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Identification of G_1 Kinase Activity for cdk6, a Novel Cyclin D Partner

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A family of vertebrate cdc2-related kinases has been identified, and these kinases are candidates for roles in cell cycle regulation. Here, we show that the human PLSTIRE gene product is a novel cyclin-dependent kinase, cdk6. The cdk6 kinase is associated with cyclins D1, D2, and D3 in lysates of human cells and is activated by coexpression with D-type cyclins in Sf9 insect cells. Furthermore, we demonstrate that endogenous cdk6 from human cell extracts is an active kinase which can phosphorylate pRB, the product of the retinoblastoma tumor suppressor gene. The activation of cdk6 kinase occurs during mid-G_1 in phytohemagglutinin-stimulated T cells, well prior to the activation of cdk2 kinase. This timing suggests that cdk6, and by analogy its homolog cdk4, links growth factor stimulation with the onset of cell cycle progression.

The cyclin-dependent protein kinases, or cdks, regulate major cell cycle transitions in eukaryotic cells. A single yeast cdk, cdc2 in *Schizosaccharomyces pombe* or CDC28 in *Saccharomyces cerevisiae*, can stimulate both the G_1/S transition and the G_2/M transition when associated with cell cycle-specific cyclins (reviewed in references 51 and 57). In contrast, a large family of cdc2-related genes in vertebrate cells has been identified and several may be involved in cell cycle regulation (7, 15, 21, 24, 31–33, 38, 41, 45, 46, 50, 52, 56, 62, 66, 72). Distinct cdks from this family are believed to regulate discrete cell cycle transitions in vertebrate cells. Among the human cdk genes, *cdc2*, *cdk2*, and *cdk3* can complement budding yeast *cdc28* mutants, suggesting that these kinases perform a role analogous to that of the yeast cdk (15, 31, 45, 50). Vertebrate cdc2 acts at the G_2/M transition and is required for entry into mitosis (14, 18, 59, 65, 69). The cdk2 kinase, in contrast, has been shown to be essential for the G_1/S transition (18, 54, 67, 69). In addition, expression of a dominant-negative *cdk3* mutant in human cells specifically arrests these cells in G_1 independently of cdk2 activity, suggesting that cdk3 activity is also necessary for cell cycle progression (69).

Activation of the cdks depends on binding to their regulatory subunits, the cyclins (14, 63). The cyclins perform cell cycle-specific functions and include cyclins which act at mitosis as well as cyclins which are essential for the G_1/S transition (reviewed in reference 61).

Several lines of evidence suggest that D-type cyclins are critical regulators of the passage of mammalian somatic cells through G_1. Cyclin D1 was initially isolated on the basis of three independent properties: rearrangement in parathyroid tumors, growth factor-dependent expression in macrophages, and ability to complement mutants of yeast G_1 cyclins (37, 43, 48, 71). When D-type cyclins were identified, it was therefore postulated that these cyclins would play a role in oncogenesis, respond to growth factor stimulation, and stimulate progress through G_1. Each of these hypotheses has been substantiated (reviewed in reference 61).

In particular, a role for D-type cyclins in G_1 progression has now been established. Overexpression of cyclin D1, D2, or D3 shortens the G_1 phase of the cell cycle in various cell types (3, 29, 58), while microinjection of antibodies against cyclin D1 or of plasmids expressing antisense cyclin D1 RNA blocks the progression of human fibroblasts through G_1 (4, 58). In addition, overexpression of D-type cyclins can overcome the G_1 growth arrest by the retinoblastoma tumor suppressor gene, RB, in Saos-2 osteosarcoma cells (16, 25). Finally, constitutive expression of cyclin D2 or D3 can prevent granulocyte differentiation (29).

Association with D-type cyclins has provided indirect evidence for cell cycle regulation by vertebrate cdc2-related kinases other than cdc2, cdk2, and cdk3. The cdk4 (previously known as PSK-J3) and cdk5 kinases, as well as cdc2 and cdk2, have been shown to associate with D-type cyclins (41, 72–74). The cdk5 kinase has been shown to be an active kinase only in terminally differentiated neurons and is not active in cycling cells (68); this pattern of activity does not suggest that cdk5 acts to promote cell cycle progression.

Several experiments suggest that cdk4 is involved in cell cycle regulation. The cdk4 kinase is activated by D-type cyclins upon baculovirus coinfection of insect Sf9 cells, and the resultant complexes can phosphorylate pRB, the product of the retinoblastoma tumor suppressor gene (16, 28, 41). In addition, cdk4 protein levels are down-regulated in transforming growth factor beta-blocked mink lung cells, and constitutive high-level expression of cdk4 in these cells overrides cell cycle arrest by transforming growth factor beta (17). However, the enzymatic activity of the cellular cdk4 protein or indeed of any cellular complexes containing D-type cyclins has not been described until now (see below and reference 42).

We have investigated the cell cycle activity of the product of the human PLSTIRE gene, the closest homolog of cdk4. The PLSTIRE product shares 71% amino acid sequence identity with cdk4, defining a distinct subfamily of the cdc2-related kinases (45). We have now shown that the PLSTIRE product associates with cyclins D1, D2, and D3 in human cells. Association of PLSTIRE with D cyclins has also been observed by Bates and colleagues (5). PLSTIRE has therefore been renamed cdk6, or cyclin-dependent kinase 6. For simplicity, we use the name cdk6 throughout this paper.

Using the product of the retinoblastoma tumor suppressor gene, pRB, as a substrate, we have identified cdk6 kinase

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activity in human cells. First, we showed that the cdk6 kinase is activated by coexpression with D-type cyclins via recombinant baculoviruses in insect cells. We then demonstrated that endogenous cdk6 exhibits kinase activity when immunoprecipitated from either primary or transformed human cells. In primary T cells which have been stimulated to enter the cell cycle, cellular cdk6 kinase activity first appears in mid-G_1, prior to the activation of any previously characterized cdk.

MATERIALS AND METHODS

Cell culture. Tissue culture cells were grown under standard conditions in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. For T-lymphocyte preparations, buffy coats from human blood were obtained from the Massachusetts General Hospital Blood Bank. The mononuclear cell layer was obtained by centrifugation on a Ficoll-Hypaque cushion (Ficoll-Paque; Pharmacia). Mononuclear cells were then resuspended at 2×10^6 to 3×10^6 cells per ml in RPMI 1640 supplemented with 10% heat-activated fetal calf serum and 1 μg of phytohemagglutinin (PHA) per ml to stimulate T-cell proliferation. Thymidine incorporation was assayed by adding 10 μCi of [^{3}H]thymidine to 1 ml of cultured T cells and continuing culture for 1 h. The cells were then lysed with 0.3 N NaOH, and the lysates were trichloroacetic acid precipitated and counted.

