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# Differential expression of proteins regulating cell cycle progression in growth vs. differentiation

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**Abstract**

The level of various G1 cyclins and cyclin-dependent kinases (cdks) present in the nuclei of synchronized ML-1 human myeloblastic leukemia cells was determined as a function of time after initiation of cell growth with insulin-like growth factor-1 (IGF-1) and transferrin (Tf), and following induction of differentiation with transforming growth factor-$\beta$1 (TGF-$\beta$1). Cyclin E and cdk2 were expressed at relatively high levels in the nuclei of proliferation-stimulated cells, whereas cyclin D1 and cdk5 were expressed at comparably high levels in the nuclei of differentiation-induced cells. In the nuclear extracts from proliferation-stimulated cells, cyclin E complexed specifically with cdk2, whereas in nuclear extracts from differentiation-induced cells, cyclin D1 bound specifically to cdk5. Increased cyclin E/cdk2 expression was accompanied by increased DNA synthesis, whereas increased cyclin D1/cdk5 levels correlated with decreased DNA synthesis. In both growth- and differentiation-induced cells, cyclin D2 expression preceded the expression of cyclin D3, and a significantly larger amount of these cyclins was present in differentiation- as compared to proliferation-induced cells. In contrast, cdk4 and cdk6 were present at similar levels in the nuclear extracts from both growth- and differentiation-induced cells. These data show that, in ML-1 cells, the proliferation-associated progression from G1 to S, as well as the differentiation-associated transit from G1 to maturation is accompanied by the expression of specific cyclin/cdk pairs, comprising cdk2/cyclin E in growth and cdk5/cyclin D1 in differentiation.

*Keywords:* Cell growth; Cell differentiation; Cyclin; Cyclin-dependent kinase; Cell cycle regulation

## 1. Introduction

It is, by now, well established that cyclins, in association with cdks, function as regulators of proliferation-related cell cycle progression [1]. However, only limited information has become available on the role these proteins may play in regulating the exit from the cell cycle onto the differentiation path. As one approach towards obtaining further such information, we compared cyclin/cdk expression in cells grown under proliferation- and under differentiation-specific conditions. Since both growth- and differen-

Abbreviations: Cdk, cyclin-dependent kinase; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tf, transferrin; TGF-$\beta$1, transforming growth factor-$\beta$1; TNF-$\alpha$, tumor necrosis factor-$\alpha$.

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tiation-inducing cytokines are present in the sera generally used for cell culturing, an assay system was required wherein serum is depleted in favor of specific cytokines.

The system we used consisted of ML-1 human myeloblastic leukemia cells [2] which, in the absence of serum, proliferate in RPMI 1640 medium when IGF-1 and Tf are supplied, or differentiate to monocytes when TGF-$\beta$1 and/or tumor necrosis factor-$\alpha$ (TNF-$\alpha$) are furnished in the presence of Tf. In long-term cultures (> 48 h), the addition of 0.5% fetal calf serum to the medium maintains cell viability at above 80%, without significantly affecting the extent of growth or differentiation. Because different regulatory proteins are expressed or activated at different stages in the cell cycle (for an overview see for example [3]), determining the type and level of such proteins present at diverse stages of the cell cycle requires the use of synchronized cell populations. Staging assures that any differences detected reflect the conditions that exist in the total cell population rather than in fractions of the population present at different stages of the cell cycle.

Additionally, because the cytoplasmic fraction contains differentially phosphorylated cdks and cyclins as well as auxiliary binding proteins that assume nuclear functions only during specific cell cycle phases [4–6], utilizing total cell extracts can lead to erroneous conclusions. We, therefore, focused our inquiry on determining the type and level of cyclins and cdks expressed in the nucleus of ML-1 cells at specific time points following induction of growth or differentiation. In so doing, we expected to obtain information concerning the regulatory proteins most immediately involved in the control of cell cycle progression.

A preliminary report on part of this work has been presented [7].

