Association of Human Cyclin E with a Periodic G_1-S Phase Protein Kinase

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G_1 cyclins control the G_1 to S phase transition in the budding yeast, *Saccharomyces cerevisiae*. Cyclin E was discovered in the course of a screen for human complementary DNAs that rescue a deficiency of G_1 cyclin function in budding yeast. The amounts of both the cyclin E protein and an associated protein kinase activity fluctuated periodically through the human cell cycle; both were maximal in late G_1 and early S phases. Cyclin E–associated kinase activity was correlated with the appearance of complexes containing cyclin E and the cyclin-dependent kinase Cdk2. Thus, the cyclin E–Cdk2 complex may constitute a human G_1-S phase–specific regulatory protein kinase.

In budding yeast, *Saccharomyces cerevisiae*, a specialized class of cyclins known as Clns activates the Cdc28 kinase, thus promoting the G_1 to S phase transition (*1–5*). Evidence for the existence of similar cyclins in vertebrates was obtained by the isolation of human cDNAs that could perform this function when expressed in yeast (*6–8*). Cyclin E was identified in this way. The amount of mRNA encoding cyclin E fluctuates through the cell cycle and is maximal near the G_1-S phase boundary. These properties indicate that cyclin E might regulate the G_1 to S phase transition in human cells (*6*).

The cyclin E protein and the associated protein kinase activity were analyzed at various stages of the cell cycle in HeLa cells synchronized by two independent methods. Extracts were prepared from cultures induced to synchrony by the double thymidine block–release procedure (Fig. 1A) (*9*) or from cultures separated according to cell size by centrifugal elutriation (Fig. 1E) (*10*). The amount of cyclin E protein in the extracts was determined by immunoblotting (Figs. 1, B and E, and 2), and the cyclin E–associated protein kinase activity was measured in immunoprecipitates prepared with antibody to cyclin E (anti-cyclin E) (Figs. 1 and 2). In the thymidine block–release experiment, the amount of cyclin E was maximal near the G_1-S phase boundary as was the amount of cyclin E–associated protein kinase activity (Fig. 1, B and C). The cyclin E–associated kinase activity had decreased to approximately one-fifth of its maximal activity by 8 hours after release from the thymidine block (*11*), at which time most cells had entered G_2. This was in marked contrast to patterns of accumulation and activation of kinase activity for cyclins A and B1 (*12*). Whereas cyclin E–associated kinase activity reached a maximum in late G_1 and early S phases and then decreased, cyclin A– and B1–associated kinase activities began to accumulate later and were maximal in the G_2 and M phases of the cell cycle (Fig. 1C). The elutriation experiment confirmed that cyclin E and cyclin E–associated kinase activity were present in G_1-enriched populations, which lacked cyclin A–associated protein kinase activity (Fig. 1, E and F). The persistence of both cyclin E and cyclin E–associated

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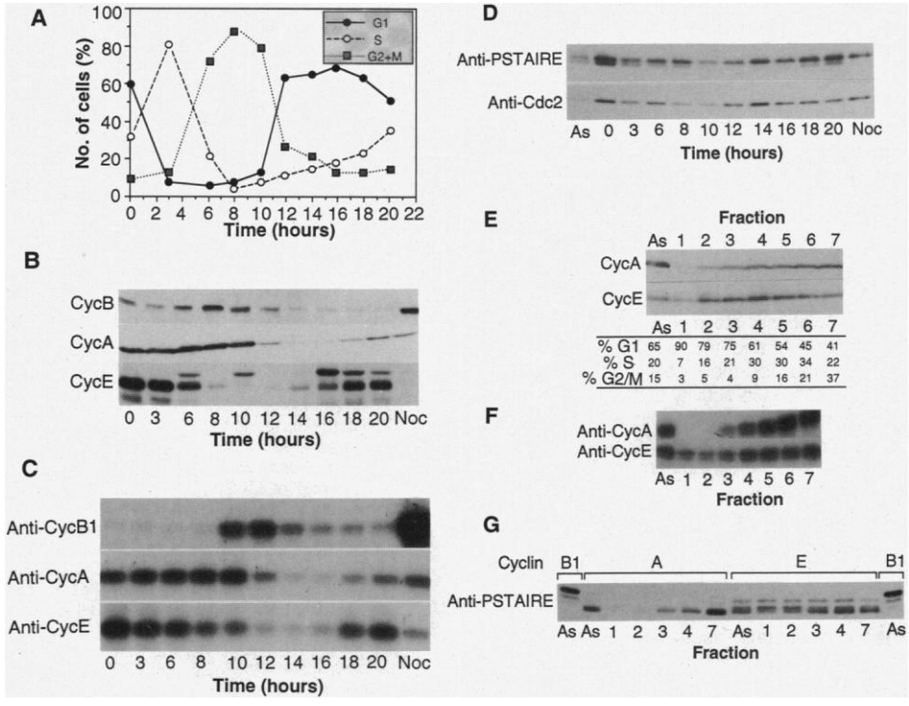

