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Regulation of G_1/S transition by cyclins D2 and D3 in hematopoietic cells

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ABSTRACT **Identification of the genes that control passage through the G_1 phase of the cell cycle in mammalian cells is of particular interest because virtually all external events that regulate proliferation act primarily or exclusively during G_1. Cyclins are likely to play a key role in controlling cell cycle progression, although their role during G_1 in higher eukaryotic cells is unclear. In the hematopoietic cell line 32Dcl3, both cyclins D2 and D3 were expressed in proliferating cells, while cyclin D1 was undetectable. Expression of D2, and to a lesser extent D3, was interleukin 3 (IL-3) dependent and declined rapidly in the absence of this growth factor. To investigate the potential role of D cyclins in regulating cell growth, cell lines overexpressing either D2 or D3 were generated by transfection. Constitutive overexpression of either D2 or D3 did not affect cell viability, rate of cell proliferation, or dependence on IL-3 for growth. However, the distribution of cells through the cell cycle was dramatically altered, with both cyclins causing an increase in the fraction of cells in S phase, apparently related to a shortening of G_1. Also, when deprived of IL-3, D3-overexpressing cells failed to arrest in G_1, and apoptotic cell death in the absence of IL-3 was delayed. These results suggest a role for cyclins D2 and D3 in controlling passage of hematopoietic cells through G_1 in the presence of growth factors and in effecting G_1 arrest in the absence of growth factors.**

Passage of eukaryotic cells through the cell cycle is a highly regulated process involving ordered expression of a series of cell cycle control genes (1–4). In hematopoietic cells, growth factors such as interleukin 3 (IL-3) or colony-stimulating factor 1 are required to induce commitment of responsive cells to enter S phase but are not again necessary until the next G_1 phase, a situation analogous to "Start" in yeast (5, 6). Deprivation of growth factors or addition of growth inhibitory cytokines such as transforming growth factor type β to hematopoietic cells causes arrest in G_1 (7), often followed by apoptosis (8). The molecular mechanisms involved in regulating passage through G_0 and G_1 remain largely unknown and are likely to be complex. In yeast, a cyclin-dependent kinase (CDK) is required to complete G_1 (9, 10), and accumulation of cyclins during G_1 is rate-limiting for transition into S phase (11). Genetic screening studies have identified three functionally redundant G_1 cyclins in *Saccharomyces cerevisiae* (CLN1, CLN2, and CLN3) (11, 12). Several potential mammalian G_1 cyclins (C, D, and E types) have also been identified (13–17).

In hematopoietic cells, the D cyclins are particularly attractive candidates for growth regulatory proteins. First, dysregulated expression of cyclin D1 may be oncogenic for lymphoid cells. The BCL1 oncogene activated by the t(11;14) translocation typical of centrocytic lymphoma maps to the cyclin D1 gene (18, 19). Second, in primary human T lymphocytes, we have previously shown that expression of both D2 and D3 cyclins is IL-2 dependent and inducible in G_1 by various mitogens (20). Cyclin D2 was the first cyclin produced after T-cell activation, preceding the appearance of cyclins E, A, D3, and B by >12 hr. There is little evidence so far, however, that any of these cyclins actually functions to regulate passage through G_1 in mammalian cells, although it has recently been shown that constitutive expression of cyclin E in fibroblasts accelerates entry into S phase (21) and that D1, D2, or E can reverse the G_1 exit block caused by introduction of the retinoblastoma gene product, Rb, into Rb-deficient cell lines (22, 23). In this study, we have examined the consequences of overexpressing cyclin D2 or D3 in a murine IL-3-dependent cell line.

MATERIALS AND METHODS

Cell Lines and Culture. 32Dcl3 is a diploid, nonleukemic, IL-3-dependent murine myeloid cell line (24), obtained from Joel Greenberger (University of Massachusetts Medical School). Cells were cultured at 2–10 × 10^5 cells per ml in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO). Medium conditioned by the WEHI-3 cell line (WEHI-CM) was used as a source of IL-3 (24). IL-3 starvation was done by washing cells twice and replating in culture medium without WEHI-CM for 16 hr.

