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Inactivation of a Cdk2 Inhibitor during Interleukin 2-Induced Proliferation of Human T Lymphocytes

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Peripheral blood T lymphocytes require two sequential mitogenic signals to reenter the cell cycle from their natural, quiescent state. One signal is provided by stimulation of the T-cell antigen receptor, and this induces the synthesis of both cyclins and cyclin-dependent kinases (CDKs) that are necessary for progression through G_1. Antigen receptor stimulation alone, however, is insufficient to promote activation of G_1 cyclin-Cdk2 complexes. This is because quiescent lymphocytes contain an inhibitor of Cdk2 that binds directly to this kinase and prevents its activation by cyclins. The second mitogenic signal, which can be provided by the cytokine interleukin 2, leads to inactivation of this inhibitor, thereby allowing Cdk2 activation and progression into S phase. Enrichment of the Cdk2 inhibitor from G_1 lymphocytes by cyclin-CDK affinity chromatography indicates that it may be $p27^{Kip1}$. These observations show how sequentially acting mitogenic signals can combine to promote activation of cell cycle proteins and thereby cause cell proliferation to start. CDK inhibitors have been shown previously to be induced by signals that negatively regulate cell proliferation. Our new observations show that similar proteins are down-regulated by positively acting signals, such as interleukin 2. This finding suggests that both positive and negative growth signals converge on common targets which are regulators of G_1 cyclin-CDK complexes. Inactivation of G_1 cyclin-CDK inhibitors by mitogenic growth factors may be one biochemical pathway underlying cell cycle commitment at the restriction point in G_1.

In recent years, a paradigm has emerged to explain the biochemical events underlying cell cycle progression. The unifying idea is that key transitions in the cell cycle of essentially all eukaryotes are controlled by the action of a unique family of protein kinases, the cyclin-dependent kinases (CDKs) (31, 53). At the transition from G_1 to S phase, CDKs are proposed to phosphorylate and thereby activate specific proteins necessary for DNA replication (3, 11, 17, 20, 32, 54, 56, 82), and at the G_2/M transition, CDKs phosphorylate and activate a different set of proteins that are necessary for mitosis and cytokinesis (2, 10, 17, 21, 43, 54, 61, 66, 68, 78).

Superimposed upon the action of the CDKs are homeostatic circuits, known as checkpoints, which coordinate the execution of independent cell cycle events with each other (33). The *RAD9* gene product, for example, is part of a pathway that delays mitosis until DNA damage is repaired (85). Other pathways ensure that DNA replication is completed before mitosis begins (7) and that the mitotic spindle has been properly assembled before the cell attempts the transition from metaphase to anaphase (36, 45).

Cell cycle progression is also dependent upon extracellular growth-controlling signals. In most eukaryotes, these extracellular signals have their most profound effects on the transition from G_1 to S phase. In *Saccharomyces cerevisiae*, for example, mating pheromones, cell size, and nutrient abundance all influence cell cycle progression at a point in G_1 called START (32). The restriction point is a physiologically analogous event in the mammalian cell cycle (58, 59, 90). The predominant effectors of the restriction point are serum growth factors, but other important regulatory signals include growth-inhibitory proteins, cell-cell interactions, and cell-substratum interactions.

Intracellular checkpoint controls can be exerted by coupling activation of the CDKs to the successful execution of specific cell cycle events. In some organisms, for example, Cdc2 activation at the onset of mitosis requires dephosphorylation of the tyrosine 15 residue, and this does not occur until DNA replication is complete (24, 74). Moreover, mutations at tyrosine 15 uncouple replication from mitosis and cause catastrophic and premature entry into M phase (24). Similarly, replication and mitosis become uncoupled if the gene encoding the tyrosine 15 phosphatase, Cdc25, is overexpressed (14, 42) or if the tyrosine 15 kinases, Wee1 and Mik1, are inactive (46).

Pathways linking extracellular signals to cell cycle progression also target CDK activation; growth-promoting signals have been shown to promote CDK activation, while growth-inhibitory signals down-regulate the CDKs. Mammalian cells arrested in G_1 by transforming growth factor β (TGF-β) or cell-cell contact are unable to assemble catalytically active cyclin-Cdk2 complexes (35, 40). The block to Cdk2 activation is caused by elevated levels of a 27-kDa protein, $p27^{Kip1}$, which binds to and inactivates G_1 cyclin-Cdk2 complexes (63). TGF-β also down-regulates expression of Cdk4 (16), and this, together with the block to Cdk2 activation, contributes to cell cycle arrest. DNA-damaging agents, such as radiation, can also restrict the transition from G_1 to S phase. One important element in this pathway is p53, which functions, at least in part, by inducing the expression of $p21^{Waf1/Cip1}$ (12). p21, like $p27^{Kip1}$, binds to and inactivates G_1 cyclin-CDK complexes (27, 30, 91–93). In yeast cells, mating pheromones cause cell cycle arrest at START, prior to activation of G_1 cyclin-CDC28 complexes (67). Pheromone-induced arrest requires the FAR1 protein, which, like $p27^{Kip1}$ and p21, binds to cyclin-CDK complexes (5, 60). Other proteins that bind to and inhibit

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cyclin-CDK complexes have been identified, but their biological roles have not yet been established (51).

Another mechanism for controlling CDK activation in response to proliferative signals is regulation of cyclin expression. Serum growth factors, for example, induce cyclin expression (1, 37, 46, 80, 86, 87). Regulated cyclin expression also has a major role in anchorage-dependent proliferation. Interactions between a cell and its substratum, a phenomenon known as anchorage dependence, are an important determinant of cell cycle progression during G_1. Cyclin A expression is one component of the anchorage requirement. Anchorage-dependent cells placed into suspension fail to express cyclin A, and enforced cyclin A expression, but not overexpression of other cyclins, is sufficient to override the anchorage requirement (28).

Growth factors positively regulate cell proliferation in part by inducing expression of many proteins, including the cyclins and CDKs, that are necessary for execution of specific cell cycle processes (1, 39, 50, 82, 88, 89). Induction of these proteins, however, is primarily associated with reentry of a quiescent cell into the cell cycle, the transition from G_0 to G_1. Most normal cells also require extracellular growth factors for the transition from G_1 to S phase (59, 94). The role of mitogenic growth factors in this second transition, from G_1 to S phase, is not as well understood. It has not been determined, for example, whether mitogenic signals might down-regulate inhibitors of G_1 progression, such as $p27^{Kip1}$ and p21.

One important example of how multiple mitogenic stimuli are necessary to complete the transition from quiescence to S phase is the activation of quiescent peripheral blood T lymphocytes by two sequential signals (6, 71, 73, 84, 86). First, stimulation of the T-cell antigen receptor releases intracellular calcium stores and triggers a series of events that culminates in expression of the interleukin 2 (IL-2) receptor and competence to respond to IL-2 (52, 65, 80, 87). Exposure of G_1 cells to IL-2 is then sufficient to cause progression into S phase (6, 73). Stimulation of quiescent lymphocytes via these two steps, therefore, uncouples cell cycle events that accompany exit from quiescence from those events that occur as G_1 cells enter S phase. By analyzing these transitions separately, we have uncovered a role for the mitogenic growth factor IL-2 in removing an inhibitor that blocks activation of the CDKs.

MATERIALS AND METHODS

Reagents and antibodies. Antibodies and other reagents used and their sources were as follows: fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) recognizing CD25 (IL-2 receptor) (Coulter Immunology, Hialeah, Fla.), FITC-conjugated anti-human transferrin receptor MAb and mouse isotype FITC-conjugated antibodies (immunoglobulins G1 or G2a) (Becton Dickinson Immunocytometry Systems, Inc., Mountain View, Calif.), antiphosphotyrosine MAb (clone G410) (Upstate Biotechnology, Inc., Lake Placid, N.Y.), antihemagglutinin (anti-HA) antibody (clone 12CA5) (Babco, Richmond, Calif.), antibodies against cyclin A, cyclin E, cyclin B, Cdc2, and Cdk2 (38, 39, 47), immobilized recombinant protein A (Repligen, Cambridge, Mass.), acridine orange (Polysciences, Inc., Warrington, Pa.), propidium iodide (PI; Sigma Chemical, St. Louis, Mo.), cell culture media (GIBCO-BRL Laboratories, Grand Island, N.Y.), fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah), [γ-^{32}P]ATP (Dupont NEN, Boston, Mass.), Trans ^{35}S-labelling reagent (1,080 Ci/mmol) (ICN Biomedicals), [^{125}I]protein A (Amersham, Arlington Heights, Ill.), enhanced chemoluminescence detection system (Amersham), and RNase (DNase free) and histone H1 (Boehringer Mannheim, Indianapolis, Ind.). Coupling of anti-cyclin A antibodies to cyanogen bromide-activated Sepharose beads (Sigma) was performed as described previously (39). Phytohemagglutinin P (PHA) (Difco Laboratories Inc., Detroit, Mich.) was reconstituted in water and kept at 4°C. Recombinant human IL-2 (Genzyme, Cambridge, Mass.) was prepared as a stock in RPMI 1640 medium with 10% FBS and stored at −70°C.

