Natl. Acad. Sci. U.S.A. **82**, 4341 (1985)], 0.5% (v/v) Triton X-100, and 25% FBS for 1 hour. The blot was incubated in 10 ml of PBS containing 10 μCi of ^{125}I-labeled goat antibodies to mouse immunoglobulin G (New England Nuclear) and 0.5% Triton X-100 for 1 hour, followed by exposure to x-ray film for 12 to 16 hours.
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Formation and Activation of a Cyclin E-cdk2 Complex During the G_1 Phase of the Human Cell Cycle

Andrew Koff, Antonio Giordano,* Dipty Desai, Katsumi Yamashita, J. Wade Harper, Stephen Elledge, Takaharu Nishimoto, David O. Morgan, B. Robert Franza, James M. Roberts

Human cyclin E, originally identified on the basis of its ability to function as a G_1 cyclin in budding yeast, associated with a cell cycle–regulated protein kinase in human cells. The cyclin E–associated kinase activity peaked during G_1, before the appearance of cyclin A, and was diminished during exit from the cell cycle after differentiation or serum withdrawal. The major cyclin E–associated kinase in human cells was Cdk2 (cyclin-dependent kinase 2). The abundance of the cyclin E protein and the cyclin E–Cdk2 complex was maximal in G_1 cells. These results provide further evidence that in all eukaryotes assembly of a cyclin-Cdk complex is an important step in the biochemical pathway that controls cell proliferation during G_1.

A major goal in studying the proliferation of eukaryotic cells is to describe the biochemical pathways that regulate progression through the G_1 phase of the cell cycle. Several cyclins have been identified that may function in G_1 regulation in higher eukaryotes. Transcription of the cyclin D gene is induced in murine macrophages in late G_1 by colony-stimulating factor–1 (*1*), and the gene is located at the breakpoint of a chromosomal rearrangement in a human parathyroid tumor (*2*). The genes encoding cyclin C, cyclin D, and cyclin E were discovered by screening human and *Drosophila* cDNA libraries for genes that could complement mutations in the *Saccharomyces cerevisae CLN* genes, which encode G_1 cyclins (*3–5*). In support of a G_1 function for cyclin E it was shown to bind and activate the Cdc2 protein kinase in extracts from human G_1 cells (*3*) and the steady state level of cyclin E mRNA is cell cycle–dependent in human cells and peaks in late G_1 (*5*).

Immunoprecipitates of cyclin E from exponentially growing MANCA cells (a human B cell line) contain a protein kinase (*3*). We examined the activity of the cyclin E–associated kinase during the cell cycle. We used centrifugal elutriation to separate exponentially growing MANCA cells into eight fractions (Fig. 1A). The cyclin E–associated kinase activity was cell cycle dependent. In three separate experiments, the maximal amount of kinase activity was between four and eight times greater than the minimum kinase activity during the cell cycle (Fig. 1B). The peak in kinase activity corresponded to those fractions containing the greatest percentage of late G_1 and early S phase cells. In some experiments we also observed a smaller second peak of cyclin E–associated kinase activity in the fraction containing cells in G_2 and M phases (*6*).

The activity of the cyclin E–associated kinase differed from that of the kinases associated with cyclin A. Monoclonal antibodies to cyclin A (C160) were used to immunoprecipitate cyclin A and its associated proteins from the same cell extracts in which the cyclin E–associated kinase had been measured (Fig. 1C). Cyclin A–associated kinase activity was first detected at the start of S phase (*7, 8*), but it continued to rise throughout S phase and peaked in G_2. The maximal activity of the cyclin A–associated kinase appeared to be approximately five to ten times that of the cyclin E–associated kinase (*6*).

We examined the kinetics with which the cyclin E–associated kinase accumulated during G_1. MANCA cells were synchronized in early G_1 and allowed to progress into S phase (*8*). Entry into S phase was determined both by flow cytometric measurement of nuclear DNA content (Fig. 1D) and by measuring [^{3}H]thymidine incorporation into chromosomal DNA (*6*). The cyclin E–associated kinase activity increased during G_1 and was maximal just as the cells entered S phase (Fig. 1E). The cyclin A–associated kinases appeared after the cyclin E–associated kinase activity; they

A. Koff and J. M. Roberts, Department of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104.
A. Giordano and B. R. Franza, Freeman Laboratory of Cancer Cell Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.
D. Desai and D. O. Morgan, Department of Physiology, University of California, San Francisco, CA 94143.
K. Yamashita and T. Nishimoto, Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka City 812, Japan.
J. W. Harper and S. Elledge, Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

*Present address: Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA 19140.

were absent in G_1 and first detected as cells entered S phase (Fig. 1E). The cyclin E–associated kinase was present in proliferating rat 208F cells but disappeared after cells became quiescent as a result of serum withdrawal (Fig. 1F). When rat PC12 cells were induced to differentiate into neurons by exposure to nerve growth factor, the cyclin E–associated kinase activity was diminished (Fig. 1F).

To characterize the proteins associated with cyclin E in vivo, we labeled exponentially growing HeLa cells with either [^{32}P]orthophosphate or [^{35}S]methionine and prepared extracts. Extracts were subjected to immunoprecipitation with affinity-purified antibodies to cyclin E (9), and antibodies to a synthetic peptide corresponding to the COOH-terminal region of human Cdk2 (anti-Cdk2) (*10*). The latter antiserum recognizes Cdk2 but not Cdc2 (*10, 11*). The immunoprecipitates were fractionated by two-dimensional polyacrylamide gel electrophoresis (PAGE) (Fig. 2), which resolves the various members of the Cdc2 protein family (*12–14*).

