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Inhibition of granulocyte differentiation by G_1 cyclins D2 and D3 but not D1

(D-type cyclins/cyclin-dependent kinases/G_1 progression)

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ABSTRACT **Growth factor-induced signals govern the expression of three D-type cyclins, which, in turn, function as regulatory subunits of cyclin-dependent kinases (cdks) to control cell cycle transitions during the late G_1 interval. 32D myeloid cells, which self-renew as uncommitted precursors in interleukin 3 (IL-3), express cyclins D2 and D3 (but not D1) in complexes with cdk4 and cdk2. When transferred to granulocyte colony-stimulating factor (G-CSF), 32D cells stop dividing and terminally differentiate to mature neutrophils. Cyclin D and cdk4 expression ceased as cells underwent growth arrest in G-CSF, but cdk2 levels were sustained. 32D cells engineered to ectopically express D-type cyclins exhibited contracted G_1 intervals with a compensatory lengthening of S phase but remained IL-3 dependent for cell growth; those overexpressing cyclins D2 and D3 (but not D1) were unable to differentiate and died in G-CSF. Cyclin D2 mutants, which cannot efficiently bind to, or functionally interact with, the retinoblastoma protein (pRb) or its relatives (p107) did not block differentiation. Conversely, the introduction of a catalytically inactive cdk4 mutant into cells overexpressing cyclin D2 restored their G-CSF response. The persistence of cdk2 and its predilection to functionally interact with cyclins D2 and D3 rather than D1 might explain the specificity of the differentiation blockade.**

Mitogenic growth factors act during the G_1 phase of the cell cycle to drive cells into S phase, after which their activities are no longer required until cells exit mitosis (reviewed in ref. 1). Unlike cyclins A and B, which are first synthesized during S phase and interact with $p34^{cdc2}$, the prototypic cyclin-dependent kinase (cdk) (reviewed in ref. 2), mammalian D-type cyclins (D1, D2, and D3) are differentially expressed during the G_1 interval in various cell types in response to growth factor stimulation. By associating with several cdks, including cdk2, -4, and -5 (3, 4), the D-type cyclins, together with the cyclin E–cdk2 complex (5, 6), are presumed to regulate phosphorylation of key substrates required for G_1 progression and S-phase entry (reviewed in ref. 7). Although their associated kinase activities have not been directly measured in mammalian cells, each of the D-type cyclins, but not cyclin A, B, or E, can activate cdk4 in insect cells (3, 8), whereas cyclins D2, D3, E, and A, but not D1, functionally interact with cdk2 (9). Constitutive ectopic expression of cyclin D1, D2, or E in mammalian fibroblasts shortens G_1 and reduces their serum requirement for S-phase entry (10, 11). Conversely, microinjection of antibodies to cyclin D1 into serum-stimulated fibroblasts during G_1 phase prevents DNA synthesis (11, 12). Thus, the functions of mammalian G_1 cyclins appear both necessary and rate limiting for cell cycle progression.

By enforcing progression into S phase, overexpression of G_1 cyclins might nullify certain commitment steps necessary for the proper execution of differentiation programs. We tested this possibility by stably overexpressing each of the D-type cyclins in 32Dcl3 myeloid precursor cells (here simply designated 32D), which self-renew in medium containing interleukin 3 (IL-3) but growth arrest and terminally differentiate to granulocytes in granulocyte colony-stimulating factor (G-CSF) (13). Constitutive expression of D-type cyclins did not affect the growth factor dependence or proliferative rate of 32D cells grown in medium containing IL-3, but cyclin D2 and D3 overexpressors were unable to differentiate in G-CSF and lost viability. Cyclin D1, which is normally not expressed in these cells, did not block differentiation. Therefore, the different D-type cyclins are unlikely to be functionally redundant, and, in an appropriate setting, their overexpression can alter growth factor-dependent differentiation responses.

MATERIALS AND METHODS

Derivation of Cell Lines and Culture Conditions. 32D cells and derivatives were maintained at 1–5 × 10^5 cells per ml in Iscove's modified Dulbecco's medium with 20% fetal bovine serum (FBS), glutamine, antibiotics, and 25% WEHI-3B conditioned medium as a source of IL-3 (13). An early passage subclone that was maximally responsive to G-CSF (>90% terminal differentiation in 12 days) was transfected with *neo*-containing pRc/RSV vectors (Invitrogen) including cDNAs encoding mouse cyclin D1, D2, or D3 (14, 15) or either of two cyclin D2 mutants (9) encoding lysine at position 7 (E7K) or deleted for residues 2–7 (Δ2–7) encompassing a Leu-Xaa-Cys-Xaa-Glu (LXCXE) motif at codons 3–7. Electroporation was performed with 80 μg of vector plasmid DNA at 270 V and 960 μF using 8 × 10^6 cells in 0.8 ml of phosphate-buffered saline (PBS). After overnight culture in medium containing IL-3, a portion was subjected to limiting dilution in medium containing IL-3 and 1 mg of G418 per ml (Sigma). These populations were used to estimate transfection efficiencies (uniformly >1 × 10^3 G418-resistant cells per electroporation) and to derive subclones from single colonies. The remaining cells were distributed into a 24-multiwell plate, and, after 2 weeks of selection, G418-resistant cells were pooled for further analysis (see Table 1, mixed pools).