Antibodies. For anti-cdk6 antibodies, a peptide CSQNTS ELNTA, was synthe sized, with the underlined residues corresponding to the C terminus of cdk6. The peptide was coupled to activated keyhole limpet hemocyanin (Pierce) and then injected into rabbits (Pocono Rabbit Farms, Canadensis, Pa.). Rabbit antisera were affinity purified by incubation overnight at 4°C with the antigenic peptide coupled to activated SulfoLink gel (Pierce) and then by elution with 100 mM glycine (pH 2.5) and neutralization with Tris (pH 8.0). A total of 10 μl of each eluted fraction was loaded onto polyacrylamide gels, separated, and subsequently stained with Coomassie brilliant blue to evaluate the presence of immunoglobulin heavy and light chains. Antibodies were then concentrated by centrifugation through Centricon 10 columns and diluted in phosphate-buffered saline containing 0.05% sodium azide to a final concentration of 0.4 mg/ml.

Anti-cyclin D1 monoclonal antibodies (HD11, HD33, and HD63) were a gift of L. Zukerberg and E. Lees (75). Polyclonal anti-cyclin D2 and anti-cyclin D3 antibodies, raised against glutathione *S*-transferase (GST) fusion proteins, were a gift of K. Ando and J. D. Griffin (1). Monoclonal antibodies to cyclins D2 and D3 were obtained from Pharmingen, Inc. Anti-cdk4 antibody and peptide were a gift of H. Zhang and D. Beach (73).

Metabolic labeling and immunoprecipitations. Cells were incubated in serum-free Dulbecco's modified Eagle medium without methionine for 30 min and then labeled with a 250-μCi/ml mixture of [^{35}S]methionine and [^{35}S]cysteine in serum-free Dulbecco's modified Eagle medium without methionine for 4 h at 37°C. Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 0.1 mM Na_2 VO_3, and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitations were performed as previously described (22). Immunoprecipitated proteins were separated on 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS), and proteins were visualized by fluorography.

For denaturing immunoprecipitations, cells were lysed in a small volume of 100 mM dithiothreitol–2% SDS–100 mM Tris (pH 6.8) and boiled for 10 min. The samples were then diluted 1:25 in lysis buffer (see above) and immunoprecipitated.

Five microliters (2 μg) of affinity-purified anti-cdk6 antibodies, 2 μl of anti-cdk4 antibodies, 3 μl of anti-cyclin D2 or anti-cyclin D3 or normal rabbit antibodies, or 100 μl of hybridoma supernatant were used to immunoprecipitate lysates corresponding to 2.5 ml of cultured cells or any other quantity specified in the text. One microgram of peptide was used to inhibit 2 μg of anti-cdk6 antibody. This represents an approximately 75-fold molar excess of peptide over antibody recognition sites. To assess the specificity of peptide competition, a titration, which showed that most of the cdk6 and associated protein signal was blocked by a 3-fold excess of peptide, was performed; even a 300-fold excess of peptide failed to eliminate background bands (data not shown).

Partial proteolytic mapping. Cells were labeled for immunoprecipitation as described above, except that 1 mCi of [^{35}S]methionine per ml was used. Samples were immunoprecipitated and then separated by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were then dried without fixation, and bands were visualized without fluorography. The bands were then excised and subjected to *Staphylococcus aureus* V8 partial proteolysis by the method of Cleveland et al. (10).

cDNA clones of cdk6 in pBSK-globin (45), cyclin D1 in pGEM7 (48), cyclin D2 in pBSK (55), and cyclin D3 in pGEM7 (49) were linearized, in vitro transcribed with T7 RNA polymerase, in vitro translated by using a rabbit reticulocyte lysate system (Promega) and [^{35}S]methionine, and gel purified. The labeled gene products were then subjected to V8 partial proteolysis as described above.

Immunoblotting. For immunoblots from whole-cell lysates, cell pellets were washed in phosphate-buffered saline, lysed in 100 mM Tris (pH 6.8)–2% SDS–100 mM dithiothreitol, sonicated, and boiled. A total of 60 μg of each cell lysate was then electrophoresed through a 10% denaturing polyacrylamide gel and transferred to Immobilon-P (Millipore). Blots were blocked in 5% milk in Tris-buffered saline with 0.2% Tween 20 (TBST) and then incubated for 1 h at room temperature with primary antibody. Dilutions of primary antibody were 1:1,500 for affinity-purified anti-cdk6 antiserum, 1:1,000 for anti-cdk4 antiserum, 1:5 for HD11 anti-cyclin D1 hybridoma supernatant, 1:10 for anti-cyclin D2 hybridoma supernatant, and 1:1,000 for affinity-purified anti-cyclin D3 monoclonal antibody (Pharmingen). The blots were then washed three times for 20 min each wash with TBST, incubated for 1 h at room temperature with secondary antibody (affinity-purified goat anti-mouse or donkey anti-rabbit antibody [Amersham] at a 1:5,000 dilution in milk-TBST), washed, and developed by using the Amersham ECL chemiluminescence reagent.

For immunoprecipitations prior to immunoblotting, antibodies were covalently cross-linked to protein A-Sepharose beads at a concentration of 2 mg of total immunoglobulin per ml of swollen beads, using 20 mM dimethyl-pimelimidate in 200 mM borate (pH 9.0). The beads were then washed with 100 mM glycine (pH 2.5) to remove any antibodies that had not been cross-linked. Antibodies used were anti-cdk6 antisera (before affinity purification) with normal rabbit serum as a negative control and anti-cyclin D1 monoclonal antibody HD63 with M73, a monoclonal antibody to adenovirus E1A (22), as a negative control. A total of 100 μl of beads was used for 500 μg of cell lysate. After immunoprecipitation, samples were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Blots were probed as described above.

Baculovirus infections. Coding sequences of cdk4 (21) and cdk6 (45) were subcloned from pCMV plasmids (69) into the

*Bam*HI sites of pVL1393 (Pharmingen, Inc.). Recombinant *Autographa californica* nuclear polyhedrosis viruses were then produced by using the BaculoGold transfection system (Pharmingen, Inc.) and subsequently amplified. Cyclin D1, D2, and D3 baculoviruses were a gift of Stefan Gruenwald (Pharmingen). Wild-type *A. californica* nuclear polyhedrosis virus (Pharmingen) was used as the baculovirus vector control.

A total of 5×10^6 Sf9 cells were infected with 0.5 ml of each tested baculovirus supernatant at approximate titers of 10^8 PFU/ml. Cells were harvested 48 h postinfection. Expression of the protein products was confirmed by immunoblotting.

Kinase assays. Sf9 cell cultures were split in half. One half was lysed by sonication in 200 μl of kinase buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES, pH 7.0], 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol); 25 μl of this lysate was then used for kinase assays. The other half was lysed and immunoprecipitated as described above under "Metabolic labeling and immunoprecipitations." Immunoprecipitations were washed once in kinase buffer prior to kinase assays.

Immunoprecipitations from human cells were performed as described above and then washed in kinase buffer before being assayed.

Samples for kinase assay were incubated for 30 min at 37°C in a final volume of 50 μl of kinase buffer containing 10 μM ATP, 0.4 mCi of [γ-^{32}P]ATP per ml, 40 μg of substrate protein per ml, and, in some experiments, 100 μg of competitor protein (bovine serum albumin or GST). The phosphorylated proteins were then electrophoresed on denaturing polyacrylamide gels; the gels were stained with Coomassie blue prior to autoradiography to permit ready excision and scintillation counting of the phosphorylated substrates.