Immunoprecipitation of proteins from ^{32}P-labeled extracts with affinity-purified antibodies to cyclin E revealed a set of six proteins that had molecular sizes and isoelectric points consistent with their being members of the Cdc2 protein family (Fig. 2A, proteins labeled 1, 2, and a to d). Two of these proteins were also seen in anti-cyclin E immunoprecipitates from cells labeled with [^{35}S]methionine (Fig. 2A) (*15*). Proteins of similar mobility were immunoprecipitated with affinity-purified antibodies to the COOH-terminus of Cdk2, from extracts of either ^{32}P-labeled (Fig. 2B) or ^{35}S-labeled cells (Fig. 2C). Mixing the anti–cyclin E and anti-Cdk2 immunoprecipitates revealed that these proteins precisely comigrated. Thus, these cyclin E–associated proteins are apparently isoforms of Cdk2 (Fig. 2, B and C). Immunoprecipitation of the cyclin E–associated Cdk2 with anti–cyclin E was blocked by recombinant glutathione-S-transferase (GST)–cyclin E fusion protein (Fig. 2, B and C) but not by excess GST (5A). Proteins 1 and 2 are similar to the isoforms of Cdk2 associated with the adenovirus E1A protein and cyclin A (*12*). However, proteins a to d have not been observed previously in association with other proteins. It appears, therefore, that we have identified at least six phosphorylated isoforms of Cdk2 bound to cyclin E. One interpretation is that the cyclin E–Cdk2 complex integrates the information provided by the many signals that control cell proliferation. Phosphorylation of multiple sites on Cdk2 could have both positive and negative effects on Cdk2 activity, and a particular phosphorylated state may be required for specific functions. The activation of the cyclin A–Cdk2 complex, which occurs after commitment to the cell cycle has been made, may be responsive to many fewer factors and therefore biochemically less elaborate.

A second prominent set of proteins in anti–cyclin E immunoprecipitates from both ^{32}P- and ^{35}S-labeled cell extracts migrated at 45 kD, the expected molecular size of the cyclin E protein (Fig. 2A) (*3, 5*). Immunoprecipitation of these proteins was blocked by GST–cyclin E but not by GST (*6*). Therefore, we have tentatively identified these proteins as isoforms of cyclin E (*16*). Two of the three putative cyclin E isoforms detected in ^{35}S-labeled cell extracts were also detected in ^{32}P-labeled extracts indicating that cyclin E might exist in multiple phosphorylated states.

Two other proteins were specifically immunoprecipitated by anti–cyclin E (*17*). One migrated in the vicinity of the Cdk2

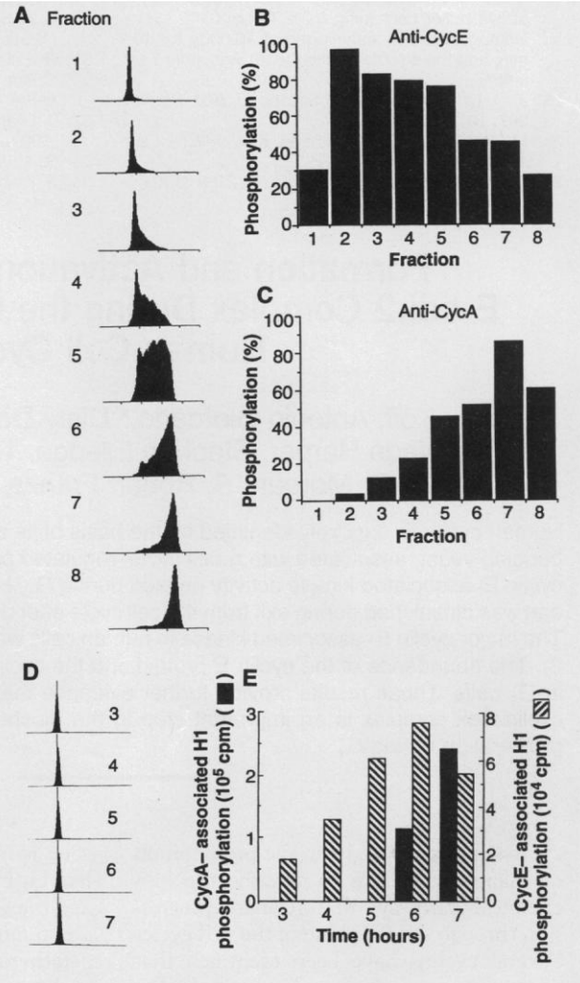

Fig. 1. Regulation of the cyclin E–associated kinase during the cell cycle, growth, and differentiation. Exponentially growing MANCA cells in different phases of the cell cycle were separated by centrifugal elutriation (*8*). Manca cells were maintained at a density of 2 × 10^5 cells/ml in RPMI containing calf serum (10%) in an atmosphere of 5% CO_2. (**A**) Position in the cell cycle. DNA content of the cells in each fraction was determined by flow cytometric analysis of propidium iodide stained nuclei. The ordinate shows cell number and the abscissa shows fluorescence intensity. (**B**) Cyclin E–associated histone H1 kinase activity. Proteins from equal numbers of cells from each fraction were immunoprecipitated with affinity purified antibodies to cyclin E (anti-CycE) and tested for the presence of histone H1 kinase (*26*). The ordinate shows the relative amount of H1 phosphorylation quantitated by phosphor imaging. (**C**) Cyclin A–associated histone H1 kinase activity. C160 monoclonal antibodies to cyclin A (anti-CycA) were used to measure cyclin A–associated kinase activity. (**D** and **E**) G_1 cells purified by elutriation were cultured at 32.5°C and portions were harvested hourly for measurement of nuclear DNA content (D) and cyclin A– and cyclin E–associated kinase activities (E) as cells approached and entered S phase. The numbers (3 to 7) in each flow cytometric histogram (D) indicate hours after release from mitotic arrest. The cyclin A– and cyclin E–associated kinase activities were quantified by phosphorimaging. (**F**) Cell lysates from growing and quiescent rat 208F cells, and growing rat PC-12 cells or rat PC-12 cells induced to differentiate with NGF were immunoprecipitated with pre-immune antiserum (lanes 1 and 4), affinity-purified anti–cyclin E (lanes 2 and 5), and affinity-purified antibodies to the COOH-terminal peptide from human Cdc2 (lanes 3 and 6) and assayed for H1 kinase activity. Rat PC-12 cells were maintained in Dulbecco's minimum essential medium (DMEM) containing fetal calf serum (5%) and horse serum (10%) in an atmosphere containing 10% CO_2. To induce neuronal differentiation confluent cells were split 1:20, and on the second day the medium was replaced with serum-free medium. Cells were incubated in serum-free medium for 24 hours and the medium was then changed to complete medium containing nerve growth factor (NGF) (50 ng/ml). NGF was added every two days and cells were harvested after 4 to 5 days. Rat 208F cells were maintained in DMEM containing 10% calf serum (CS) in an atmosphere containing 5% CO_2. To induce quiescence the cells were washed twice with phosphate-buffered saline and subsequently grown in DMEM with 0.1% calf serum for 48 hours.