Isolation of T lymphocytes. Human peripheral blood mononuclear (PBM) cells from normal healthy adults were isolated from leukocyte source packs (American Red Cross Blood Services, Pacific Northwest Division, Portland, Ore.). The content of each pack (ca. 40 ml) was washed two times with 160 ml of prewarmed Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution (HBSS) and centrifuged at 1,000 × *g* for 5 min at room temperature. PBM cells were fractionated by flotation on a Ficoll-Hypaque gradient (1.077 g/ml) after dilution of the washed leukocyte pellets with 200 ml of HBSS (4). Gradients were centrifuged at 1,000 × *g* for 30 min at room temperature; the cells at the interface were removed and washed once at 800 × *g* for 10 min and twice at 600 × *g* for 10 min with HBSS. The cellular pellet was resuspended in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 100 U of penicillin per ml, and 100 μg of streptomycin per ml). Resting lymphocytes were separated from preactivated lymphocytes, monocytes, remaining erythrocytes, and dead cells by layering the PBM cell suspension onto preformed discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradients (40 to 62.5%) and spun at 1,500 × *g* for 20 min (62). The dense fraction (62.5%) and the cell interface immediately above it were removed, diluted three times with complete RPMI, and centrifuged at 600 × *g* for 5 min. To deplete B cells and remaining monocytes, pelleted resting lymphocytes were resuspended in prewarmed RPMI 1640 and layered onto a nylon wool (Polysciences) fiber column (25). After incubation of the column for 30 to 40 min at 37°C, nonadherent cells were collected by passing 2 void volumes of prewarmed RPMI 1640 and centrifuged at 600 × *g* for 5 min. The cell fraction recovered after the nylon wool column represent our resting population of human T cells (G_0 fraction). The presence of contaminating cells was detected by using fluorochrome-conjugated MAbs against specific cell surface markers present in monocytes and B cells, including FITC-conjugated CD4 (T4) and CD8 (T8) and phycoerythrin-conjugated CD20 (B1) for B cells (Coulter Immunology) and FITC-conjugated CD14 (Leu M3) for monocytes (Becton Dickinson). Fluorescence emission from stained cells was analyzed by flow cytometry. A standard G_0 preparation of T cells contained less than 0.4% B cells and no more than 0.2% monocytes. Viability was more than 99% by trypan blue exclusion.

Flow cytometry. All flow cytometric analyses of stained intact cells or nuclei were performed with a Becton Dickinson FACScan equipped with an argon ion laser at 488 nm and 250-mW light output. For analysis, 10,000 events were collected in list mode fashion and stored by Lysis II version 1.0 (November 1990) (Becton Dickinson, San Jose, Calif.). For data acquisition of PI emission signals, the doublet discrimination module was used, and a polygonal gating was drawn on dot plot histograms from events collected in FL2-width (*x* axis) and FL2-area (*y* axis) that exclude the presence of doublets. The PI-gated regions then were plotted in one-parameter histograms with red fluorescence emission values on a linear *x*-axis scale, and cell number was plotted on the *y* axis. Percentages of cells in each region were obtained by using the

histogram statistical data (H-stats) of the Lysis II system. For fluorescence emission of intact cells staining with fluorochrome-conjugated MAbs, an open gate (nongated data) was used on data collected in forward scattering on a linear amplification scale (*x* axis) and green or red fluorescence on a log amplification scale (*y* axis). DNA content analysis of stained nuclei was performed as follows (62). A total of 5×10^5 T lymphocytes were washed once in cold RPMI 1640 medium, resuspended in 125 μl of a hypotonic solution (10 mM Tris-HCl [pH 7.4], 20 mM NaCl, 20 mM $MgCl_2$ [solution A]) containing 2.5 μg of RNase, and kept on ice for 5 min. Then, 125 μl of a lysis solution (solution A plus 1% Nonidet P-40 [NP-40]) was added, and the suspension was kept on ice for another 5 min. Next, cells were incubated for an additional 30 min at 37°C, after which 250 μl of a PI solution (100 μg of PI per ml in phosphate-buffered saline [PBS] containing 0.1% Triton X-100) was added. To quantify IL-2 and transferrin receptors, 5×10^5 T lymphocytes were washed in cold PBS plus 3% FBS and 0.02% sodium azide (SB buffer), resuspended in 50 μl of SB buffer containing fluorescein-conjugated MAb to the p55 IL-2 receptor (CD25), and incubated for 20 min on ice. The cells were washed once with cold SB buffer and fixed in 0.5 ml of PBS containing 1% paraformaldehyde. Simultaneous analysis of DNA and RNA content by acridine orange staining was performed as described previously (79), with some modifications. A total of 10^6 cells were resuspended with 0.5 ml of a solution containing 0.1% Triton X-100, 0.1 N HCl, 150 mM NaCl, and 2% FBS. After 1 min at room temperature, 1.5 ml of a solution containing 0.2 M disodium phosphate, 0.1 M citric acid buffer (pH 6.0), 1 mM Na_2EDTA, 150 mM NaCl, and 15 μg of acridine orange per ml was added to the cell suspension. Cell sorting was done on an EPICS flow cytometer and MDADS computer (Coulter Electronics). A total of 5×10^7 G_1 T cells were stained with FITC-conjugated anti-IL-2 receptor MAb with the following modification: after being washed once with cold RPMI 1640 medium, the cell pellet was resuspended in 1 ml of SB buffer containing FITC–IL-2 receptor MAb and incubated for 30 min on ice. Cells were washed two times with an excess of SB buffer and resuspended in RPMI 1640 medium containing 3% FBS at a final density of 3×10^6 to 4×10^6 cells per ml. Two different cell populations were selected according to their levels of fluorescence emitted on the green scale (*y* axis). Both type of cells were simultaneously gated according to their forward angle light scatter, registered on the *x* axis, selecting only the smaller cells (69). Between 5×10^6 and 6×10^6 cells were collected for each gate.

Cell culture and preparation of cell extracts. T-cell extracts were prepared as follows. Freshly isolated human T lymphocytes (G_0 cells) were resuspended at 10^6 cells per ml in complete RPMI 1640 medium with (G_1 cells) or without (unstimulated cells) 0.8 μg of PHA per ml. Cells were transferred to 175-cm^2 tissue culture flasks (1×10^8 to 1.5×10^8 total cells per flask) and incubated in 5% CO_2 at 37°C. After 72 h, cells were harvested by centrifugation (600 × *g*, 4°C, 10 min), washed once with cold HBSS, and lysed in a NP-40 buffer (0.3 ml per 10^8-cell pellet) at 4°C. NP-40 buffer contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.05% NP-40, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg of leupeptin per ml, 10 μg of aprotinin per ml, 50 mM NaF, 0.5 mM sodium orthovanadate, 80 mM β-glycerophosphate, and 5 μM mycrocistin. Cell extracts were sonicated for 30 s and centrifuged at 100,000 × *g* for 25 min at 4°C. Supernatants were stored at −70°C. Protein concentrations were determined by using the Bio-Rad protein assay. Lysates from proliferating T cells were prepared by the same protocol as described above except that recombinant IL-2 (100 U/ml) was added. In some experiments, IL-2 was added together with a high concentration of PHA (8 μg/ml) to ensure maximal cell proliferation. In other experiments IL-2 was added subsequent to prior incubation with 0.8 μg of PHA per ml as described above. For metabolic labelling, 10^7 cells per ml were incubated for 30 min in RPMI 1640 minus Met and Cys supplemented with 2 mM glutamine, 10% dialyzed FBS, 25 mM HEPES, and 1 mM sodium pyruvate. Trans ^{35}S label was then added at a final concentration of 0.25 mCi/ml, and the cells were incubated for 12 to 14 hours at 37°C. Metabolically labelled cell extracts were precleared twice by incubation for 20 min at 4°C with protein A-Sepharose and stored at −70°C. Insect cell lysates overexpressing cyclins and CDKs were prepared in the following manner. Sf9 cells (clonal isolate of *Spodoptera frugiperda*) were infected with recombinant baculoviruses expressing either cyclin A, B, or E, Cdc2, or Cdk2, the last two epitope tagged with the influenza virus HA epitope (all gifts from D. Morgan, University of California, San Francisco). Infected cells were harvested (200 to 300 × *g*, 5 min), and cell pellets were resuspended with 1 ml of insect cell lysis buffer containing 10 mM HEPES-KOH (pH 7.5), 75 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg of leupeptin per ml, 25 μg of aprotinin per ml, 50 mM NaF, 0.5 mM sodium orthovanadate, 80 mM β-glycerophosphate, and 5 μM mycrocistin. Cell lysates were sonicated for 30 s and centrifuged (100,000 × *g*, 25 min, 4°C). Supernatants were stored at −70°C. Insect cell lysates overexpressing cyclins D1, D2, and D3 and cyclin D1-, D2-, and D3-Cdk4 (gifts from J.-Y. Kato and C. Sherr) were prepared as described previously (40). In cases in which cyclin-CDK complexes were assembled from separately expressed subunits (see Fig. 6 and 7), the complexes were assembled and activated by using p34^{Cdc2}-activating kinase (CAK) as described previously (63).

