) or 1 μg of histone H1 as described above, and the products were separated by SDS/PAGE before autoradiography. Homogeneous RF-A consisting of 14-, 34-, and 70-kDa subunits was provided by Anindya Dutta and Bruce Stillman (Cold Spring Harbor Laboratory). Homogeneous Ckshs-1 protein (36) was coupled to Affi-Gel-10 at a concentration of 5 mg/ml of resin.

RESULTS

Cdk2 Associates with Cyclin A in HeLa Cells. Previous studies indicate that the α-cyclin A monoclonal antibody C160 coimmunoprecipitates a Cdc2-related protein, p33, in HeLa cells (23, 29). Although p33 reacts with antibodies directed toward the α-PSTAIRE motif of Cdc2Hs, it does not react with C-terminal-specific Cdc2Hs antibodies (29) and displays a tryptic peptide map distinct from Cdc2Hs (23). The sequence similarity of Cdk2 and Cdc2Hs (17) led us to examine whether *CDK2* encodes the cyclin A-associated p33. Using affinity purification, we generated polyclonal antibodies specific for Cdk2. We determined their specificity by showing that they recognize epitope-tagged Cdk2 (HA–Cdk2) but not epitope-tagged Cdc2Hs (HA–Cdc2Hs) expressed in yeast (Fig. 1*A*, *Left*). α-HA antibodies were used as a positive control for HA–Cdc2Hs and HA–Cdk2 expression (Fig. 1*A*, *Middle*). In HeLa cell extracts, these antibodies recognize a doublet of proteins of ≈33–34 kDa that comigrate with *E. coli*-expressed Cdk2 (Fig. 1*A*, *Right*). The faster migrating band (≈33 kDa) is ≈5% of total Cdk2. When blots are overdeveloped to enhance staining of weakly crossreacting proteins, a HeLa cell protein near 45 kDa is detected. This blot could reflect common epitopes in the two proteins or the presence of impurities in our affinity-purified α-Cdk2 preparations. Because these affinity-purified α-Cdk2 antibodies

