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Activation of the p34 CDC2 Protein Kinase at the Start of S Phase in the Human Cell Cycle

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Using a protocol for selecting cells on the basis of both size and age (with respect to the preceding mitosis), we isolated highly synchronous human G1 cells. With this procedure, we demonstrated that the p34 CDC2 kinase was activated at the start of S phase. Cyclin A synthesis began at the same time, and activation of the p34 CDC2 kinase at the start of S phase was, at least in part, due to its association with cyclin A. Furthermore, cells synchronized in late G1 by exposure to the drug mimosine contain active cyclin A/p34 CDC2 kinase, indicating that p34 CDC2 activation can occur before DNA synthesis begins. Thus, the cyclin A/CDC2 complex, which previously has been shown to be sufficient to start SV40 DNA synthesis in vitro, assembles and is activated at the start of S phase in vivo.

INTRODUCTION

The CDC2 protein in the yeast *Schizosaccharomyces pombe* and the homologous CDC28 protein in *Saccharomyces cerevisiae* are necessary for entry into S phase and mitosis (Nurse and Bisset, 1981; Piggot *et al.*, 1982; Reed and Wittenberg, 1990; Ghiara *et al.*, 1991; Surana *et al.*, 1991). The CDC2 protein is probably present in all eukaryotes (for review, see Cross *et al.*, 1989). Direct biochemical observations established that the mitotic role of the CDC2 protein kinase has been evolutionarily conserved (for review, see Nurse, 1990). However, the function of this protein regulating the transition from G1 to S phase in higher eukaryotes is not well understood.

In the G1 phase of the cell cycle of *S. cerevisiae*, the CDC28 protein kinase is required at a position called START, where cells become committed to the program of cell duplication (Hartwell *et al.*, 1974). At START, the yeast cell responds to specific extracellular signals, such as mating pheromone, and intracellular signals related to cell growth and either begins DNA synthesis or withdraws from the cell cycle and adopts alternative fates, such as mating or quiescence (Bucking-Throm *et al.*, 1973; Hereford and Hartwell, 1974; Hartwell and Unger, 1977; Johnston *et al.*, 1977). Some features of G1 regulation in the mammalian cell cycle closely resemble START control in the yeast cell cycle, suggesting that the molecular components of these pathways might be similar. In mammalian cells the kinetics of progression through G1 suggest that cell proliferation is controlled at the restriction point (Pardee, 1974, 1989; Zetterberg and Larson, 1985). Passage through the restriction point requires specific extracellular growth factors and a certain rate of intracellular protein synthesis. On passage through the restriction point, the cell becomes committed to complete the remainder of the cell cycle (for review, see Zetterberg, 1990).

A requirement for the CDC2 protein kinase in the G1 to S transition in higher eukaryotes has received some direct experimental support. The human *CDC2* gene can substitute for the *S. cerevisiae* CDC28 gene and the *S. pombe* cdc 2^+ gene for both their G1/S and mitotic functions (Lee and Nurse, 1987; Wittenberg and Reed, 1989). CDC2 may be required for DNA replication, because the CDC2 kinase isolated from human S-phase cells can activate SV40 DNA replication in extracts from human G1 cells in vitro (D'Urso *et al.*, 1990). Conversely, depletion of CDC2, or the related cyclin-dependent kinase 2 (CDK2) kinase from *Xenopus* extracts, can prevent DNA synthesis (Blow and Nurse, 1990; Fang and Newport, 1991). Also, inhibition of CDC2 synthesis in primary human T lymphocytes with anti-sense oligonucleotides blocks entry into S phase (Furakawa *et al.*, 1990).

Activation of the CDC2 protein kinase requires its association with a member of the cyclin protein family (for reviews, see Hunt, 1989; Murray and Kirschner, 1989). The CLN proteins are the cyclins that activate CDC28 at START (Sudbery *et al.*, 1980; Cross, 1988, 1990; Nash *et al.*, 1988; Hadwiger *et al.*, 1989; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990), and some signals that regulate START may act by modulating CLN

protein function (Chang and Herskowitz, 1990; Wittenberg *et al.*, 1990; Cross and Tinkelenberg, 1991). It is not known which cyclins are required for the start of S phase in higher eukaryotic cells; however, prominent candidates include cyclins A, C, D, and E (for review, see Pines and Hunter, 1991). Cyclin A is synthesized in S phase and is associated with the CDC2 protein in S-phase cells (Giordano *et al.*, 1989; Pines and Hunter, 1990). Perturbations of cyclin A metabolism correlate with altered regulation of cell proliferation, thereby indirectly implicating cyclin A in G1 control. For instance, the cyclin A gene is an integration site for the hepatitis B viral genome in a hepatocellular carcinoma (Wang *et al.*, 1990). Also, cyclin A is bound by the adenovirus E1A transforming protein, and mutations that disrupt this interaction eliminate the oncogenic potential of E1A (Giordano *et al.*, 1989, 1991; Pines and Hunter, 1990). Cyclin A also may have a direct role in DNA replication, because the addition of cyclin A to an extract from G1 cells is sufficient to activate SV40 DNA replication in vitro (D'Urso *et al.*, 1990).

It has been shown that both a cyclin A-associated kinase and the p34 CDC2 kinase are active during S phase; however, the precise relationship between the start of DNA synthesis, formation of a cyclin A/p34 CDC2 complex, and the activation of the p34 CDC2 kinase has not been analyzed. Using new protocols for cell synchronization, we have studied the timing of p34 CDC2 protein kinase activation during the G1 to S phase transition and studied the role of cyclin A in activating p34 CDC2. We show that cyclin A activates the p34 CDC2 kinase shortly before S phase begins.

MATERIALS AND METHODS

The monoclonal antibody, C160, to cyclin A (Giordano *et al.*, 1989) and rabbit anti-mouse antisera was provided by P. Whyte (McMaster University, Hamilton, Ontario, Canada). The rabbit polyclonal antiserum to cyclin A and DNA clone for cyclin A used for in vitro translation reactions were a gift from J. Pines and T. Hunter (Salk Institute for Biological Studies, San Diego, CA). The in vitro translation reaction of the cyclin A-containing plasmid was performed as described (Koff *et al.*, 1991). A peptide, EPVKEEKISPEP, of human cyclin B from amino acids 108–119 of the carboxy-terminus of human CDC2, YLDNQIKKM, and of the PSTAIRE domain of CDC2, CEGVPSTAIREISLLKE, was synthesized and rabbit antisera was purified as previously described (Koff *et al.*, 1991). All cell culture media was from GIBCO (Grand Island, NY), and bovine calf serum was obtained from Biocell (Carson, CA). γ-[^{32}P]ATP (3000 Ci/mmol) and [^{3}H]thymidine (80 Ci/mmol) were from Dupont New England Nuclear (Boston, MA), and [^{125}I]protein A was from Amersham (Arlington Heights, IL). The Superose 12 gel filtration column (24 ml, 10 × 300 mm) and fast-performance liquid chromatography (FPLC) system were supplied by Pharmacia (Piscataway, NJ).

Cell Culture and Synchrony

Human Burkitt lymphoma cells, MANCA, were grown in RPMI-1640 medium supplemented with L-glutamine (2 mM) and 10% bovine calf serum in an atmosphere of 5% CO_2 and 95% air mixture at 37°C. MANCA suspension cultures were maintained at a density of 2–3 × 10^5 cells/ml. The culture was mycoplasma free.

MANCA cell culture (4 l) was synchronized in metaphase of mitosis by the addition of nocodazole (Sigma Chemical, St. Louis, MO) at 100 ng/ml for 10 h. Approximately 80% of the population after 10 h was blocked in mitosis. These arrested cells were collected by centrifugation (10^3 × *g*, 5 min.) at 22°C and washed twice in growth media at 37°C. Cells were inoculated as a suspension culture into growth media (1 l) at 37°C until 50–75% of the population had a cell volume corresponding to cells in the G1 phase of the cell cycle (~2 h). The cell volume was determined by a Coulter Counter and Channelyzer (Coulter Electronics, Hialeah, FL). An enriched population of G1 cells was obtained from this culture that was released from nocodazole by modified method of centrifugal elutriation as previously described (Keng *et al.*, 1981).