RESULTS

cdk6 forms cellular complexes with cyclins D1, D2, and D3. To characterize the activities of the cdk6 protein, polyclonal antisera were generated by immunizing rabbits with a peptide corresponding to the carboxy-terminal 10 amino acids of the predicted cdk6 gene product. These anti-cdk6 antisera were then affinity purified by using the C-terminal peptide immunogen.

The anti-cdk6 antisera immunoprecipitated two prominent protein bands with apparent molecular masses of 40 and 34 kDa from [^{35}S]methionine-labeled myeloid leukemia ML-1 cells under mild, nondenaturing conditions (Fig. 1A, lane 1). These molecules were not precipitated when the antisera were preincubated with an excess of the immunizing peptide as competitor (Fig. 1A, lane 2). Several additional protein bands of lesser intensity were likewise abolished by the peptide.

To establish which protein was directly recognized by the anti-cdk6 antibody and thus might correspond to cdk6, we lysed and boiled ML-1 cells in a denaturing buffer prior to immunoprecipitation. Under these conditions, only the 40-kDa protein was recognized by the anti-cdk6 antisera (Fig. 1A, lane 3). Binding of the antisera to this protein was again abolished by competition with the peptide (Fig. 1A, lane 4). The 40-kDa protein from anti-cdk6 immunoprecipitates comigrated with in vitro-translated *cdk6* mRNA (not shown). We were able to confirm the identity of the 40-kDa protein recognized by anti-cdk6 antisera with the in vitro-translated product of the *cdk6* gene by partial proteolytic mapping with V8 protease (Fig. 1B). Thus, the 40-kDa protein recognized by anti-cdk6 antisera was identified as the cellular cdk6 protein.

We postulated that the 34-kDa protein present in anti-cdk6 immunoprecipitates from ML-1 cells under native but not denaturing conditions might be a cyclin subunit. In light of their known association with cdk4 (41, 72), D-type cyclins were good candidates for cdk6-associated proteins in the 35-kDa range. Therefore, we compared the cdk6-associated protein of ML-1 cells with the known D-type cyclins. The 34-kDa protein coprecipitated by anti-cdk6 antibodies from ML-1 lysates (Fig. 2A, lane 1) migrated at the same velocity as cellular cyclin D3 precipitated with anti-cyclin D3 antibody (Fig. 2A, lane 5). No cyclin D1 or D2 was detected by immunoprecipitation from ML-1 cells (Fig. 2A, lanes 3 and 4). Partial proteolytic digestion with V8 protease showed that the 34-kDa protein from an anti-cdk6 immunoprecipitation (Fig. 2B, lanes 1 to 3) was indistinguishable from cellular cyclin D3 (Fig. 2B, lanes 4 to 6) and very similar to in vitro-translated cyclin D3 (not shown). Thus, the prominent band in anti-cdk6 immunoprecipitates from ML-1 cells is cyclin D3. Since the anti-cdk6 antibodies failed to recognize cyclin D3 in denatured form (Fig. 1A), we infer that cdk6 and cyclin D3 form heterodimers in ML-1 cells. Although anti-cyclin D3 antibodies did not coprecipitate significant amounts of labeled cdk6 from lysates of ML-1 cells labeled for 30 min (Fig. 2A, lane 5), cdk6 could be detected in anti-cyclin D3 immunoprecipitates when ML-1 cells were labeled for 4 or 6 h (not shown). These data suggest that cyclin D3 turns over more rapidly than cdk6 and/or that cdk6 is in excess over cyclin D3 in ML-1 cells.

We wondered whether other D cyclins might also be associated with cdk6 in the cells that expressed the other D cyclins. To identify cell-specific expression patterns, we immunoblotted lysates from a panel of human cells and probed them with antibodies to cyclins D1, D2, and D3 as well as to cdk6 and

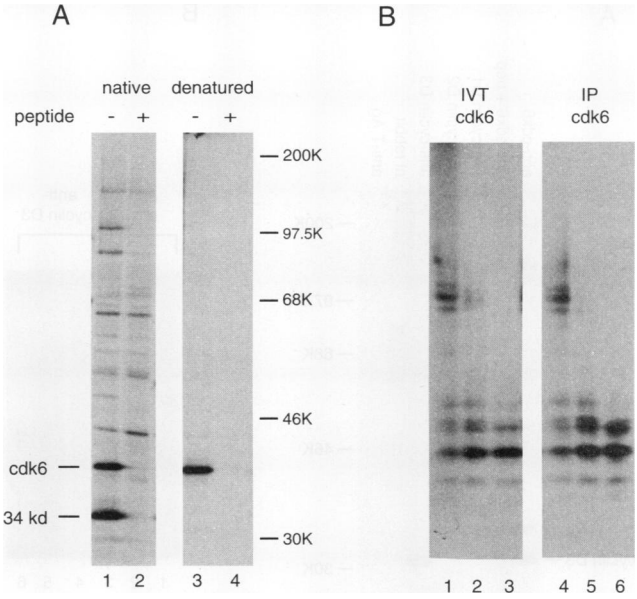

FIG. 1. Characterization of cellular cdk6 protein and anti-cdk6 antisera. (A) Immunoprecipitation with anti-cdk6 antisera (lanes 1 and 3) or with anti-cdk6 antisera preincubated with antigenic peptide (lanes 2 and 4), from lysates of ^{35}S-labeled ML-1 cells prepared under nondenaturing (lanes 1 and 2) or denaturing (lanes 3 and 4) conditions. (B) V8 partial proteolytic digestion of ^{35}S-labeled cdk6 proteins. In vitro-translated cdk6 (IVT; lanes 1 to 3) was compared with the 40-kDa band from an anti-cdk6 immunoprecipitation (IP; lanes 4 to 6). V8 protease was added at 50 ng (lanes 1 and 4), 500 ng (lanes 2 and 5), or 5,000 ng (lanes 3 and 6) per lane. Figures were prepared electronically with a LaCie Silverscan scanner and the Adobe Illustrator and Adobe Photoshop programs.