To generate sublines of 32Dcl3 that constitutively express cyclins D2 and D3, cyclin D2 and D3 cDNAs (16) were cloned into the pGD retroviral vector (25), which uses the 5′ long terminal repeat of Moloney leukemia virus as a promoter and contains the neomycin phosphotransferase gene as a selectable marker. A series of cell lines stably expressing cyclin D2 or D3 were obtained by selection in G418-containing medium under limiting-dilution conditions. Seven cell lines expressing each cyclin were chosen for study, and all experiments were performed on at least two lines and typically on all lines. Cell viability was determined by exclusion of trypan blue. Apoptosis was assessed by detection of "laddering" of DNA in agarose gels stained with ethidium bromide (26).

Cell Cycle Analysis and Proliferation Assays. Cells (5 × 10^5 cells) were resuspended in 0.5 ml of propidium iodide solution [propidium iodide (50 μg/ml)/sodium citrate (0.1%)/Nonidet P-40 (NP-40; 0.1%)] (26). DNA histograms were analyzed by using the Multicycle computer program (Phenix Flow Systems, San Diego). [^{3}H]Thymidine incorporation was measured after incubating cultures with 0.2 mCi of [^{3}H]thymidine (2 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) for 16 hr.

Direct Measurement of Cell Cycle Times. Nonsynchronized cell cultures were pulse labeled with 10 μM bromodeoxyuridine (BrdUrd) for 30 min, washed, and recultured. Aliquots were removed hourly for 19 hr and the number of BrdUrd-labeled mitotic cells was counted as described (27) by detecting BrdUrd with a monoclonal antibody (Boehringer

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Abbreviations: CDK, cyclin-dependent kinase; IL-3, interleukin 3.

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Mannheim). The durations of G_1, S, and total cell cycle time were calculated as described (21).

RNA Extraction and Northern Blotting. Total cellular RNA was extracted from 2×10^7 cells by the guanidium thiocyanate method. Samples (10 μg) were subjected to 1% Mops/formaldehyde agarose gel electrophoresis and blotted onto nitrocellulose membranes. The blots were hybridized with ^{32}P-labeled cDNA probes. Murine cyclins D1, D2, and D3 (16) and CDK4 (28) cDNAs were gifts from Charles Sherr (St. Judes Children's Research Hospital, Memphis, TN). A CDK2-HS cDNA (29) in pADNS and a human cyclin E cDNA (15) in pGEX-2T were gifts from A. Koff and J. M. Roberts (Fred Hutchinson Cancer Research Center, Seattle). A cyclin C cDNA (14) was a gift from Steve Reed (Scripps Research Institute, La Jolla, CA). Inserts were labeled with [^{32}P]dCTP by the hexanucleotide primer technique.

Generation of Antibodies to Cyclins D2 and D3 and Immunoprecipitation. Cyclins D2 and D3 were expressed as glutathione *S*-transferase fusion proteins in *Escherichia coli* transformed by plasmids pGEX3XN9 or pGEX3XN2, respectively (from Charles Sherr). Purified fusion proteins were used to immunize rabbits, and high titer, affinity-purified antisera against D2 and D3 were prepared as described (20). Cells were labeled with [^{35}S]methionine (1200 Ci/mmol; New England Nuclear) for 3 hr in methionine-free RPMI 1640 medium (Flow Laboratories) and lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 137 mM NaCl, 10% (vol/vol) glycerol, 1.0% NP-40 (Sigma), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit of aprotinin per ml, 10 mg of leupeptin per ml, 1 mM sodium orthovanadate, and 100 mM NaF (Sigma). The cell lysates were incubated with 5 μl of preimmune or immune serum and protein A-Sepharose beads (Sigma) for 2 hr at 4°C. The beads were washed five times in the same buffer, suspended in sample buffer as described (20), and heated at 95°C for 5 min; the eluted proteins were applied to SDS/polyacrylamide gels and detected by autoradiography.