For centrifugal elutriation, JE.6-B centrifuge, JE.5 rotor, and 30-ml chamber, which were manufactured by Beckman (Palo Alto, CA), were used. All subsequent procedures were done at 4°C. The cells, released from nocodazole after 2 h, were collected by centrifugation and resuspended in 20 ml of growth media. These cells were loaded in the chamber of the elutriator rotor at 1600 rpm with a flow rate of 20 ml/min using RPMI 1640 with 1% bovine calf serum. The flow rate was increased to 28 ml/min and remained constant throughout the procedure. The rotor speed was decreased in increments of 50–100 rpms until population with a volume of G1 cells exited from the chamber. Successive 250-ml fractions were combined and yielded an enriched G1 population of 10^8 cells. The purity of the G1 population was determined by flow cytometric analysis of DNA content and was >98% homogeneous. These G1 cells were inoculated into a suspension culture and harvested hourly. To arrest cells in late G1 of the cell cycle as described (Lalande, 1990), 300 μM of mimosine (Sigma Chemical) was added to the suspension culture of elutriated G1 cells.

For elutriation of exponential cells, G1 cells were collected from an asynchronous culture using the same rotor speeds and flow rates described above, but successive fractions were not combined. The flow rate was then increased at increments of 5 ml/min, and fractions were collected in the range of 30–60 ml/min.

Analysis of Cell Synchrony

For flow cytometric analysis, 10^6 cells were fixed in a solution of 95% ethanol and isotonic Tris(hydroxymethyl)amino methane (Tris)-buffered saline, pH 7.5, (4/1 vol/vol) at 4°C and stored for 3–5 d at 4°C. These fixed cells were stained with propidium iodide as described (Tate *et al.*, 1983). The fluorescence intensity from propidium iodide, which is proportional to the cellular DNA content, was quantitated using an EPICS flow cytometry and MDADS computer (Coulter Electronics). To determine the onset of cellular DNA synthesis as cells progressed from G1 to S phase of the cell cycle, [^{3}H]thymidine incorporation into DNA was measured as described (Roberts and D'Urso, 1988).

Cellular Extract Preparation

Cells (5 × 10^7) were harvested after centrifugal elutriation at the times indicated, washed twice in Tris-buffered saline, pH 7.5, and lysed in a hypotonic buffer A (0.2 ml) at 4°C. Buffer A contained 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5), 5 mM KCL, 0.5 mM $MgCl_2$, 0.25 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), pepstatin (0.5 μg/ml), 50 mM sodium fluoride, and 0.1 mM sodium orthovanadate. Alternatively, 0.1% Triton X-100 detergent was also added to buffer A but did not affect the results. On lysis, 200 mM NaCl was added to the extracts and incubated an additional 10 min. Cellular extracts were centrifuged at 10^5 × *g* for 30 min at 4°C, and supernatant was frozen in liquid nitrogen. The cellular extract was stored at −70°C.

Extracts from an equivalent amount of cells were applied to a Superose 12, FPLC gel filtration column at a rate of 0.35 ml/min, and 0.5-ml fractions were collected. The gel filtration column was equilibrated in buffer B, which contained 20 mM HEPES-KOH (pH 7.5), 0.25 mM EDTA, 0.25 mM ethylene glycol-bis(β-aminoethyl ether)-

N,N,N',N'-tetraacetic acid, 200 mM NaCl, 10% (vol/vol) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), pepstatin (0.5 μg/ml), 50 mM sodium fluoride, and 0.1 mM sodium orthovanadate. Each collected fraction was concentrated 10-fold with a Centricon 30-PM (Amicon, Danvers, MA).

Phosphorylation and Immunoprecipitation

For immunoprecipitation, either 45 μl of fractions from gel filtration chromatography of extracts or 150 μl of extract from 5×10^7 cells were diluted in buffer B with 0.1% Nonidet P-40 (NP40) to a final volume of 250 μl and incubated with 5 μg of affinity-purified antibodies to the carboxy-terminus of human p34 protein CDC2 or 15 μl of hybridoma supernatant containing monoclonal antibody to cyclin A (C160) for 30 min at 4°C. A secondary rabbit anti-mouse sera (5 μl) was added to reactions that contained cyclin A antibodies and incubated for an additional 30 min. The antibody and antigen complexes were immunoprecipitated with 1/10 volume of protein A-Sepharose (Repligen, Cambridge, MA) for 30 min at 4°C and washed three times with buffer B with 0.1% NP40. The immunoprecipitates were then washed in buffers used for the phosphorylation assay. For the experiments involving immunoprecipitations followed by immunoblotting, the hybridoma supernatant containing C160 monoclonal antibody was first coupled to CNBr-Sepharose 4B (Sigma Chemical) according to manufacturer's instructions. After coupling, the C160-Sepharose 4B was incubated with 1 M ethanolamine (pH 8.0) to block unreacted groups on the Sepharose.

For the phosphorylation of histone H1, the immunoprecipitates were resuspended in buffer B with 0.1% NP40 and 10 mM $MnCl_2$ and washed twice. The immunoprecipitates were resuspended in a final volume of 50 μl of buffer B with 10 mM $MnCl_2$, 30 μM ATP, 10 μCi of γ-[^{32}P]ATP, and 1 μg of histone H1 and incubated 30 min at 37°C. The phosphorylation products of H1 histone were analyzed by electrophoresis of a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel as described (Laemmli, 1970), followed by autoradiography.

The phosphorylation of proteins in the immunoprecipitates was assayed as described for histone H1 except that buffer B containing 0.1% NP40 and 10 mM $MgCl_2$ was used for the final two washings, and the resuspension was in a final volume of 50 μl of buffer B with 10 mM $MgCl_2$, 30 μM ATP, and 10 μCi of [γ-^{32}P]ATP. The phosphorylated proteins in the immune complexes were resolved by electrophoresis of a 12% SDS polyacrylamide gel and visualized by autoradiography. Phosphorylated proteins were also excised from undried gels and digested with Lys-C endoprotease according to the directions of the manufacturer (Promega, Madison, WI).

For peptide kinase assays, cellular extracts (5 μl) from concentrated gel filtration fractions were added to a final volume of 20 μl containing 25 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 mM NaCl, 50 μM ATP, 5 μ Ci of [γ-^{32}P]ATP, and 200 μM of the T-antigen peptide, CSH103, and assayed as described (Marshak *et al.*, 1991).

Western Blot Analysis

Proteins resolved by electrophoresis in a 12% SDS-polyacrylamide gel were transferred to a polyvinyldifluoride membranes (Immobilon-P, Millipore, Bedford, MA) at 0.8 mA/cm2 for 2 h using a semi-dry electroblotter (Ellard Instruments, Seattle, WA). After transfer, the membranes were incubated for 1 h in 25 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 (buffer C) with the addition of 1% nonfat dry milk or, when the anti-CDC2 C terminus antibody was used, 1% gelatin (cold fish skin, Sigma Chemical) and then rinsed twice. A 1/200 volume of each rabbit antisera to cyclins A and B and the PSTAIRE and carboxy-terminal domains of human CDC2 protein was added to buffer C and dilution of antisera was incubated separately with the immunoblots overnight. Membranes were washed three times for 10 min in buffer C, followed by the addition of 5 μCi [^{125}I]protein A/15 cm^2 for 1. The excess radioactive protein A was removed by washing five times for 10 min in buffer C, and the membranes were dried followed by autoradiography.