Fig. 1. Analysis of cyclin E protein and associated protein kinase activities in synchronized HeLa cells. (**A**) HeLa cells were synchronized at the G_1 to S phase boundary by means of a double thymidine block (*6, 9*). Samples were taken at the indicated times after release from the block. The synchrony of the cycling populations was monitored by subjecting the samples to flow cytometric analysis after staining of nuclear DNA with propidium iodide (*24*). (**B**) HeLa cells were released from the double thymidine block and total cell lysates were subjected to immunoblot analysis at the indicated times (*25*). Extracts were prepared from cells incubated with nocodazole (Noc; 100 ng/ml) after release from the block, as a mitotic control. Blots were probed with affinity-purified antibodies to cyclin B1 and cyclin E and crude antiserum to cyclin A (Fig. 2) (*26, 27*). (**C**) Histone H1 kinase assays were done on anti–cyclin B1, anti–cyclin A, and anti–cyclin E immune complexes prepared from extracts at each time point (*28*). (**D**) Cdk subunits coimmunoprecipitated with cyclin E. Proteins were immunoprecipitated from lysates of cells released from double thymidine block (*28*) with anti–cyclin E coupled to protein A beads (*29*), separated on an SDS-polyacrylamide gel (11%), and immunoblotted with a monoclonal antibody (MAb) to Cdc2 and an MAb to PSTAIRE (*26, 27*). (**E**) HeLa cells were separated according to size by centrifugal elutriation (*10*). The relative percentages of G_1, S, and G_2 or M cells in asynchronous culture (As) and in elutriated fractions were determined by flow cytometric analysis of nuclear DNA content (*24*). Total cell lysates were subjected to immunoblot analysis with antisera to cyclin A and cyclin E (*25–27*). (**F**) Histone H1 kinase assays were performed on anti–cyclin A and anti–cyclin E immune complexes prepared from each elutriated fraction (*28*). (**G**) Cdk subunits co-immunoprecipitated from lysates of elutriated cells. Immunoblots of anti-cyclin A, anti–cyclin B1, and anti–cyclin E immunoprecipitates were developed with anti-PSTAIRE as described (Fig. 1D) (*24, 25*).

kinase in subsequent fractions was likely due to the universal presence of late (and therefore larger) G_1 cells in these fractions; in HeLa cells there is not a strong correlation between cell size and entry into S phase. Larger G_1 cells, however, have a higher probability of being near the G_1-S phase boundary and therefore are expected to have high levels of cyclin E and cyclin E–associated kinase activity. Conversely, fractions 1 and 2, consisting primarily of small G_1 cells, not yet close to the G_1-S phase boundary, are expected to yield comparatively lower amounts of cyclin E–associated kinase activity.