RESULTS

Expression of Cyclins and CDKs in 32D Cells. 32Dcl3 cells proliferating in response to IL-3 were found to express cyclin D2 and cyclin D3 RNA by Northern blot analysis (Fig. 1*A*, lane C). Cyclin D1 was not detected. After removal of IL-3 from the culture medium for 16 hr, >80% of cells arrested in G_1. Cyclin D2 RNA declined rapidly and was undetectable at 20 hr, while cyclin D3 RNA levels decreased by only 50% during this time (data not shown). Immunoprecipitation of cyclins D2 and D3 from [^{35}S]methionine-labeled cells showed that protein levels correlated well with RNA levels (data not shown). Longer starvation times induced cell death through apoptosis. When IL-3-deprived cells were retreated with IL-3, cyclin D2 was induced rapidly (within 1 hr), but cyclin D3 RNA increased only modestly (Fig. 1*A*). Cyclins A and E and cdk2 RNAs were induced ≈15 hr after stimulation (at the G_1/S boundary), whereas cdk4 was induced in mid-G_1 phase (Fig. 1*A*). Protein expression of cyclins D2 and D3 was compatible with the RNA kinetics (Fig. 1*B*). Expression of cyclins D2 and D3 was not affected by the level of serum in the culture medium.

Generation of Factor-Dependent Cell Lines Overexpressing Cyclins D2 and D3. To investigate the biological functions of cyclins D2 and D3 in hematopoietic cells, we generated 32D cell lines constitutively expressing cyclin D2 or D3. Cells were transfected with retroviral expression vector pGD, pGD-D2, or pGD-D3 by electroporation, and individual clones were selected in G418-containing medium. Seven independent clones expressing cyclin D2 (designated as clones 20, 21, and 24–28) and seven independent clones (designated as clones 30, 31, and 35–39) expressing cyclin D3 were obtained (Fig. 2). The accumulation of cyclin protein in these cell lines was