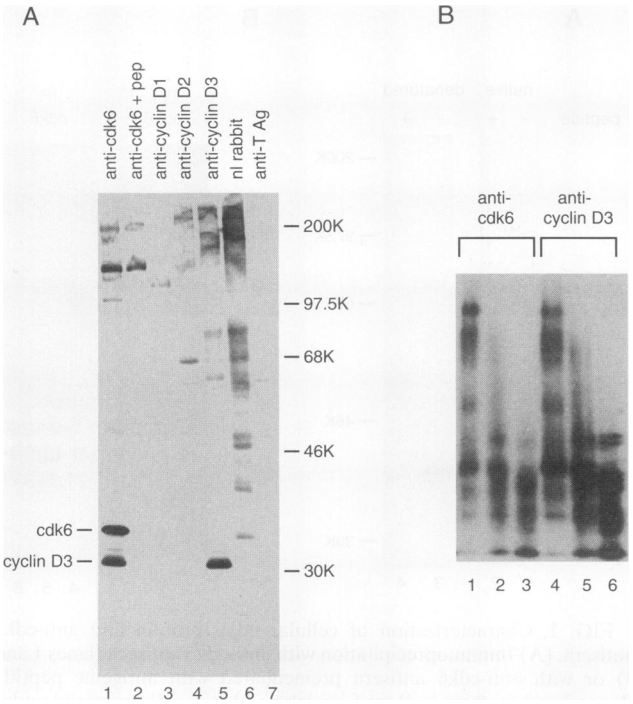

FIG. 2. Association of cdk6 with cyclin D3 in ML-1 cells. (A) Comigration of the prominent cdk6-associated protein with cyclin D3. Immunoprecipitation from lysates of [^{35}S]methionine-labeled ML-1 cells with affinity-purified anti-cdk6 antibody (lane 1; plus peptide competitor (+ pep) in lane 2), anti-cyclin D1 monoclonal antibody HD33 (lane 3), anti-cyclin D2 antiserum (lane 4), anti-cyclin D3 antiserum (lane 5), normal (nl) rabbit serum (lane 6), or Pab419 control monoclonal antibody against simian virus 40 T antigen (T Ag; lane 7). (B). V8 partial proteolytic digestion of ^{35}S-labeled cyclin D3 proteins. The 34-kDa band from an anti-cdk6 immunoprecipitation (lanes 1 to 3) was compared with cellular cyclin D3 from an anti-cyclin D3 immunoprecipitation (lanes 4 to 6). A total of 50 ng (lanes 1 and 4), 500 ng (lanes 2 and 5), or 5,000 ng (lanes 3 and 6) of V8 protease was used for digestion.

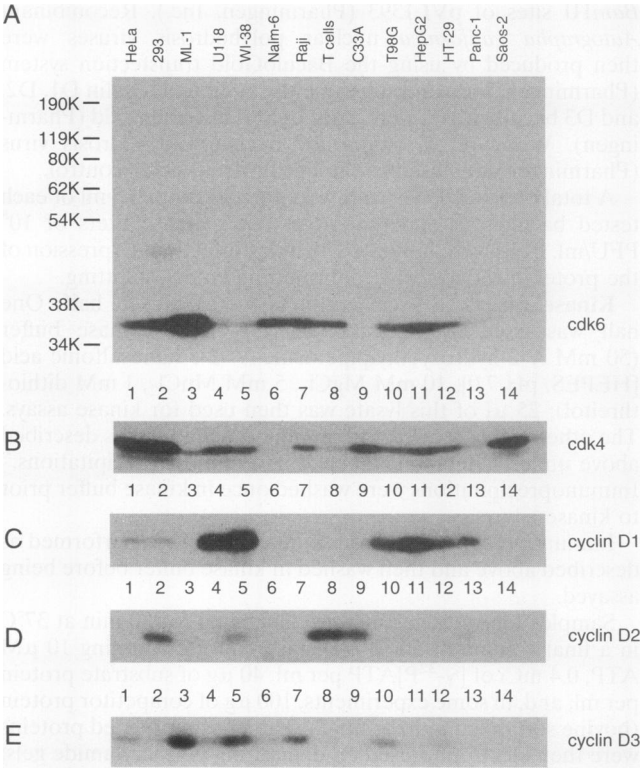

FIG. 3. Immunoblots of human cell lysates probed with antibodies to cdk6, cdk4, and D-type cyclins. A total of 60 μg of the following cell lysate was loaded per lane and then blotted: HeLa cervical carcinoma cells (lanes 1), 293 adenovirus-transformed embryonic kidney cells (lanes 2), ML-1 myeloid leukemia cells (lanes 3), U118 glioblastoma cells (lanes 4), WI-38 diploid fibroblasts (lanes 5), Nalm-6 pre-B-leukemia cells (lanes 6), Raji T-cell lymphoma cells (lanes 7), primary peripheral blood T lymphocytes (lanes 8), C33A cervical carcinoma cells (lanes 9), T98G glioblastoma cells (lanes 10), HepG2 hepatoma cells (lanes 11), HT-29 colon carcinoma cells (lanes 12), Panc-1 pancreatic adenocarcinoma cells (lanes 13), and Saos-2 osteosarcoma cells (lanes 14). The immunoblots were then probed with affinity-purified anti-cdk6 antisera (A), anti-cdk4 antisera (B), anti-cyclin D1 monoclonal antibody HD11 (C), anti-cyclin D2 monoclonal antibody (D), and anti-cyclin D3 monoclonal antibody (E).

cdk4 (Fig. 3). The cdk6 and cdk4 proteins are differentially expressed in distinct cell types (Fig. 3A and B), as had been found for the corresponding mRNAs (45). The cdk6 protein was most abundant in ML-1 cells (Fig. 3A), consistent with the elevated mRNA expression seen in this cell line (45). Similarly, D cyclins were expressed in a cell-type-specific manner (Fig. 3C to E), as had been previously described (1, 30, 43, 55, 70). Cells which expressed the highest levels of a given D-type cyclin were chosen to study cdk6 association with this specific cyclin.

Primary human peripheral blood T lymphocytes were selected to assess the association of cdk6 with cyclin D2. Anti-cdk6 antibodies precipitated the 40-kDa cdk6 protein as well as proteins of 35 and 34 kDa from labeled T cells (Fig. 4A, lane 1); these protein bands were abolished by competitor peptide (Fig. 4A, lane 2). The 35-kDa band comigrated with cellular cyclin D2 (Fig. 4A, lane 4), while the 34-kDa band comigrated with cellular cyclin D3 (Fig. 4A, lane 5). Partial proteolytic mapping with V8 protease showed that the 35-kDa protein from anti-cdk6 immunoprecipitates is cyclin D2 (Fig. 4B). The 34-kDa protein in anti-cdk6 immunoprecipitates was likewise confirmed to be cyclin D3 by proteolytic mapping (not shown). Several additional bands in the anti-cdk6 immunoprecipitation (Fig. 4A, lane 1) have not been identified; these include a band at 50 to 55 kDa, also seen in WI-38 cells (Fig. 5A, lane 2), which has not been reproducible, and two bands in the 20- to 25-kDa range, one of which may correspond to the previously characterized p21 protein associated with cyclins and cdks (72–74).

Cellular complexes of cdk6 with cyclin D1 in WI-38 cells were identified. Anti-cdk6 antibodies precipitated the 40-kDa cdk6 protein as well as a 36-kDa cellular protein from lysates of WI-38 cells (Fig. 5A, lane 2). The 36-kDa protein comigrated with cyclin D1; in addition, anti-cyclin D1 antibodies coprecipitated a 40-kDa protein from WI-38 cells which comigrated with cdk6 (Fig. 5A, lane 4). The pattern produced by partial V8 digestion of the 36-kDa band in the anti-cdk6 immunoprecipitation was indistinguishable from the patterns produced by digestion of either in vitro-translated cyclin D1 or cyclin D1 immunoprecipitated with anti-cyclin D1 monoclonal antibodies (Fig. 5B). In addition, the 40-kDa band in a cyclin D1 immunoprecipitation from WI-38 cells gave rise to a partial V8 proteolytic digestion pattern virtually identical to that of cellular cdk6 (not shown). The association between cdk6 and