Western blot (immunoblot) and kinase assays. Immunoblotting was performed as described previously (47, 63). Immunoprecipitations and histone H1 kinase assays were performed as described elsewhere (39, 63). Quantitation of radiolabeled histone H1 bands on dried sodium dodecyl sulfate-polyacrylamide gels was determined with a PhosphorImager and ImageQuant software (Molecular Dynamics).

RESULTS

Mitogenic activation of peripheral blood T lymphocytes. Human T lymphocytes purified from peripheral blood were greater than 99% viable and were contaminated with less than 1% monocytes and less than 2% B lymphocytes (see Materials and Methods). Lymphocyte activation was performed in stages by treating cells sequentially with PHA and IL-2. Exposure of freshly isolated lymphocytes to 0.8 μg of purified PHA per ml, or to soluble antibodies to CD3 (a component of the antigen receptor complex) (83), initiated cell cycle progression by stimulation of the T-cell antigen receptor. These treatments induced low levels of expression of both the IL-2 and transferrin receptors, but typically 3% or fewer of the cells entered S phase (Fig. 1A). In addition to induction of IL-2 and transferrin receptors, stimulation of the antigen receptor also altered other parameters indicative of G_0 exit and entry into G_1, including increased total RNA per cell and increased cell size (Fig. 1B). Expression of the transferrin receptor indicated that a low level of endogenous IL-2 expression might have been induced by these experimental conditions, but this was below the threshold necessary to cause progression from G_1 to S phase. Entry into S phase was then induced by exposure of cells to 100 U of recombinant IL-2 per ml (Fig. 1C). On the

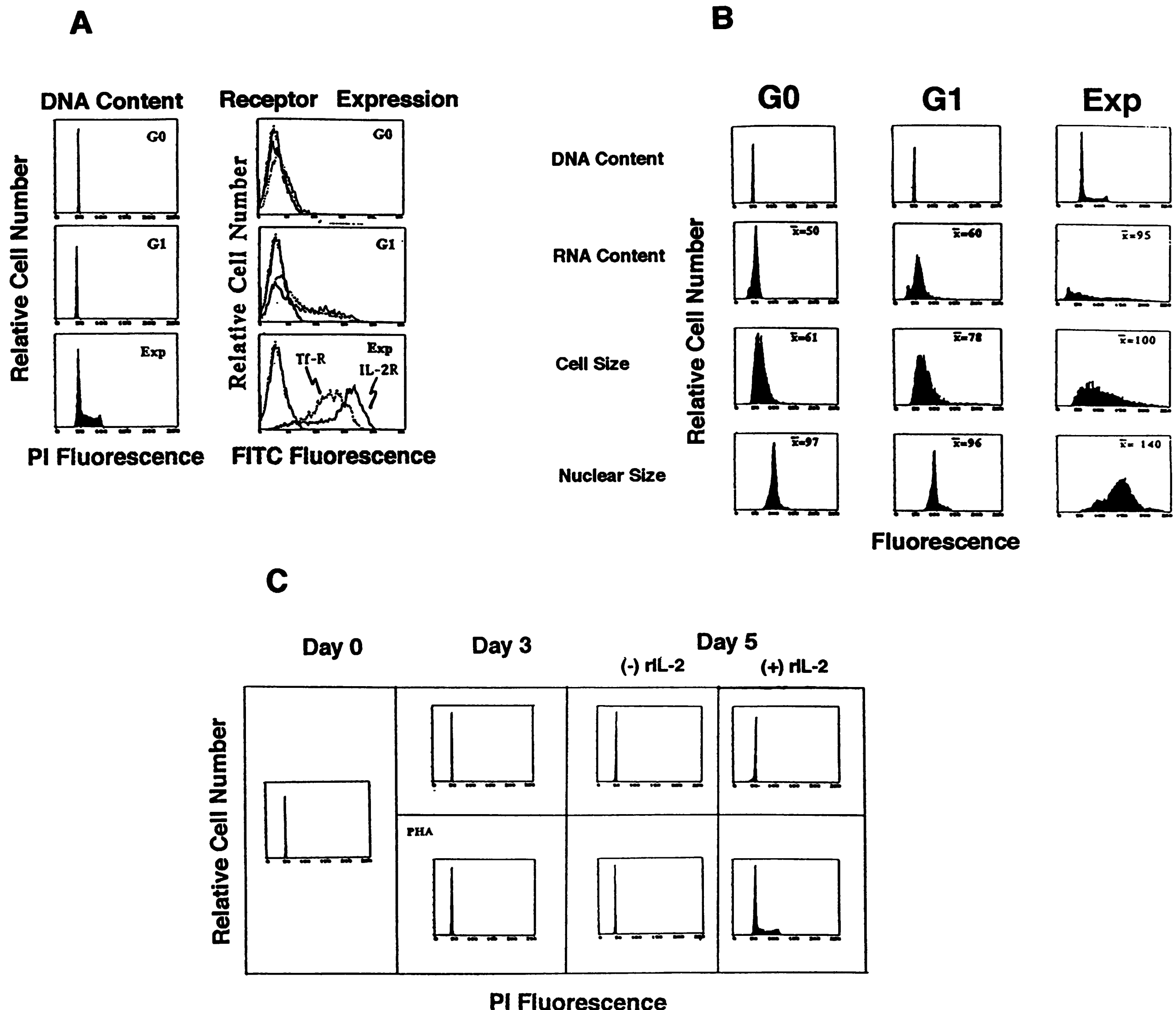

FIG. 1. Activation of peripheral blood T lymphocytes. (A) Freshly isolated peripheral blood T lymphocytes were maintained in vitro for 72 h in medium containing serum (G_0), serum plus 0.8 μg of PHA per ml (G_1), or serum plus PHA and 100 U of recombinant IL-2 per ml (Exp). Cell cycle progression was monitored by flow cytometric measurements of nuclear DNA content. Expression of the IL-2 and transferrin receptors was measured by flow cytometry using MAbs specific to those receptors. (B) Various parameters indicative of cell cycle progression were measured in cells exposed to serum (G_0), serum plus PHA (G_1), and serum plus PHA and IL-2 (Exp). G_1 cells exhibit increased cell size and increased RNA content per cell but do not show an increase in either nuclear size or DNA content per cell. In this experiment, cells were stained with acridine orange to simultaneously measure DNA and RNA content per cell. (C) PHA and IL-2 act sequentially. Peripheral blood T lymphocytes were treated with serum or serum plus PHA for 3 days. The cells were then washed extensively and placed into fresh medium containing serum or serum plus recombinant IL-2 (rIL-2). Entry into S phase was determined by flow cytometric measurements of nuclear DNA content. Only cells first receiving PHA and then IL-2 entered S phase.

basis of these results, we refer to freshly isolated lymphocytes as G_0 cells, lymphocytes cultured in medium containing only serum as unstimulated cells, lymphocytes cultured in serum plus PHA as G_1 cells, and lymphocytes receiving serum, PHA, and IL-2 as exponentially proliferating cells. Exposure to IL-2 without previous stimulation of the antigen receptor did not cause quiescent lymphocytes to enter S phase (Fig. 1C), nor could S-phase entry be induced by sequential treatment with first IL-2 followed by PHA (not shown). Thus, the transition from quiescence to S phase required two mitogenic signals, and these must occur in a specific order.

Cyclin and CDK expression during T-cell activation. Expression of cyclins A, E, and B, Cdc2, and Cdk2 was examined during staged T-cell activation by immunoblotting (Fig. 2A). These experiments describe the inductive effects of partial mitogenic stimulation on cyclin and CDK protein expression. The results, understandably, differ in some respects from those obtained when the time course of cyclin expression is measured after resting lymphocytes receive a complete mitogenic signal (1).

Cyclin E was not detected in freshly isolated lymphocytes, but a small amount of cyclin E typically was observed in