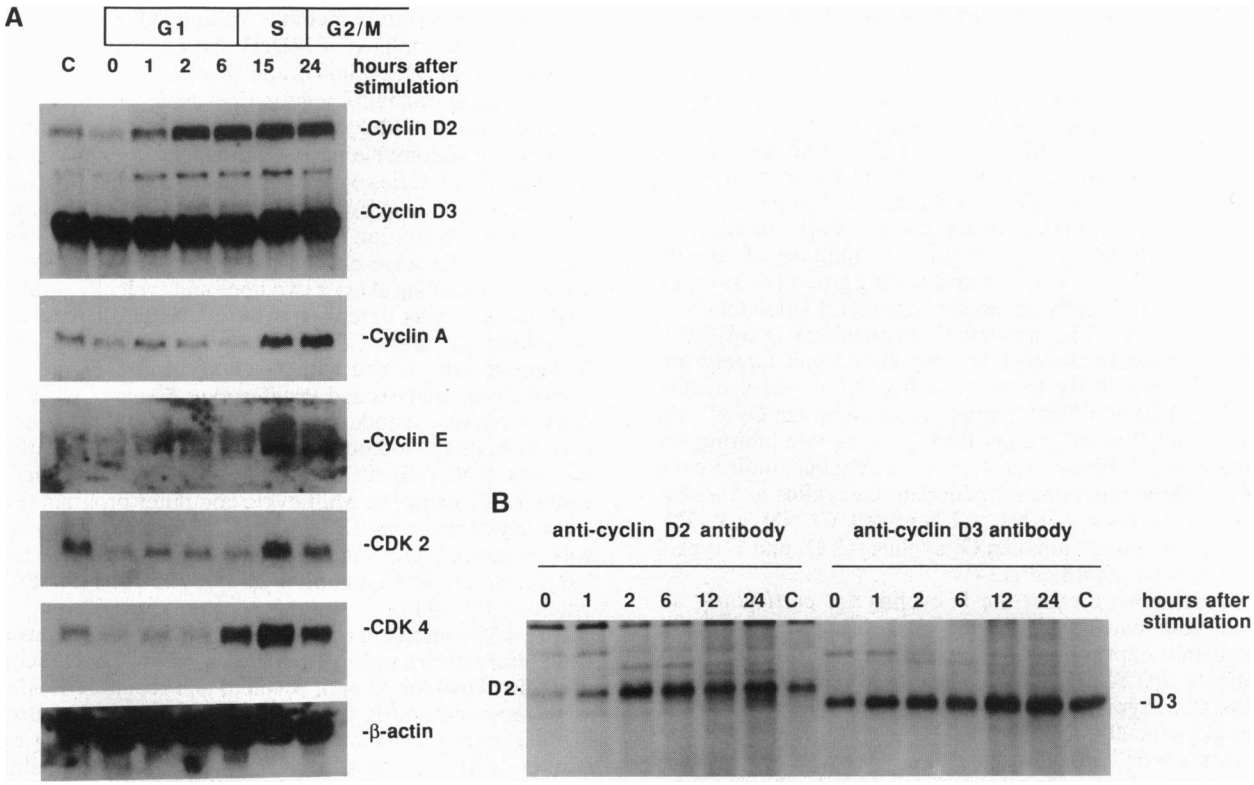

FIG. 1. Expression of cyclins and CDKs in 32Dcl3 cells. (*A*) Northern analysis. Cells were deprived of IL-3 for 16 hr and then stimulated with 15% WEHI-CM for the indicated times (hours). Each lane contains 10 μg of total RNA. Sample C is from a rapidly growing culture. (*B*) Cyclin D2 and D3 protein induction by IL-3 in factor-deprived cells. [^{35}S]Methionine-labeled cell lysates were immunoprecipitated with anti-cyclin D2 or D3 antibody and analyzed as described. Positions of cyclin D2 and D3 bands are indicated.

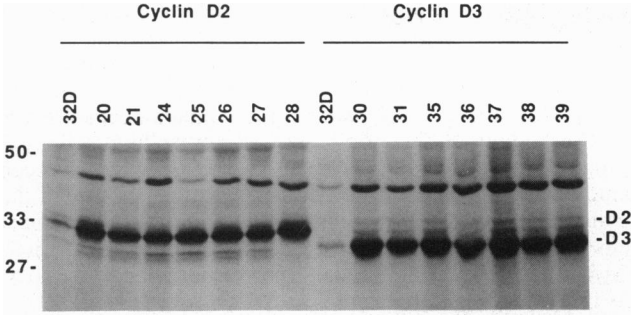

FIG. 2. Expression of cyclin D2 and D3 proteins in 14 independent transformants with cyclin D2 or D3 expression vectors. Immunoprecipitation with anti-cyclin D2 or D3 antibody in seven independent transformants for cyclin D2 (clones 20, 21, and 24–28), seven for D3 (clones 30, 31, and 35–39), and parent 32D cells metabolically labeled with [^{35}S]methionine. Numbers on left indicate positions of molecular mass markers (kDa).

variable and ranged from approximately wild-type levels to a 3- to 4-fold increase. In contrast to wild-type cells, the loss of cyclin protein following IL-3 deprivation was delayed or absent in transfected cell lines (Fig. 3).

Altered Cell Cycle Kinetics in Cell Lines Constitutively Expressing Cyclin D2 or D3. The fraction of cells in each phase of the cell cycle was measured by staining DNA with propidium iodide. Cyclin D2- or D3-expressing cell lines had a strikingly increased fraction of cells in S phase and a decreased fraction in G_1 phase compared with wild-type 32D empty vector transformants (Fig. 4). This phenomenon was observed in each of the 14 clones examined expressing cyclin D2 or D3. The increment in S phase cells was confirmed by measuring the uptake of BrdUrd with an anti-BrdUrd antibody and flow cytometry. In a typical experiment, 26% of wild-type or vector-only transformants incorporated BrdUrd, while 43% of clone 27 cells and 48% of clone 36 cells incorporated BrdUrd. This technique produces a lower estimate of S phase fraction compared to propidium iodide staining, but the overall results are the same. Constitutive expression of either cyclin D2 or D3 did not affect the rate of cell growth or viability (data not shown). This suggested that the observed accumulation of cells in S phase could be due to a shortened time in G_1. We therefore directly measured the duration of G_1 and S phases by pulse-labeling cells with BrdUrd and serially enumerating mitotic cells that were BrdUrd positive hourly for 19 hr (Fig. 5). Total cell cycle times were identical for 32D cells, clone 27, and clone 36. However, G_1 phase was decreased from 4.9 to 1.5 and 1.7 hr, respectively, and S phase was increased from 6.6 to 10.0 and 9.4 hr, respectively, in the experiment shown. Labeling index and mitotic index in the 1-hr samples were, respectively, 33% and 3% in 32D, 57% and 4% in clone 27, and 53% and 3% in clone 36.