A clone derived from a single cell (designated D2-8 in Table 1) was reelectroporated with a pRc/RSV vector encoding catalytically inactive cdk4, in which lysine at the ATP binding site (position 35) was converted to methionine (K35M) (3, 8). We used an equal mixture of cdk4 vector and a plasmid (pHEBo) (16) containing the hygromycin B phosphotransferase gene (*hygro*) and selected transfectants with 1000 units of hygromycin B per ml (Calbiochem). For culture in G-CSF, cells were washed twice in PBS and transferred to medium

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Abbreviations: IL-3, interleukin 3; G-CSF, granulocyte colony-stimulating factor; cdk, cyclin-dependent kinase.

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containing 25 ng of recombinant human G-CSF per ml (Amgen Biologicals) in lieu of IL-3.

Antisera to D-Type Cyclins and cdks. Rabbit antisera to mouse D-type cyclins, $p34^{cdk4}$, and $p34^{cdc2}$, each preferentially reactive with the cognate protein, were raised to engineered bacterial polypeptides (3, 14). Specific antisera to $p33^{cdk2}$, $p36^{cdk3}$, and $p31^{cdk5}$ were raised against synthetic C-terminal peptides (anti-$p33^{cdk2}$ from Giulio Draetta, Mitotix, Cambridge, MA). Although the antisera to cyclins D1 and D2 crossreact, the two proteins can be distinguished by their different molecular masses (3, 8, 15).

Protein Analysis. Cells (1×10^7) were metabolically labeled for 1 hr with 200 μCi of [^{35}S]methionine per ml (1000 Ci/mmol; 1 Ci = 37 GBq; Tran^{35}S-label, ICN) in methionine-free medium containing 20% dialyzed FBS and the indicated hemopoietins. Cell lysis, immunoprecipitation, immunoblotting, and gel electrophoresis were performed as described (17, 18). For detection of cyclin D–cdk complexes, cell lysates were precipitated with antisera to cdk, and the denatured immunoprecipitates were separated on polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antisera to cyclins.

RESULTS

Cyclin D and cdk4 Synthesis in 32D Cells. Mouse myeloid 32D cells grown in IL-3 express only cyclins D2 and D3, but transfer to G-CSF for 20 hr led to their reduced synthesis (Fig. 1*A*) and eventual disappearance, with cyclin D2 being much more labile than D3 (Fig. 1*B*). Synthesis of the major catalytic subunit for D-type cyclins, cdk4 (3, 4), was also diminished in cells undergoing granulocytic differentiation (Fig. 1*A*; cf. Fig. 4*A*). Reduced synthesis of these proteins preceded G-CSF-induced growth arrest observed within 48–72 hr (Fig. 2*A*) and occurred well in advance of morphologic changes typifying granulocytic differentiation (Fig. 3*A* vs. Fig. 3*B*).

Irreversible commitment of >80% of the cells, manifested by an inability to respond to IL-3, requires 3–5 days in G-CSF (13, 19), after which terminal differentiation to mature neutrophils takes 5–7 more days. The cells ultimately arrest with a 2*n* DNA content, and their viability is maintained for 10–12 days, after which the terminally differentiated cells die. In the presence of both IL-3 and G-CSF, cyclin D2 and D3 levels are maintained, and the cells do not differentiate, implying that persistent growth signals override the commitment step(s) (13) and sustain cyclin D synthesis. The failure to completely remove IL-3 retards differentiation in G-CSF and may account for the inability of some cells to arrest immediately. Persistence of cyclin D3 during the initial commitment stage might also help maintain cells in cycle.