Both yeast and mammalian cyclins activate protein kinase catalytic subunits classified as cyclin-dependent kinases (Cdks) (*13*). At least three of the human Cdk family members—Cdc2 (*14*), Cdk2 (*15–17*), and Cdk3 (*18*)—are recognized by a monoclonal antibody (anti-PSTAIRE) specific for the PSTAIRE (Pro, Ser, Thr, Ala, Ile, Arg, Glu) motif, an epitope conserved in known Cdk family members. The association of cyclin E with Cdk subunits at various stages of the cell cycle and the amount of kinase activity associated with cyclin E through the cell cycle were analyzed by immunoblotting anti–cyclin E immunoprecipitates with anti-PSTAIRE (Fig. 1, D and G). Cyclin E was associated with three proteins recognized by anti-PSTAIRE; the middle band corresponds to Cdc2 (Fig. 3C). This was established by reacting the same immunoblot sequentially with anti-Cdc2 and anti-PSTAIRE. Cyclin E–associated kinase activity through the cell cycle was correlated with co-precipitation of the most rapidly migrating species (Fig. 1, D and G) but not the others, including Cdc2 (*19*). The most rapidly migrating cyclin E–associated species comigrates with Cdk2 immunoprecipitated with cyclin A (Fig. 1G) (*12, 17*). Furthermore, immunodepletion of Cdk2 removed most (75 to 80%) of the cyclin E–associated kinase activity from

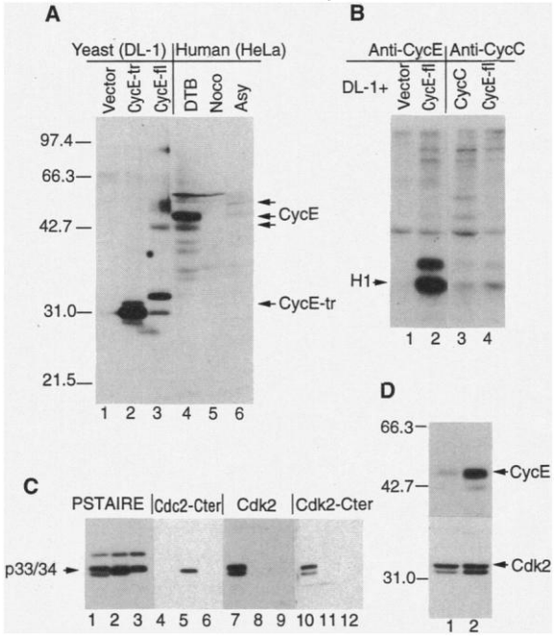

Fig. 2. Characterization of antibodies to cyclin E and Cdk2. (**A**) Antibodies to bacterially produced truncated cyclin E recognized the cyclin E gene product in yeast (lanes 2 and 3) and HeLa cells (lanes 4 to 6). Proteins in lysates from yeast DL-1 cells (*6*) containing pDB20 vector (*6*) alone (lane 1) or plasmids encoding truncated (CycE-tr, lane 2) or full-length (CycE-fl, lane 3) human cyclin E, from HeLa cells arrested in G_1-S by a double thymidine block (DTB, lane 4), or in mitosis by treatment with nocodazole (Noco, lane 5), and from asynchronous cells (Asy, lane 6) were separated by SDS-PAGE (11% gel), transferred to Immobilon membrane, and probed with affinity-purified antibodies to cyclin E. (**B**) Immunoprecipitation of cyclin E–associated histone H1 kinase with anti–cyclin E. Proteins in lysates of DL-1 cells containing vector alone (lane 1) or genes encoding cyclin E (lanes 2 and 4) or cyclin C (lane 3) (*6*) genes were immunoprecipitated either with anti–cyclin E (lanes 1 and 2) or with anti–cyclin C (lanes 3 and 4). Immunoprecipitated complexes were assayed for histone H1 kinase activity (*28*). (**C**) Rabbit antibodies to bacterially produced human Cdk2 specifically recognized Cdk2 in yeast cells. Proteins in lysates from the strains expressing human Cdk2 (lanes 1, 4, 7, and 10), human Cdc2 (lanes 2, 5, 8, and 11), and endogeneous Cdc28 (lanes 3, 6, 9, and 12) were separated by SDS-PAGE (11% gel) and transferred to an Immobilon membrane. The blots were probed with anti-PSTAIRE (lanes 1 to 3), antibody to the COOH-terminus of Cdc2 (Cdc2-Cter) (lanes 4 to 6), and with affinity-purified rabbit antisera to human Cdk2 (lanes 7 to 9) or to the COOH-terminus of *Xenopus* Cdk2 (Cdk2-Cter) (lanes 10 to 12) (*25*). (**D**) Anti–cyclin E and anti-Cdk2 recognize cyclin E and Cdk2, respectively, in ML-1 cells. Protein extracts from asynchronous (lane 1) and G_1-S arrested (lane 2) ML-1 cells were separated by SDS-PAGE polyacrylamide gels (11% gel) and transferred to an Immobilon membrane. The blot was cut and the upper half was probed with affinity-purified anti–cyclin E. The lower half was probed with affinity-purified anti-Cdk2. The proteins were detected as described.