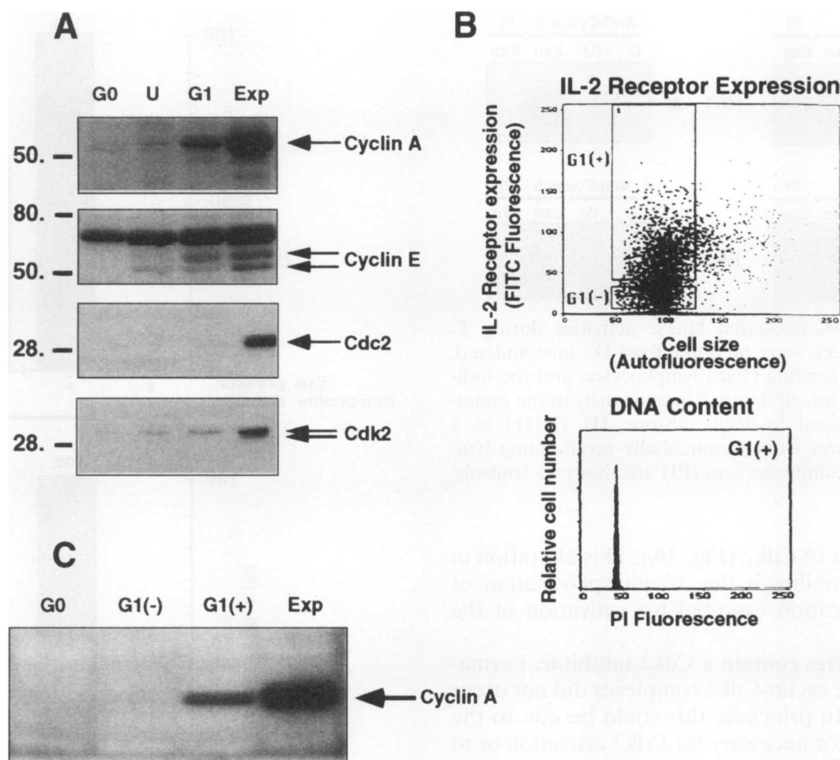

FIG. 2. Expression of cyclins and CDKs during T-lymphocyte activation. (A) Extracts were prepared from G_0, unstimulated (U), G_1, and exponentially proliferating (Exp) lymphocytes. Expression of the indicated proteins was measured by immunoblotting. The prominent band in the cyclin E immunoblots, which migrates just more slowly than cyclin E, is nonspecific and is not seen with use of affinity-purified antibodies. Sizes are indicated in kilodaltons. (B) G1 lymphocytes were sorted by flow cytometry on the basis of size and IL-2 receptor expression. Small IL-2 receptor-positive cells [G1(+)] were separated from small IL-2 receptor-negative cells [G1(−)] and collected. DNA content was measured by flow cytometry. The G1(+) fractionated cells were greater than 99.5% G_1, as determined by measurement of nuclear DNA content. (C) Expression of cyclin A was compared in G_0, proliferating, and G_1 lymphocytes expressing [G1(+)] or not expressing [G1(−)] the IL-2 receptor.

cultured, unstimulated lymphocytes. The two cyclin E isoforms differ at their amino termini and derive from alternatively spliced mRNAs (38, 44, 69). Following stimulation of the antigen receptor with PHA, both cyclin E isoforms increased in abundance, and the levels of both showed further moderate increases in response to IL-2.

Cyclin A was not detected in G_0 or unstimulated lymphocytes. Surprisingly, cyclin A expression was induced by stimulation of the antigen receptor alone, a treatment that is insufficient to promote S-phase entry. We further purified the G_1 lymphocytes to exclude the possibility that the cyclin A protein in apparently G_1 lymphocytes was from contaminating cells located in other phases of the cell cycle. Cell sorting was used to isolate the smallest, IL-2 receptor-positive cells from the population of lymphocytes that had been stimulated with PHA alone (Fig. 2B). Flow cytometry showed that these were greater than 99.5% G_1 cells (Fig. 2B). The purified G_1 cells contained cyclin A protein, essentially ruling out the possibility that cyclin A was derived entirely from contaminating S-, G_2-, or M-phase cells (Fig. 2C). The fact that cyclin A expression occurred in G_1 cells is consistent with the fact that cyclin A is necessary for S-phase entry (22, 57) and with earlier results showing that cyclin A is expressed in cells arrested in late G_1 by treatment with mimosine (47). We emphasize, however, that the level of cyclin A expression undergoes a substantial increase once cells enter S phase and that induction of cyclin A expression is likely to be an important component of the cellular response to IL-2.

Cdk2 was present in quiescent, G_1, and proliferating lymphocytes. IL-2 induced about a threefold increase in the total abundance of Cdk2 (Fig. 2A). Pulse-labelling and immunoprecipitation experiments showed, however, that IL-2 induced a more substantial, approximately five- to sevenfold increase in the rate of Cdk2 synthesis (not shown).

Cdc2 could not be detected in freshly isolated T lymphocytes or lymphocytes maintained in culture for 72 h without mitogenic stimuli (Fig. 2A). In most cases, stimulation of the antigen receptor alone did not induce Cdc2 expression, although this was somewhat variable. Cyclin B expression also was not induced by stimulation of the antigen receptor alone (not shown). Subsequent addition of IL-2 induced S-phase entry, and this was accompanied by expression of both cyclin B and Cdc2.

Cyclin- and CDK-associated kinase activities. G_1 cells that had been stimulated by PHA alone contained at least two cyclins that control S-phase entry, cyclins A (22, 57) and E (55), as well as their catalytic partner, Cdk2 (9, 13, 39, 70, 81). PHA stimulation, however, was not sufficient to promote formation of catalytically active cyclin A-Cdk2 or cyclin E-Cdk2 complexes. Cyclin A, cyclin E, and Cdk2 were immunoprecipitated from cells during each stage of mitogenic activation, and kinase activity in the immunoprecipitates was measured by using histone H1 as a substrate (Fig. 3). Kinase activity was undetectable until cells had received the full complement of mitogenic stimuli. Activation of Cdk2-associated kinase in cells receiving IL-2 correlated with the appearance of a more

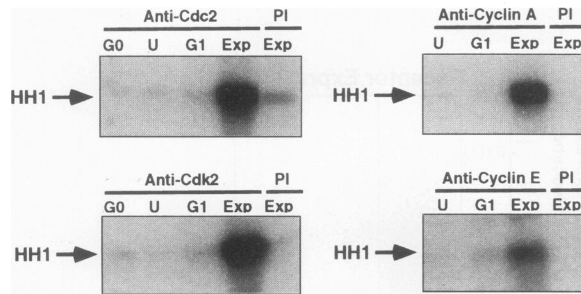

FIG. 3. Cyclin- and CDK-associated kinase activities during T-lymphocyte activation. Extracts were prepared from G_0, unstimulated, G_1, and exponentially proliferating (Exp) lymphocytes, and the indicated proteins were immunoprecipitated. Kinase activity in the immunoprecipitates was determined by using histone H1 (HH1) as a substrate. Immunoprecipitates from exponentially proliferating lymphocyte extracts by using preimmune sera (PI) are shown as controls.

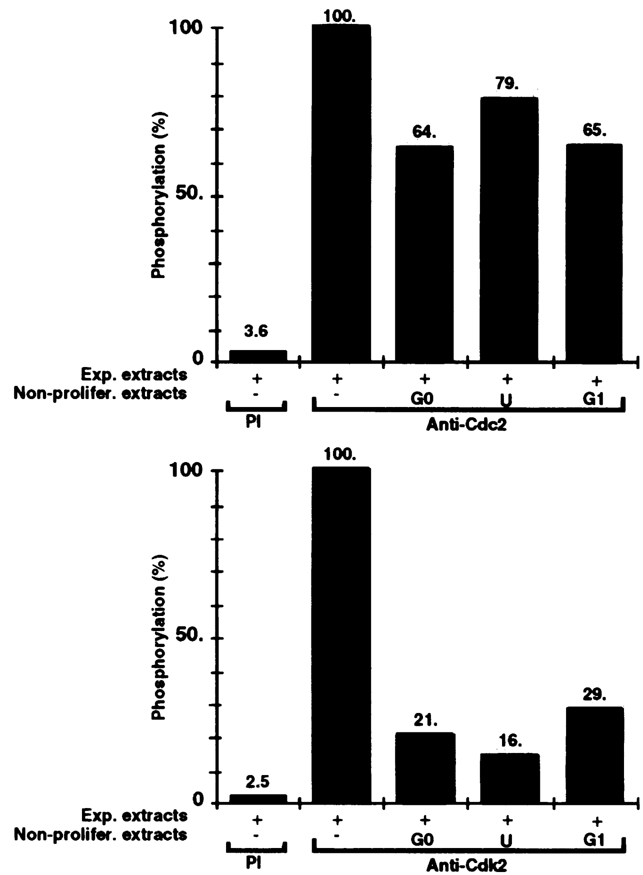

FIG. 4. Nonproliferating lymphocytes contain a dominant Cdk2 inhibitor. Extracts prepared from proliferating lymphocytes were mixed with equal amounts of G_0, unstimulated, and G_1 cell extracts or with buffer containing bovine serum albumin (−). Cdc2 and Cdk2 were immunoprecipitated from the mixed extracts, and kinase activity was measured by using histone H1 as a substrate. Results were quantitated with a PhosphorImager and plotted relative to the amount of kinase activity (Cdc2 or Cdk2) recovered from immunoprecipitates from proliferating lymphocytes. Titrations of the nonproliferating extracts indicated that this experiment was performed within the linear range of the inhibitor dose response.

rapidly migrating isoform of Cdk2 (Fig. 2A). This alteration in Cdk2 electrophoretic mobility is due to phosphorylation of threonine 160, a modification essential for activation of the Cdk2 kinase (26).

G_0 and G_1 T lymphocytes contain a Cdk2 inhibitor. Formation of catalytically active cyclin-Cdk2 complexes did not occur in the absence of IL-2. In principle, this could be due to the absence of a positive factor necessary for Cdk2 activation or to the presence of a Cdk2 inhibitor. These alternatives were tested by mixing extracts from proliferating lymphocytes (stimulated with PHA plus IL-2) with extracts from G_0 or G_1 lymphocytes (unstimulated or stimulated with PHA alone). Cdk2 was active in proliferating lymphocytes, but it was inhibited approximately 80% in the mixed lysates. Thus, G_0 and G_1 lymphocytes contained a factor(s) that inactivated Cdk2. Cdc2 on the other hand, was only partially inhibited in mixed lysates (Fig. 4; see below). CAK was initially identified and purified on the basis of its ability to phosphorylate Cdc2 and activate cyclin B-Cdc2 complexes. This same enzyme can phosphorylate Cdk2 and activate cyclin E-Cdk2 and cyclin A-Cdk2 complexes (18, 19, 64, 76). Addition of purified CAK to G_0 and G_1 extracts did not override the Cdk2 inhibition and allow activation of cyclin-Cdk2 complexes (not shown). This finding supported the idea that these lysates contained a dominant Cdk2 inhibitor and were not simply deficient in CAK or a CAK-like activity.