RESULTS

Cell Synchronization by Mitotic Release Followed by Elutriation

For our studies of the p34 CDC2 and cyclin A proteins, standard protocols were not adequate to obtain large quantities of cells that progressed synchronously from mitosis to the start of S phase (see DISCUSSION). Optimal synchronization was achieved by obtaining early G1 cells that were homogeneous for both cell size and cell age with respect to the preceding mitosis. An exponentially growing MANCA cell culture was arrested in metaphase using nocodazole, a microtubule destabilizing agent. After nocodazole was removed from the culture, the cells completed mitosis and cytokinesis. At the time when 50% of the cells had completed cytokinesis (~2 h after nocodazole removal), a homogeneous size fraction of newborn G1 cells was separated from residual M-phase cells by centrifugal elutriation. The elutriation procedure was completed in ~1 h. The newborn G1 cells were returned to cell culture where the population progressed synchronously through G1 and entered S phase 6.5 h after release from the mitotic block. The synchrony of S phase entry was established both by flow cytometry to measure cellular DNA content and by [^{3}H]thymidine incorporation to measure cellular DNA synthesis (Figure 1).

Synthesis of Cyclin A and p34 CDC2 During the G1 to S Interval of the Cell Cycle

Our experiments followed the synthesis and activation of p34 CDC2, cyclin A, and cyclin B during the G1 and S phases of the human cell cycle. Antisera against these proteins were characterized by immunoblotting (Figure 2). The cyclin B antiserum was raised against a peptide from the amino terminus of human cyclin B (see MATERIALS AND METHODS). This antisera recognized a protein with an apparent molecular mass of ~60 kDa, which is consistent with the known molecular mass of cyclin B (Figure 2A). To confirm that this protein was cyclin B, we examined its abundance during the cell cycle. Exponentially growing MANCA cells were fractionated within the cell cycle by centrifugal elutriation (Figure 2B). Extracts from these fractions were immunoblotted using our cyclin B antiserum (Figure 2C). This antiserum recognized a 60-kDa protein that was absent from G1 cells, increased in abundance during the cell cycle, and peaked in G2/M; this demonstrated that our antiserum detected the human cyclin B protein. The PSTAIRE antiserum should recognize all known members of the CDC2 protein family, whereas the antiserum against the peptide corresponding to the p34 CDC2 carboxy terminus will be specific for the detection of

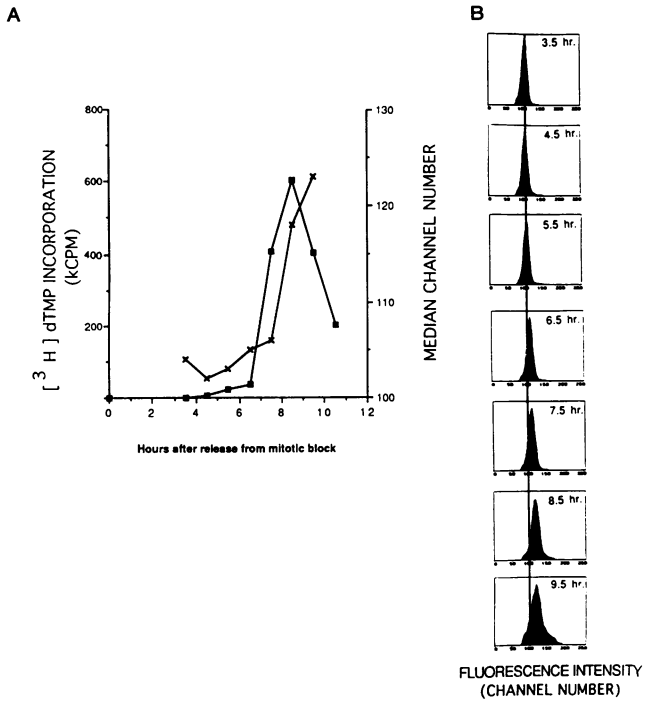

Figure 1. Synchronization of MANCA cells by mitotic release/elutriation. G1 cells were obtained by elutriation 3.5 h after release from a mitotic arrest (see MATERIALS AND METHODS) and were grown for 6 h (9.5 h after mitotic release). Samples of the cell population were harvested hourly as indicated and were incubated with [^{3}H]thymidine or stained with propidium iodide for flow cytometry. (A) The time course incorporation of [^{3}H]thymidine into cellular DNA (■). (B) For flow cytometric analysis, the DNA content of cells is proportional to the fluorescence intensity of propidium iodide. The fluorescence intensity of propidium iodide from stained cells is shown in the histograms and expressed in arbitrary units in a linear scale (channel number) on the horizontal axis and the cell number on vertical axis. The median channel number of each population (×) is graphed in (A).

the p34 CDC2 protein. Both antisera recognized the multiple forms of p34 CDC2, which are known to differ in their phosphorylation state (Simanis and Nurse, 1986; Draetta and Beach, 1988; Solomon *et al.*, 1990). Both antisera also recognized another protein of 45 kDa (see Figure 5). This protein was bound with lower affinity than authentic p34 CDC2 because it was not seen in immunoblots using greater dilutions of the antisera nor was it immunoprecipitated by these antisera. The cyclin A antiserum has been described previously (Pines and Hunter, 1990).

We determined the steady-state levels of cyclin A, and p34 CDC2 proteins as cells progressed from the conclusion of mitosis to the middle of S phase. Extracts were prepared hourly and immunoblotted using an antiserum against the human cyclin A protein (Pines and Hunter, 1990). Cyclin A protein accumulated to detectable levels just before, or coincidentally with, the start of cellular DNA synthesis and continued to increase in abundance throughout S phase (Figure 3). Total cellular levels of cyclin A were maximal in G2/M (Pines and Hunter, 1990;). Identical samples were also im-

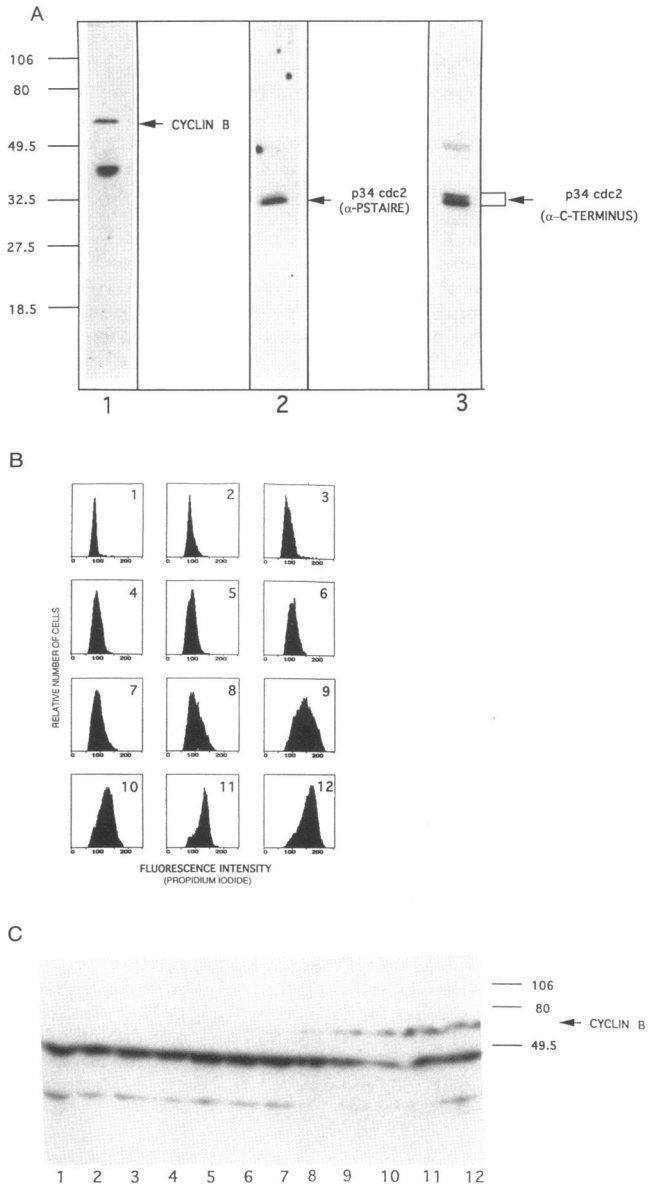

Figure 2. Characterization of cyclin B and CDC2 antisera. (A) Extracts from exponentially growing MANCA cells were immunoblotted with antisera against an N-terminal peptide of human cyclin B (lane 1), the PSTAIRE domain of CDC2 (lane 2), and a peptide from the C-terminus of p34 CDC2 (lane 3). Each antiserum was used at a dilution of 1:1000, and autoradiography was used for 18 h at −70°C with an intensifying screen. (B) Exponentially growing MANCA cells were separated into 12 fractions by centrifugal elutriation, and the cell cycle position of each fraction was determined by flow cytometric measurement of cellular DNA content. The DNA histograms are labeled numerically and correspond to each lane of the immunoblot. (C) Extracts from each fraction were immunoblotted using the N-terminus-specific cyclin B antiserum. The bands representing the cyclin B and p34 CDC2 proteins are indicated.