## 2. Materials and methods

### *2.1. Cytokines and antibodies*

TGF-$\beta$1 was kindly provided by Bristol-Myers Squibb, Seattle, WA., and IGF-1 was purchased from Austral Biologicals, San Ramon, CA. The following antibodies were obtained from Santa Cruz Biotechnology: HD11, a mouse $IgG_1$ monoclonal antibody derived by fusion of NS/1 mouse myeloma cells with spleen cells from a mouse immunized with recombinant human cyclin D1. PRAD1, a rabbit polyclonal antibody raised against a peptide corresponding to amino acids 9–37 of the human cyclin D1 gene product [8–10]. C-17, a rabbit polyclonal antibody targeted to a peptide containing the human cyclin D2 amino acid residues 274–290 [10]. The antibody is characterized as not being reactive with either cyclin D1 or cyclin D3. Antibody 34B1-3, a rat $IgG_{2a}$ monoclonal antibody, derived by fusion of rat myeloma cells with spleen cells from a rat immunized with recombinant human cyclin D2. The antibody is specified to lack detectable cross-reactivity with either cyclin D1 or D3. Antibody 18B6-10, a rat $IgG_{2a}$ monoclonal antibody derived by fusion of rat myeloma cells with spleen cells from a rat immunized with recombinant human cyclin D3. It is stated to lack detectable cross-reactivity with either cyclin D1 or D2 [10–13]. C-16, an affinity-purified rabbit polyclonal antibody raised against the peptide containing amino acid residues 277–292 of human cyclin D3 [10,12]. That antibody is represented as being non-cross-reactive with either cyclin D1 or D2. Cyclin E was detected with antibody HE12 and C-19. HE12 is a mouse $IgG_{2b}$ monoclonal antibody derived by fusion of NS/1 mouse cells with spleen cells from a mouse immunized with recombinant human cyclin E protein, whereas C19 is a rabbit polyclonal antibody raised to a peptide incorporating the human cyclin E amino acid residues 377–395 [14,15]. The cdk5-directed antibody DC-17, used in this study, is a mouse $IgG_1$ monoclonal antibody, derived by fusion of NS/1 mouse cells with spleen cells from a mouse immunized with human recombinant cdk5 protein [16]. Another cdk5-directed antibody used, C-8, is a rabbit polyclonal antibody raised against the peptide containing the amino acid residues 284–291 mapping at the carboxy terminus of cdk5 [17,18]. Cdk2 was detected by antibody M2, a rabbit polyclonal antibody raised to a peptide containing the amino acids 283–298 mapping at the carboxy terminus of human cdk2 [15,17,19]. Antibody C-22 is a rabbit polyclonal antibody raised against a peptide containing the residues 282–303 that map at the carboxy terminus of of human cdk4 p34 (psk-J3p34) [17,18]. The antibody is stated to be not cross-reactive with other

cyclin-dependent kinases. Similarly, C-21, a rabbit polyclonal antibody raised against a peptide corresponding to amino acid residues 303–326 mapping at the carboxy terminus of cdk6 (PLSTIRE) [17,20], is characterized as being non cross-reactive with other cdks. A cyclin D2-directed rat monoclonal antibody (Ab-1), purchased from Oncogene Science, Inc., Cambridge, MA, was also used in this study.

### 2.2. Reagents

Goat anti-mouse, goat anti-rat and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology; and immobilized protein A from Pierce, Rockford, IL. Molecular standards, used in sodium dodecil sulfate polyacrylamide gel electrophoresis (SDS-PAGE), were obtained from Bio-Rad Labs., Hercules, CA and western blotting reagents were purchased from Amersham International, Arlington Heights, IL.

### 2.3. Cell cultures

Suspension cultures of ML-1 human myeloblastic leukemia cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) 100 units/ml of penicillin and 100 $\mu$g/ml of streptomycin [2]. The cells were incubated at 37°C, in a humidified 5% $CO_2$ atmosphere. For assessing the expression of cell cycle-regulating proteins, $6–7 \times 10^5$ cells/ml were removed from the maintenance culture, washed with RPMI 1640 medium and synchronized by incubation for 2 days at 37°C in RPMI 1640 medium containing 0.05% FBS. Following this staging, from 80–85% of the cells were in G1. After washing twice with serum-free RPMI 1640 medium, the cells were resuspended in RPMI 1640 medium containing 0.5% FBS and were treated with 40 ng/ml IGF-1 plus 0.5 $\mu$g/ml transferrin for growth, or with 60 ng/ml of TGF-$\beta$1 plus 0.5 $\mu$g/ml TF for differentiation. The 0.5% serum supplement was added to preserve the viability of the cultures over the 72 h incubation period employed. Cell numbers were determined by hemocytometer, viability was assessed by trypan blue exclusion, and differentiation monitored by counting the number of cells displaying differentiated morphology [2].