isoforms (Fig. 2A, panel II, spot 3), but was not detected in anti-Cdk2 immunoprecipitates (Fig. 2C). Immunoprecipitation of this protein was blocked by GST–cyclin E (Fig. 2C) but not by GST (6). ^{32}P-labeling of this protein was not detected in extracts from ^{32}P-labeled cells (Fig. 2A). This protein appeared to precisely comigrate with a protein immunoprecipitated by an antiserum (G8) raised against the *Schizosaccharomyces pombe* Cdc2 protein (6). The G8 antiserum recognizes many members of the

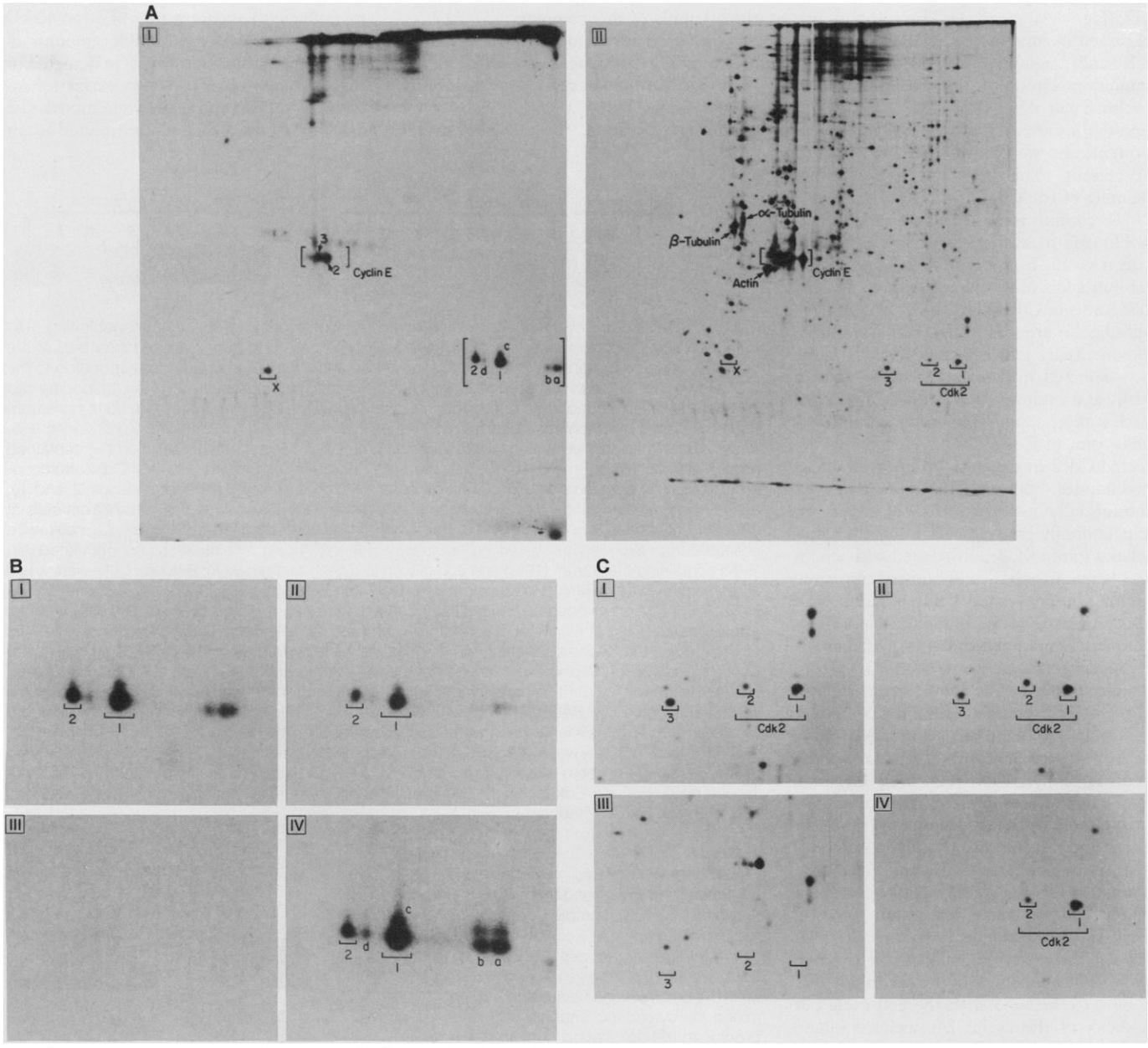

Fig. 2. Two-dimensional gel analysis of cyclin E–associated proteins (*28*). Extracts from exponentially growing HeLa cells were labeled with either [^{32}P]orthophosphate or [^{35}S]methionine and proteins were immunoprecipitated with the indicated antibodies. Immunoprecipitates were fractionated by two-dimensional high-resolution PAGE. HeLa cells were grown and extracts prepared as described (*12, 13*). We used affinity-purified anti–cyclin E (2 μg) and, in some instances, 20 μg of competing antigenic protein. Cdk2 was precipitated with 1 to 5 μg of affinity-purified anti-Cdk2 (*10*). Cell extracts were incubated with antibody for 1 hour at 0°C, and protein A Sepharose (40 μl) was added. The mixture was incubated on a rotator for 20 min and then centrifuged. The sedimented material was washed three times in lysis buffer. Conditions for two-dimensional gel electrophoresis were as described (*12*). Isoelectric focusing (horizontal dimension) was achieved between pH 3.5 (left) to pH 10 (right) and electrophoresis in the second dimension (vertical) was on a 10% polyacrylamide gel. (**A**) Panel I, anti–cyclin E immunoprecipitate of [^{32}P]orthophosphate-labeled extract; spots corresponding to Cdk2 isoforms are indicated by 1 and 2, and a to d. The phosphorylated isoforms of cyclin are indicated by 2 and 3. The other cyclin E–associated protein is indicated by x. Panel II, anti–cyclin E immunoprecipitate of [^{35}S]methionine-labeled extract. Tubulins and actin are indicated for purposes of orientation. Cyclin E isoforms are indicated in brackets. Cdk2 isoforms are indicated by 1 and 2. The other cyclin E–associated protein is indicated by x. (**B**) Close-up of the region of the gel containing the labeled isoforms of Cdk2 from ^{32}P-labeled extract. Panel I, anti–cyclin E immunoprecipitate; panel II, mixture of anti–cyclin E and anti-Cdk2 immunoprecipitates; panel III, anti–cyclin E immunoprecipitate prepared in the presence of unlabeled recombinant cyclin E; panel IV, anti-Cdk2 immunoprecipitate. (**C**) As in (B) except that ^{35}S-labeled extracts were used.

Cdc2 protein family, suggesting that this protein might be Cdc2 or a protein related to Cdc2.

Anti–cyclin E immunoprecipitates also contained a relatively acidic phosphoprotein with an apparent molecular size of 32 kD (Fig. 2A, spot x). This protein was observed in immunoprecipitates from both ^{32}P- and ^{35}S-labeled cell extracts and its immunoprecipitation was blocked by GST–cyclin E but not by GST (6). We do not know the identity of the acidic 32-kD protein, and we do not have any evidence at present to suggest that it is another member of the Cdc2 family (*18*).

To confirm these results we used immunoblotting to examine the association between cyclin E and both Cdk2 and Cdc2. Immunoblots of whole-cell extracts showed two forms of Cdk2 (Fig. 3A) (*10*). In both aphidicolin-arrested cells (Fig. 3) and in exponentially growing cells (Fig. 4) Cdk2 was detected in association with cyclin E (*19*), and cyclin E preferentially associated with a more rapidly migrating phosphorylated form of Cdk2 (*10, 20*). We also detected Cdc2 in the anti–cyclin E immunoprecipitates, although its abundance was substantially less than that of Cdk2. In exponentially growing cells a less phosphorylated form of Cdc2 associated with cyclin E, whereas in aphidicolin-arrested cells more highly phosphorylated forms of Cdc2 were also present (Fig. 3). Immunodepletion experiments confirmed that the major cyclin E–associated kinase was Cdk2 (*21*).

We examined the formation of the cyclin E–Cdk2 complex during the MANCA cell cycle. Exponentially growing MANCA cells were separated into eight fractions by centrifugal elutriation (Fig. 4A). Cyclin E and its associated proteins were immunoprecipitated with affinity-purified anti–cyclin E and the presence of Cdk2 was detected by immunoblotting with anti-Cdk2. The amount of the cyclin E–Cdk2 complex reached a maximum during late G_1 and early S phase and declined as cells progressed through the remainder of the cell cycle. The abundance of the cyclin E–Cdk2 complex correlated with the cell cycle periodicity of the cyclin E–associated kinase activity. In MANCA cells, the cyclin E–Cdk2 complex did not accumulate in an inactive form before its activation in late G_1.

We determined the abundance of the cyclin E protein during the cell cycle by immunoblotting the proteins in anti-cyclin E immunoprecipitates with anti–cyclin E. The amount of the cyclin E protein was maximal in late G_1 and declined in S, G2, and M phases (Fig. 4B). The amount of cyclin E protein detected was linearly dependent on the amount of cell extract subjected to immunoprecipitation (6). These observations suggested that the abundance of the cyclin E–Cdk2 complex, and hence the periodicity of the cyclin E–associated kinase activity, may be directly regulated by the abundance of the cyclin E protein. It is also possible that the phosphorylation state of cyclin E contributes to the assembly of this complex.