All cell lines remained dependent on IL-3 for proliferation, and the dose–response to IL-3 (measured by [^{3}H]thymidine

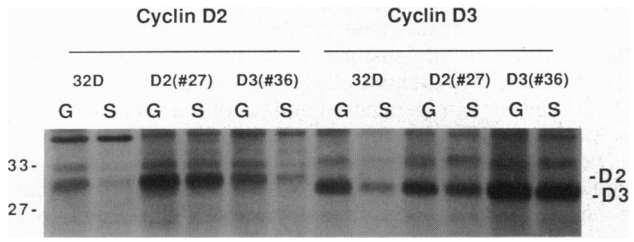

FIG. 3. Expression of cyclins D2 and D3 in wild-type cells and clones 27 and 36. Exponentially growing cells (G) and IL-3-deprived cells (16 hr) (S) were analyzed for expression of cyclins D2 and D3 by immunoprecipitation.

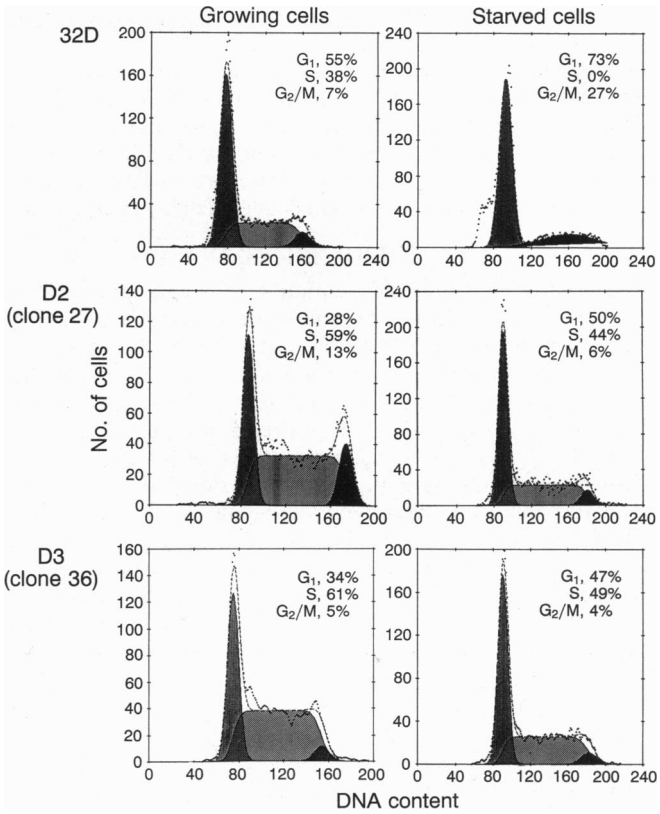

FIG. 4. DNA histograms of wild-type cells and clones 27 and 36. Exponentially growing or IL-3-deprived cells (16 hr) (5×10^5 cells) were stained with propidium iodide and DNA histograms were analyzed by flow cytometry. *x* axis, DNA content in arbitrary fluorescence units; *y* axis, cell number in arbitrary units.

incorporation) was found to be identical to wild-type cells (data not shown). After IL-3 deprivation, the wild-type cells rapidly arrested in G_1, while cyclin D2- and D3-expressing lines were found to have a delay in G_1 arrest (Fig. 4).

Overexpression of Cyclin D3 Delays Apoptotic Death in Growth Factor-Deprived Cell Lines. 32D cells die within 24–48 hr in the absence of IL-3 through the process of

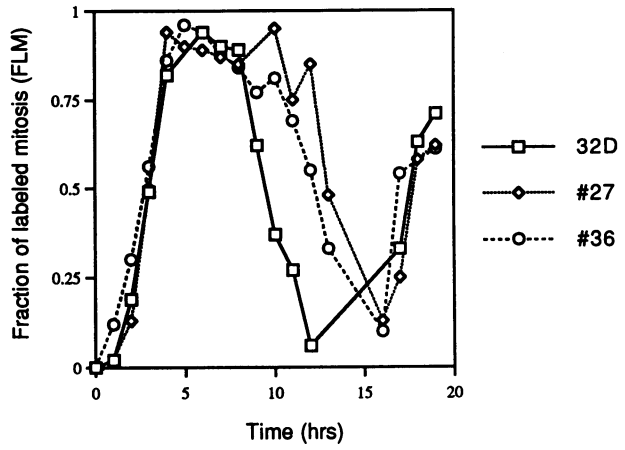

FIG. 5. Cyclins D2 and D3 shorten G_1 and lengthen S phase. Asynchronously growing cultures of 32D, clone 27, and clone 36 were pulse labeled with BrdUrd and the fraction of labeled mitoses was determined hourly for 19 hr. Cell cycle times were calculated as described in the text. In this representative experiment, total cell cycle time was 14.4, 14.4, and 14.0 hr, respectively (32D, clone 27, clone 36). G_1 phase was 4.9, 1.5, and 1.7 hr, and S phase was 6.6, 10.0, and 9.4 hr.