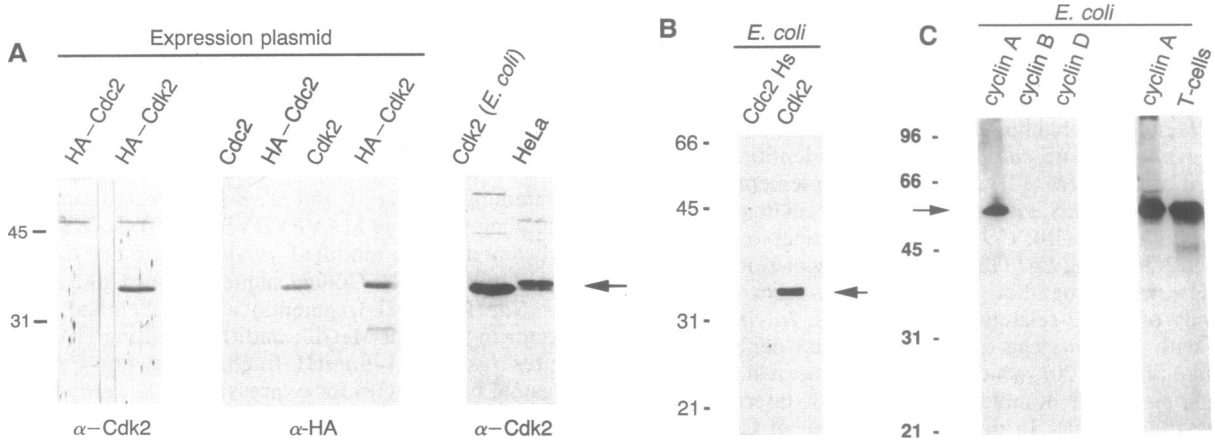

FIG. 1. Characterization of α-Cdk2 and α-cyclin A antibodies. (*A Left*) Proteins (100 μg) from strain Y61 (17) expressing epitope-tagged HA–Cdk2 and HA–Cdc2Hs were immunoblotted with α-Cdk2 (1:2000) antibodies. (*Middle*) Proteins from yeast expressing Cdk2, HA–Cdk2, Cdc2Hs, and HA–Cdc2Hs were immunoblotted with α-HA antibodies (Babco, Emeryville, CA; 1:1000). (*Right*) HeLa cell proteins (25 μg) and an *E. coli* lysate containing 100 ng of Cdk2 were immunoblotted with α-Cdk2. Arrow indicates position of Cdk2 expressed in *E. coli*. (*B*) Extracts from *E. coli* expressing indicated protein (≈30 ng each) were immunoblotted with α-$Cdk2^{CT}$ (1:5000). (*C*) Total proteins from *E. coli* expressing indicated human cyclins (60 ng of cyclin per lane) and from T cells (50 μg) were immunoblotted with α-cyclin A^{NT} (1:20,000). Arrow indicates position of cyclin A expressed in *E. coli*. Detection was accomplished by using alkaline phosphatase-labeled secondary antibodies for *A* and *B* and chemiluminescence (Amersham) for *C*. Molecular mass markers are shown in kDa.