Cells Overexpressing Cyclins D2 and D3 Do Not Differentiate. 32D cells electroporated with a control *neo* expression vector or with vectors encoding D-type cyclins were selected in medium containing G418 and IL-3. Comparable transfection efficiencies were obtained for each vector, and pooled drug-resistant populations synthesized 5- to 8-fold higher levels of the corresponding cyclins (Fig. 1*C*), which were sustained when cells were transferred into G-CSF (Fig. 1*D*). When grown in IL-3, each population remained undifferentiated and proliferated at rates indistinguishable from those of parental 32D cells or control transfectants (Fig. 2*B*). Asynchronously growing cells overexpressing D-type cyclins had increased S-phase fractions (range, 52–64% calculated by flow cytometric analysis of DNA content) compared to *neo*-resistant controls (40–45%) but displayed no changes in their G_2/M fractions. Because >98% of cells remained viable when propagated in IL-3, their G_1 intervals must have been contracted to maintain equal generation times. A 15% decrease in the G_1 fraction reflects a shortening of G_1 phase by

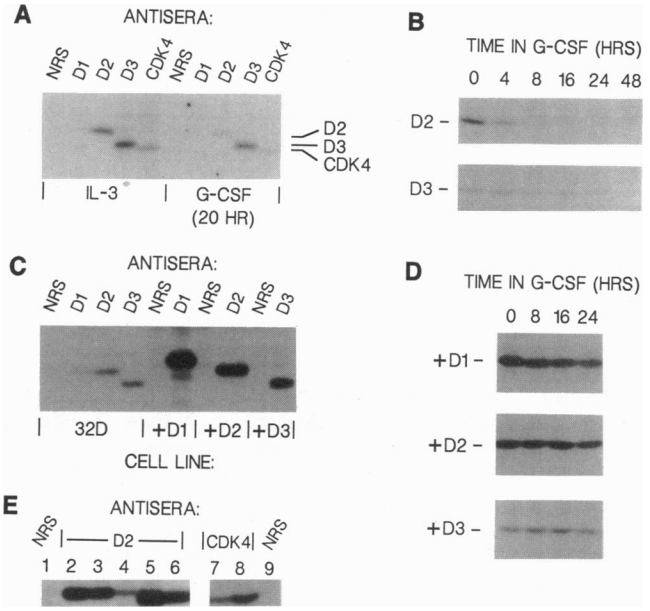

FIG. 1. Expression of D-type cyclins and cdk4. (*A*) Parental 32D cells in IL-3 or in G-CSF for 20 hr were labeled with [^{35}S]methionine, and immunoprecipitates prepared with nonimmune rabbit serum (NRS) or antisera were separated on denaturing gels and detected by autoradiography. (*B*) Parental 32D cells were shifted from IL-3 to G-CSF, and immunoprecipitates prepared using antisera to either cyclin D2 or D3 were separated on denaturing gels, transferred to nitrocellulose, and immunoblotted with the same antibodies. Although D2 synthesis persists for up to 20 hr (*A*), >90% of the total D2 protein is degraded by 8–16 hr. (*C*) Metabolically labeled cell lysates from parental 32D cells or from mixed cell pools transfected with D-type cyclin genes (lanes +D1, +D2, and +D3) were precipitated with antisera as indicated, and labeled proteins were resolved on denaturing gels. (*D*) Lysates from cells overexpressing D-type cyclins were obtained at different times after culture in G-CSF. Immunoprecipitated cyclins were immunoblotted with the same antisera as in *B*. (*E*) Parental 32D cells (lanes 1 and 4), subclone D2-8 (Table 1) expressing cyclin D2 (lane 2), pooled *hygro*-resistant D2-8 derivatives transfected with catalytically inactive cdk4 (K35M) (lane 3), or parental 32D cells transfected with cyclin D2 mutants encoding disrupted (lane 5) or deleted (lane 6) LXCXE segments were labeled with [^{35}S]methionine, and lysates were precipitated with nonimmune serum (NRS; lane 1) or antiserum to cyclin D2 (lanes 2–6). Both cyclin D2 mutants migrated somewhat faster than the wild-type protein on denaturing gels (lanes 5 and 6). Lysates from parental 32D cells (lane 7) or from the D2-8 subclone transfected with catalytically inactive cdk4 (lanes 8 and 9) were immunoprecipitated with antiserum to cdk4 (lanes 7 and 8) or with NRS (lane 9). Separated proteins transferred to nitrocellulose were blotted with antiserum to cdk4. In all panels, immunoprecipitates were prepared from equivalent numbers of cells. To facilitate quantitative comparisons, the immunoblots shown in *B* and *D* were derived from parallel cultures in a single experiment. All autoradiographic exposure times were matched (16 hr).

≈28%, consistent with the intervals calculated from rates of incorporation of 5-bromodeoxyuridine into mitotic chromosomal DNA (ref. 10; data not shown).