Our observations indicate that cyclin E preferentially associates with Cdk2 rather than Cdc2 in human cells. One possibility is that cyclin E has a greater affinity for Cdk2 than for Cdc2. We expressed cyclin E, Cdc2, and Cdk2 from baculovirus vectors (*22*). The formation of cyclin E–Cdc2 and cyclin E–Cdk2 complexes was measured by gel filtration chromatography. As monomers, both Cdc2 and Cdk2 eluted during Superose-12 gel filtration chromatography with apparent sizes of 30 to 40 kD (Fig. 5, A and B). Negligible amounts of histone H1 kinase activity were detected in these lysates (6, *27*). When extracts containing cyclin E and Cdk2 were mixed, the majority of the Cdk2 protein eluted at an

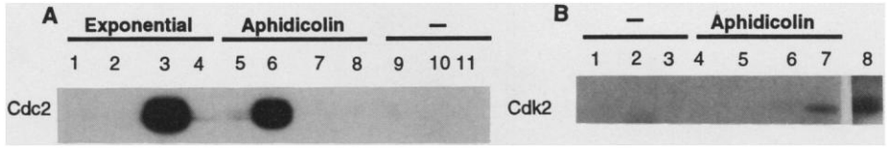

Fig. 3. Detection of cyclin E–Cdc2 and cyclin E–Cdk2 complexes by immunoblotting. (**A**) Immunoprecipitates from exponentially growing MANCA cells and from cells synchronized at the start of S phase with aphidicolin were probed with an antiserum to a peptide corresponding to the COOH-terminal region of human Cdc2 (anti-Cdc2). For all immunoprecipitations the antibodies had been cross-linked to Sepharose. Immunoprecipitations were done with the pre-immune antiserum (lanes 1, 8, and 9), Sepharose beads alone (lanes 2 and 7), affinity-purified anti-Cdc2 (lanes 3, 6, and 10), or affinity-purified anti–cyclin E (lanes 4, 5, and 11). The set of lanes labeled (–) contained no cell extract. (**B**) As in (A) except that the immunoblots were probed with anti-Cdk2. Immunoprecipitations were done with pre-immune serum (lanes 1 and 4), anti-Cdc2 (lanes 2 and 6), anti–cyclin E (lanes 3 and 7), or Sepharose beads alone (lane 5). A whole-cell extract from cells at the start of S phase is shown in lane 8. For synchronization at the start of S phase, G_1 cells were collected from exponentially growing populations of MANCA cells by elutriation and inoculated into RPMI containing calf serum (10%) and aphidicolin (5 μg/ml) and grown for 8 hours. Cells were lysed by sonication in SDS-RIPA (1% deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM tris (pH 8.0), 0.3 M NaCl, 0.1 mM orthovanadate, 50 mM NaF) containing protease inhibitors. For these experiments, approximately 1 mg of affinity-purified antibody, or 1 ml of pre-immune serum was coupled to 1 ml of CNBr-activated Sepharose. Immunoprecipitations were performed with 2.5×10^7 cells and 100 μl of antibody-linked Sepharose. Immune complexes were formed for 3 hours at 4°C and were then washed twice with SDS-RIPA containing BSA (5 mg/ml) and three times with SDS-RIPA. Samples were fractionated by PAGE (12% gels). Gels were transferred to nitrocellulose by semi-dry electroblotting and the membranes were blocked with either 2% milk in TNT [25 mM tris (pH 7.5), 150 mM NaCl, 0.05% Tween-20] for Cdc2 or Cdk2, or 1% gelatin in TNT for cyclin E. Blots were probed overnight at room temperature with either a 1:300 dilution of affinity-purified anti-Cdc2, or 1:1000 dilution of anti-Cdk2, or a 1:1000 dilution of affinity-purified anti–cyclin E. Bound antibody was detected with I^{125}-labeled protein A.

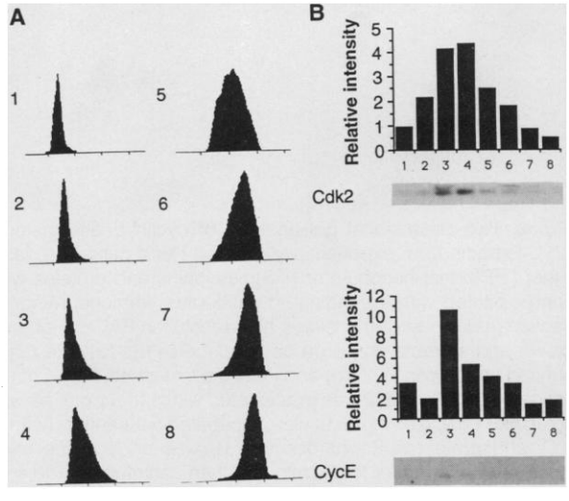

Fig. 4. Regulation of cyclin E concentration and the abundance of the cyclin E–Cdk2 complex during the cell cycle. (**A**) Position of cell fractions in the cell cycle. DNA content of the elutriated MANCA cell fractions was determined by cytometric analysis of propidium iodide–stained nuclei. (**B**) Lysates were subjected to immunoprecipitation with affinity-purified antibodies to cyclin E coupled to Sepharose and immunoblotted with either anti-Cdk2 (top panel) or affinity-purified anti-cyclin E (bottom panel). A non-specific band was seen above the Cdk2 band in some lanes. The histograms were generated by quantitating the amount of bound ^{125}I-labeled protein A with a phosphorimager. The actual autoradiogram is shown below each histogram (CycE, cyclin E). Extracts were prepared from 10^7 cells from each fraction and immunoprecipitated with 30 μl of anti–cyclin E Sepharose (30 μl).

approximate molecular size of 160 kD, indicating that it had formed a complex with cyclin E (Fig. 5D). In contrast, when extracts containing a similar amount of Cdc2 were mixed with cyclin E only a small fraction of the Cdc2 protein stably associated with cyclin E (Fig. 5C). Both the cyclin E–Cdc2 and cyclin E–Cdk2 complexes were active kinases (Fig. 5, C and D) (*23*). Although the simplest explanation for these results is that the affinity of cyclin E for Cdk2 is greater than its affinity for Cdc2, other explanations remain possible.