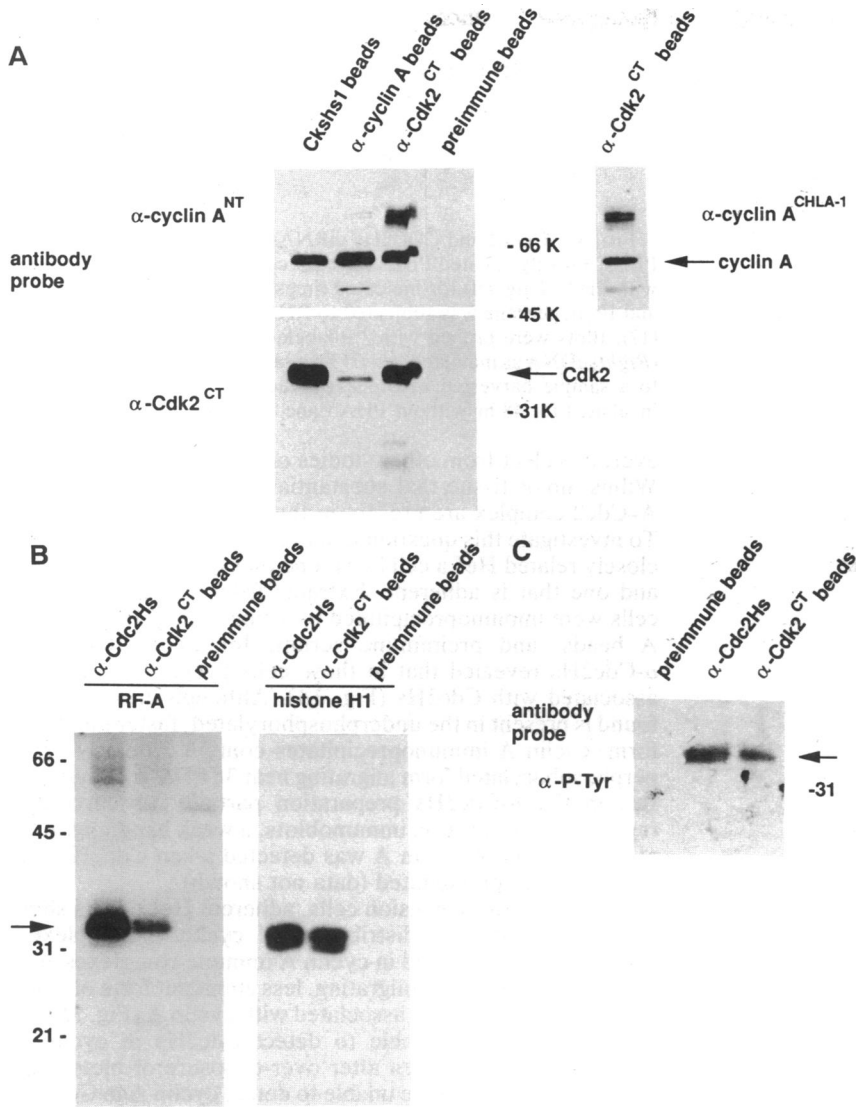

FIG. 2. *CDK2* encodes a 33-kDa cyclin A-associated protein kinase. (*A*) Extracts (2 mg of protein) from suspension HeLa cells were immunoprecipitated with either Ckshs1 beads, α-cyclin A^{NT} beads, α-$Cdk2^{CT}$ beads, or preimmune beads, immunoblotted, and probed with either α-cyclin A^{NT}, α-$Cdk2^{CT}$, or α-cyclin A^{CHLA-1} as indicated, by using chemiluminescence detection. The preimmune gel lane was from the same blot but was not adjacent to other lanes. (*B*) Kinase activity of Cdk2 and Cdc2Hs immune complexes toward histone H1 and 34-kDa subunit of RF-A (see *Materials and Methods*). RF-A exposure was 16 hr; histone H1 exposure was 3 hr. (*C*) Cdk2, Cdc2Hs, and preimmune immunoprecipitates were immunoblotted by using α-phosphotyrosine antibodies (1:1000).

do not immunoprecipitate Cdk2 from HeLa extracts, we also generated α-$Cdk2^{CT}$ (Fig. 1*B*), which immunoprecipitates Cdk2 (see below). Antibodies recognizing epitopes within residues 1–114 of cyclin A, α-cyclin A^{NT}, were also generated. These antibodies detect a protein of 60 kDa in human T cells (and HeLa cells, data not shown) that comigrates with cyclin A expressed in *E. coli* but do not react with human cyclin B or cyclin D (Fig. 1*C*).

HeLa cell extracts from cells grown in suspension were immunoprecipitated using α-cyclin A^{NT} beads, α-$Cdk2^{CT}$ beads, or preimmune beads and were then immunoblotted. As a positive control, extracts were incubated with immobilized Ckshs-1, the human homolog of $p13^{suc1}$ from *S. pombe* (36), which binds Cdc2Hs and related proteins including p33 (23) and allows affinity purification of monomeric p34 and p34–cyclin complexes. When the upper portion of the blot (>45 kDa) was probed with α-cyclin A^{NT} (Fig. 2*A*), a protein of 60 kDa was detected in the α-$Cdk2^{CT}$ immunoprecipitate that comigrated with cyclin A found in both α-cyclin A immune complex and in proteins bound by Ckshs-1 beads. The immunoreactivity near 85 kDa in lanes derived from immunoprecipitates is due to an IgG fragment released from the antibody beads by SDS. The identity of the 60-kDa protein recognized by α-cyclin A^{NT} in Cdk2 immune complexes was confirmed by the finding that a second, independently produced α-cyclin A antibody (α-cyclin A^{CHLA-1}, ref. 35) directed against a C-terminal epitope also reacted with the 60-kDa protein (Fig. 2*A*).

α-$Cdk2^{CT}$ recognizes a single protein migrating at ≈33 kDa in the cyclin A immune complex, which is absent in the preimmune sample (Fig. 2*A*). In proteins bound by Ckshs1 beads or α-$Cdk2^{CT}$ beads, two distinct forms of Cdk2 that migrate as a 33- to 34-kDa doublet similar to that found in total HeLa cells (Fig. 1*A*) were detected upon shorter blot exposure (Fig. 2*A*). This finding may reflect alternatively phosphorylated forms of the protein, as seen for $p34^{cdc2}$. Interestingly, cyclin A associates only with the faster migrating 33-kDa form of Cdk2, which is present at ≈20-fold lower levels than the slower migrating form.