When switched to G-CSF, parental 32D cells or those transfected with the control vector underwent growth arrest (Fig. 2*A*) and differentiated to neutrophils (Fig. 3*B*). The percentage with 2*n* DNA content (G_0/G_1) increased from 40% to 80% within 3 days (data not shown). Cells overexpressing cyclin D1 also arrested and differentiated (Figs. 2*A* and 3*C*), but those overexpressing cyclins D2 and D3 gradually lost viability (Fig. 2*A*) and failed to exhibit neutrophilic features (see below). Although most of the latter did not survive G-CSF treatment, their viability was prolonged as compared

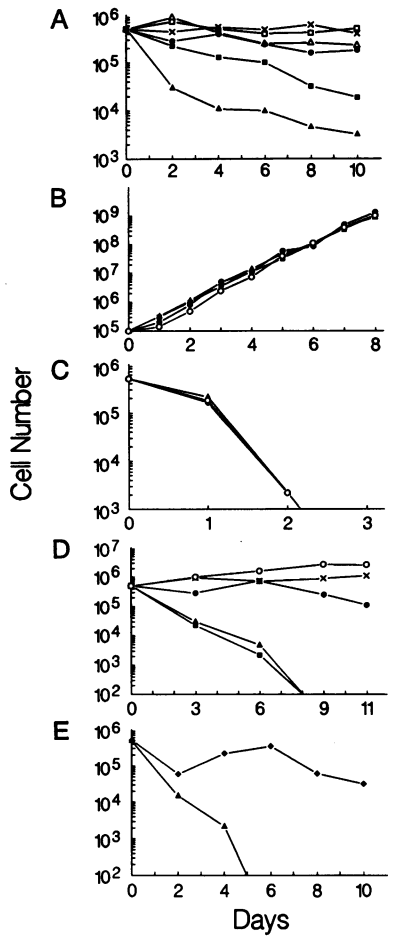

FIG. 2. Growth of 32D cells and transformants in media containing IL-3, G-CSF, or no hemopoietin. Mixed pools of transfected cells (*A*) or representative subclones (*B–D*) were cultured in medium containing G-CSF (*A*, *D*, and *E*), IL-3 (*B*), or no exogenous hemopoietin (*C*), and the numbers of viable cells were determined at the indicated intervals. Cells maintained in IL-3 were washed twice with PBS and reseeded at 1×10^5 cells per ml in IL-3 or at 5×10^5 cells per ml in G-CSF or no added growth factor. Cells expanding in IL-3 were diluted 1:10 at 2-day intervals; G-CSF-containing cultures were split 1:2 every 4 days. ○, Parental 32Dcl3 cells; ×, cells transfected with the control *neo* vector (either the mixed pool in *A* or subclone 5 in *B–D*); ●, cells transfected with cyclin D1 (either the mixed pool in *A* or subclone D1-5 in *B–D*); ▲, cells transfected with cyclin D2 (either the mixed pool in *A*, subclone D2-8 in *B–D*, or hygro-resistant D2-8 cells in *E*); ■, cells transfected with cyclin D3 (either the mixed pool in *A* or subclone D3-2 in *B–D*); □, cells transfected with the cyclin D2 (E7K) mutant (mixed pool in *A*); △, cells transfected with the cyclin D2 (Δ2–7) mutant (mixed pool in *A*); ◆, subclone D2-8 transfected with catalytically inactive cdk4 (K35M) (mixed pool in *E*).

to cells deprived of all hemopoietins (Fig. 2*A* vs. Fig. 2*C*). Cell death during prolonged culture in G-CSF might reflect the inability of blocked cells to upregulate G-CSF receptors or other components of the G-CSF signaling pathway.

Subclones derived in IL-3 retained the characteristics of parental transfectants. None showed altered growth rates in IL-3 nor grew in its absence, but those overexpressing cyclin D2 could not be maintained in G-CSF (Fig. 2*D*; Table 1) and did not differentiate to neutrophils, as judged by cell morphology (Fig. 3*D*), expression of histochemical markers, and granulocytic cell surface antigens (Table 1). Less prominent effects were seen with cyclin D3 (Figs. 2*A* and 3*E*; Table 1), whereas cyclin D1 subclones exhibited no perturbations (Figs. 2 *A* and *D* and 3*C*; Table 1). Immunoblotting did not reveal significant variations in cyclin protein expression