### 2.4. Nuclear extracts

To obtain nuclear extracts, $2–5 \times 10^7$ cells were washed with serum-free, ice-cold RPMI 1640 medium, followed by suspension in 0.3 ml of a nuclear buffer consisting of 2 mM $MgCl_2$, 5 mM $K_2HPO_4$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF), 20 $\mu$g/ml aprotinin, 20 $\mu$g/ml leupeptin and 0.1 mM $Na_3VO_4$. An additional 0.3 ml of nuclear buffer, containing 0.7% of Triton X-100, was then added and, after standing on ice for 8 min, the cells were homogenized using a Dounce tissue grinder, and the homogenate centrifuged at $150 \times$ g. The pellet was washed once with nuclear buffer and the nuclear extract prepared using the method of Dignam et al. [21]. Briefly, the pellet was resuspended in buffer containing 20 mM N-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid (Hepes), pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT), 0.1 mM $Na_3VO_4$, 50 mM NaF, 30 mM pyrophosphate, 20 $\mu$g/ml leupeptin and 20 $\mu$g/ml aprotinin, and homogenization was carried out using a Kontes Duall tissue grinder. After centrifugation ($12\,000 \times g$) of the homogenate, the supernatant, designated the nuclear extract, was frozen and stored at $-70$°C.

### 2.5. Western blotting

The cyclin and cdk proteins were analysed by western blotting. Fractions containing 30–50 $\mu$g of nuclear protein were loaded onto 10% polyacrylamide SDS gel, and after electrophoresis in discontinuous buffer, the proteins were transferred to nitrocellulose membranes using a trans-blot electrophoretic transfer cell. The membranes were blocked for 2 h with 5% milk in phosphate buffered saline (PBS), followed by incubation with primary antibody for 1–2 h. After washing once for 15 min and three times for 5 min with PBS containg 0.1% Tween 20, the membranes were incubated with secondary antibody for 40–60 min, washed, and incubated for 1 minute in Amersham ECL reagent. Kodak x-omat AR or Dupont NEN films were used for autoradiography. Developed films were scanned by computing densitometer, and relative levels determined by reference to the stated controls.

*2.6. Immunoprecipitation and immunoblotting*

Immunoprecipitation was performed by established procedures. [15,18,22]. Briefly, excess nuclear extract (200 $\mu$g protein) from synchronized ML-1 cells incubated in the absence or presence of IGF-1 or TGF-$\beta$1 was added to 300 $\mu$l of immunoprecipitation buffer containing 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 20 mM EDTA, 0.1 mM $Na_3VO_4$, 50 mM NaF, 1 mM PMSF, 1 mM benzamidine, 10 $\mu$g/ml trypsin inhibitor, 20 $\mu$g/ml leupeptin and 20 $\mu$g/ml aprotinin. To each sample, 1 $\mu$g of affinity-purified antibody was added in the presence or absence of peptide controls. After rocking at 4°C for 1–2 h, immobilized protein A was added to each sample, and rocking continued overnight. Immunoprecipitates were collected by Microfuge centrifugation, and after washing four times with 1 ml aliquots of immunoprecipitation buffer, the pellets were resuspended in 40 $\mu$l aliquots of sample buffer [23]. After boiling for 3 min, the proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and detected by ECL as described above.

*2.7. [$^3$H]thymidine incorporation*

$5 \times 10^5$ synchronized ML-1 cells/ml were treated for the indicated periods of time with the amounts of growth or differentiation factors specified. 1 h before harvest, [$^3$H]thymidine (1 $\mu$Ci/ml; specific activity 87 Ci/mmol) was added to each culture, and incubation continued for an additional h. The cells were collected by centrifugation at $200 \times g$, and 1 ml of 10% trichloroacetic acid (TCA) was added to the pellet. After resuspension, the cells were collected on glass microfiber filters, washed with 5% TCA/ethanol, and the radioactivity present on the dried filters determined by scintillation spectrometer.