apoptosis (8, 30). Although deregulated expression of cyclin D2 or D3 did not make 32D cells IL-3 independent, the rate of cell death in IL-3-free media was delayed, especially in lines expressing cyclin D3 (Fig. 6). Even after 7 days of IL-3 deprivation, up to 20% of cells were alive, and these cells could be readily induced to proliferate by IL-3 (Fig. 6). The effects of cyclin D2 expression were more variable, as only two of six clones showed the same level of delay in apoptosis.

DISCUSSION

The proliferation and differentiation of hematopoietic progenitor cells is controlled by a network of growth stimulatory and inhibitory cytokines that act primarily or exclusively in G_0/G_1. After each round of replication of myeloid lineage cells, exposure to an adequate level of lineage-specific growth factors such as IL-3 is required or the cells will rapidly arrest in G_1 and die through apoptosis. Although much is known about the growth factors of myeloid cells, their receptors, and the initial events of signaling, little is known about the events that actually cause cell cycle arrest or lead to apoptosis. Previous studies in budding yeasts and other cells have shown that cyclins and CDKs participate in forming "checkpoints" at which cells must make critical decisions such as advancement from G_1 into S phase

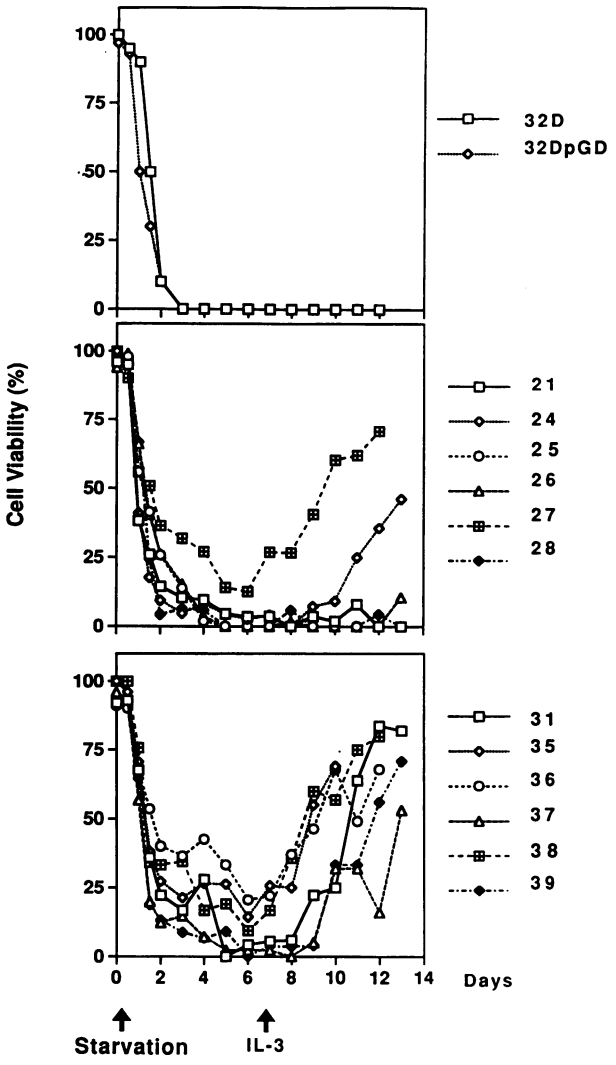

FIG. 6. Cell viability curves after deprivation of IL-3. Cell viabilities were assessed by exclusion of trypan blue at each time point (days). Seven days after deprivation of IL-3, 15% WEHI-CM was added as indicated.