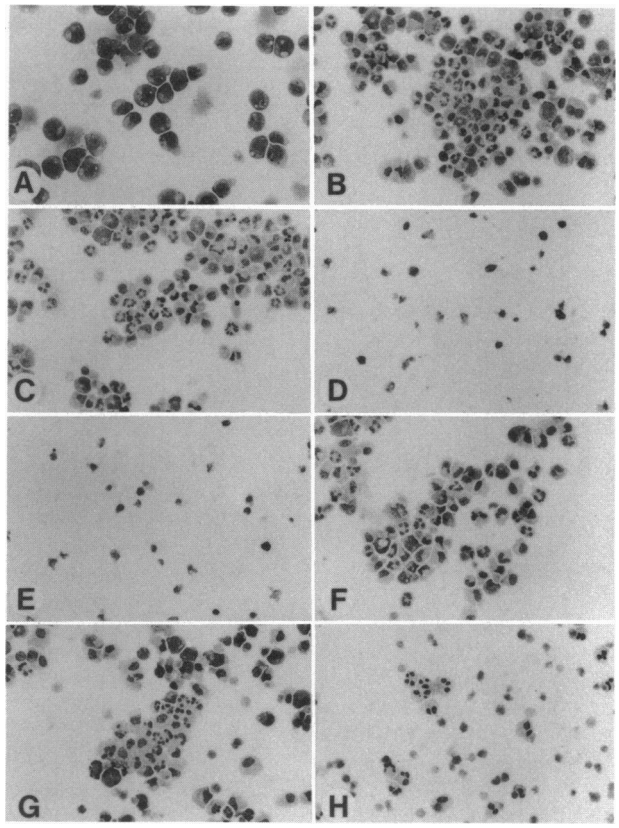

FIG. 3. Granulocytic differentiation in medium containing G-CSF. Cells grown in IL-3 (*A*) or G-CSF (*B–H*) for 12 days were cytocentrifuged and stained with Giemsa. (*A*) Parental 32D cells. (*B*) Parental cells induced to differentiate. (*C*) Cyclin D1 overexpressor (clone D1-5). (*D*) Cyclin D2 overexpressor (clone D2-8). (*E*) Cyclin D3 overexpressor (clone D3-2). (*F* and *G*) Cells transfected with cyclin D2 (Δ2–7) or D2 (E7K) mutants, respectively. (*H*) Cyclin D2 overexpressors (clone D2-8) transfected with inactivated cdk4 (K35M) (mixed pool).

between groups of subclones overexpressing cyclins D1 and D2 (cf. Fig. 1 *C* and *D*), arguing that their effects reflect functional differences between them.

D2 Mutants Disrupted in Their LXCXE Motif Do Not Block Differentiation. D-type cyclins have a LXCXE motif at their N termini, which is found in DNA tumor virus oncoproteins that bind to pRb and other pRb-like proteins (e.g., p107). D-type cyclins can bind pRb and p107 (8, 9, 22) and can override the ability of pRb to suppress G_1 exit in human *RB*-negative Saos-2 cells (9, 22), but cyclin D2 mutants disrupted in their LXCXE motif bind pRb poorly and cannot abrogate its growth suppressive function (9). When cyclin D2 mutants containing an E to K substitution or lacking the entire LXCXE pentapeptide were introduced into 32D cells, the proteins were overexpressed (Fig. 1*E*, lanes 4–6), and their synthesis was sustained when cells were cultured in G-CSF (data not shown). These cells remained viable (Table 1; Fig. 2*A*) and differentiated to granulocytes (Fig. 3 *F* and *G*), possibly implicating pRb or a pRb-like protein in the differentiation process.

Cyclin D2-Mediated Differentiation Block Is Reversible. Electroporation of a catalytically inactive cdk4 mutant that retains the ability to bind D-type cyclins (3, 8) into parental 32D cells led to a 10-fold reduction in G418-resistant colonies, which in turn failed to overexpress the protein. However, when the inactive *cdk4* gene was introduced together with a plasmid conferring resistance to hygromycin B into the D2-8 subclone overexpressing cyclin D2, the number of drug-resistant cells was similar to that obtained with the control *hygro* vector alone. These pooled derivatives overexpressed

Table 1. Survival and differentiation of transfected myeloid cells in G-CSF

Transfection	Cell number
None (parental cells)	3.3×10^6
neo alone, mixed pool	7.5×10^5
Clone 1	2.3×10^5
Clone 2	3.1×10^5
Clone 3	5.2×10^4
Clone 4	2.6×10^6
Clone 5	1.1×10^6
Cyclin D1, mixed pool	1.8×10^5
Clone D1-1	5.8×10^4
Clone D1-2	1.9×10^5
Clone D1-3	9.0×10^4
Clone D1-4	5.6×10^4
Clone D1-5	1.2×10^5
Cyclin D2, mixed pool	3.2×10^3
Clone D2-1	$<8.0 \times 10^2$
Clone D2-2	$<8.0 \times 10^2$
Clone D2-3	$<8.0 \times 10^2$
Clone D2-4	$<8.0 \times 10^2$
Clone D2-5	$<8.0 \times 10^2$
Clone D2-6	$<8.0 \times 10^2$
Clone D2-7	$<8.0 \times 10^2$
Clone D2-8	$<8.0 \times 10^2$
Clone D2-8 (transfected with *hygro*)	$<8.0 \times 10^2$
Clone D2-8 [*cdk4* (K35M) plus *hygro*]	3.2×10^4
Cyclin D2 (E7K), mixed pool	5.2×10^5
Clone D2 (E7K)-1	4.9×10^5
Cyclin D2 (Δ2–7), mixed pool	2.3×10^5
Clone D2 (Δ2–7)-1	1.8×10^6
Clone D2 (Δ2–7)-2	1.2×10^6
Cyclin D3, mixed pool	1.9×10^4
Clone D3-1	2.2×10^3
Clone D3-2	$<8.0 \times 10^2$
Clone D3-3	2.6×10^3
Clone D3-4	5.5×10^3