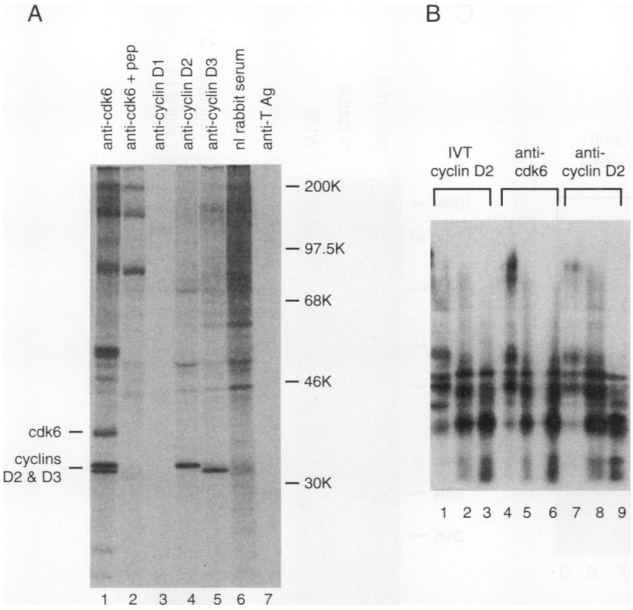

FIG. 4. Association of cdk6 with cyclins D2 and D3 in T cells. (A) Comigration of cdk6-associated proteins with cyclin D2 and cyclin D3. Immunoprecipitation from lysates of [^{35}S]methionine-labeled T cells (96 h after PHA stimulation) with affinity-purified anti-cdk6 antibody (lane 1; plus peptide competitor (+ pep) in lane 2), anti-cyclin D1 monoclonal antibody HD33 (lane 3), anti-cyclin D2 antiserum (lane 4), anti-cyclin D3 antiserum (lane 5), normal (nl) rabbit serum (lane 6), or Pab419 control monoclonal antibody against simian virus 40 T antigen (Ag; lane 7). (B) V8 partial proteolytic digestion of ^{35}S-labeled cyclin D2 proteins. In vitro-translated (IVT) cyclin D2 (lanes 1 to 3) was compared with the 35-kDa band from an anti-cdk6 immunoprecipitation (lanes 4 to 6) and cellular cyclin D2 from an anti-cyclin D2 immunoprecipitation (lanes 7 to 9). A total of 50 ng (lanes 1, 4, and 7), 500 ng (lanes 2, 5, and 8), or 5,000 ng (lanes 3, 6, and 9) of V8 protease was used for digestion.

cyclin D1 was confirmed further by reciprocal immunoprecipitations and immunoblots (Fig. 5C).

The above data show that cdk6 is associated with cyclins D1, D2, and D3 in cells which express the given cyclin together with cdk6.

Reconstituted and endogenous cdk6 immune complexes are active as pRB kinases. When a cdk-cyclin complex is identified, it is important to determine whether the complex exhibits kinase activity, and if so, which substrates it can phosphorylate. Coinfection of insect Sf9 cells with recombinant baculoviruses has shown that cdk4 is activated as a kinase by D-type cyclins (16, 28, 41). However, immunoprecipitation of this reconstituted kinase activity or of endogenous cellular kinase activity has not been reported for cdk4 or for D-type cyclins.

First, we wanted to determine whether the coexpression of cdk6 with D-type cyclins in Sf9 cells gave rise to kinase activity. Sf9 cells were coinfected with baculoviruses encoding cdks or vector controls together with baculoviruses encoding D-type cyclins or vector controls. Kinase assays were then performed on crude lysates of these Sf9 cells. A number of substrates were tried for these assays; among these, only fusion proteins with the retinoblastoma tumor suppressor gene product, pRB, were good substrates for cdk6. For these experiments, the chosen substrate was a fusion protein between GST and amino acids 792 to 928 from the C terminus of pRB, GST-RB-Cterm (19); GST-RB60 containing amino acids 379 to 928 from pRB (27) was also a suitable substrate, while GST alone was not phosphorylated (not shown).

D-type cyclins activated both cdk6 and cdk4 (used as positive control) in Sf9 coinfection experiments. Lysates from cells which coexpressed cyclin D1 with cdk4 (Fig. 6A, lane 4) or with cdk6 (Fig. 6A, lane 6) exhibited pRB kinase activity; no activity was seen in cells infected with vector control, cyclin D1, cdk4, or cdk6 alone. The significance of the higher level of kinase activity seen with cdk6 is unclear. The increased activity may be due to the relative amounts of protein expressed in the baculovirus-infected cells, as expression levels were assayed only by immunoblotting and were not strictly controlled. Cyclin D2 and cyclin D3 were also able to activate both cdk4 and cdk6 kinase activity (not shown). The ability of other cyclins to activate cdk6 has not yet been tested.

When lysates of baculovirus-infected cells were immunoprecipitated and then assayed for kinase activity, affinity-purified anti-cdk6 antisera were able to precipitate the pRB kinase activity from cells coinfected with cdk6 and cyclin D1 baculoviruses (Fig. 6B, lane 8) but not from cells expressing cdk4 and cyclin D1 (Fig. 6B, lane 4). Anti-cdk6 antibodies also recognized the kinase activity produced by coexpression of cdk6 with cyclins D2 and D3 (not shown). This shows that the cdk6-D cyclin complexes can directly phosphorylate the pRB protein and that the antibodies do not interfere with kinase activity. In contrast, anti-cdk4 antibodies did not precipitate high levels of kinase activity from cells coexpressing cdk4 with cyclin D1 (Fig. 6B, lane 2), although weak activity was seen on a longer exposure (not shown). The different abilities of anti-cdk4 and anti-cdk6 antibodies to precipitate active kinase in these experiments may be due to the probably higher titer of the affinity-purified anti-cdk6 antibody or to the detergent conditions required for precipitating active complexes of cdk4 and D-type cyclins (42).

To test whether endogenous cdk6 protein complexes from human cells also exhibited kinase activity, lysates of ML-1 cells and primary T cells were precipitated with anti-cdk6 antibodies or with anti-cdk2 antibodies as a positive control. The immune complexes were then assayed for the ability to phosphorylate both GST-RB-Cterm and histone H1.

When GST-RB-Cterm was used as a substrate, anti-cdk6 immunoprecipitations from lysates of both ML-1 cells (Fig. 7A, lane 1) and T cells (Fig. 7A, lane 5) exhibited significant kinase activity. In contrast, cdk6 did not appear to phosphorylate histone H1 appreciably. The pRB phosphorylation by cdk6 immune complexes was abolished by preincubating the antibodies with competitor peptide (Fig. 7A, lanes 2 and 6). In contrast, anti-cdk2 immunoprecipitations phosphorylated both GST-RB-Cterm and histone H1 (Fig. 7A, lanes 3 and 7).

The above results suggest that the cellular cdk6 kinase exhibits substrate specificity, preferring pRB to histone H1 as a substrate. Similar specificity has been reported for cdk4 kinase reconstituted in insect cells (41). To confirm the preference of cellular cdk6 kinase for pRB, the Coomassie blue-stained bands containing the 2.5 μg of GST-RB-Cterm and of histone H1 used as a substrate for the kinase reactions were excised and counted. The counts were corrected by subtracting the values for the peptide competition lanes, and the ratio of pRB to histone H1 phosphorylation was then determined. In both ML-1 cells and T cells, cdk6 exhibited a greater preference for pRB phosphorylation than did cdk2 (Fig. 7B), suggesting that these two kinases exhibit distinct substrate specificities.