("Start") and determine the timing of the initiation of mitosis (reviewed in ref. 31). Furthermore, there is evidence that some factors that regulate both G_1/S and G_2/M transition do so by controlling the synthesis or activity of cyclins. Although the functions of cyclins in mammalian cells are well established at G_2/M, their role in controlling cell cycle progression in G_1 is largely hypothetical. However, a recent study from Ohtsubo and Roberts (21) has demonstrated that constitutive overexpression of cyclin E in fibroblasts shortens G_1, lengthens S, and decreases the serum requirement for G_1/S transition. Cyclin E has therefore been suggested to act as a G_1 cyclin (21, 32).

In primary human T lymphocytes, we have recently shown that G_0 lymphocytes express low levels of cyclins D2 and C but lack expression of cyclins D1, D3, E, A, and B (20). After mitogenic stimulation, there is an IL-2-dependent induction of cyclins D2 and C in early G_1, followed 12 or more hr later by cyclins D3, E, and A (in late G_1 or early S). This differential regulation suggested that "G_1 cyclins" in hematopoietic cells may actually participate in more than one checkpoint in G_1, perhaps an early checkpoint near G_0/G_1, and a later event near the mammalian equivalent of Start (20).

In this study, two D cyclins were overexpressed in an IL-3-dependent cell line, 32Dcl3 (8, 24). Overexpression of either cyclin had no effect on growth rate or dose–response to IL-3 but had profound effects on the cell cycle, reducing the number of cells in G_1 and increasing the number of cells in S phase by 60% or more. This effect appears to be due to a shortening of G_1 and lengthening of S phase, without a change in the overall length of the cell cycle. It is possible that the primary effect is premature entry into S phase, with secondary lengthening of S phase because other factors necessary for optimum rates of DNA synthesis have not yet been induced. Ohtsubo and Roberts (21) demonstrated a similar phenotype in fibroblasts that were made to constitutively overexpress cyclin E. Overall, these results suggest that cyclin accumulation in G_1 is a limiting event in determining the timing or rate of entry into S phase in cells growing in optimal concentrations of IL-3.

We also examined the effects of cyclin overexpression during factor deprivation. Expression of either cyclin D2 or D3 dramatically delayed G_1 arrest in the absence of IL-3. However, cyclins D2 and D3 had different effects on apoptotic death. Wild-type cells entering G_1 in the absence of IL-3 immediately arrest and then undergo apoptosis in a matter of hours. In unsynchronized cultures, the majority of cells are dead in 24 hr, and virtually no viable cells can be recovered after 48 hr. Constitutive expression of cyclin D3 delayed apoptosis substantially in six of six lines studied, while D2 lines were more variable. Up to 20% of cyclin D3-overexpressing cells remained viable for up to 7 days in multiple experiments, and readdition of IL-3 led to rapid proliferation of these cells. These results suggest that down regulation of the level of D cyclins is likely to contribute to the rapid G_1 arrest characteristic of factor-deprived hematopoietic cells. Since overexpression of D cyclins only delays, rather than blocks, G_1 arrest, other factors are involved in controlling this event. One candidate would be the CDK4 kinase shown by Matsushime *et al.* (28) to interact with D cyclins. As shown in this study, expression of CDK4 RNA in 32Dcl3 cells is IL-3 dependent and rapidly disappears during factor deprivation.

Our results suggest that the level of D cyclins can directly regulate the rate or timing of progression from G_1 to S phase in rapidly growing IL-3-dependent cells and may also be critically involved in causing G_1 arrest in cells deprived of growth factors. In normal hematopoietic cells, the level of D cyclin expression and activity is directly dependent on the level of lineage-specific growth factors such as IL-3 (in myeloid cells) and IL-2 (in T cells). Accumulation of D

cyclins to critical levels may be necessary to exit from G_1. Our results also suggest the possibility that D cyclins could participate in the G_1 arrest that is characteristic of the terminal differentiation of myeloid cells, and differentiation-related regulation of D cyclins should be examined. Finally, since the regulation of growth and differentiation of hematopoietic cells by cytokines involves a careful balance of proliferation and cell death, our results provide a framework in which to understand the apparently oncogenic effects of dysregulated cyclin D expression in human parathyroid adenomas, centrocytic lymphomas, and other tumors (17). The anti-apoptotic effects of cyclin D overexpression could disrupt the normally absolute ability of cytokines to control cell proliferation and viability.

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