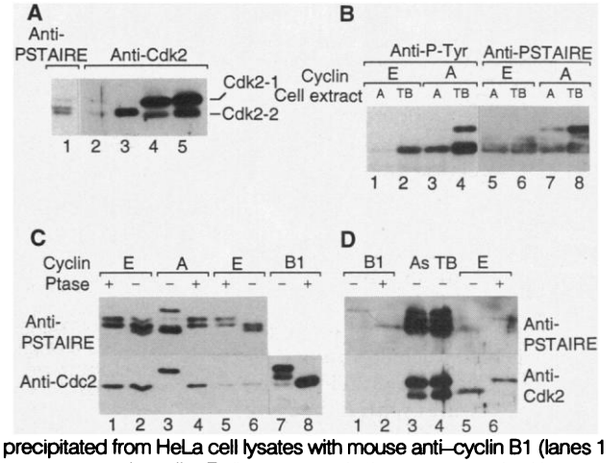

Fig. 3. Analysis of cyclin-dependent kinase subunits associated with cyclin E. (**A**) Immunoblot analysis of anti–cyclin E immune complexes with antiserum to Cdk2. Immunoblots of proteins immunoprecipitated with anti–cyclin E (lanes 1 to 3), or total protein from extracts of asynchronous (lane 4) or G_1-S–enriched (lane 5) HeLa cells, were probed with anti-PSTAIRE (lane 1) or rabbit antibodies to Cdk2 (lanes 2 to 5) (*27, 36*). Immunoprecipitates were prepared with either rabbit (lane 1) or mouse (lanes 2 and 3) anti–cyclin E (*26*) from asynchronous (lanes 1 and 2) or G_1-S–arrested (thymidine-blocked; lane 3) HeLa cells. (**B**) Immunoblot analysis of anti–cyclin E immune complexes with MAb to phosphotyrosine. Immunoprecipitates prepared from asynchronous (A) and thymidine-blocked (TB) HeLa cells were immunoblotted first with antibodies to phosphotyrosine (lanes 1 to 4) and then with anti-PSTAIRE (lanes 5 to 8) (*26, 27*). The reprobing does not represent a quantitative analysis because the signal exceeded the saturation point. However, all proteins expected to react with anti-PSTAIRE were observed. (**C**) Phosphatase treatment of anti–cyclin E immune complexes. Proteins immunoprecipitated from HeLa cell lysates (*28*) with rabbit anti–cyclin E (lanes 1, 2, 5, and 6), anti–cyclin A (lanes 3 and 4), or anti–cyclin B1 (lanes 7 and 8) were treated with potato acid phosphatase (lanes 1, 4, 5, and 8) or phosphatase buffer (lanes 2, 3, 6, and 7) (*28*). The same immunoblot was probed with mouse MAb to Cdc2 and anti-PSTAIRE. The lysates were from asynchronous (lanes 1, 2, 7, and 8) or thymidine-blocked (lanes 3 to 6) cells. The anti–cyclin B1 immune complex was not immunoblotted with anti-PSTAIRE. (**D**) Phosphatase treatment of Cdk2 in cyclin E immune complexes. Proteins immunoprecipitated from HeLa cell lysates with mouse anti–cyclin B1 (lanes 1 and 2) or mouse anti–cyclin E (lanes 5 and 6) were treated with potato acid phosphatase (lanes 2 and 6) or with phosphatase buffer alone (lanes 1 and 5) (*30*). Total protein extracts of asynchronous (lane 3) and thymidine-blocked (lane 4) HeLa cells were used as standards. The immunoblot was probed sequentially with rabbit anti-Cdk2 and anti-PSTAIRE.

lysates, whereas immunodepletion of Cdc2 removed none of this activity (*20*). Thus, cyclin E complexes having protein kinase activity are likely to contain Cdk2 but not Cdc2.