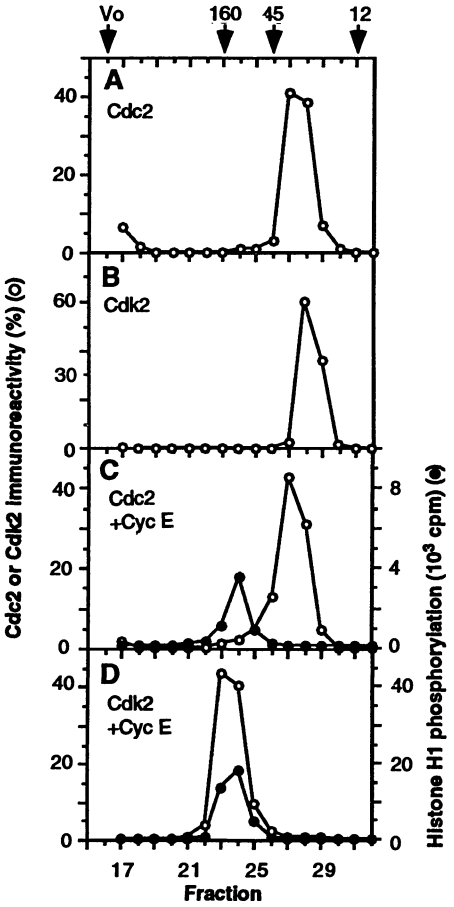

Fig. 5. Cyclin E binding to Cdc2 and Cdk2 in vitro. Diluted Sf-9 cells lysates containing Cdc2 (**A** and **C**) or Cdk2 (**B** and **D**) were incubated alone (A and B) or with cyclin E (C and D) for 20 minutes at 24°C. Cdc2, Cdk2, and cyclin E concentrations in these mixtures were all approximately 0.2 μM. Mixtures were subjected to gel filtration on a Superose-12 column and fractions were analyzed for the presence of Cdc2 or Cdk2 by immunoblotting with antibodies that recognize a peptide conserved among the Cdc2-related proteins (anti-PSTAIRE). Immunoblots were probed with ^{125}I-labeled secondary antibodies and quantified with a phosphorimager (open symbols). Histone H1 kinase activity was also measured in the fractions but is shown only for the two active combinations (C and D); activity in (A) and (B) was negligible. Kinase activity was quantitated by Cerenkov counting of excised gel bands (closed symbols). Note the change in phosphorylation scales in the different panels. Molecular sizes (in kilodations) of marker proteins, determined in parallel runs, are indicated (Vo, void volume; 160 kD, immunoglobulin G; 45 kD, ovalbumin; 12 kD, cytochrome C). Complex formation between human cyclin E and human Cdc2 or human Cdk2 and the kinase activity of the respective complexes was measured by gel filtration analysis exactly as described (*27*).

The evidence that cyclin E functions during the G_1 phase of the human cell cycle can be summarized as follows: Cyclin E can perform the G_1 functions of the yeast CLN proteins; it can complement mutations in the yeast *CLN* genes (*3*, *5*). Furthermore, cyclin E in combination with either human Cdc2 or human Cdk2 can rescue yeast strains that are mutated for both CLN and CDC28 function (*3*). We found that cyclin E associated with the Cdk2 protein kinase in human cells. The activity of the cyclin E–associated protein kinase, the abundance of the cyclin E protein, and the abundance of the cyclin E–Cdk2 complex were maximal during late G_1 and then declined as cells progressed through S, G_2, and mitosis. Activity of this kinase was also absent from cells that had exited the cell cycle and differentiated or become quiescent. In contrast to cyclin E, the cyclin A protein and cyclin A–associated kinase activities are not detectable until S phase starts (*7*, *8*). These actions of cyclin E during G_1 suggest that its physiological function precedes the S phase role of cyclin A (*24*). As a direct test of this hypothesis we have found that constitutive expression of cyclin E diminishes the growth factor requirements for proliferation of human cells and accelerates progression through the G_1 phase of the cell cycle (*25*). Our results offer further evidence that in eukaryotes assembly of a cyclin-Cdk complex is a critical step in the biochemical pathway that controls cell proliferation during the G_1 phase of the cell cycle.