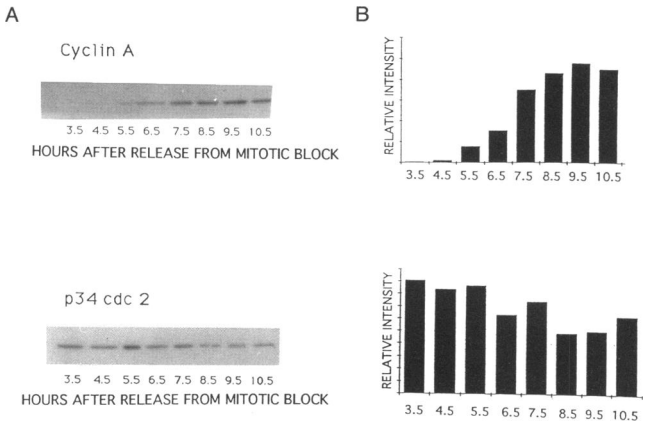

Figure 3. Expression of human p34 CDC2 and cyclin A protein during the G1 to S phase interval of the cell cycle. Synchronous newborn G1 cells were inoculated into fresh medium, and lysates were prepared from 10^6 cells at the indicated times. (A) Proteins from these lysates were resolved on an SDS-polyacrylamide gel, transferred to an Immobilon-P membrane. Membranes were incubated with either antiserum to cyclin A (C160) or human p34CDC2 (carboxy-terminus), followed by [^{125}I]protein A. The presence of cyclin A or human CDC2 is visualized by autoradiography. (B) The amount of cyclin A (top) and human cdc 2 (bottom) was quantitated from the immunoblots with phosphor imaging plates using a Molecular Dynamics 400 A instrument and is expressed as relative intensity on the vertical axis for each sample of harvested cells.

munoblotted using antisera raised against either the C-terminal 8 amino acids of the p34 CDC2 protein family (Figure 3) or the conserved PSTAIRE domain of the CDC2 protein. As others have observed (Durkacz *et al.*, 1986; Simanis and Nurse, 1986; Draetta and Beach, 1988), the abundance of the p34 CDC2 protein is invariant during this interval of the cell cycle.

Formation of a Cyclin A/p34 CDC2 Complex at the Start of S Phase

Using the protocol described above, we determined whether a cyclin A/p34 CDC2 complex assembled at the start of S phase. Extracts from cells at the start of S phase and from exponentially growing cells were immunoprecipitated with either anti-C terminus p34 CDC2 antiserum or C160 anti-cyclin A monoclonal antibodies coupled to Sepharose 4B using conditions that did not disrupt cyclin/CDC2 complexes (Figure 4). Immunoblotting demonstrated the presence of cyclin A in p34 CDC2 immunoprecipitates (Figure 4, lanes 5 and 6) and, conversely, the presence of p34 CDC2 in cyclin A immunoprecipitates (Figure 4, lanes 3 and 4). Interestingly, in exponentially growing cells more than half of the p34 CDC2 protein associated with cyclin A exhibited a retarded electrophoretic mobility (Figure 4, lane 3). This is consistent with phosphorylation of threonine 14 and/or tyrosine 15, two modifications that inactivate the p34 CDC2 kinase (Gould and Nurse, 1989; Solomon *et al.*, 1990; Krek and Nigg, 1991; Norbury *et al.*, 1991). In contrast, at the start of S phase almost all of the p34 CDC2 associated with cyclin A was in the most rapidly migrating form (Figure 4, lane 4), suggesting that the cyclin A/p34 CDC2 complex was an active kinase (see below). Our experiments did not address the possibility that other cyclins also played roles in activating p34 CDC2 so it remains to be determined if other cyclin/CDC2 and CDK complexes assemble at this point in the cell cycle.

Activation of the p34 CDC2 Protein Kinase at the Start of S Phase

We determined the activity of the CDC2 protein kinase and its association with cyclins A and B in newborn G1 cells, in cells at the start of S phase, and in metaphase-arrested cells. Activation of the p34 CDC2 protein kinase activity requires its association with cyclin and formation of the cyclin/CDC2 complexes can be observed by gel-filtration chromatography of cell extracts. A population of newborn G1 cells was prepared as described above

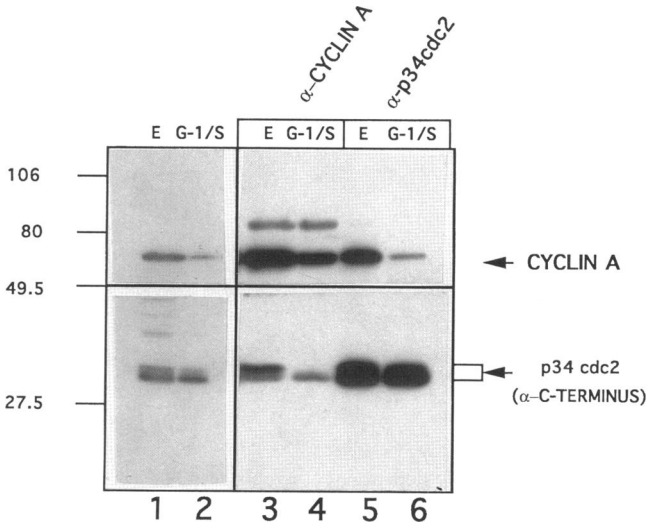

Figure 4. Association of cyclin A with p34 CDC2 in exponentially growing cells and in cells at the start of S phase. Extracts from exponentially growing cells (lanes 1, 3, and 5) and cells at the start of S phase (lanes 2, 4, and 6) were immunoprecipitated with the C160 monoclonal antibody coupled to Sepharose 4B, for cyclin A (lanes 3 and 4) or an antiserum specific for the carboxy-terminus of p34 CDC2 (lanes 5 and 6). The immunoprecipitates, as well as the starting extracts (lanes 1 and 2), were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted. The immunoblot was divided at the position of the 49.5-kDa molecular standard, and the relevant portions of each membrane were incubated with either rabbit antiserum to cyclin A (top) or carboxy-terminus of human p34 CDC2 (bottom), followed by [^{125}I]protein A. The multiple phosphorylated forms of p34 CDC2 are indicated. Note that at the start of S phase only the fastest migrating form of p34 CDC2 is associated with cyclin A. The additional band above cyclin A in lanes 3 and 4 is nonspecific, as it was seen in mock immunoprecipitations that used buffer instead of cell extract (not shown).