In summary, we have shown that cdk6 kinase activity is activated by D cyclins in Sf9 insect cells. Furthermore, endogenous cdk6 immune complexes from lysates of both primary

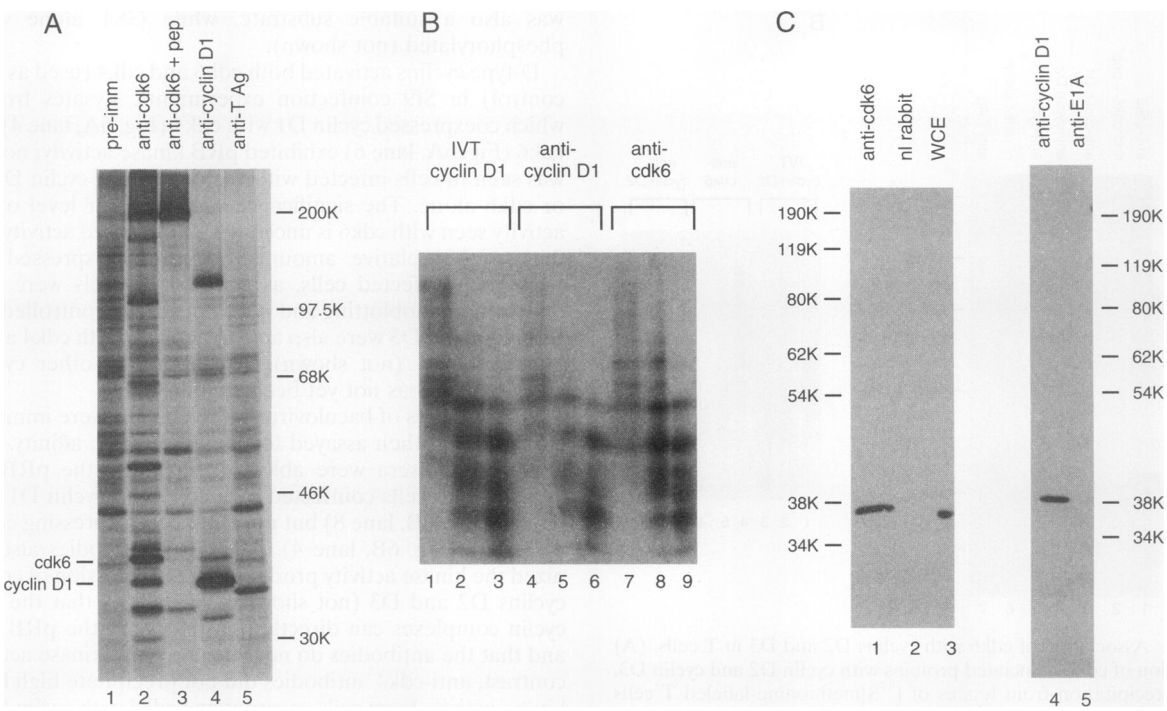

FIG. 5. Association of cdk6 with cyclin D1. (A) ^{35}S-labeled cell lysates from WI-38 cells were immunoprecipitated with preimmune sera (pre-imm; lane 1), anti-cdk6 antisera (lane 2), anti-cdk6 antisera preincubated with peptide (+ pep; lane 3), anti-cyclin D1 monoclonal antibody HD33 (lane 4), and negative control monoclonal antibody Pab419 against simian virus 40 T antigen (T Ag; lane 5). (B) V8 partial proteolytic digestion of ^{35}S-labeled cyclin D1 proteins. In vitro-translated (IVT) cyclin D1 (lanes 1 to 3) was compared with the 36-kDa band from anti-cyclin D1 immunoprecipitations (lanes 4 to 6) and the 36-kDa band from anti-cdk6 immunoprecipitations (lanes 7 to 9). A total of 50 ng (lanes 1, 4, and 7), 500 ng (lanes 2, 5, and 8), or 5,000 ng (lanes 3, 6, and 9) of V8 protease was used for digestion. (C) Immunoprecipitation and immunoblots. Anti-cdk6 (lane 1) and normal (nl) rabbit (lane 2) immunoprecipitates from 1 mg of cell lysate and 20 μg of whole-cell extract (WCE; lane 3) were blotted and probed with anti-cyclin D1 monoclonal antibody HD11 (lanes 1 to 3). Immunoprecipitations from 1 mg of cell lysate with the anti-cyclin D1 monoclonal antibody HD33 (lane 4) and with the control anti-E1A monoclonal antibody M73 (lane 5) were probed with anti-cdk6 antisera (lanes 4 and 5).

and transformed human cells exhibit significant kinase activity, phosphorylating pRB as a candidate substrate.

D-cyclin association and kinase activation of cdk6 occur during G_1 in activated T cells. When peripheral blood mononuclear cells are treated with PHA, the T lymphocytes progress from a resting state into the cell cycle in roughly synchronous fashion. Human T lymphocytes prepared in this fashion expressed significant levels of both cdk6 and cyclin D2 (Fig. 3). In these T cells, cdk6 was associated with both cyclin D2 and cyclin D3 (Fig. 4), and cdk6 immune complexes from T cells could phosphorylate pRB (Fig. 7). Finally, levels of cdk6 in these T cells were significantly higher than cdk4 levels, as demonstrated by immunoprecipitation (not shown) and immunoblotting (Fig. 3). Therefore, PHA-stimulated T cells were chosen as a model system to study the cell cycle regulation of cdk6 association with cyclins and cdk6 kinase activity.

In the experiment whose results are shown in Fig. 8, the T lymphocytes underwent blastogenesis between 4 and 8 h after PHA stimulation, as displayed by the appearance of clusters of enlarged cells. The T cells began to enter S phase at the 31-h time point, as shown by thymidine incorporation (Fig. 8A). When the same cell populations were analyzed by flow cytometry, a significant fraction of S-phase cells was first observed at 36 h, and G_2/M cells were first detected at 48 h. The cells did not appear to be highly synchronous at later time points, whether they were analyzed by thymidine incorporation (Fig. 8A) or by flow cytometry.

The association of cyclins D2 and D3 with cdk6 occurs during G_1. When stimulated T cells from various time points were labeled with [^{35}S]methionine, lysed, and then immunoprecipitated with anti-cdk6 antibodies, cdk6 could be visualized as soon as 5 h after PHA stimulation (Fig. 8B). At this 5-h time point, cdk6 was complexed with cyclin D2. Subsequently, at 16 to 20 h, prior to the onset of DNA synthesis, cdk6 was also associated with cyclin D3 (Fig. 8B). The timing of cyclin D2 and cyclin D3 association with cdk6 is consistent with the previously reported pattern of synthesis of these cyclins in activated T cells (1).