## 3. Results

As shown in Fig. 1a, synchronized ML-1 cells stimulated to proliferate with IGF-1 plus Tf, underwent division between 60–70 h of incubation. A small increase in cell number was also observed in the control population which, like all assay samples, was cultured in the presence of 0.5% fetal calf serum, added to maintain cell viability at above 80% during the 72 h incubation period. The small amount of growth factor present in the serum suffices to stimulate the growth of this small fraction of cells, as is indicated by the fact that no such increase in cell number occurred in the cell population treated with the differentiation-factor TGF-$\beta$1, the elevated level

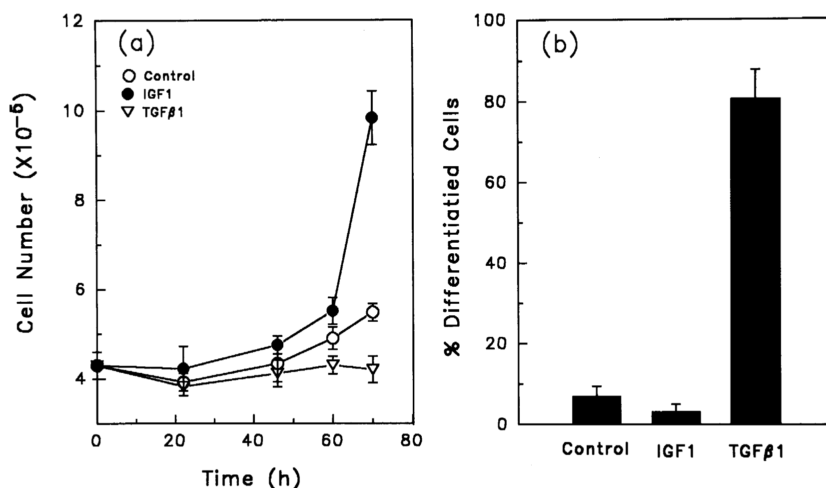


Fig. 1. (a) Total cell number and (b) percent of differentiated cells obtained after incubating, at 37°C, a population of synchronized ML-1 cells suspended in RPMI 1640 medium containing 0.5% FBS and 40 ng/ml IGF-1 or 60 ng/ml TGF-$\beta$1 together with 0.5 $\mu$g/ml transferrin. Cell numbers were counted by hemocytometer and the percent of differentiated monocytes determined on the basis of morphology. The data shown are the mean $\pm$ SD derived from three separate experiments, at least 500 cells being scored in each experiment.

of differentiation factor antagonizing the low level of growth signal that is present [24].

As shown in Fig. 1b, approximately 80% of the cell population treated with TGF-$\beta$1 matured to monocytes, whereas only a small fraction (approximately 8%) of the cells matured in control and an