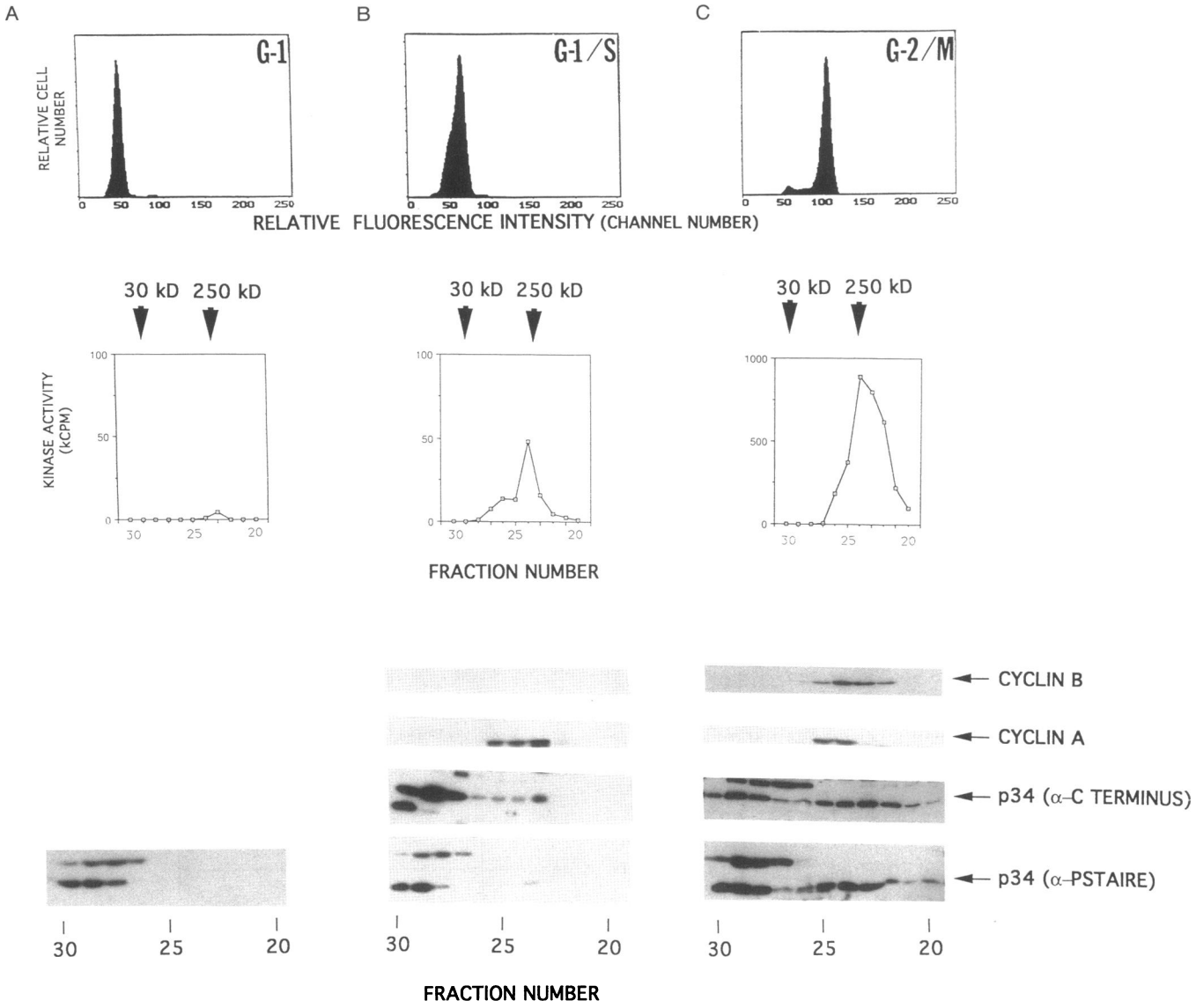

Figure 5. Cell cycle dependent assembly of p34 CDC2 protein into high molecular weight complexes is associated with activation of the CDC2 kinase. G1 cells (A), cells at the start of S phase (B), and cells arrested at G2/M by nocodazole (C) were obtained as described in text. (Top) A sample of each population was stained with propidium iodide and analyzed by flow cytometry. Histograms representing the DNA content of each population is shown. (Middle) Lysates from an equivalent number of each population were prepared and fractionated separately by gel filtration chromatography. A volume (1:10) of each gel filtration fraction was assayed for CDC2 kinase activity using a synthetic peptide of T antigen. The amount of [^{32}P]incorporation for each kinase reaction is depicted. (Bottom) The remaining portion of each gel filtration fraction was divided equally, resolved on two SDS-polyacrylamide gels, and transferred to Immobilon-P membranes. The relevant portions of each membrane were incubated with either rabbit antiserum to cyclin A, cyclin B, human p34 CDC2 (carboxy-terminus), or the PSTAIRE domain of p34 CDC2, followed by [^{125}I]protein A. Cyclins A and B and p34CDC2 are identified.

(Figure 5A, top) and fractionated by Superose 12 gel filtration chromatography. Each fraction was assayed for kinase activity toward a T-antigen peptide that is a specific substrate of the p34 CDC2 protein kinase (Marshak *et al.*, 1991). Also, each fraction was immunoblotted using antisera to either the PSTAIRE domain or C-terminus of the human CDC2 protein. In addition, the same fractions were immunoblotted using the cyclin A antiserum or an anti-peptide antiserum against human cyclin B. In newborn G1 cells, a very small amount of CDC2 protein kinase activity was detected (Figure 5A, middle). Immunoblotting, using either PSTAIRE or C-terminus antisera, showed that all the CDC2 protein was in a monomeric state, eluting from the gel filtration column at an apparent molecular mass of 30–40 kDa (Figure 5A, bottom). Neither cyclin A nor cyclin B

could be detected in these early G1 cells (see Figures 2 and 3).

An equivalent amount of G1 cells was returned to culture and harvested at the start of S phase (Figure 5B, top). Gel filtration chromatography of extracts from these cells revealed a peak of CDC2 protein kinase activity with a molecular mass of ~250 kDa (Figure 5B, middle). Immunoblotting showed that assembly of ~5% of the CDC2 protein into a 250 kDa protein complex correlated with the appearance of CDC2 protein kinase activity (Figure 5B, bottom). This portion of the CDC2 protein could be detected with both PSTAIRE and C-terminus antisera showing that at least some complexed form of the CDC2 protein was p34 CDC2. Also, cyclin A protein was present in cells at the start of S phase and cofractionated with the kinase active portion of the CDC2 protein. Cyclin B protein was not detected at this stage of the cell cycle (Figure 5B, bottom; see also Figure 2).

A population enriched for G2/M cells was prepared by nocodazole-induced metaphase arrest (Figure 5C, top), and cell extracts were fractionated by Superose 12 gel filtration chromatography. Consistent with previous results (Draetta *et al.*, 1989), mitotic cells had a broad peak of CDC2 protein kinase activity eluting from the gel filtration column with proteins in the range of 250 kDa (Figure 5C, middle). Note that the level of CDC2 protein kinase in M phase cells was ~20-fold higher than in cells from the start of S phase. Immunoblotting showed that the p34 CDC2 protein exists in at least two states in the enriched mitotic cell population (Figure 5C, bottom). Approximately half of the protein appeared to be monomeric, eluting from the gel filtration column at 30–40 kDa. This portion of the CDC2 protein had no detectable kinase activity on the T-antigen peptide substrate. The remainder of the CDC2 protein was found in higher molecular mass complexes and cofractionated with the observed CDC2 protein kinase activity. Note that the cyclin A/CDC2 complex showed a lower apparent molecular mass than the cyclin B/CDC2 complex (Figure 5C, bottom).

Activation of the Cyclin A/p34 CDC2 Complex at the Start of S Phase

The above experiment did not directly demonstrate activation of the p34 CDC2 kinase at the start of S phase because it was possible that kinases related to CDC2, such as CDK2 (Elledge and Spottswood, 1991; Koff *et al.*, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991), would also phosphorylate the T-antigen peptide kinase substrate. Also, it was not apparent whether the cyclin A-associated form of p34 CDC2 was active. To address these questions, we fractionated extracts from newborn G1 cells and from cells at the start of S phase by Superose 12 gel filtration chromatography and immunoprecipitated the fractions containing the high molecular mass complex and monomeric forms of CDC2 with either anti-cyclin A, C160, monoclonal antibodies, or C-terminus specific p34 CDC2 antiserum. Both the cyclin A and CDC2 immunoprecipitates from the early S phase cells showed histone H1 kinase activity (Figure 6B), whereas neither immunoprecipitate from newborn G1 cells showed H1 kinase activity (Figure 6A). In neither case did monomeric CDC2 show H1 kinase activity. Therefore, both cyclin A and p34 CDC2 were associated with a histone H1 kinase activity at the start of S phase.