The mechanism of Cdk2 inhibition in mixed extracts was explored by examining the association of Cdk2 with cyclin A and the phosphorylation state of Cdk2. These initial experiments focused on cyclin A because the majority of Cdk2-associated kinase activity in proliferating cells was due to cyclin A-Cdk2 complexes (not shown). Inhibition of Cdk2 activity was not caused by dissociation of cyclin A-Cdk2 complexes because the amount of Cdk2 bound to cyclin A did not decrease in mixed lysates (Fig. 5A). Moreover, inhibition of Cdk2 was not accompanied by an increase in tyrosine-phosphorylated Cdk2 in cyclin A immune complexes (Fig. 5B). Finally, inhibition of Cdk2 activity was not associated with an alteration in Cdk2 electrophoretic mobility (Fig. 5C). Despite greater than 80% inhibition of the Cdk2 kinase, no decrease in the abundance of the rapidly migrating isoform of Cdk2 was observed in mixed lysates. This form of Cdk2 has been shown to be phosphorylated on threonine 160, which is the only covalent modification of Cdk2 known to be essential for its activation. In support of this conclusion, multiple phosphatase inhibitors had no effect on the inactivation of cyclin A-Cdk2 complexes in G_0 or G_1 extracts (see Materials and Methods). In sum, neither of the parameters known to correlate with Cdk2 activity—cyclin binding or Cdk2 phosphorylation—appeared to be altered when Cdk2 was inactivated by factors present in nonproliferating lymphocyte extracts.

The results obtained with mixed cell extracts were confirmed and extended by testing the inhibitory effects of G_0 and G_1 lysates on specific cyclin-CDK complexes. Cyclin-CDK complexes were assembled and activated by mixing lysates from Sf9 cells expressing recombinant cyclins and kinases together with purified CAK. Nonproliferating lymphocytes completely inhibited cyclin A-, E-, and B-Cdk2 complexes (Fig. 6). Cyclin A-Cdc2 complexes were also inhibited, while cyclin B-Cdc2 complexes were unaffected. The relative insensitivity of cyclin B-Cdc2 to inhibition also explained why total Cdc2-associated kinase activity was only slightly decreased when lysates from proliferating cells were mixed with lysates from G_1 lymphocytes. Titrations of cell extracts showed that the specific inhibitory activities toward each of the various cyclin-CDK complexes were equally abundant and that their levels were

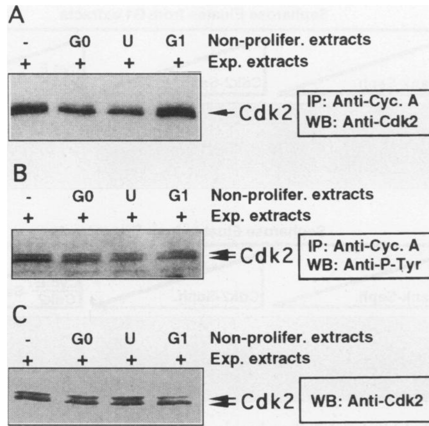

FIG. 5. Analysis of Cdk2 in mixed lysates. Extracts from proliferating lymphocytes were mixed with extracts from nonproliferating lymphocytes or with bovine serum albumin as described in the legend to Fig. 4. (A) Cyclin A-Cdk2 complexes were immunoprecipitated (IP) with a MAb against cyclin A. The immunoprecipitates were then immunoblotted (WB) with antibodies against Cdk2. No change in the amount of Cdk2 bound to cyclin A was observed in mixed lysates. (B) Cyclin A-Cdk2 complexes were immunoprecipitated with a MAb against cyclin A. The immunoprecipitates were then immunoblotted with antiphosphotyrosine (Anti-P-Tyr) antibodies. No increase in tyrosine phosphorylation of Cdk2 could be detected in mixed lysates. (C) Immunoblot of Cdk2 protein. The more rapidly migrating isoform of Cdk2 reflects phosphorylation of threonine 160. The distribution of Cdk2 between the two isoforms is not altered in mixed lysates.

not substantially changed during the transition from G_0 to G_1 (not shown). No inhibitory activity toward any complex could be detected in extracts from proliferating lymphocytes (Fig. 6).

During characterization of the Cdk2 inhibitor in G_1 lymphocytes, it became apparent that it was heat stable. G_1 lymphocyte extracts were boiled for 5 min and then added to an extract from proliferating lymphocytes. The boiled extract was just as effective as unboiled extract in inhibiting Cdk2 in the proliferating cell extract (Fig. 7A). Moreover, boiled G_1 lysates were able to completely inactive cyclin A-Cdk2 and cyclin E-Cdk2 complexes, while no effect on cyclin B-Cdc2 complexes was detected (Fig. 7B). Thus, the specificity of the heat-stable inhibitor was similar, if not identical, to the specificity of the inhibitory activity detected in untreated lysates. The heat-stable inhibitor was, at least in part, a protein because it could be completely destroyed by treatment with trypsin (not shown). In related experiments, we have shown that epithelial cells arrested in G_1 either by contact inhibition or by exposure to TGF-β contain a Cdk2 inhibitor (40, 63). That inhibitory protein has been purified and also shown to be heat stable (63).

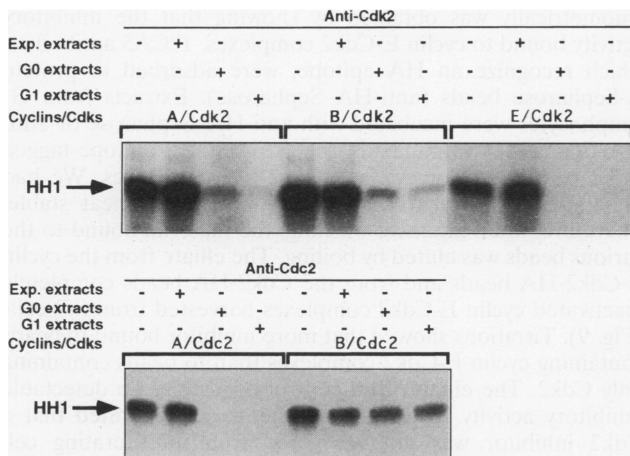

FIG. 6. Inhibitor specificity. Specific cyclin-CDK complexes were assembled and activated as described in the text. Active complexes were then added to the indicated cell extracts or to buffer containing bovine serum albumin, and histone H1 (HH1) kinase activity was measured following immunoprecipitation. None of the cyclin-CDK complexes were inhibited by proliferating cell extracts, and all except cyclin B-Cdc2 complexes were inactivated by G_0 or G_1 cell extracts. Extract titrations demonstrated that an excess of inhibitor was present in this experiment.

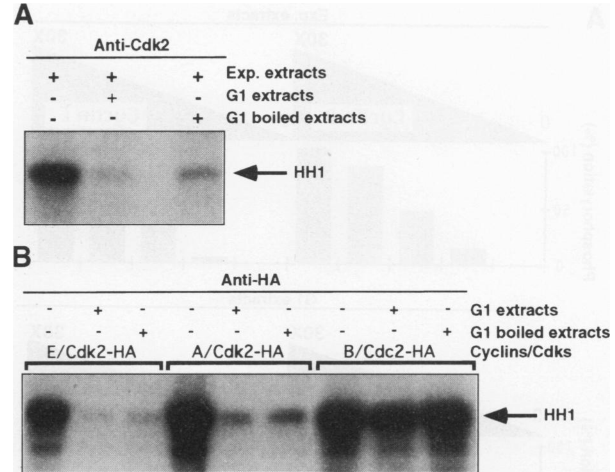

FIG. 7. Heat stability of the Cdk2 inhibitor. (A) Extracts from proliferating lymphocytes (Exp.) were mixed with a G_1 lymphocyte extract or a G_1 lymphocyte extract that had been boiled for 5 min. Cdk2 was immunoprecipitated, and histone H1 (HH1) kinase activity was determined. (B) Cyclin-CDK complexes containing an HA-tagged CDK subunit were mixed with the indicated extracts. Complexes were immunoprecipitated with an antiserum against the HA epitope and tested for kinase activity, using histone H1 as a substrate.

Cyclin activation of Cdk2. To investigate further the effects of the Cdk2 inhibitor, we tested the ability of exogenously added cyclins to activate the endogenous Cdk2 present in G_0, G_1, and proliferating lymphocyte extracts. Cyclins A and E were expressed in Sf9 cells by using baculoviral vectors, and the amount of each cyclin present in the Sf9 cell lysates was compared by immunoblotting with the amount present in proliferating lymphocyte extracts. A range of cyclins A and E, from 1- to 30-fold physiological levels, was added to lymphocyte lysates. In extracts from proliferating cells, this gave an approximately linear increase in cyclin-associated kinase activity (Fig. 8A). In contrast, when the cyclins were added to G_0 (not shown) or G_1 (Fig. 8A) lymphocyte lysates, no kinase activity was detected in cyclin immunoprecipitates. Under these conditions, the added cyclin bound to the endogenous Cdk2 protein (Fig. 8B), but the Cdk2 protein within these complexes was not catalytically active.