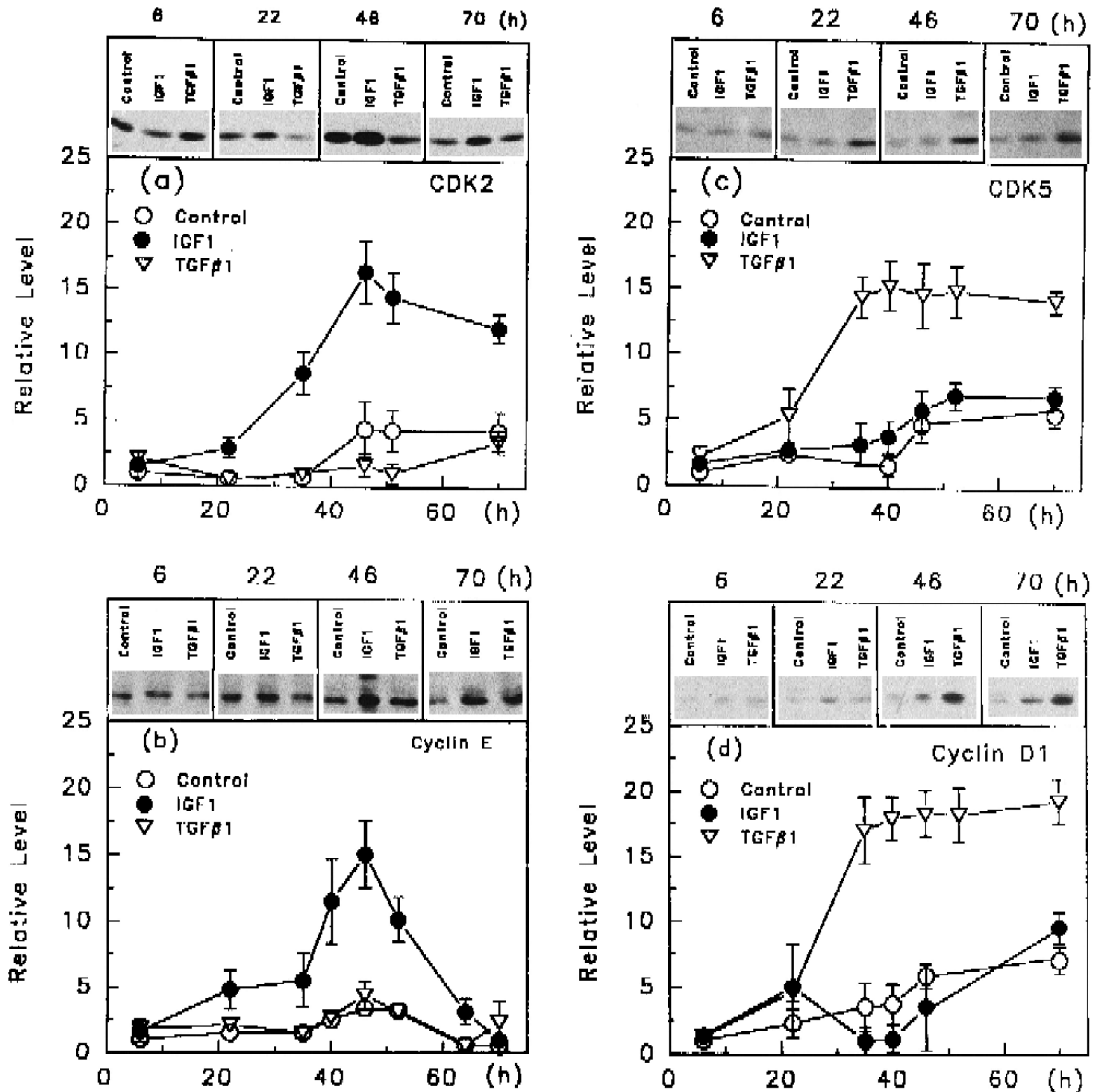


Fig. 2. Levels of cdk2 (a), cyclin E (b), cdk5 (c) and cyclin D1 (d) proteins present in nuclear extracts from synchronized ML-1 cells incubated, at 37°C, in RPMI 1640 medium containing 0.5% FBS, 40 ng/ml of the growth factor IGF-1, or 60 ng/ml of the differentiation factor TGF-$\beta$1 and 0.5 $\mu$g/ml transferrin. Aliquots of the nuclear extracts, containing 30 $\mu$g protein, were immunoblotted, and the blots probed with monoclonal or with affinity-purified polyclonal antibodies. Cells incubated in the absence of IGF-1 or TGF-$\beta$1 served as control. All values obtained were normalized to the 6 h control value, which was set equal to 1. The 6 h control value did not differ significantly from the value at 0 time. The data shown are means ± SD derived from three separate experiments.

even smaller number (2%) in the IGF-1-treated cultures. The small difference between control and the IGF1-treated culture is real, reflecting the competition that exists between the elevated level of IGF-1 added to the proliferation- stimulated culture and the small amount of differentiation factor that is contained in the 0.5% amount of serum supplied [24].

To obtain information on the possible role cell cycle regulating proteins may play in directing proliferating cells towards the differentiation path, we compared the identity and the level of the cyclins and cdks present in the nuclei from proliferation- and from differentiation-induced ML-1 cells.

The transition from the G1 phase of the cell cycle to the S phase constitutes a well characterized locus for regulating the cycle progression of proliferating cells. That transit has been demonstrated to involve the cdk2/cyclin E pair [14,25,26]. As shown in Fig. 2a, the level of cdk2 present in the nuclei of proliferation-stimulated ML-1 cells increased approx. 10-fold after 46 h of incubation and declined slowly thereafter. A small increase in cdk2 level was also observed in control cells, that increase likely reflecting the small amount of IGF-1 present in the 0.5% serum supplement that was added. In contrast, in the maturation stimulated cells, no significant elevation of the cdk2 level was observed at that time interval.

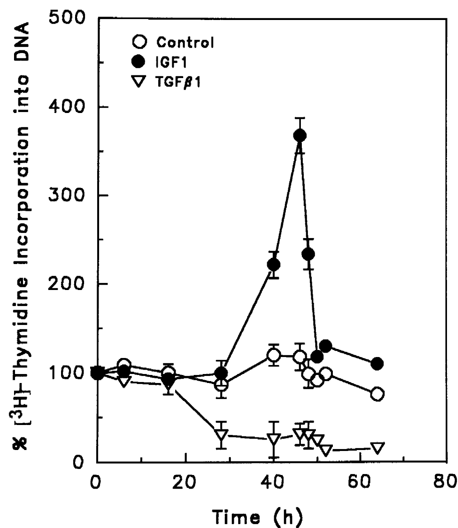


Fig. 3. DNA synthesis, as determined by [$^3$H]thymidine incorporation, in synchronized ML-1 cells incubated, at 37°C, in serum-depleted medium containing the growth factor IGF-1 or the differentiation factor TGF-$\beta$1. The amount of thymidine incorporated is given relative to 0 h control. The values shown represent the mean ± SD of three separate experiments.