Cells derived and maintained in medium containing IL-3 and G418 were cultured for 1 week without G418. Cells (5×10^5) were washed twice with PBS, seeded in 1-ml cultures containing G-CSF, and fed every 3 days thereafter. Twelve days after initiating the cultures, viable cells were enumerated by using trypan blue vital dye. The indicated values represent averages from duplicate cultures. More than 90% of surviving cells exhibited a granulocytic phenotype, as judged by cell morphology (see Fig. 3), histochemical staining, and immunofluorocytometric analyses of granulocyte-specific antigens (13, 19–21). Markers that are not present in IL-3-dependent myeloid precursors but that appeared in the terminally differentiated cells included chloroacetate esterase, the 8C5 granulocyte antigen, and myeloperoxidase.

cyclin D2 (Fig. 1*E*, lanes 2 and 3) and produced 2-fold higher levels of cdk4 (Fig. 1*E*, lanes 7 and 8). A significant proportion of these cells survived in G-CSF (Fig. 2*E*; Table 1) and showed clear evidence of granulocytic differentiation (Fig. 3*H*). Thus, the failure of wild-type D2 overexpressors to differentiate in G-CSF was not due to an irreversible clonal selection of cells that had lost the ability to respond.

Overexpressed Cyclins Form Complexes with cdk4 and cdk2. When expressed in insect cells, all D-type cyclins can functionally interact with cdk4, whereas cyclins D2 and D3, but not D1, activate cdk2 (8, 9). In parental G-CSF-treated cells, $p34^{cdk4}$ was rapidly degraded; in contrast, $p33^{cdk2}$ levels were sustained as cells underwent differentiation (Fig. 4*A*). Surprisingly, two forms of cdk2 were observed, the larger 38-kDa protein arising from translation of an alternatively spliced cdk2 mRNA (Fig. 4*A Right*). A cDNA encoding the 38-kDa species was cloned from a myeloid cell library, and it contains a 48-residue insert between amino acids 196 and 197. When this cDNA was coexpressed with cyclins in insect Sf9

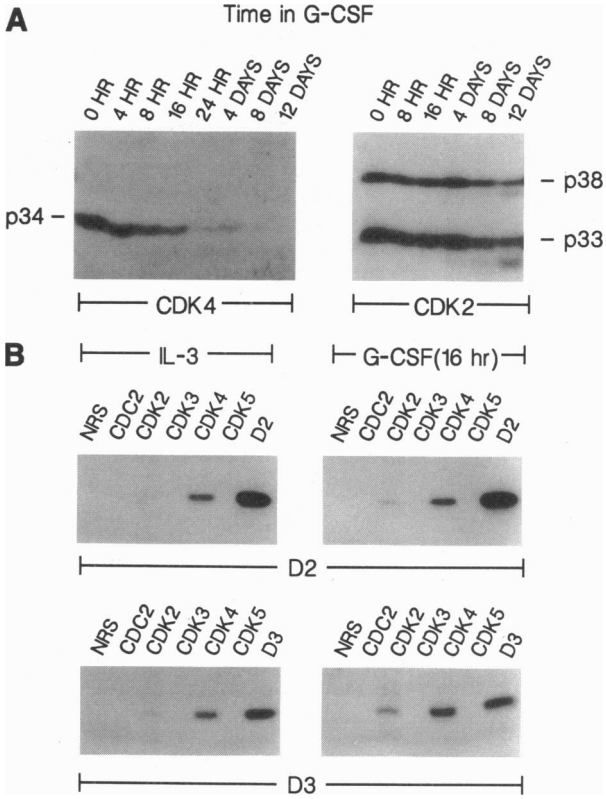

FIG. 4. Complex formation between D-type cyclins and cdks. (*A*) Lysates from parental 32D cells grown in G-CSF for the indicated times were immunoprecipitated and blotted with antisera to cdk4 and cdk2. (*B*) Cells overexpressing cyclin D2 or D3 were grown in IL-3 or in G-CSF for 16 hr and then lysed and precipitated with antisera to the indicated cdk or to cyclin D. Proteins separated on denaturing gels were blotted with antisera to cyclin D2 or D3. To normalize for the loss of cell viability in G-CSF, protein from the same number of living cells was precipitated with each antiserum.

cells, $p38^{cdk2}$ was activated by cyclins D2, E, and A, but not by D1, just like the $p33^{cdk2}$ isoform (H. Matsushime, J.-y.K. and C.J.S., unpublished data). Experiments similar to those in Fig. 4*A* could not be performed with cells overexpressing D-type cyclins, which die in G-CSF (Fig. 2).