The most rapidly and least rapidly migrating proteins of the cyclin E–associated Cdk triplet were identified as Cdk2. Proteins from HeLa cells were immunoprecipitated with anti–cyclin E and immunoblotted with a Cdk2-specific antiserum (anti-Cdk2) (Fig. 3A). The most rapidly migrating and least rapidly migrating proteins reacted with anti-Cdk2, whereas the middle band identified as Cdc2 did not. Comparable amounts of both forms of Cdk2 (Cdk2-1 and Cdk2-2) were observed when proteins from unfractionated HeLa cell extracts were immunoblotted directly with anti-Cdk2 (Fig. 3, A and D).

The two forms of Cdk2 differed both qualitatively and quantitatively in their states of phosphorylation. Only the more rapidly migrating form (Cdk2-2) contained a detectable amount of phosphotyrosine (Fig. 3B). Furthermore, treatment of anti-cyclin E immunoprecipitates and anti–cyclin A immunoprecipitates with a general phosphatase converted the Cdk2-2 to the low-mobility form (Cdk2-1) (Fig. 3, C and D). The cyclin E–associated Cdc2 did not change in mobility after treatment with the phosphatase and comigrated with dephosphorylated Cdc2 derived from anti–cyclin B1 immunoprecipitates (Fig. 3C). Thus, Cdk2-2, which is associated with cyclin E (and with cyclin A; Fig. 3C) in active complexes, is a phosphorylated derivative of Cdk2-1. Although some or all of the Cdk2-2 contained phosphotyrosine, other phosphorylated amino acids may also be present in this species and responsible for its increased electrophoretic mobility.

These data suggest that cyclin E–associated kinase activity may be regulated by phosphorylation of Cdk2 and by accumulation of cyclin E. Cyclin E also underwent changes in electrophoretic mobility that correlated with associated kinase activity (Fig. 1B). Whereas the predominant species in late G_1 and S phases, in which kinase activity was high, migrated at 51 kD, a more slowly migrating protein (55 kD) was observed in G_2 and early to mid-G_1, when kinase activity was low. Also, the electrophoretic mobility of the 51-kD form appeared to change in late G_1 cells (Fig. 1B). Thus, the protein kinase activity associated with cyclin E may also be regulated by phosphorylation of cyclin E itself.

Our results indicate that, like control of mitosis, control of the G_1 to S phase transition may be similar in yeast and vertebrates. One possible target of a G_1-S phase–specific kinase in human cells is the retinoblastoma susceptibility protein, p105-Rb. Phosphorylation of p105-Rb in G_1 has been proposed to be a key factor in the G_1 to S phase transition (*21*). The cyclin E–associated kinase described above can efficiently phosphorylate p105-Rb in vitro (*22*). Furthermore, exogenous cyclin E stimulates phosphorylation and inactivation of p105-Rb in vivo (*23*). Thus, growth regulatory signals may affect the cell cycle by controlling cyclin E–mediated phosphorylation of p105-Rb or other regulators of cell cycle progression.