Cdk2 Is a Protein Kinase and Displays Substrate Preferences Distinct from Cdc2Hs. Cdk2 immune complexes from suspension HeLa cell extracts display histone H1 kinase activity comparable to Cdc2Hs immune complexes (Fig. 2*B*). RF-A was also examined as a substrate. The 34-kDa subunit of RF-A becomes phosphorylated at the G_1-to-S transition (37) and has been shown to be a substrate for Cdc2Hs *in vitro* (A. Dutta and B. Stillman, personal communication). When equal portions of immune complexes were used for RF-A and histone H1 kinase assays, Cdk2 kinase complexes displayed ≈10-fold lower relative activity toward RF-A than the Cdc2Hs kinase complexes (Fig. 2*B*).

Cdk2 Contains Phosphotyrosine. To examine whether or not Cdk2 contains phosphotyrosine, Cdk2 immune complexes were immunoblotted with α-phosphotyrosine antibodies; Cdc2Hs immune complexes served as a positive control. A band was identified in the Cdk2 (Fig. 2*C*) that had

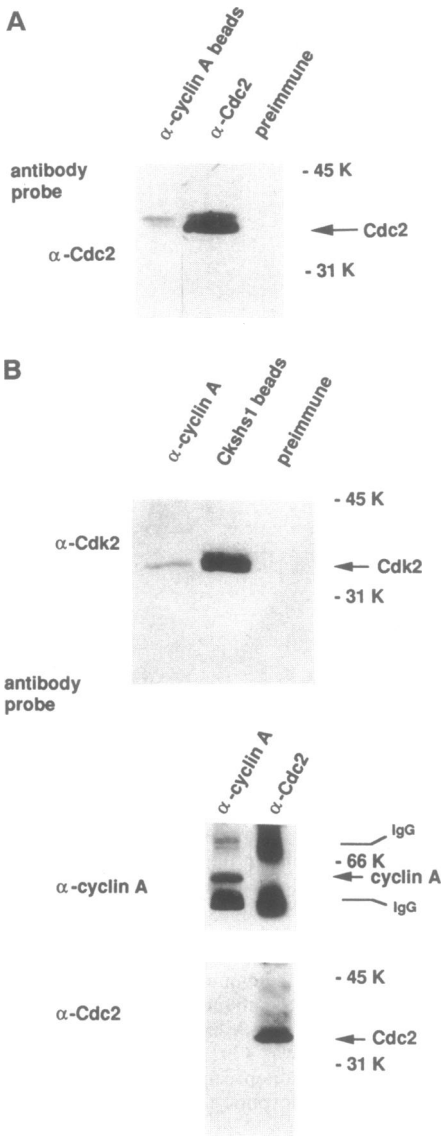

FIG. 3. (A) Differential association of cyclin A with Cdk2 and Cdc2. (*A*) Extracts (2 mg of protein) from HeLa cells grown in suspension were incubated with either α-Cdc2Hs (5 μg), α-cyclin A^{NT} beads, or preimmune serum and immunoblotted with α-Cdc2Hs (1:4000) by using chemiluminescence detection. Gel lanes used in this composite figure were derived from a single blot within each experiment but were not adjacent in original gel. (*B*) Extracts (2 mg of protein) from adherent HeLa cells were incubated with either α-cyclin A^{NT}, preimmune serum, α-Cdc2Hs (10 μg), or Ckshs-1 beads, and the resulting complexes were immunoblotted with either α-Cdk2, α-cyclin A^{NT}, or α-Cdc2Hs antibodies. *N*-ethylmaleimide was not used in sample buffer for this experiment, and bands located at ≈85 and 50 kDa are from IgG released from protein A beads by SDS.

essentially comigrated with the major phosphotyrosine-containing form of Cdc2Hs under the gel conditions used.