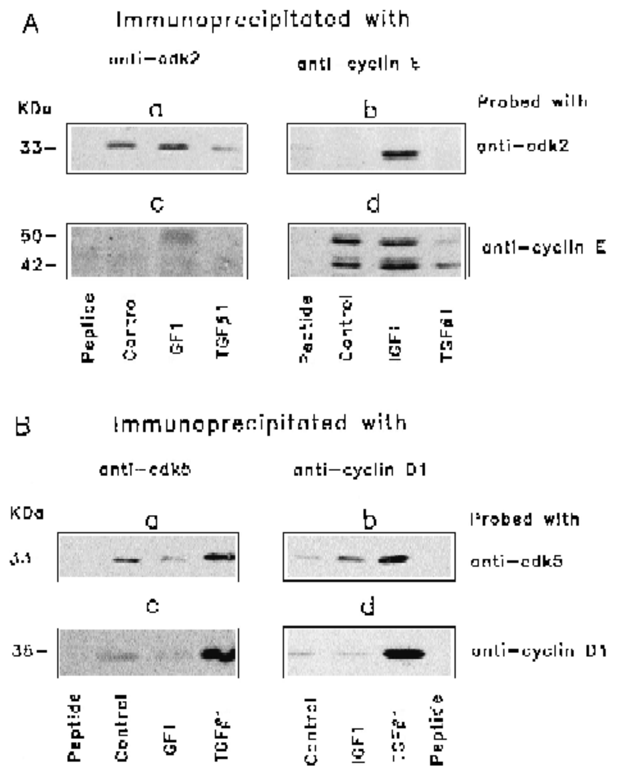


Fig. 4. Complexation of cdk2 with cyclin E, and of cdk5 with cyclin D1, in nuclear extracts from synchronized ML-1 cells incubated for 46 h (A) or for 40 h (B) in RPMI 1640 medium supplemented with 0.5% FBS and with 40 ng/ml of the growth factor IGF-1 or with 60 ng/ml of the differentiation factor TGF-$\beta$1, together with 0.5 $\mu$g/ml of transferrin. Nuclear extracts (200 $\mu$g protein) from these cells were immunoprecipitated with aliquots (1 $\mu$g protein) of anti-cdk2 (A, a, c), anti-cyclin E (A, b, d), anti-cdk5 (B, a, c), or anti-cyclin D1 (B, b, d) antibodies. Following immunoprecipitation, the precipitates were probed with antibodies to cdk2 and cyclin E (A), and with antibodies to cdk5 and cyclin D1 (B). In (A), control peptides for cdk2 or for cyclin E were added to the IGF-1- treated immunoprecipitates, whereas in (B), control peptides for cdk5 or for cyclin D1 were added to the TGF-$\beta$1-treated immunoprecipitates.

In parallel with cdk2 (Fig. 2a), cyclin E, the regulatory subunit of cdk2, was maximally expressed at the same time point at which cdk2 reached its optimal level (Fig. 2b). However, while the cdk2 level declined slowly, the level of cyclin E decreased much more rapidly, in keeping with the notion that the cyclins regulate the activity of their target cdks. In control and in differentiation-induced cells, the cyclin E levels increased only marginally.

Whereas the cdk 2/cyclin E levels were elevated in the nuclei of proliferation-stimulated cells (Fig. 2a,b), it was the cdk5/cyclin D1 levels that were increased in the nuclei of differentiation-induced cells (Fig. 2c,d).