A Cdk2 inhibitor binds to cyclin-Cdk2 complexes. Two observations suggested that the Cdk2 inhibitor present in G_0 and G_1 lymphocytes functioned stoichiometrically rather than catalytically. First, the inhibitory activity was titratable with excess cyclin-CDK complexes. Increasing amounts of cyclin E together with increasing amounts of Cdk2 were added to G1 extracts. This resulted in assembly of proportionately increas-

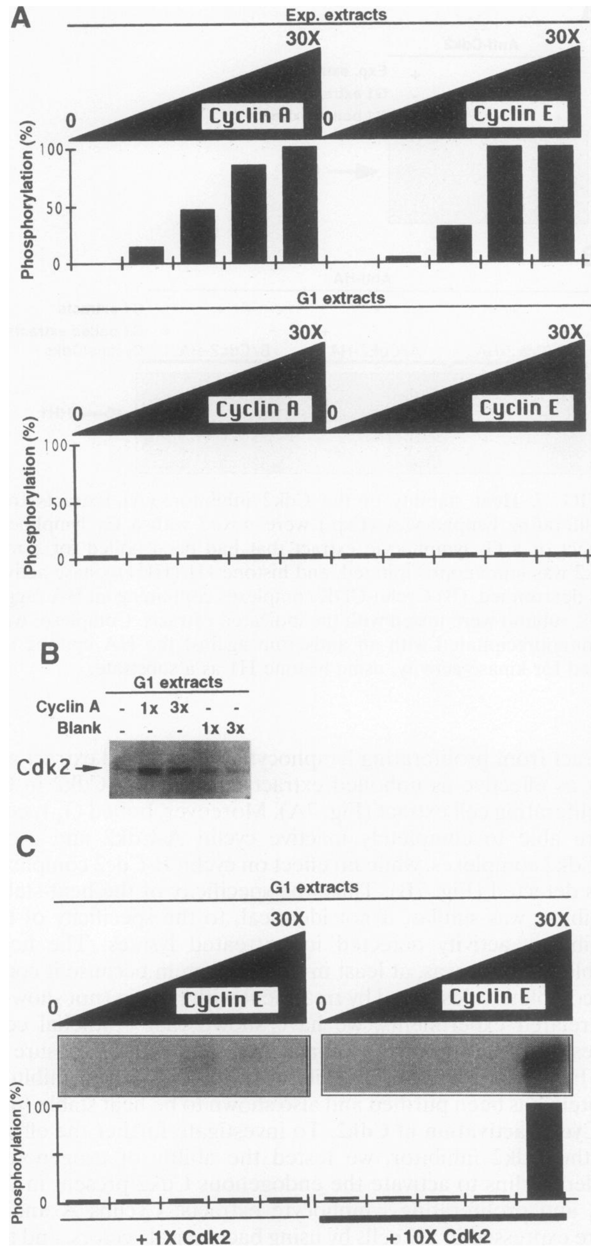

FIG. 8. Assembly and activation of cyclin-Cdk2 complexes in lymphocyte extracts. (A) Increasing amounts of cyclin E or cyclin A were added to G_1 and proliferating lymphocyte extracts. The amounts of cyclins A and E added were 0-, 1-, 3-, 10-, and 30-fold physiological levels. Immunoprecipitates obtained by using antibodies against the added cyclin were assayed for histone H1 kinase activity. The results were quantitated with a PhosphorImager. In the experiment in which cyclins were added to proliferating cell extracts, the results are plotted after subtraction of the kinase activity detected in the cell extract without addition of exogenous cyclin. (B) Cyclin A was added to G_1 lymphocyte extracts. Cyclin A-Cdk2 complexes were immunoprecipitated with antibodies against cyclin A, and the immunoprecipitates were immunoblotted with Cdk2 antisera. Cyclin A-Cdk2 complexes were present in G_1 cell extracts, and addition of extra cyclin A protein to those extracts promoted the formation of more cyclin A-Cdk2 complexes. Blank lanes received lysate from Sf9 cells infected with wild-type nonrecombinant baculovirus. Cyclin A lanes received lysate from Sf9 cells infected with recombinant baculovirus expressing human cyclin A. (C) Increasing amounts of cyclin E were added to G_1 lymphocyte extracts as in panel A except that extracts were also supplemented with physiological (1×) or 10 times physiological (10×) amounts of Cdk2. Immunoprecipitates obtained by using antibodies against the added cyclin were assayed for histone H1 (HH1) kinase activity.

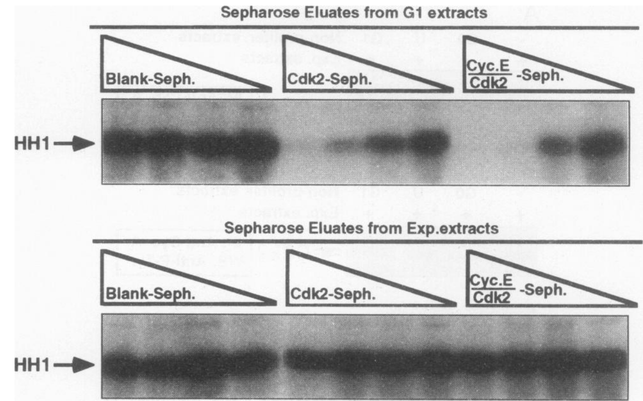

FIG. 9. A Cdk2 inhibitor binds to Cdk2 and cyclin E-Cdk2 complexes. G_1 or proliferating cell extracts were incubated with anti-HA control-Sepharose beads (blank) or anti-HA beads adsorbed to Cdk2-HA or cyclin E-Cdk2-HA complexes (see Materials and Methods). After extensive washing, the protein bound to the beads was eluted by boiling, and diminishing amounts of the eluates were mixed with active cyclin E-Cdk2 complexes assembled from recombinant proteins. The amounts added at each point correspond to 60, 25, 10, and 5% of the total recovered eluate. Both Cdk2-Sepharose and cyclin E-Cdk2-Sepharose bound an inhibitory activity in extracts from G_1 lymphocytes but not in extracts from proliferating lymphocytes. HH1, histone H1.

ing amounts of cyclin-Cdk2 complexes (not shown). A threshold was crossed with 30-fold excess cyclin and 10-fold excess Cdk2 (Fig. 8C). At that point, activation of Cdk2 kinase was observed, consistent with the idea that a saturable inhibitor had been titrated out. Once this threshold was crossed, further increases in the amount of added cyclin produced a directly proportionate increase in the amount of cyclin-associated kinase (not shown). Addition of purified CAK to the G_1 lysates did not lower the threshold level of cyclin E or A necessary to activate Cdk2 (not shown).

Further evidence that the Cdk2 inhibitor functioned stoichiometrically was obtained by showing that the inhibitory activity bound to cyclin E-Cdk2 complexes. 12CA5 antibodies, which recognize an HA epitope, were adsorbed to protein A-Sepharose beads (anti-HA–Sepharose). Extracts from G_1 lymphocytes were incubated with anti-HA–Sepharose or anti-HA–Sepharose immunoadsorbed to either epitope-tagged Cdk2 (Cdk2-HA) or cyclin E-Cdk2-HA complexes. We had previously shown that the Cdk2 inhibitor was heat stable. Therefore, after extensive washing, the material bound to the various beads was eluted by boiling. The eluate from the cyclin E-Cdk2-HA beads and from the Cdk2-HA beads completely inactivated cyclin E-Cdk2 complexes harvested from Sf9 cells (Fig. 9). Titrations showed that more inhibitor bound to beads containing cyclin E-Cdk2 complexes than to beads containing only Cdk2. The eluate from control beads had no detectable inhibitory activity. Parallel experiments demonstrated that a Cdk2 inhibitor was not recovered from proliferating cell extracts, using either Cdk2-Sepharose or cyclin E-Cdk2-Sepharose (Fig. 9).