To study complexes between endogenous cdk and overexpressed D-type cyclins, lysates from cells grown in IL-3 or treated with G-CSF for 16 hr were precipitated with antisera to different cdk, and denatured complexes were immunoblotted with antisera to the cyclins. In both IL-3 and G-CSF-stimulated cells, cdk2 and cdk4 formed complexes with cyclins D2 and D3, whereas none was detected with antisera to cdc2, cdk3, or cdk5 (Fig. 4*B*). As expected, cdk4 was the major D-type cyclin partner (3, 4). Because 30–50% of cyclin D2 and D3 overexpressors lose viability after 16 hr in G-CSF (Fig. 2), we normalized for total cyclin synthesis by using protein from equal numbers of living cells in each lane of the immunoblots. A somewhat greater percentage of total cyclin was bound to cdk2 when cells were shifted to G-CSF (Fig. 4*B*). At later times, only cdk2 should be available to bind the overexpressed cyclins (Fig. 4*A*), but the continued loss of cell viability precluded such analyses.

DISCUSSION

32D cells self-renew in IL-3 but terminally differentiate to neutrophils in G-CSF (13). In this system, G-CSF is at best poorly mitogenic and instead serves to support the viability of cells that undergo growth arrest and commit to the granulocytic lineage. Differentiation commitment may be a

stochastic process, which involves a loss of proliferative potential but necessitates a survival signal, and the death of the cells in the absence of any hemopoietins might simply reflect the latter requirement. Because cells grown in IL-3 plus G-CSF continue to proliferate as immature blasts, mitogenic signals can override the commitment step(s). Cells grown in IL-3 express cyclins D2 and D3, but not D1, and when transferred to G-CSF, cyclin D synthesis terminates, leading to a loss of the proteins as the cells undergo growth arrest. The synthesis of cdk4, the major D-type cyclin partner, also ceases in G-CSF-treated cells, but cdk2 levels are sustained, presumably as inactive monomers.

Ectopic overexpression of cyclins D1, D2, and D3 contracted the G_1 intervals and correspondingly lengthened the S phases of 32D cells grown in IL-3, but only cyclins D2 and D3 prevented their differentiation in G-CSF. Because the demonstrated effects of cyclin D1 on the cell cycle were insufficient to block differentiation, we speculate that overexpressed cyclin D1 was functionally inactivated when 32D cells were transferred to G-CSF, whereas cyclins D2 and D3 continued to provide constitutive cdk-mediated signals. Although each of the D-type cyclins can activate cdk4, its synthesis depends on IL-3, and, in G-CSF, cdk4 is degraded and cannot collaborate with ectopically expressed cyclins to inhibit commitment. In contrast, cdk2, which also formed complexes with overexpressed D-type cyclins, persisted as the cells differentiated. Although the inability of cyclin D2 overexpressors to differentiate could be rescued by catalytically inactive cdk4, the inhibitory effects of cyclins D2 and D3 could well be mediated by cdk2, and the failure of D1 to functionally interact with this catalytic partner (9) might explain its inability to inhibit differentiation. Cells blocked in terminal differentiation by overexpression of cyclins D2 and D3 were unable to survive in G-CSF, although they died less rapidly than those deprived of hemopoietins. The phenotype induced by the cyclins differs from that provoked by v-*abl* and v-*src* (20, 23), which abrogate the requirement for IL-3, or by v-*ras* and v-*myb* (24, 25), which block differentiation by enabling proliferation in G-CSF.

The inability of cyclin D2 mutants disrupted in their LXCXE motif to prevent differentiation may also implicate pRb or pRb-like proteins in this process. Here, however, we cannot exclude the possibility that such mutants, although able to functionally interact with cdk, might be perturbed in their ability to phosphorylate other substrates. Introduction of pRb into RB-negative Saos-2 cells can facilitate their myogenic determination in response to myoD (26), and transgenic mice lacking functional *RB* genes arrest relatively late in fetal development with specific hematopoietic and neurogenic defects (27–29), consistent with the notion that pRb can govern differentiation. Whatever the exact mechanisms, the fact that cyclins D2 and D3, but not D1, can act in a lineage-specific manner to disrupt granulocytic differentiation underscores functional differences between them and suggests that they can negatively regulate programs that may in part depend on G_0/G_1 arrest for their execution. Although they do not induce oncogenic transformation when overexpressed in fibroblasts (11), the D-type cyclins might contribute to tumor formation by disrupting differentiation in instances in which they are deregulated by chromosomal translocations or gene amplification (30–33).