Cyclin A Differentially Associates with Cdk2 and Cdc2 in Different Cell Types. Results of several studies in a variety of cell lines aimed at determining whether cyclin A associates with p33 or $p34^{cdc2}$ have shown inconsistencies difficult to interpret. One study using the cyclin A monoclonal antibody C160 indicated that in adherent ^{35}S-labeled HeLa cells cyclin A is associated with p33 and not detectably with $p34^{cdc2}$ (23). Similar results were obtained in an independent study with C160 in conjunction with adherent HeLa cells (see figures 3 and 5 in ref. 29), but, in addition, very small amounts of cyclin A were detected in Cdc2Hs immune complexes (29). However, it is clear from other studies on mouse FM3A cells and Wilms tumor tissue that substantial quantities of the cyclin A–Cdc2 complex are present in these cells and tissues (35). To investigate this question in more detail, we examined two closely related HeLa cell lines, one that grows in suspension and one that is adherent. Extracts from suspension HeLa cells were immunoprecipitated by using α-Cdc2Hs, α-cyclin A beads, and preimmune serum. Immunoblotting with α-Cdc2Hs revealed that in these cells cyclin A is, indeed, associated with Cdc2Hs (Fig. 3*A*). Although most Cdc2Hs found is present in the underphosphorylated, faster migrating form, cyclin A immunoprecipitates contain primarily a hyperphosphorylated form migrating near 35 kDa. Although the IgG in the α-Cdc2Hs preparation partially obscured the region near cyclin A on immunoblots, a weak band migrating at the position of cyclin A was detected when Cdc2Hs was quantitatively precipitated (data not shown).

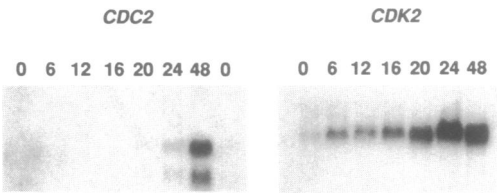

FIG. 4. CDK2 and CDC2Hs mRNAs in PHA-stimulated human PBLs. Freshly isolated PBLs (2×10^6 cells per ml) were stimulated with PHA (1 μg/ml) for indicated times (in hr), RNA was isolated, and 10 μg per lane was subjected to RNA blot analysis as described (17). Blots were probed with ^{32}P-labeled CDC2Hs (*Left*) or CDK2 (*Right*) cDNA as indicated. (*Left*) Two samples indicated as 0 hr refer to a sample harvested before PHA addition (lane 1) or a sample incubated for 48 hr without PHA (lane 8).

In contrast to suspension cells, adherent HeLa cells show a strikingly different distribution of cyclin A complexes. Cdk2 is readily detected in cyclin A immune complexes and, as expected, the faster migrating, less abundant form of Cdk2 at 33 kDa is exclusively associated with cyclin A (Fig. 3*B*). In contrast, we were unable to detect Cdc2Hs in cyclin A immune complexes even after over-exposure of blots (Fig. 3*B*). Moreover, we were unable to detect cyclin A in Cdc2Hs immune complexes from this cell line (Fig. 3*B*). In two other cell lines examined, human T lymphocytes and mouse FM3A cells, both Cdk2 and Cdc2 are readily detected in cyclin A immune complexes (data not shown). These data suggest that in different transformed or developmental states, the proportion of cyclin A in complexes with Cdc2, Cdk2, and perhaps other Cdc2-related proteins can vary.

Expression of *CDK2* in Human PBLs. Previous studies indicated that Cdc2 mRNA is first detected at or near the G_1/S boundary when PBLs are induced to re-enter the cell cycle (38). As cyclin A mRNA also begins to accumulate just before entry into S phase in this system (data not shown), it was of interest to examine temporal expression of Cdc2Hs and Cdk2 with the activated lymphocyte system (38). In unstimulated PBLs, CDC2Hs mRNA levels are very low, but CDK2 message is readily detected in G_0 (Fig. 4). Increase in CDC2Hs mRNA is first detected 24 hr after stimulation (Fig. 4 *Left*) (38). In contrast, CDK2 mRNA abundance increases above the G_0 level 16-hr after stimulation and peaks near 24 hr (Fig. 4 *Right*). Flow cytometry analysis (5) of these cells showed that entry into S phase occurred between 20 and 24 hr, consistent with previous studies (38). Thus, induction of *CDK2* expression precedes *CDC2Hs* induction, occurring before initiation of S phase and, therefore, these genes are regulated differently at the level of transcription.