To determine the identity of the protein that might be

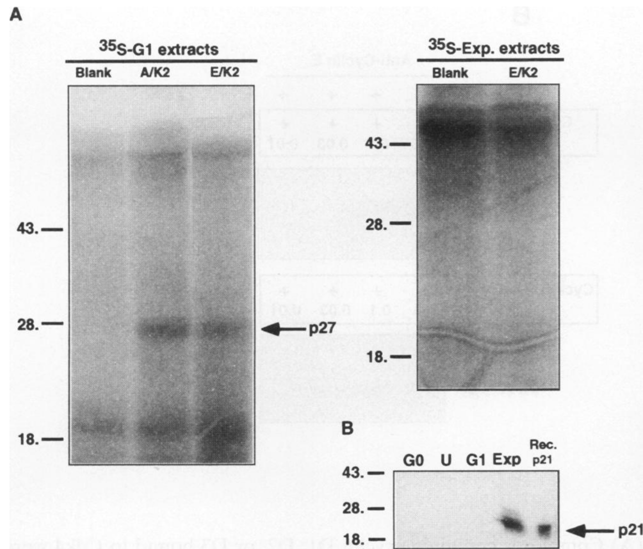

FIG. 10. A 27-kDa cyclin-CDK binding protein in nonproliferating lymphocytes. (A) The experiment described in the legend to Fig. 9 was repeated using metabolically labelled G_1 or exponential cell extracts. In addition, beads containing cyclin A-Cdk2-HA complexes were tested. Proteins were eluted by heat treatment from anti-HA beads (blank) or anti-HA beads adsorbed to either cyclin A- or cyclin E-Cdk2-HA (A/K2 or E/K2). The eluates were subjected to polyacrylamide gel electrophoresis and visualized by autoradiography. (B) Extracts from proliferating and nonproliferating T lymphocytes were immunoblotted with a MAb the recognizes human $p21^{Waf1}$. Recombinant p21 purified from *E. coli* is shown as a positive control. Sizes are indicated in kilodaltons.

responsible for Cdk2 inhibition, we repeated the experiment described above, using metabolically labelled cell extracts. The only metabolically labelled protein specifically eluted from the cyclin E-Cdk2-HA beads had a molecular size of 27 kDa (Fig. 10A). Parallel experiments using cyclin A-Cdk2-HA beads also yielded a protein of similar size, consistent with the observation that the lymphocyte inhibitor inactivated both cyclin E- and cyclin A-Cdk2 complexes. This protein was not eluted from control beads or from cyclin E-Cdk2 beads incubated in an extract from proliferating cells (Fig. 10A). Two Cdk2-inhibitory proteins have recently been discovered, $p27^{Kip1}$ (40, 63) and $p21^{Waf1/Cip1}$ (12, 27, 30, 91), both of which inhibit cell cycle progression in G_1 by binding to and inactivating G1 cyclin-Cdk2 complexes. The data described above suggest that $p27^{Kip1}$ may be the Cdk2 inhibitor in growth-arrested lymphocytes, although immunologic reagents are not yet available to directly demonstrate that the 27-kDa protein detected in these experiments is the human homolog of the mink $p27^{Kip1}$. On the other hand, p21 was not detected in extracts from nonproliferating lymphocytes, although it was found at relatively high levels in actively proliferating T cells (Fig. 10B). Thus, p21 did not appear to be responsible for controlling Cdk2 activity during mitogenic activation of quiescent T lymphocytes.

Regulation of a Cdk2 inhibitor by IL-2. DNA replication began approximately 12 h after exposure of G_1 lymphocytes to IL-2. The loss of Cdk2-inhibitory activity was measured during this 12-h interval. Cyclin A was added to lymphocyte extracts prepared every 2 h as G_1 cells progressed into S phase following exposure to IL-2. Cdk2 remained refractory to activation by cyclin A until 1 to 2 h before S phase began (not shown). Therefore, the cells' competence to replicate DNA correlated closely with inactivation of the Cdk2 inhibitor. The delayed inactivation of the Cdk2 inhibitor suggested that this occurred downstream of earlier IL-2-induced events. Since the levels of cyclins A, E, D2, and D3 all increase in IL-2-treated cells, we tested whether cyclins themselves might have roles in inactivating the Cdk2 inhibitor.

Cells progressing from G_2 into mitosis activate cyclin B-Cdc2 via a posttranslational positive feedback loop whereby a small amount of catalytically active Cdc2 activates a large pool of latent cyclin B-Cdc2 complexes. It seemed unlikely that cyclin A or E would be responsible for its own activation during G_1 via an analogous pathway because, as shown above, titration of the Cdk2 inhibitor required substantially greater than physiological amounts of cyclin A, cyclin E, or Cdk2. In addition, we tested whether a small amount of active cyclin A- or cyclin E-Cdk2 complexes could activate the endogenous, inactive pool of cyclin E- and cyclin A-Cdk2 complexes in G_1 lymphocytes. Addition of either active cyclin A-Cdk2 or active cyclin E-Cdk2 complexes had no detectable effect on the activity of the endogenous cyclin-Cdk2 complexes, nor did addition of small amounts of active complexes significantly lower the threshold level of cyclin E required for Cdk2 activation (not shown).

Syntheses of cyclins D2 and D3 have also been shown to be IL-2 dependent (1). Remarkably, cyclin D2-Cdk4 complexes were able to block the Cdk2 inhibitor and allow Cdk2 activation by cyclin E or cyclin A. Complexes containing cyclin D1, D2, or D3 together with their catalytic subunit, Cdk4, were assembled by coinfection of Sf9 cells with baculoviral vectors expressing these proteins (37, 48). The D cyclins were present at equivalent levels in these lysates. The ability of cyclin A and cyclin E to activate Cdk2 was tested in G_1 lymphocyte extracts into which cyclin D1-, D2-, or D3-Cdk4 complexes had been added. Activation of Cdk2 by either cyclin A or cyclin E was fully restored by cyclin D2-Cdk4 complexes (Fig. 11A), while cyclin D1 or D3 complexes were less active. Titrations suggested that inactivation of the Cdk2 inhibitor may require, at most, physiological amounts of cyclin D2-Cdk4 complexes (Fig. 11). Addition of either cyclin D2 alone or Cdk4 alone had no effect on the ability of cyclin A or E to activate Cdk2 (not shown). Thus, removal of the Cdk2 inhibitor was observed only in the presence of the intact cyclin D2-Cdk4 complex.

Cyclin D-Cdk4 complexes are active kinases, strongly phosphorylating the retinoblastoma protein in vitro (37, 48, 49) and probably in vivo (15, 34). However, the ability of cyclin D2-Cdk4 complexes to promote Cdk2 activation did not require the catalytic activity of Cdk4, because cyclin D2 in combination with a catalytically inactive mutant of Cdk4 was equally effective (Fig. 11B). It was most likely, therefore, that cyclin D2-Cdk4 sequestered the Cdk2 inhibitor and thereby prevented it from binding to and inactivating cyclin A- and cyclin E-Cdk2 complexes. These results showed that the Cdk2 protein present in G_1 lymphocytes had not been covalently and irreversibly inactivated, but rather was unavailable for activation because of association with an inhibitory and titratable binding protein. They also suggested that G_1 progression could be facilitated by a feed-forward mechanism in which early-acting cyclin-CDK complexes, such as cyclin D2-Cdk4, promote activation of later-acting cyclin-CDK complexes, such as cyclin E-Cdk2, through interactions with common regulatory proteins (see Discussion). From these results, we speculated that the Cdk2 inhibitor might be present in proliferating lymphocytes, but sequestered and unavailable to block Cdk2 activation. This idea is supported by the observation that Cdk2-inhibitory activity can be recovered from proliferating lymphocytes by brief heat treatment (Fig. 12). Active cyclin-

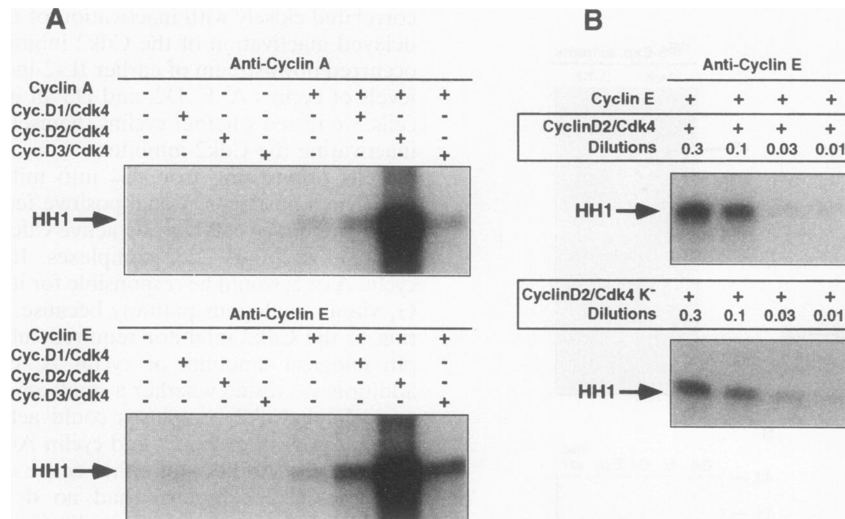

FIG. 11. Cyclin D2-Cdk4 complexes facilitate cyclin E-Cdk2 activation. (A) Complexes containing cyclin D1, D2, or D3 bound to Cdk4 were added to G_1 lymphocyte extracts. Cyclin A or cyclin E was then added. Cyclin A or cyclin E was immunoprecipitated, and histone H1 (HH1) kinase activity was determined. (B) Dilutions of cyclin D2 complexed with active (cyclin D2/Cdk4) or catalytically inactive (cyclin D2/Cdk4 K^-) Cdk4 was added to G_1 lymphocyte extracts. Cyclin E was then added, the mixture was immunoprecipitated, and cyclin E-associated histone H1 kinase activity was measured.

CDK complexes harvested from Sf9 cell lysates were incubated with extracts from proliferating lymphocytes and with identical extracts that had been heated to 100°C. Heating released an inhibitory activity that inactivated cyclin E-Cdk2 complexes but had no effect on cyclin B-Cdc2 complexes.