Using pRB as a substrate, we were able to detect cdk6 kinase activity beginning 12 to 16 h after PHA stimulation (Fig. 8C). The cdk6 kinase activity reached a peak at 36 h as cells were continuing to enter S phase and then persisted throughout the stimulation experiment. In contrast, cdk2 kinase activity did not appear until 31 h after PHA stimulation, coincident with the onset of DNA synthesis (Fig. 8D). This is consistent with previous reports that cdk2 is activated at or just prior to the G_1/S transition (53, 60, 67).

Finally, in addition to the D cyclins, a 28-kDa protein, p28, first appeared in anti-cdk6 immunoprecipitates at 12 h after PHA stimulation (Fig. 8B). Strikingly, p28 was present in cdk6 immune complexes for only a discrete window of time and disappeared by 58 h after PHA addition. It is unlikely that p28 is responsible for cdk6 kinase activity, since the p28 band was not detectable at later time points, when cdk6 kinase activity persisted. The p28 protein has not been observed in cdk6

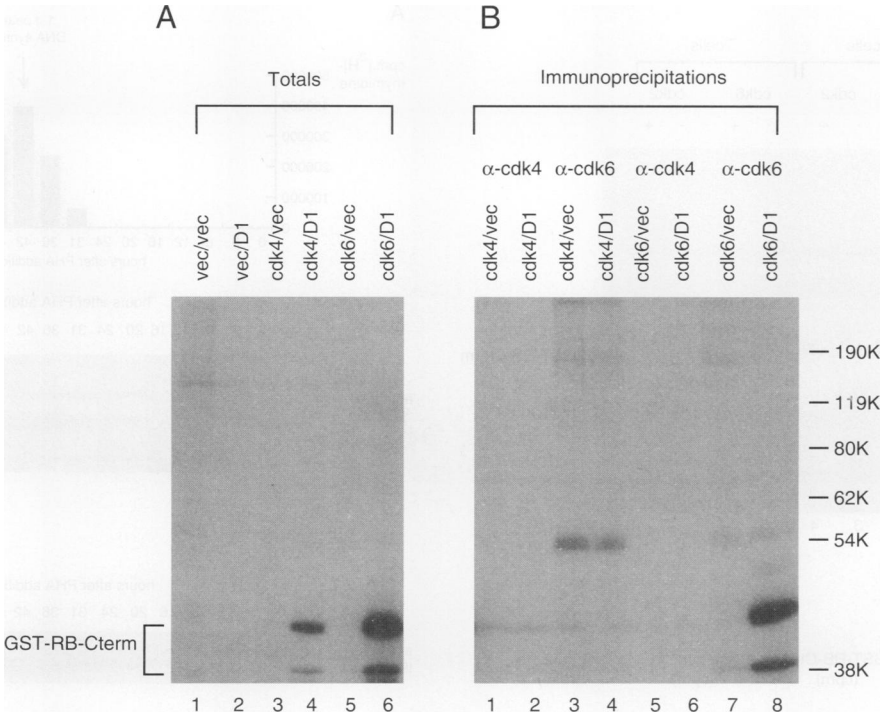

FIG. 6. Coexpression of cdk6 and cyclin D1 in Sf9 cells produces active cdk6 kinase. Sf9 insect cells were infected with the baculoviruses indicated in the figure. vec, control vector. (A) Total lysates of baculovirus-infected Sf9 insect cells were subjected to kinase assays with GST-RB-Cterm as a substrate. (B) Immunoprecipitations were performed from Sf9 cells with the indicated antibodies. The resulting immune complexes were assayed for kinase activity towards GST-RB-Cterm.

immunoprecipitates from cycling cells, and its identity is unknown.

In summary, cdk6 is activated as a pRB kinase during the G_1 phase of PHA-stimulated T cells, prior to the activation of cdk2. Our data suggest that the complex of cdk6 with D cyclins is responsible for this G_1-phase kinase activity.

DISCUSSION

We have shown that cdk6 is associated with and activated by D-type cyclins. The cdk6 kinase from human T lymphocytes exhibits kinase activity during the cell cycle at a point prior to the activation of any other known cdk. Furthermore, the cdk6 kinase can phosphorylate pRB near the time when pRB phosphorylation is initiated in T cells (11) and is thus a good candidate for the initial pRB kinase.

The missing piece of the puzzle for understanding D-type cyclin function has been the absence of identified kinase activity associated with D cyclins or their catalytic partners in mammalian cells. In consequence, a variety of alternative hypotheses has been generated. These include the suggestion that cellular D-type cyclins fail to activate cdks because of an associated inhibitor of kinase activity and the proposal that D cyclins exert their activity by retaining other proteins in the nucleus.

We have now demonstrated an endogenous human kinase activity associated with cdk6, a D-type cyclin partner. This kinase activity is initiated during G_1, well prior to the onset of DNA synthesis, and shows specificity for pRB relative to histone H1. While we have not yet definitively proven the dependence of cellular cdk6 kinase activity upon association with D-type cyclins, the following evidence strongly suggests that this is the case. First, coexpression of D cyclins with cdk6 in Sf9 cells is required to activate cdk6 kinase. Second, D-type cyclins are the only proteins found in stoichiometric ratios in the anti-cdk6 immunoprecipitates. Finally, preclearing with anti-cyclin D antibodies eliminates the majority of cdk6 kinase activity (44). Thus, while it remains a formal possibility that cdk6 kinase activity in human cells is activated by a molecule other than a D-type cyclin, D-type cyclins are likely to be the positive regulatory subunits.

Previous difficulties in detecting mammalian cell kinase activity associated with D-type cyclins now appear to be technical. Matsushime and colleagues have independently identified a pRB kinase activity specifically associated with anti-D-type cyclin immunoprecipitates (42). The catalytic subunit for this D-cyclin-dependent kinase activity, identified in macrophages and fibroblasts, is cdk4, while cdk6-cyclin D kinase activity, in contrast, is most prominent in lymphocytes. Although the identification of kinase activity for D-type cyclins associated with cdk6 and with cdk4 does not rule out alternative models for cyclin D function as discussed above, it strongly suggests that D-type cyclins are likely to regulate cell cycle progression by activating the cdk6 and cdk4 kinases.

The ability of endogenous human cdk6 to phosphorylate pRB raises the question of whether cdk6 may regulate pRB function in vivo. Phosphorylation of pRB appears to be an important element in cell cycle control by this tumor suppressor protein. The pRB protein is unphosphorylated in G_1, becomes phosphorylated prior to the G_1/S transition, and is then dephosphorylated during mitosis (6, 9, 12, 47). Phosphorylation of pRB is believed to down-regulate its growth-inhibitory activities (8, 23, 40). Thus, the kinases that phosphorylate pRB are likely to be growth-promoting elements.