Due to the diversity of potential cyclin/CDC2 or CDK complexes, it is difficult to be certain that one specific complex is an active kinase at a given point in the cell cycle. In the experiments described above it was possible that the active p34 CDC2 kinase, at the start of S phase, was associated with a cyclin other than cyclin A and that the cyclin A-associated kinase was not p34 CDC2. Thus, the cyclin A/p34 CDC2 complex that assembled at the start of S phase (Figure 4) might have accumulated in an inactive form. We addressed this issue by taking advantage of the fact that autophosphorylation of the associated cyclin is characteristic of active cyclin/CDC2 complexes (Lohka *et al.*, 1988). Specifically, we demonstrated that cyclin A was autophosphorylated in p34

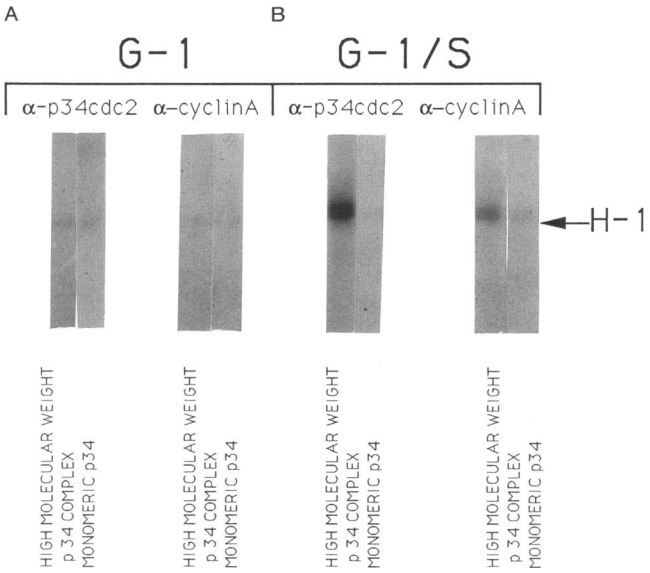

Figure 6. Histone H1 kinase activity immunoprecipitated from G1 cells and cells at the G1/S boundary. Lysates from an equal number of G1 cells (A) or cells at the G1/S boundary (B) were prepared and fractionated separately by gel filtration chromatography. Gel filtration fractions, which should contain a high molecular mass complex of p34CDC2, ~250 kDa (fraction 24), and monomeric p34CDC2 (fraction 27) were collected from each population and divided equally. Antisera to the carboxy-terminus of human p34 (α-p34CDC2) or cyclin A, C160 (α-cyclin A), were added to indicated samples for immunoprecipitations. The immunoprecipitations were assayed for histone H1 kinase activity. The kinase reactions were resolved on a SDS-polyacrylamide gels, and the phosphorylated histone H1 is visualized by autoradiography.

CDC2 immunoprecipitates and, therefore, that the cyclin A/p34 CDC2 complex was an active kinase. We immunoprecipitated the p34 CDC2 protein from cells at the start of S phase using the C-terminus specific p34 CDC2 antiserum and incubated the immunoprecipitate with [γ-^{32}P]ATP. One band at ~60 kDa was reproducibly detected (band A, Figure 7A, lane 1), in addition to several others at molecular mass > 70 kDa that have not been further characterized. The ~60-kDa phosphoprotein precisely comigrated with in vitro-translated human cyclin A protein and, as shown below, this phosphoprotein was cyclin A. In these reactions two others phosphoproteins of 45 and 34 kDa were often observed, although their intensity of labeling was variable (bands B and C—compare Figure 7A, lane 1, with Figure 7B, lane 3). The identities of the other phosphoproteins have not been established.

To show that the ~60-kDa phosphoprotein was cyclin A, we compared the phosphoproteins observed in autophosphorylation reactions using either p34 CDC2 or cyclin A immunoprecipitates from cells at the start of S phase (Figure 7B). Both cyclin A and p34 CDC2 immunoprecipitates autophosphorylated a band migrating at ~60 kDa. The ~60-kDa phosphoprotein from both reactions was mapped by one-dimensional partial proteolysis using the lys-C endoprotease. The partial proteolytic digestion patterns were virtually identical (Figure 7C). These experiments showed that the cyclin A/p34 CDC2 complex that assembled at the start of S phase was an active kinase because it was able to autophosphorylate the cyclin A subunit. These experiments did not address the possibility that cyclin A also associated with other members of the CDC2 protein family at this point in the cell cycle, such as CDK2 (Pines and Hunter, 1990; Tsai *et al.*, 1991), and that some of the cyclin A-associated histone H1 kinase activity was due to these other complexes.

Formation of the Cyclin A/p34 CDC2 Complex Preceded the Start of S Phase

We used the drug mimosine, which arrests cells in late G1 (Watson *et al.*, 1991), to show that the accumulation of active cyclin A/CDC2 complexes did not require that the cells begin DNA synthesis but actually preceded the start of S phase. MANCA cells arrested by mimosine treatment have a G1 DNA content, as determined by flow cytometry (Figure 8A), and enter S phase ~30 min after removal of the drug. Cells released from an aphidicolin-induced early S phase arrest are not sensitive to mimosine until the following G1 period (Lalande, 1990; Marracino, unpublished observations). Conversely, cells released from a mimosine block show a first cycle arrest on exposure to aphidicolin. These observations demonstrate that mimosine arrests cell cycle progression before the start of DNA synthesis and not during S phase. The mechanism of mimosine action is not known, although mimosine may effect the function of the eukaryotic translation initiation factor 4D (Hoffman *et al.*, 1991).

Synchronous newborn G1 cells, prepared as above, were cultured in the absence or presence of mimosine. After 3 h, the cells without mimosine had entered S phase, whereas the mimosine-treated cells were blocked

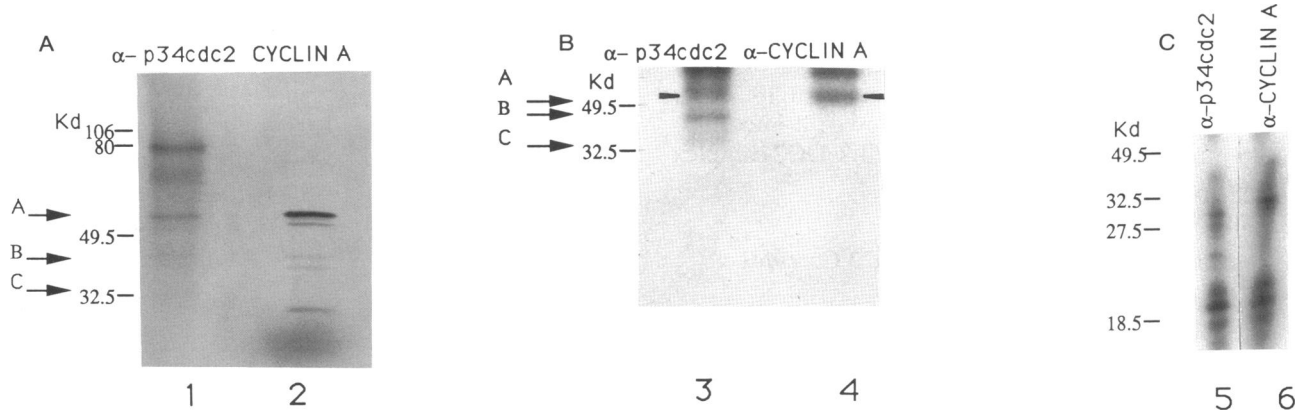

Figure 7. Autophosphorylation of p34 CDC2 and Cyclin A immunoprecipitates. (A) Whole-cell extracts from cells at the G1/S boundary were prepared, and p34CDC2 was immunoprecipitated with antiserum to the carboxy-terminus of human CDC2. Immunoprecipitates were incubated with γ-[^{32}P]ATP in a kinase reaction, and the products were resolved by SDS-polyacrylamide gel electrophoresis. An autoradiography of a dried gel is shown. The positions of specific phosphorylated proteins immunoprecipitated are indicated (lane 1, A–C), and the positions of molecular mass standards are shown. An in vitro translated [^{35}S]labeled human cyclin A is resolved in lane 2 and migrates to a position indicated as A. (B) Extracts were prepared as described in A, but immunoprecipitations were done with either antiserum to the carboxy-terminus to human CDC2 (lane 3) or a monoclonal antibody (C160) to cyclin A (lane 4). An autoradiography of the undried gel is shown. (C) Gel slices containing the phosphorylated proteins migrating to position A in lanes 3 and 4 were excised and incubated with 2.5 U/ml lys-C endoprotease, and the proteolytic digestion products were separated on an 12% SDS-polyacrylamide gel, lanes 5 and 6, respectively. The digestion products were visualized by autoradiography from a dried gel.