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9. Antibodies to cyclin E were affinity-purified on columns of glutathione-*S*-transferase (GST)–cyclin E fusion protein. *Escherichia coli* containing the plasmids pGEX-2T or pGEX-2TcycE (*3*) were grown to an optical density at 600 nm of 0.4 and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 4 hours at 30°C. Cells were harvested, washed once in phosphate-buffered saline (PBS) and stored at −70°C. GST encoded by pGEX-2T was prepared as described (*3*). GST–cyclin E encoded by pGEX-2TcycE was prepared by a modification of a method described previously [M. Glotzer, A. W. Murray, M. Kirschner, *Nature* **349**, 132 (1991)]. The cells from a 500-ml culture were sonicated in 7 ml of 10 mM tris (pH 7.4), 0.1 M NaCl, 1 mM $MgCl_2$, 5 mM dithiothreitol (DTT) with protease inhibitors. The extract was centrifuged at 13,000*g* and the sediment was resuspended in 7 ml TN buffer [0.2 M tris (pH 8.2), 0.5 M NaCl] containing 5 mM DTT and centrifuged again. The sediment was suspended in 8 M urea containing 5 mM DTT and mixed gently at 4°C for 4 hours. The resulting extract was centrifuged at 13,000*g* for 10 min and the supernatant was dialyzed against TN buffer containing 1 mM DTT. At least 2.5 mg of either GST or GST–cyclin E was incubated with 1 ml of glutathione agarose beads for 2 hours at 4°C, centrifuged at 1000*g*, and washed 3 times with TN buffer containing 1 mM DTT. To couple the GST or GST fusion protein to the glutathione-agarose support, we transferred the support to a column and washed it with 0.1 M borate buffer (pH 8.0) and then 0.2 M triethanolamine (pH 8.2), and then added dimethylpimelimidate (DMP) cross-linker [40 mM DMP, 0.2 M triethanolamine (pH 8.2)]. Coupling was continued for 1 hour at room temperature. The column was then washed first with 40 mM ethanolamine (pH 8.2) at 4°C, then with 0.1 M borate buffer (pH 8.0). Uncoupled protein was eluted by washing the column with PBS containing 20 mM glutathione (pH 7.5), and the column was stored in PBS containing 0.5% azide. For affinity purification of antibodies to cyclin E, rabbit antiserum (100 ml) was precipitated with 50% ammonium sulfate. The precipitate was centrifuged at 8000*g* and suspended in 10 mM sodium phosphate (pH 8.0) and dialyzed against PBS. The dialysate was adjusted to 10% glycerol and passed over a GST column. The flow-through was collected and the column regenerated by washing with 0.2 M glycine (pH 2.2). The column was equilibrated with PBS and this process was repeated three times. The treated antiserum was applied to a GST–cyclin E column. The column was then washed first with PBS and then with PBS containing 2 M KCl, and bound antibody was eluted with NaI and sodium thiosulfate (*3*). The eluate was dialyzed against coupling buffer [0.1 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl] and concentrated five- to tenfold in Centricon 10 concentrators (Amicon). The affinity-purified anti–cyclin E specifically recognizes cyclin E and no other known cyclins. These antibodies do not immunoprecipitate or immunoblot cyclin A or B (*3*) and two-dimensional gel analyses indicate that none of the proteins immunoprecipitated by these antibodies is cyclin D (R.F., unpublished observation). The specificity of the antibodies results from the lack of recognition of motifs within the "cyclin box," a domain conserved among all known cyclins. The protein preparation used for immunization contained protein (~90% pure) from the amino NH_2-terminal 5 kD of the cyclin E protein, and therefore lacked the cyclin box domain. This NH_2-terminal region contains no similarity to other known cyclins.
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11. Antisera to Cdk2 and Cdc2 were prepared against peptides corresponding to the COOH-terminal portions of the respective proteins. The COOH-terminal portion of the Cdc2-related proteins is not highly conserved. Affinity-purified antibody to the COOH-terminal portion of Cdc2 and to the conserved PSTAIRE (Phe, Ser, Thr, Alu, Ile, Arg, Glu) domain of Cdc2 have been described (*8*). Antibodies to Cdk2 were raised against a peptide corresponding to the 15 COOH-terminal amino acids of human Cdk2 coupled to keyhole limpet hemocyanin. Two other antisera to a peptide corresponding to the nine COOH-terminal amino acids of human Cdk2 were also used in the course of these experiments (*10*, *20*). We have

used immunoprecipitation and immunoblotting to show that the antibodies to the COOH-terminal peptide from Cdc2 recognized Cdc2 but not Cdk2, and conversely that the antibodies to the COOH-terminal peptide from Cdk2 recognized Cdk2, but not Cdc2 (A.K. and J.M.R., unpublished observations).
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14. Essentially the same profile of cyclin E–associated proteins was observed by two-dimensional PAGE of immunoprecipitates from ^{35}S-labeled extracts of MANCA cells (B. Robert Franza, Antonio Giordano, Andrew Koff, James M. Roberts, unpublished data).