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11. Cyclin E–associated histone H1 kinase activity was quantitated by Cerenkov counting of the histone H1 bands excised from the gel used in Fig. 1C.
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19. In Fig. 1D, the triplet of bands that react with anti-PSTAIRE is not apparent. The gel was not run under conditions optimized for resolution of proteins of this molecular size such as those used in other experiments (Fig. 1G). Thus, the middle band was not resolved from the upper band of the triplet. Also, the same blot was first probed with anti-Cdc2 (lower panel) and then with anti-PSTAIRE (upper panel). In this experiment, the antibody concentrations used led to detection of some of the signal from anti-Cdc2 in the anti-PSTAIRE panel; this obscured the upper and faintest band of the triplet. However, the most rapidly migrating band and the middle band (detected with anti-Cdc2) of the triplet are apparent in this figure.
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25. For immunoblotting, the cells were lysed in sample buffer containing SDS (without reducing agent) and sonicated. Proteins in the equivalent of 0.15 to 0.3 A_{280} of the lysate were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (usually on 11% gels). The proteins were then transferred to Immobilon membranes (Millipore) at 150 mA (90 min) with a semidry blotting apparatus containing transfer buffer (48 mM tris-base, 39 mM glycine, 0.037% SDS, 20% methanol). Except for phosphotyrosine blots, filters were blocked in TBST [0.2 M NaCl, 0.2% Tween-20, 10 mM tris (pH 7.4)] containing nonfat dry milk (5%) and 0.02% NaN_3 (blotto), incubated with antibody in the same buffer, washed several times with TBST for a total of 1 hour, incubated with secondary antibody in blotto, and washed for 1 hour in TBST. We used the following primary antibodies: affinity-purified antibody from rabbit to cyclin E (anti–cyclin E, dilution 1:500), affinity-purified antibody from rabbit to cyclin B1 (1:500), whole rabbit antiserum to cyclin A (1:20,000), mouse antiserum to cyclin E (1:1,000), monoclonal antibodies to Cdc2 (1:2,000; Zymed), monoclonal antibodies to phosphotyrosine (1:1,000; UBI), and monoclonal antibodies to PSTAIRE (1:5,000). Secondary antibodies were either goat antibodies to rabbit IgG-HRP (immunoglobulin G–horseradish peroxidase) or goat antibodies to mouse IgG-HRP (1:5,000; both from Pharmingen). The detection system used was enhanced chemiluminiscence (ECL; Amersham and Kodak XAR-5 film).
26. The antisera to cyclin E and Cdk2 were raised in New Zealand White rabbits immunized with truncated cyclin E (residue 129 to the COOH-terminus) and full-length human Cdk2, respectively. Cyclin E and Cdk2 were produced in *Escherichia coli* (*31*). The mouse polyclonal antisera to the same polypeptide from cyclin E were described (*32*). Truncated cyclin E cDNA containing the open reading frame from residue 129 to the COOH-terminus (*6*) was mutagenized with the polymerase chain reaction (*33*) to introduce an Nde I site at position 313 to 319 and a Bam HI site 99 nucleotides downstream of the translation termination codon. The cyclin E coding region was placed under control of the bacteriophage T7 promoter by introduction of the resulting Nde I to Bam HI fragment into plasmid pRK171. The plasmid pRK171-cycE was transformed into *E. coli* strain BLysS, and the expression of the gene was induced by addition of 0.4 mM IPTG at a culture density of $A_{600} = 0.4$. The cyclin E protein was enriched by preparation of an insoluble fraction (*34*), purified by preparative SDS-PAGE, and electroeluted from the gel. The rabbit antiserum to cyclin E was affinity purified by adsorption to and elution from nitrocellulose strips to which bacterially produced cyclin E polypeptide was bound (*35*). Nitrocellulose strips were eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with one-tenth volume of 1 M tris. The same procedure was followed to generate Cdk2-specific antibody with the cloned human *CDK2* cDNA (*16*). Other antibodies and their sources were: rabbit antiserum to cyclin A (*12*), J. Pines (Salk Institute, La Jolla, CA); affinity-purified rabbit antibody to *Xenopus* Cdk2 (Eg-1), J. M. Maller (University of Colorado, Denver); rabbit antisera to cyclin B1 and Cdc2 (COOH-terminal), C. McGowan and P. Russell (Scripps Research Institute, La Jolla); mouse MAb to PSTAIRE (1.5 mg/ml) (which recognizes Cdc2, Cdk2, and probably other PSTAIRE-containing proteins), M. Yamashita and Y. Nagahama (Okazaki, Japan); rabbit polyclonal antiserum to PSTAIRE, P. Russell (Scripps Research Institute); mouse polyclonal antiserum to human cyclin B1, S. Schiff and E. Harlow (MGH Cancer Center); Cdc2-specific mouse MAb (1 mg/ml), Zymed; purified rabbit IgG to Cdk2 (2.5 mg/ml), and mouse MAb to phosphotyrosine (1 mg/ml), UBI, Lake Placid, NY. Preimmune sera were obtained from rabbits before immunization.
27. The antisera we used appear to be specific for cyclin E (Fig. 2, A, B, and D). Two independently prepared rabbit sera and three mouse sera de-