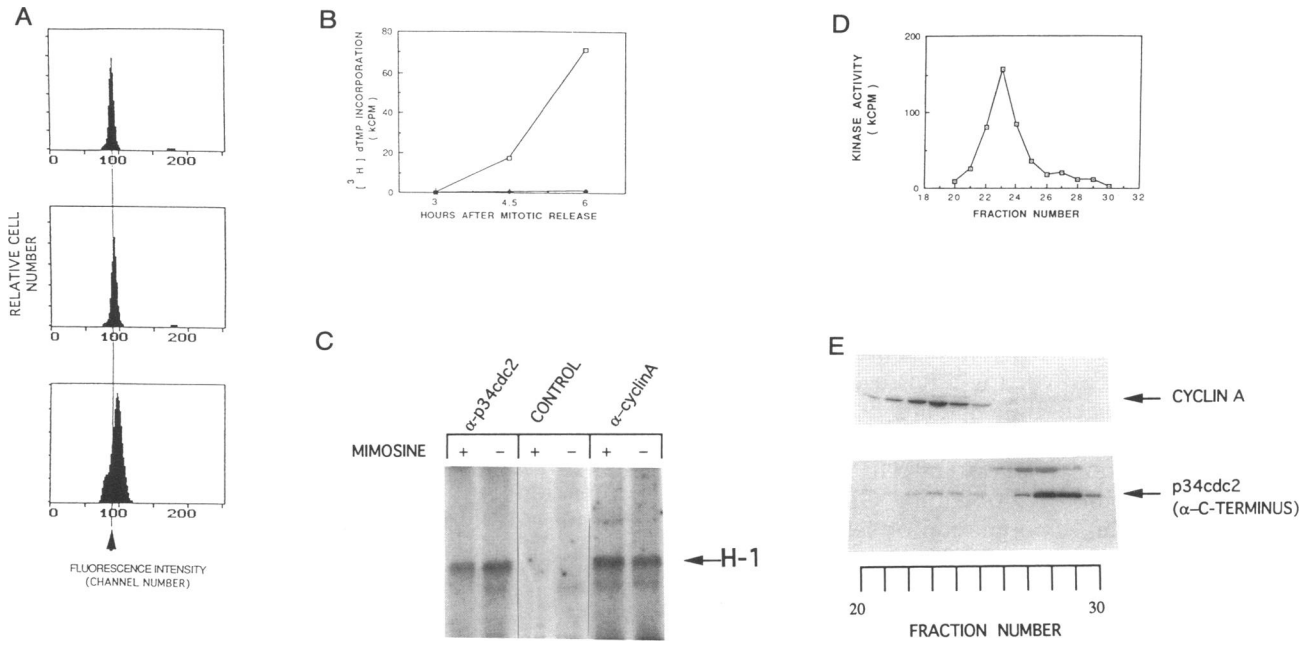

Figure 8. Expression of cyclin A, p34CDC2, and appearance of CDC2 kinase activity in cells arrested in late G1. G1 cells were grown for 3 h either in the presence or absence of mimosine. (A) A sample of each population was stained with propidium iodide and analyzed by flow cytometry. Histograms representing the DNA content of the G1 population (top) and cells grown in the presence (middle) or absence of mimosine (bottom) are shown. (B) The time course incorporation of [^{3}H]thymidine into cellular DNA is graphed as G1 cells were grown in the presence (closed symbols) or absence (open symbols) of mimosine. (C) Lysates from an equivalent number of cells grown for 3 h in the presence (+) or absence (−) of mimosine were prepared. Equivalent volumes of each lysate were incubated with either the antisera to the carboxy-terminus human CDC2 (α-C-terminus), a nonspecific rabbit antisera (control), or cyclin A, C160 (α-cyclin A), and immune complexes were precipitated. The immunoprecipitates were assayed for histone H1 kinase activity. (D) Lysates from a cell population grown for 3 h with mimosine were prepared and fractionated separately by gel filtration chromatography. A volume (1:10) of each gel filtration fraction was assayed for kinase activity using a synthetic peptide of T antigen. The peptide kinase reaction were quantitated by scintillation counting. The amount of [^{32}P]incorporation for each kinase reaction is depicted. Note that the orientation of the gel filtration profile is opposite to that shown in Figure 3. (E) The remaining portion of each gel filtration fraction was resolved on an SDS-polyacrylamide gel and transferred to an Immobilon-P membrane. The relevant portions of each membrane were incubated with either antiserum to cyclin A (C160) or human p34CDC2 (carboxy-terminus), followed by [^{125}I]protein A. The immunoblot were visualized by autoradiography, and cyclins A and human CDC2 are identified.

in G1, as assayed by either flow cytometry (Figure 8A) or [^{3}-H]thymidine incorporation into cellular DNA (Figure 8B). Extracts from these cells were immunoprecipitated using an anti-cyclin A monoclonal antibody (C160) and the C-terminus specific anti-p34 CDC2 serum. Both immunoprecipitates showed H1 kinase activity (Figure 8C). In addition, extracts from the mimosine-treated cells were fractionated by Superose 12 gel filtration chromatography and immunoblotted using cyclin A and p34 CDC2 antisera and assayed for CDC2 protein kinase. Immunoblotting showed the assembly of the CDC2 protein into a high molecular mass complex at ~250 kDa, the appearance of cyclin A protein, and the cofractionation of the cyclin A protein with the kinase active fraction of the CDC2 protein (Figure 8D; note that the orientation of this gel filtration profile is opposite to that in Figure 5). Cyclin B was not detected in mimosine-arrested cells. We also observed a peak of T-antigen peptide kinase activity at ~250 kDa (Figure 8E). The overall results were indistinguishable from what was observed in untreated cells that were harvested at the start of S phase (Figure 5B) and therefore suggested that activation of a cyclin A/p34 CDC2 complex occurred in the late G1 phase of the cell cycle before the start of DNA replication.

DISCUSSION

Do the cyclins and the CDC2-related kinases regulate G1 progression and the start of DNA synthesis in higher eukaryotic cells as they do in yeast? This question was first approached experimentally using cell free assays to study the events that control the start of DNA replication (Blow and Nurse, 1990; D'Urso *et al.*, 1990; Fang and Newport, 1991). Although these assays showed that the cyclins and CDC2-like kinases could activate DNA replication in vitro, they could not show that they do so in vivo. Consequently, an essential step in understanding CDC2 and cyclin function is to determine the relationship between the time of their action in vivo and the occurrence of specific cell cycle events.

Using Cell Synchronization to Study the G1 to S Phase Transition

It is difficult to determine precisely the relative timing of the various events that comprise the cell cycle in higher eukaryotes. This problem is particularly acute when attempts are made to correlate events that occur on a cellular scale (e.g., the start of S phase) with those that occur on a molecular scale (e.g., formation of the cyclin A/p34 CDC2 complex) because most molecular events cannot be studied in single cell assays. The relationship between cellular and molecular events can be studied by using cell synchronization to produce large numbers of cells that occupy a narrow cell cycle window. In this approach, the reliability of the conclusions is limited by the degree of cell synchrony.

Serum stimulation of quiescent cells is the most frequently used method for obtaining G1 cells, but it is the least adequate. A population of serum-stimulated cells enters S phase over the course of many hours, making it impossible to establish the relationship between particular molecular events and cell cycle progression. Furthermore, the pathways connecting either G1 to S phase or quiescence to S phase are likely to have significant differences.

A second common method of cell synchronization involves collection of mitotic cells from monolayer culture. When returned to culture, mitotic cells enter S phase more synchronously than serum-stimulated cells. It is not possible, however, to collect sufficient quantities of mitotic cells from monolayer cultures to perform many types of biochemical analyses.