15. Spots a and b were also seen in longer exposures of immunoprecipitates from [^{35}S]methionine-labeled extracts.
16. Anti–cyclin E immunoprecipitates from MANCA and HeLa cells also contained a 45-kD protein that was detected in immunoblots with affinity-purified anti–cyclin E, and this protein precisely comigrated on PAGE with in vitro–translated cyclin E (A.K. and J.M.R., unpublished observations).
17. Immunoblots of anti–cyclin E immunoprecipitates with the anti–cyclin E detected a single protein at 45 kD. Therefore, the other proteins found in anti–cyclin E immunoprecipitates—phosphoprotein x and spot 3—were most likely bound to cyclin E and not simply detected because of cross-reactivity with this antibody.
18. A similar spot has not been observed in two-dimensional PAGE of proteins that bind the peptide p13 nor has it been seen in immunoprecipitates with G8 antiserum, which recognizes many members of the Cdc2 protein family (*13*). We have not observed a phosphoprotein of similar molecular size in immunoprecipitates with an antiserum to the conserved PSTAIRE domain of the Cdc2 protein family.
19. The identification of Cdk2 in cyclin E immunoprecipitates has been confirmed with three different antisera independently raised against peptides corresponding to the COOH-terminal portion of human Cdk2. All three antisera recognize Cdk2 but not Cdc2 (*10, 11, 20*).
20. S. Elledge *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2907 (1992).
21. Immunodepletion of Cdc2 protein from MANCA cell extracts had no effect on the amount of cyclin E–associated kinase whereas depletion of Cdk2 protein removed 50 to 80% of the cyclin E–associated kinase (A.K. and J.M.R., unpublished observations).
22. When these proteins were overexpressed in insect cells infected with baculovirus vectors, their intracellular concentrations were approximately 5 to 10 μM. To study the binding of cyclin E to Cdc2 and Cdk2, we diluted the extracts so that the concentration of these proteins was closer to normal physiologic levels, 0.2 μM.
23. The absolute amounts of kinase activity detected in these experiments did not correlate with the abundance of the particular cyclin-Cdk complexes and was probably limited by the abundance of a kinase that activates Cdk (*27*). Therefore an estimation of the specific activities of these particular complexes is not yet possible.
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26. For H1 kinase assays, 8.3×10^6 cells were sonicated in 100 μl of H1 lysis buffer [50 mM tris (pH 7.4), 0.25 M NaCl, 0.5% Nonidet P-40 containing the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml *N*-thosylphenylalanine chloromethyl ketone (TPCK), 20 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin]. Lysates were centrifuged at 13,000*g* for 10 min at 4°C and the supernatant was diluted with an equal volume of H1 lysis buffer. Extract (50 μl) was subjected to immunoprecipitation with anti–cyclin E (2 μl) or anti-Cdc2 (2 μl) for 1 hour, or with the C160 monoclonal antibody (5 μl) for 30 min. Rabbit antibodies to mouse immunoglobulin G (2 μl) were added and the mixture was incubated for 30 min. Immune complexes were collected on protein A Sepharose (Pierce) and then washed twice with lysis buffer and four times with H1 kinase buffer [20 mM tris (pH 7.4), 7.5 mM $MgCl_2$, 1 mM DTT]. Kinase reactions were performed as described (*3*).
27. D. Desai, Y. Gu, D. Morgan, *Molec. Biol. Cell* **3**, 571 (1992).
28. The QUEST regulatory protein quantitative database coordinates for two-dimensional gel-resolved spots in this report are: cyclin E isoforms: 1, HH11; 2, HH21; and 3, GH11. Cyclin E–associated protein: *x*, EE11; and Cdk2 isoforms: 1, RE41; 2, QE21; 3, NE11; a, TE31; b, TE41; c, RE51; and d, QE31. J. I. Garrels and B. R. Franza, *J. Biol. Chem.* **264**, 5823 (1989).
29. We thank R. Marraccino and M. Ohtsubo for help and advice during the course of this work. We also thank K. Keegan and S. Halegoua for contributing to our analysis of PC12 cell differentiation, and J. Schumacher, K. Carlson and R. Richman for help preparing antisera. A.G. and R.F. acknowledge support from the Freeman Charitable Trust and from James Watson. A.G. was supported by a fellowship from the Irvington Foundation. *QUEST* 2D gel facility and P. Renna and J. Duffy are thanked for performing the two-dimensional gels and preparing the photography and artwork, respectively. D.M. was supported by a Searle Scholars Award from the Chicago community trust and by a grant from the NIH. K.Y. and T.N. were supported by Grants-in-aid for Scientific Research and for Cancer Research from the Ministry of Education, Science and Culture and the Science and Technology Agency of Japan. S.E. is a PEW scholar in the biomedical sciences and this work was supported in part by a grant from the NIH to S.E. J.W.H. is the recipient of an American Cancer Society Junior Faculty Award. A.K. was supported by a virology training grant from the NIH. J.R. is a Lucille P. Markey scholar in the biomedical sciences. This work was supported in part by grants from the NIH and from the Lucille P. Markey Charitable Trust.