tected the same spectrum of peptides in human cell lysates prepared under a number of different conditions. One of the mouse antisera to cyclin E has been further characterized by partial proteolysis analysis of immunoprecipitated metabolically labeled protein (*32*). Furthermore, when lysates prepared from recombinant yeast cells expressing either a truncated or full-length cyclin E were analyzed by immunoblotting, bands of the appropriate mobility were detected (Fig. 2A). Also, cyclin E–associated kinase activity was only detected in immunoprecipitates of yeast cells expressing cyclin E (Fig. 2B). Antisera to another cyclin did not immunoprecipitate histone kinase activity from these yeast cells. These observations indicate that the antisera used in this study are specific for cyclin E.

The various Cdk antiserum were characterized with three recombinant yeast strains—one expressing only human Cdk2 (*16*), one expressing only human Cdc2, and one expressing only the endogenous protein Cdc28. The MAb to PSTAIRE reacted with human Cdk2, human Cdc2, and yeast Cdc28 (Fig. 2C). The MAb to human Cdc2 reacted only with lysate prepared from yeast expressing human Cdc2. The rabbit polyclonal antiserum to human Cdk2, and affinity-purified antibody to *Xenopus* Cdk2, reacted only with lysate prepared from yeast expressing human Cdk2.

To confirm that our results could be generalized to other human cell lines, lysates were analyzed from asynchronous and thymidine-blocked ML-1 cells derived from a human myeloid leukemia. Similar cyclin E species were detected and enhanced by the thymidine block, as were seen in HeLa cell lysates (Fig. 2D). Also, the same characteristic Cdk2 doublet was observed in both cell lysates.

28. We immunoprecipitated cyclins from cell extracts (30 to 50 μl at A_{280} of 15 to 30) by adding antiserum to cyclin E (5 μl), cyclin A (1 μl), or cyclin B1 (1 μl) and incubating the mixtures at 4°C for 1 hour. Immune complexes were collected by adding 25 to 30 μl (0.1 g/ml) of protein A–Sepharose beads (Sigma); samples were then incubated for 1 hour with frequent mixing at 4°C. The beads were then washed three times with RIPA buffer [1% deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM tris-HCl (pH 7.5), 1 mM sodium pyrophosphate], once in RIPA without SDS and pyrophosphate, and three times in reaction buffer [20 mM tris-HCl (pH 7.5), 4 mM $MgCl_2$]. The beads were then resuspended in the kinase assay mixture or, alternatively, dissolved in SDS–sample buffer (*36*). When high resolution was required, the SDS-PAGE (11%) was done at 60 mA (constant current) on long (20 cm) gel slabs. For protein kinase assays, the cyclin complexes were immunoprecipitated with Protein A–Sepharose beads. The beads were then mixed with 5 μl of a mixture consisting of 2× reaction buffer with 80 μM [γ-^{32}P]ATP (adenosine triphosphate) and histone H1 (2 μg) (Sigma). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 2× Laemmli gel sample buffer (*36*). Proteins were separated by electrophoresis on an 11% polyacrylamide gel. Histone H1 was visualized by Coomassie blue staining and the gel was boiled in 5% trichloracetic acid for 20 min and then dried. Bands were detected by autoradiography of dried gels with XAR-5 film (Kodak).
29. Antibodies to cyclin E were coupled to protein A–Sepharose beads (Pharmacia) with dimethylpimelimidate (DMP) (Pierce) (*35*).
30. Immune complexes on protein A–Sepharose beads were washed once with acid phosphatase buffer [100 mM (2-[*N*-morpholino]ethanesulfonic acid (MES) (pH 6.0)] and then incubated in 100 μl of the same buffer containing potato acid phosphatase (1 unit) (Boehringer Mannheim) for 15 min at 37°C. The supernatant was then removed by aspiration, and the beads were suspended in 2× SDS-PAGE sample buffer (*36*).
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