DISCUSSION

The primary observations of our studies are (*i*) *CDK2* encodes a 33-kDa cyclin A-associated protein kinase; (*ii*) Cdk2

contains phosphotyrosine, suggesting a mechanism for its regulation in the cell cycle; (*iii*) in many cell types including suspension HeLa cells, human T cells, and mouse FM3A cells cyclin A associates with both Cdk2 and Cdc2, whereas in adherent HeLa cells, cyclin A appears to be primarily, if not exclusively, associated with Cdk2; (*iv*) two electrophoretically distinct forms of Cdk2 can bind the Ckshs1 protein, although only the faster migrating species is found associated with cyclin A; (*v*) we have shown that the Cdc2Hs and Cdk2 kinase complexes have different substrate preferences *in vitro*; and (*vi*) CDK2 message is present in G_0 cells, and its mRNA levels increase upon entry into the cell cycle before an increase in CDC2 mRNA.

While this work was in progress, an independent study (40) also identifying p33 as Cdk2 found that the cyclin A–Cdk2 complex, as opposed to the cyclin A–Cdc2 complex, is the target for E1a. Cdk2 may also interact with other cyclins, as is the case when expressed in *S. cerevisiae*, (17). In addition, *Xenopus* Cdk2 appears to interact primarily with proteins distinct from cyclin A in activated egg extracts (21). The finding that cyclin A-associated kinase subunits differ in different cells suggests that cells of different developmental or transformed states may use distinct mechanisms of cell cycle regulation.

Immune complexes containing Cdk2 and Cdc2Hs have different reactivities toward RF-A and histone H1, suggesting the improbability of completely overlapping specificities *in vivo*. Although the relevance of *in vitro* phosphorylation of RF-A by Cdk2 and Cdc2Hs is unknown, the reduced activity of Cdk2 toward RF-A is contrary to what might be expected by its putative role in S phase. However, it is premature to conclude that Cdk2 has less *in vivo* activity toward RF-A than does Cdc2Hs for four reasons. (*i*) Each immunoprecipitate is likely to contain a mixture of cyclins complexed to a variety of modified kinase forms that represent an average of complexes present throughout the cell cycle. The only complexes potentially relevant to RF-A activation are those present and active at the correct time and in the proper cellular compartment to interact with RF-A. (*ii*) The total amounts of active complex in these immune precipitates are unknown, and therefore absolute specific activities are impossible to calculate. For example, the activity of Cdk2 could actually be quite high toward RF-A and it also could have a higher specific activity toward histone H1 than does Cdc2Hs. (*iii*) The sites of phosphorylation by Cdk2 are unknown, and phosphorylation of distinct sites may have different ramifications for RF-A function. (*iv*) The presence of IgGs bound to the kinases may provide steric interference that differentially alters activity toward RF-A.

The discovery of phosphotyrosine in Cdk2 points toward a regulatory role for phosphorylation. The Cdc2–cyclin B complex is negatively regulated by phosphorylation of Tyr-15, located in the ATP-binding site (11). There are eight tyrosines in Cdk2, seven of which are conserved with Eg1. Identification of the position(s) of phosphorylation, and correlation of cell cycle timing and kinase activity will be required to establish the significance of this modification; it may be responsible for the two distinctly migrating forms seen in immunoblots.

That the CDK2 subfamily diverges considerably from CDC2 subfamily likely reflects different roles in regulation of the mammalian cell cycle. Depletion of *Xenopus* Cdk2 (Eg1, Cdc2b) but not of Cdc2 (Cdc2a) from activated egg extracts interferes with DNA synthesis (21), suggesting that the highly homologous *CDK2* may also play an integral role in S phase in human cells, perhaps controlling the G_1-to-S-phase transition. This result is consistent with the timing of *CDK2* expression. However, analysis of the protein levels must be done to determine significance of the mRNA expression pattern. The timing of $p33^{cdk2}$ association with cyclin A and possibly other proteins during the cell cycle and regulation of the resulting kinase activities may be critical features in regulating mammalian cell proliferation and deserve further study.

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