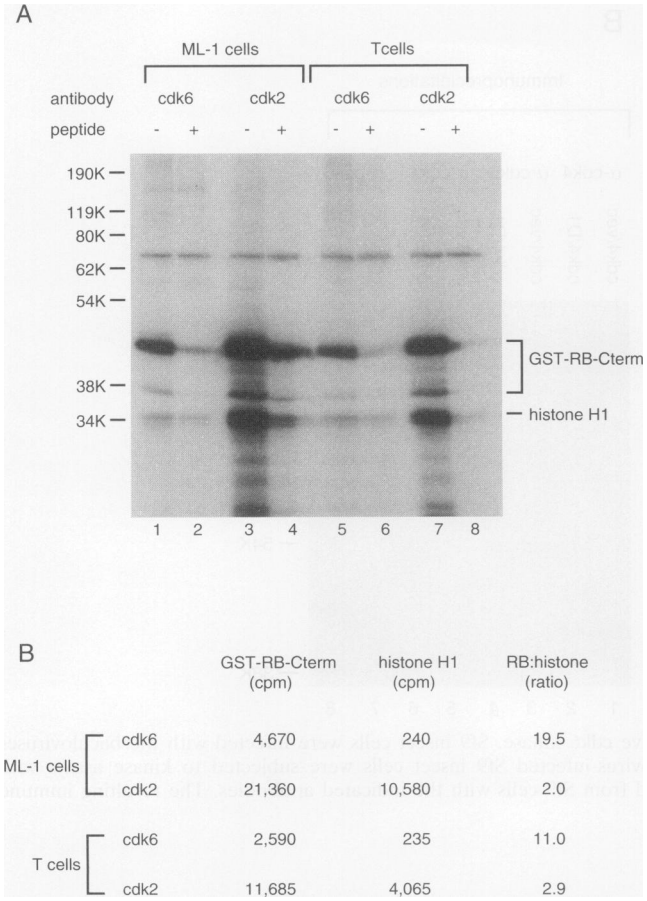

FIG. 7. Kinase activity of cellular cdk6 immune complexes. (A) Immunoprecipitations were performed from lysates of ML-1 cells (lanes 1 to 4) or T cells (lanes 5 to 8) with anti-cdk6 antibodies (lanes 1 and 5), anti-cdk6 antibodies blocked with peptide (lanes 2 and 6), anti-cdk2 antibodies (lanes 3 and 7), or anti-cdk2 antibodies blocked with peptide (lanes 4 and 8). The immune complexes were then incubated in kinase buffer containing [γ-^{32}P]ATP, histone H1, and GST-RB-Cterm to assess kinase activity. (B) Substrate preference of cdk6. GST-RB-Cterm and histone H1 bands were excised from immune complex kinase assay gels and counted. Counts for each kinase were corrected by subtracting the values for peptide competitor lanes. Ratios were obtained by dividing the counts per minute for GST-RB-Cterm by those for histone H1.

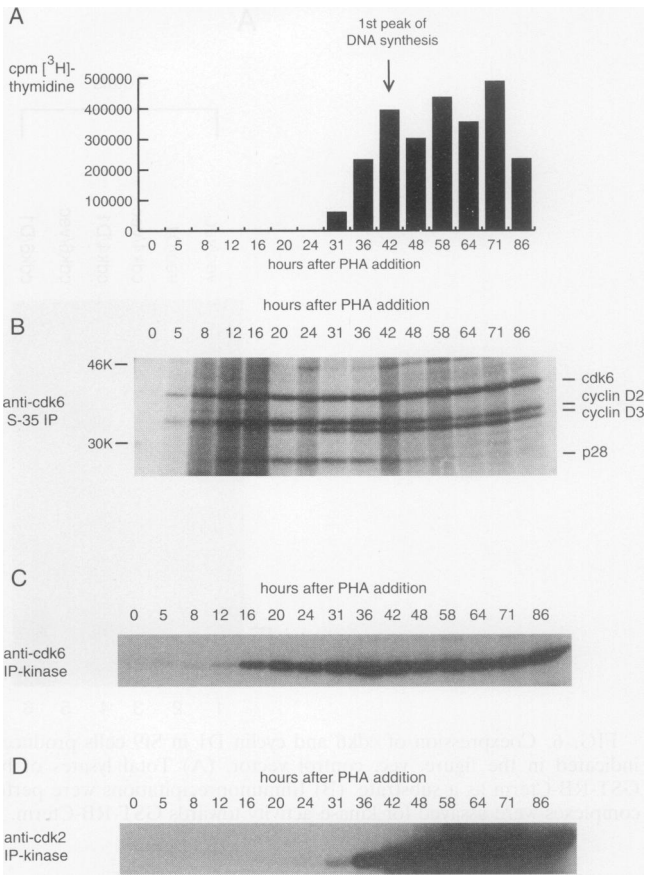

FIG. 8. Cell cycle regulation of the cyclin association and kinase activity of cdk6 in activated T cells. (A) [^{3}H]thymidine incorporation into T cells at the indicated times following the addition of PHA. (B) Immunoprecipitation with affinity-purified anti-cdk6 antiserum from activated T cells labeled with [^{35}S]methionine at the indicated times following PHA stimulation. Each immunoprecipitation was performed from lysate containing 2×10^6 cpm of trichloroacetic acid-precipitable ^{35}S (140-h exposure). Identities of the proteins are indicated; precipitation of all proteins shown was abolished by competitor peptide. (C and D) Immunoprecipitations with affinity-purified antisera from activated T cells at the indicated time points were subjected to kinase assays with 2.5 μg of GST-RB-Cterm as a substrate. Each immunoprecipitation was performed from 135 μg of cell lysate. Significant kinase activity was not seen in control immune complexes formed by antibodies preincubated with competitor peptide. (C) Immune complex kinase assays with anti-cdk6 immunoprecipitates. Film was exposed for 4 h at room temperature. (D) Immune complex kinase assays with anti-cdk2 immunoprecipitates. Film was exposed for 1 h at room temperature.

A cdc2 family kinase or a close relative is likely to be the physiological pRB kinase. Both human cdc2 and cdk2 can phosphorylate pRB at sites corresponding to the sites phosphorylated in vivo (2, 35, 36, 39, 64) and are physically associated with pRB, albeit in small quantities (26, 34). However, in T cells, the timing for cdc2 and cdk2 kinase activation follows the initial phosphorylation of pRB (11, 20) (Fig. 8D). The recent observation that cdk4 coexpression with D cyclins in insect cells gives rise to a pRB kinase (16, 28, 41) and the reports of physical interactions between D cyclins and pRB (13, 16, 28) suggest that D cyclins and their partners might also regulate pRB function.

Our data now demonstrate cell cycle-dependent cdk6 kinase activity towards pRB. The onset of this kinase activity coincides with the timing of the first wave of pRB phosphorylation in T cells (11). Moreover, cellular cdk6 kinase appears to prefer pRB to histone H1 as a substrate. Therefore, the cdk6 kinase is a candidate for the initial pRB kinase in T cells. Like cdk6, cellular cdk4 kinase becomes active in mid-G_1 in macrophages and fibroblasts, also preferring pRB to histone H1 as a substrate (42). Further experiments should determine whether cdk6, or cdk4 in appropriate cell types, is indeed the physiological pRB kinase.

Finally, the timing of cdk6 activation during G_1 in human T cells, as well as the timing of cdk4 activation in fibroblasts and macrophages (42), precedes the activation of any other cdk which has been tested. Thus, cdk6 and cdk4 may be the first cdks to be activated in response to growth factor stimulation, perhaps in distinct cell types. If so, cdk4 and cdk6, in associa-

tion with their D-cyclin partners, may serve to link growth factor stimulation with cell cycle progression.

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