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Trypsin-Sensitive, Rapid Inactivation of a Calcium-Activated Potassium Channel

Christopher R. Solaro and Christopher J. Lingle*

Most calcium-activated potassium channels couple changes in intracellular calcium to membrane excitability by conducting a current with a probability that depends directly on submembrane calcium concentration. In rat adrenal chromaffin cells, however, a large conductance, voltage- and calcium-activated potassium channel (BK) undergoes rapid inactivation, suggesting that this channel has a physiological role different than that of other BK channels. The inactivation of the BK channel, like that of the voltage-gated Shaker B potassium channel, is removed by trypsin digestion and channels are blocked by the Shaker B amino-terminal inactivating domain. Thus, this BK channel shares functional and possibly structural homologies with other inactivating voltage-gated potassium channels.

Calcium-activated K^+ channels provide a link between elevations of cytosolic Ca^{2+} and membrane excitability (*1*). The physiological functions of different types of Ca^{2+}-activated K^+ channels are thought to reflect intrinsic differences in kinetics of activation, voltage dependence, and Ca^{2+} sensitivity (*2*). However, at a given membrane potential, Ca^{2+}-activated K^+ channels are, in general, thought to function as persistent sensors of the submembrane Ca^{2+} concentration (*2, 3*). Here, we report that a large conductance, voltage- and Ca^{2+}-activated K^+ channel, often termed the BK channel (*4*), can undergo rapid and virtually complete inactivation, suggesting a new physiological role for this channel distinct from that of noninactivating BK channels (*5*). Although the relation between BK channels and the family of voltage-gated K^+ channels is not known, BK channel inactivation and inactivation of some voltage-gated K^+ channels (*6–8*) share a number of striking similarities.

Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO 63110.

*To whom correspondence should be addressed.

Unlike most other BK channels (*3*), BK channels (275 ± 9 pS; mean ± SD; $n = 6$) in rat adrenal chromaffin cells undergo rapid inactivation (Fig. 1). The cytoplasmic face of excised, inside-out membrane patches (*9*) containing BK channels was exposed to saline containing a fixed concentration of calcium ($[Ca^{2+}]_i$). We activated BK channels by stepping the patch potential from −40 to +60 mV for 680 ms every 3 s. After opening in the presence of 5 μM $[Ca^{2+}]_i$, BK channels inactivate before the end of the voltage step (Fig. 1A). Single-channel openings were not observed when the patch was bathed in a Ca^{2+}-free solution (Fig. 1B). Ensemble averages generated from idealized single-channel records show that during the voltage step the probability of channels being open (P_O) rises and then decays with a time course reflecting single BK channel activation and subsequent inactivation (Fig. 1C). Consistent with effects of tetraethylammonium (TEA) on other BK channels (*10*), 1 mM TEA applied to an outside-out patch inhibited more than 90% of the single-channel ensemble current (Fig. 1D), whereas 50% inhibition was observed between 200 and