DISCUSSION

We have used human T lymphocytes as a model to explore how mitogenic signals activate the cell cycle. Like most types of cells, T lymphocytes require more than one signal to reenter the cell cycle from a quiescent state. In the specific case of T cells, those signals are provided, sequentially, by antigen receptor stimulation and mitogenic cytokines. First, stimulation of the T-cell antigen receptor promotes synthesis of the cyclins and CDKs that are necessary for G_1 progression and entry into S phase. The activities of these proteins are kept in check, however, by an inhibitor that binds to Cdk2 and

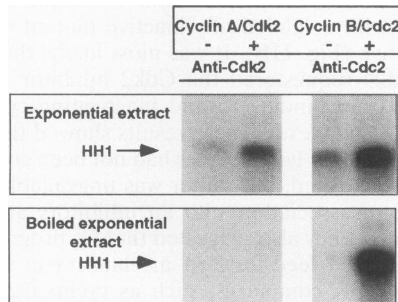

FIG. 12. Recovery of a Cdk2 inhibitor from proliferating cell extracts. An extract was prepared from proliferating lymphocytes, and a portion of the extract was then heated to 100°C for 5 min. The native and heated extracts were then assayed for CDK-inhibitory activity by adding active cyclin A-Cdk2 or cyclin B-Cdc2 complex. Kinase activity was measured in the indicated immunoprecipitates. Boiling uncovered a Cdk2 inhibitor, but not a Cdc2 inhibitor, in proliferating cell extracts. HH1, histone H1.

cyclin-Cdk2 complexes. This inhibitory activity persists until the lymphocyte has received the second of the two signals that are required for cell proliferation to start, in this case IL-2. Thus, one function of the cytokine IL-2 is to inactivate this inhibitor and activate Cdk2. Since Cdk2 is necessary for entering S phase (56, 82), removing the Cdk2 inhibitor is an essential step in progression from G_1 to S phase. These observations raise the possibility that in general, mitogenic growth signals activate the cell cycle by independent but complementary effects on cell cycle proteins. This would be one mechanism to integrate the multiple stimuli necessary to induce cell proliferation.

The presence of a Cdk2 inhibitor in nonproliferating lymphocytes was deduced by a number of independent observations. First, G_1 lymphocytes contain cyclin A, cyclin E, and Cdk2 but lack catalytically active complexes containing those proteins. Second, extracts from these cells will inactivate both cyclin-CDK complexes which had been assembled and activated by using recombinant proteins as well as the active cyclin-CDK complexes which are present in extracts from proliferating lymphocytes. Third, extracts from nonproliferating lymphocytes support the formation of cyclin-CDK complexes from separate cyclin and CDK subunits but do not allow activation of those complexes. Fourth, a protein that inhibits the kinase activity of cyclin-Cdk2 complexes can be recovered from arrested but not from proliferating lymphocytes, using cyclin-Cdk2 affinity chromatography. Fifth, S phase begins 10 to 12 h after exposure of cells to IL-2, and this correlates with loss of the Cdk2 inhibitor and assembly of active cyclin-Cdk2 complexes.

There are various mechanisms by which an inhibitor could modulate Cdk2 kinase activity. Cdk2 is positively regulated by cyclin binding and by phosphorylation of threonine 160 (8, 18, 23, 26, 41, 64, 76, 77); Cdk2 is negatively regulated by phosphorylation of tyrosine 15 (8, 26) and by binding of inhibitory proteins such as $p27^{Kip1}$ (40, 63) and p21 (27, 30, 91). Both $p27^{Kip1}$ and p21 are cyclin-CDK binding proteins whose activities are increased by signals that inhibit cell proliferation. $p27^{Kip1}$ activity is induced by TGF-β and contact inhibition

(63), and p21 is induced by p53 (12). p21 could not be detected in growth-arrested lymphocytes, suggesting that it is unlikely to play a role in this biological context. The lymphocyte inhibitor does, however, have a number of properties in common with $p27^{Kip1}$. Neither the lymphocyte inhibitor nor $p27^{Kip1}$ disrupts cyclin-Cdk interactions or alters the phosphorylation state of Cdk2. Both bind to cyclin-CDK complexes, are heat stable, preferentially effect Cdk2 as opposed to Cdc2 activity, are sequestered specifically by cyclin D2-Cdk4 complexes, are present in proliferating cells but unavailable to inhibit Cdk2, and can block the activation of newly assembled cyclin-Cdk2 complexes as well as inactivate previously activated complexes. Finally, a 27-kDa protein was specifically recovered from cyclin E-Cdk2 complexes after incubation in an extract from growth-arrested lymphocytes. This observation, together with the functional similarities to $p27^{Kip1}$, suggests that the lymphocyte inhibitor described here is likely to be the human homolog of mink $p27^{Kip1}$.

We have shown that lymphocytes stimulated in the absence of IL-2 arrest in G_1 and accumulate a pool of inactive G_1 cyclin-Cdk2 complexes. This is physiologically analogous to the events that occur at G_2/M, where cells also accumulate inactive cyclin-CDK complexes until previous cell cycle events are complete (53). Uncoupling the gradual assembly of cyclin-CDK complexes from the sudden activation of those complexes is thought to allow for rapid, irreversible transitions between discrete cellular states (75). In contrast to the G_2/M transition, where activation of cyclin B-Cdc2 occurs by an autocatalytic feedback loop, our results suggest that Cdk2 activation in G_1 may be controlled, at least in part, by a noncatalytic feed-forward mechanism—sequestration of an inhibitor by upstream cyclin-CDK complexes. One possibility is that this difference reflects a requirement that the cell coordinate the time of action of multiple cyclin-CDK complexes during G_1. Progression through G_1 is characterized by the sequential appearance of distinct G_1 cyclin-CDK complexes (72). In a normal cell cycle, cyclin D-Cdk4 complexes are active prior to cyclin E-Cdk2 (49). Our in vitro experiments show that the Cdk2 inhibitor can be effectively sequestered, and its activity thereby down-regulated, by cyclin D2-Cdk4. A potential consequence of this is that cyclin D-Cdk4 complexes might need to be present before Cdk2 can be activated in vivo. In fact, cyclin D2 synthesis has been shown to be IL-2 dependent in vivo (1), and this could render Cdk2 activation dependent upon IL-2 by the pathway discussed above. This is one example of how a feed-forward mechanism could integrate the multiple extracellular signals required for S-phase entry and determine the order in which different G_1 cyclin-CDK complexes act during G_1.

Our results from studies using cell extracts suggest that cyclin D2-Cdk4 complexes may contribute to the activation of Cdk2, although we have not yet determined the extent to which this pathway is important for Cdk2 activation in vivo. Indeed, induction of cyclin D2 expression may not entirely explain the effect of IL-2 on cyclin E-Cdk2 activity. In vitro, the cyclin D2 subunit did not sequester the Cdk2 inhibitor, even though the G_1 cell extracts contained endogenous Cdk4 (19). One possibility is that assembly of cyclin D2-Cdk4 complexes is inefficient, at least in vitro, and may even be regulated by IL-2 in vivo. Alternatively, posttranslation modification of the cyclin D2-Cdk4 complex may be necessary for it to sequester the Cdk2 inhibitor. In addition, IL-2 might decrease expression of the Cdk2 inhibitor. The potential abilities of other cyclin-CDK complexes to inactivate the Cdk2 inhibitor also needs further investigation.

A curious aspect of our results is that cyclin A is expressed in G_1 lymphocytes. Similar observations have been made in other cell types, but only in cells exposed to metabolic inhibitors that can potentially alter normal patterns of gene expression (47). Cells arrested in G_1 by cell-cell contact and TGF-β exhibit properties similar to the ones we describe for lymphocytes deprived of IL-2 (63). They express cyclin E and Cdk2, but inhibitory Cdk2-binding proteins prevent formation of active cyclin E-Cdk2 complexes. In those cases, cyclin A is not expressed, indicating that in some cases, cyclin A expression may be dependent upon activation of cyclin E-Cdk2. In contrast, in T cells exposed to partial mitogenic stimuli, cyclin A expression can begin before activation of cyclin E-Cdk2, although a significant increase in cyclin A expression does occur after cells are exposed to IL-2. The fact that the normal timing of cyclin expression can be altered by changes in the cellular environment reinforces the notion that a mechanism might be required to safeguard against potential consequences of those alterations and ensure that cyclin-Cdk complexes are activated in a specific order. Inhibitory proteins, like the ones described here, are likely to have important roles in that pathway.

Inhibition of Cdk2 by proteins that bind to cyclin-Cdk2 complexes is a general mechanism by which proliferative signals control cell cycle progression during G_1. Earlier work had implicated inhibitory binding proteins, such as $p27^{Kip1}$ and p21, in mediating the effects of negative growth signals, including TGF-β, cell-cell contact, and DNA damage (12, 63). Our new results show that the same or functionally similar molecules are down-regulated by positively acting growth factors. The picture that emerges from these results is that growth-inhibitory and growth-stimulatory signals converge on common biochemical targets, CDK inhibitors, which control activation of G_1 cyclin-CDK complexes. Inactivation of a Cdk2 inhibitor in response to a specific growth factor could result in cellular commitment to S-phase entry and may be one biochemical pathway responsible for the physiological event called the restriction point (58).

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