The large numbers of cells that can be grown in suspension culture overcome the quantitative limitations of monolayer cultures, and centrifugal elutriation can be used to fractionate suspension cells according to their position in the cell cycle. Cells smoothly increase in volume as they progress through the cell cycle, and centrifugal elutriation separates cells according to cell volume. An essentially homogeneous population of G1 cells can be obtained in this way. When these G1 cells are cultured, however, the synchrony with which they enter S phase is not always adequate to establish the timing of specific cell cycle events. This is because cell size alone is not a precise determinant of a cell's position within G1. For example, early and late G1 cells can have the same size. For the cells described here, the age of the cell with respect to the preceding mitosis is at least as important as cell size in determining position within G1 (Roberts, unpublished observations). Our protocol for cell synchronization combined drug-induced mitotic arrest with centrifugal elutriation to select cells that were homogeneous with respect to both cell size and time from the preceding mitosis. This method produced early G1 cells that progressed synchronously into S phase.

Our observations demonstrated that p34 CDC2 was activated at the start of S phase and therefore support the idea that this kinase regulates the G1 to S phase transition in humans, as it does in yeast. Using cells that progressed synchronously into S phase, we showed that the p34 CDC2 protein kinase was relatively inactive in newborn G1 cells and was activated concomitantly with the start of DNA replication. Synthesis of the cyclin A protein paralleled activation of the p34 CDC2 protein kinase—cyclin A was absent from newborn G1 cells and accumulated to detectable levels as soon as cellular DNA synthesis could be detected. Association of cyclin A with p34 CDC2 was responsible, at least in part, for activation of the CDC2 kinase as cells entered S phase.

These experiments showed that activation of the p34 CDC2 kinase closely corresponded to the start of cellular DNA synthesis. The point at which DNA synthesis can first be detected is commonly referred to as the G1/S transition, but it should be kept in mind that this does not necessarily reflect the point when regulation of DNA synthesis occurs. The biochemical pathways that control the cell's ability to replicate its DNA have not yet been described, but we anticipate that onset of DNA replication will be regulated at a presynthetic step, for example, unwinding of the replication origin (Roberts and D'Urso, 1988). Activators of DNA replication might be expected to regulate these presynthetic events. Accordingly, we have begun to examine the events that occur just before the beginning of DNA synthesis by blocking cell cycle progression with the drug mimosine. Mimosine arrests the cell cycle shortly before DNA synthesis begins; DNA replication is first detected 15 min after removal of mimosine. Other drugs, such as aphidicolin, can block the start of DNA replication by directly inhibiting replication enzymes. In contrast, mimosine specifically blocks a pre-S phase event, because S phase cells exposed to the drug complete DNA replication and mitosis before arresting in the following G1 period (Watson *et al.*, 1991). By using mimosine to prevent the start of DNA synthesis, we showed that formation and activation of the cyclin A/p34 CDC2 complex can occur before the start of S phase. This is consistent with a role for the CDC2 kinase in regulating the biochemical processes that allow DNA synthesis to begin.

Drug-induced synchronization has the advantage of yielding large numbers of cells at a uniform cell cycle position. However, there are many reasons why the phenotype of the arrested cells might not accurately represent a normal cellular phenotype corresponding to a particular cell cycle position. In particular, blocking one component of the cell cycle does not always inhibit the progression of other cell cycle processes. For example, the cell cycle of *S. cerevisiae* has been depicted as a set of parallel pathways that are regulated, to some extent, independently of one another (see Cross *et al.*, 1989). The cdc4 mutation, for instance, prevents the start of S phase but does not inhibit the parallel budding pathway. In human cells, cyclin B continues to accumulate in hydroxyurea-arrested cells, reaching much

higher levels than during a normal S phase (Marraccino and Roberts, unpublished observations). Therefore, activation of the cyclin A/CDC2 kinase in mimosine-arrested cells may not mean that this complex is activated during the G1 period of an unperturbed cell cycle. However, our inability to observe cyclin B accumulation in mimosine-arrested cells supports the idea that these cells accurately represent the late G1 state.

In light of the issues raised above, it will be important to turn to other systems where the molecular events that comprise G1 progression can be investigated without external perturbations of the cell cycle. One useful model system will be activation of primary T cells. T-cell activation requires a succession of mitogenic stimuli each of which causes the T cell to progress further through G1 toward S phase. The events that are associated with each of the stimuli can be studied and the biochemical pathways between quiescence and S phase established. Our initial experiments with this system show that cyclin A and CDC2 expression begin during the G1 phase of the human T cell cycle and correlate with the point at which the T cell becomes competent to respond to interleukin 2 (Firpo and Roberts, unpublished data).

An S Phase Role for CDC2

In conclusion, our data suggest that the CDC2 kinase might function at the start of S phase in the human cell cycle. This was not anticipated on basis of genetic analyses of the yeast cell cycle that showed that the CDC2 or CDC28 kinases functioned at START, before the G1/S transition. In *S. cerevisiae*, START is separated from S phase by at least the two steps defined by the execution points of the CDC4 and CDC7 genes (Hartwell *et al.*, 1973, 1974). In *S. pombe*, the execution point for CDC2 can occur early in G1, before the CDC10 execution point and before the onset of DNA synthesis (Novak and Mitchison, 1987). Also, the serum restriction point in the mammalian cell cycle, which may be functionally and biochemically analogous to START, can occur hours before S phase (Pardee, 1974). In contrast to these observations, we have suggested previously that the CDC2 kinase might directly activate DNA replication. Extracts from human G1 cells are deficient in their ability to initiate DNA synthesis at the SV40 replication origin (Roberts and D'Urso, 1988), and addition of the CDC2 kinase to G1 extracts can overcome this deficiency and activate DNA synthesis in vitro (D'Urso *et al.*, 1990). Recently, it has been demonstrated that a specific replication initiation protein, RPA, will not associate with the SV40 replication origin in G1 cell extracts (Fotedar and Roberts, unpublished data), and others have shown that RPA is a substrate of the p34 CDC2 protein kinase (Dutta and Stillman, personal communication). One interpretation of these results is that the p34 CDC2 kinase can directly activate S phase by modulating the activity of specific replication proteins, such as RPA, that are necessary to initiate DNA synthesis. In related experiments, addition of cyclin A to a G1 extract was sufficient to activate SV40 DNA replication. This showed that accumulation of a cyclin, perhaps cyclin A, was the limiting step for activation of the CDC2 kinase and the start of DNA synthesis (D'Urso *et al.*, 1990). The experiments presented here show that the CDC2 kinase is activated in vivo by cyclin A just before the start of S phase. Therefore, we have now shown that the cyclin A/CDC2 complex, which is sufficient to start SV40 DNA replication in vitro, is present and active at the G1/S phase transition in vivo.

Together these data indicate that G1 progression might have at least two execution points for CDC2 or related kinases, one event involving the conventional idea of START and another that is more directly related to activating the replication machinery. The p34 CDC2 protein recently has been shown to be one member of a family of related proteins (Elledge and Spottswood, 1991; Koff *et al.*, 1991; Paris *et al.*, 1991), and one of these proteins, CDK2, has been implicated in G1/S phase regulation (Fang and Newport, 1991; Koff *et al.*, 1991; Tsai *et al.*, 1991). Also, three new human cyclins have been cloned, and all can substitute for the *S. cerevisiae* CLN proteins in functional assays (Koff *et al.*, 1991; Lew *et al.*, 1991; Motokura *et al.*, 1991; Xiong *et al.*, 1991). In proposing an S-phase role for CDC2 and the cyclin A/CDC2 complex, we are not excluding the possibility that this role is shared among more than one type of cyclin/CDC2 or CDK complex. Indeed, a substantial fraction of cyclin A associates with CDK2 during S phase (Pines and Hunter, 1990; Tsai *et al.*, 1991). Other facets of G1 regulation, such as the response to extracellular growth factors, also might involve the CDC2-like family of kinases. These G1 functions of the CDC2-like kinases may require their interaction with other members of the cyclin family and may be more closely analogous to START.

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