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1. Pardee, A. B. (1989) *Science* **246**, 603–608.
2. Nurse, P. (1990) *Nature (London)* **344**, 503–508.
3. Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J.-y., Hanks, S. K., Roussel, M. F. & Sherr, C. J. (1992) *Cell* **71**, 323–334.
4. Xiong, Y., Zhang, H. & Beach, D. (1992) *Cell* **71**, 505–514.
5. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R. & Roberts, J. M. (1992) *Science* **257**, 1689–1694.
6. Dulic, V., Lees, E. & Reed, S. I. (1992) *Science* **257**, 1958–1961.
7. Sherr, C. J. (1993) *Cell* **73**, 1059–1065.
8. Kato, J.-y., Matsushime, H., Hiebert, S. W., Ewen, M. E. & Sherr, C. J. (1993) *Genes Dev.* **7**, 331–342.
9. Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J.-y. & Livingston, D. M. (1993) *Cell* **73**, 487–497.
10. Ohtsubo, M. & Roberts, J. M. (1993) *Science* **259**, 1908–1912.
11. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J.-y., Bar-Sagi, D., Roussel, M. F. & Sherr, C. J. (1993) *Genes Dev.* **7**, 1559–1571.
12. Baldin, V., Likas, J., Marcote, J. M., Pagano, M., Bartek, J. & Draetta, G. (1993) *Genes Dev.* **7**, 812–821.
13. Valtieri, M., Tweardy, D., Caracciolo, D., Johnson, K., Mavilio, F., Altmann, S., Santoli, D. & Rovera, G. (1987) *J. Immunol.* **138**, 3829–3835.
14. Matsushime, H., Roussel, M. F., Ashmun, R. A. & Sherr, C. J. (1991) *Cell* **65**, 701–713.
15. Matsushime, H., Roussel, M. F. & Sherr, C. J. (1991) *Cold Spring Harbor Symp. Quant. Biol.* **56**, 69–74.
16. Sugden, B., Marsh, K. & Yates, J. (1985) *Mol. Cell. Biol.* **5**, 410–413.
17. Anderson, S. G., Gonda, M. A., Rettenmier, C. A. & Sherr, C. J. (1984) *J. Virol.* **51**, 730–741.
18. Downing, J. R., Rettenmier, C. W. & Sherr, C. J. (1988) *Mol. Cell. Biol.* **8**, 1795–1799.
19. Morishita, K., Parganas, E., Matsugi, T. & Ihle, J. N. (1992) *Mol. Cell. Biol.* **12**, 183–189.
20. Rovera, G., Valtieri, M., Mavilio, F. & Reddy, P. (1987) *Oncogene* **1**, 29–35.
21. Kato, J.-y. & Sherr, C. J. (1990) *Blood* **75**, 1780–1787.
22. Dowdy, S. F., Hinds, P. W., Louie, K., Reed, S. I., Arnold, A. & Weinberg, R. (1993) *Cell* **73**, 499–511.
23. Kruger, A. & Anderson, S. M. (1991) *Oncogene* **6**, 245–256.
24. Mavilio, F., Kreider, B. L., Valtieri, M., Naso, G., Shirsat, N., Venturelli, D., Reddy, E. P. & Rovera, G. (1989) *Oncogene* **4**, 301–308.
25. Patel, G., Kreider, B., Rovera, G. & Reddy, P. (1993) *Mol. Cell. Biol.* **13**, 2269–2276.
26. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V. & Nadal-Ginard, B. (1993) *Cell* **72**, 309–324.
27. Lee, E. Y.-H. P., Chang, C.-Y., Hu, N., Wang, Y.-C. J., Lai, C.-C., Herrup, K., Lee, W.-H. & Bradley, A. (1992) *Nature (London)* **359**, 288–294.
28. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A. & Weinberg, R. A. (1992) *Nature (London)* **359**, 295–300.
29. Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M. T., van der Valk, M., Hooper, M. L., Berns, A. & te Riele, H. (1992) *Nature (London)* **359**, 328–330.
30. Motokura, T., Bloom, T., Kim, H. G., Juppner, H., Ruderman, J. V., Kronenberg, H. M. & Arnold, A. (1991) *Nature (London)* **350**, 512–515.
31. Withers, D. A., Harvey, R. C., Faust, J. B., Melnyk, O., Carey, K. & Meeker, T. C. (1991) *Mol. Cell. Biol.* **11**, 4846–4853.
32. Rosenberg, C. L., Wong, E., Petty, E. M., Bale, A. E., Tsujimoto, Y., Harris, N. L. & Arnold, A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9638–9642.
33. Lammie, G. A., Fantl, V., Smith, R., Schuuring, E., Brookes, S., Michalides, R., Dickson, C., Arnold, A. & Peters, G. (1991) *Oncogene* **6**, 439–444.