The kinetics of cdk5 expression in the maturation-induced cells (Fig. 2c) differed significantly from the kinetics with which cdk2 was expressed in proliferating cells (Fig. 2a). The maximal level of cdk5 was reached after about 35 h of incubation, whereas cdk2 expression reached its optimal level after approximately 46 h. Further, while elevated cdk5 levels were maintained over the remainder of the 72 h incubation period (Fig. 2c), the cdk2 levels began to decrease soon after reaching their maximum (Fig. 2a). Analogously, while the cyclin D1 level remained elevated upon incubation past 35 h (Fig. 2d), the cyclin E level decreased sharply following the 46 h time point (Fig. 2b). That difference may reflect the fact that the cdk2/cyclin E complex regulates a transient but recurring event involving the transition of the cells from G1 to the S-phase, whereas the cdk5/cyclin D1 complex appears to be involved in a more permanent event related to the non-recurring expression of the differentiation program.

That interpretation receives support from the data shown in Fig. 3. In proliferation-stimulated cells, maximal cdk2/cyclin E expression coincides with maximal DNA synthesis required for cell division, whereas, in differentiation-induced cells, optimal expression of cdk5/cyclin D1 is associated with the loss of that synthesis, expected from cells that are slated to move towards functional maturity rather than towards division.

To assess whether the simultaneous increase in the levels of cdk2 and cyclin E in growing cells, and of cdk5 and cyclin D1 in differentiating cells is paralleled by the ability of these proteins to bind, immunoprecipitates generated in nuclear extracts from growth- or from differentiation-induced cells were probed for their constituent proteins. As shown in Fig. 4A, b and c, complexation between cdk2 and cyclin E occurred only in nuclear extracts from proliferation-stimulated cells, even though small amounts of these two proteins were also present in nuclear extracts from control and from differentiation-induced cells (Fig. 4A, a

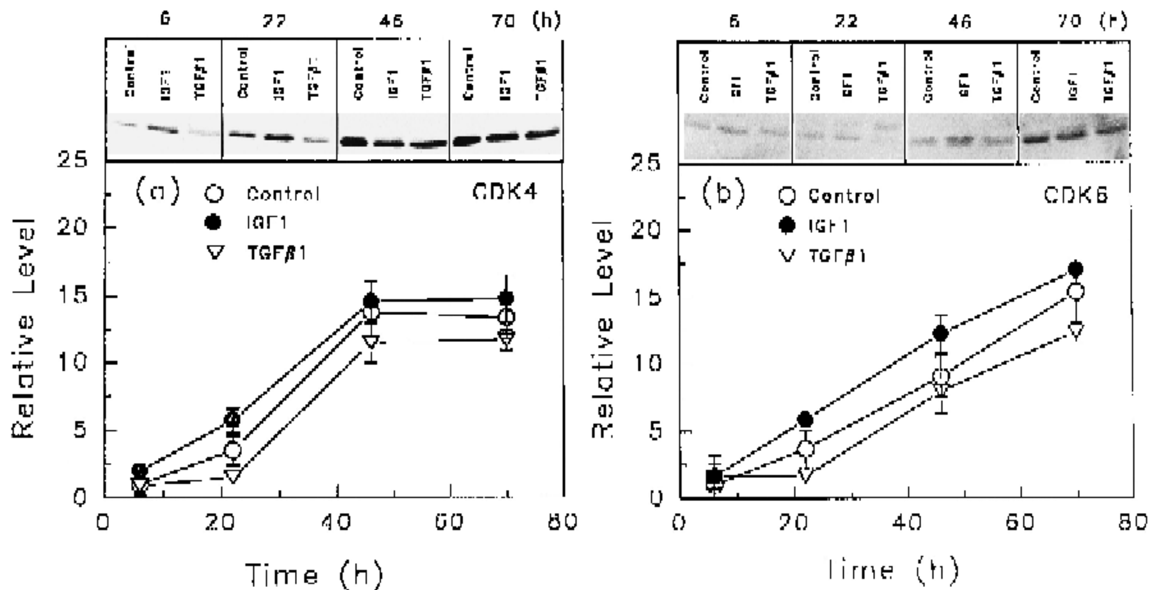


Fig. 5. Changes in the levels of cdk4 and cdk6 proteins in nuclear extracts from synchronized ML-1 cells incubated, at 37°C, in RPMI 1640 medium containing 0.5% FBS and 40 ng/ml of the growth factor IGF-1 or 60 ng/ml of the differentiation factor TGF-$\beta$1. The extracts were subjected to immunoblotting, and the blots probed with polyclonal antibodies to cdk4 and cdk6. Cells incubated in the absence of either IGF-1 or TGF-$\beta$1 served as control. All values obtained were normalized to the 6 h control value. Each experimental point represents the mean ± SD derived from three separate experiments.

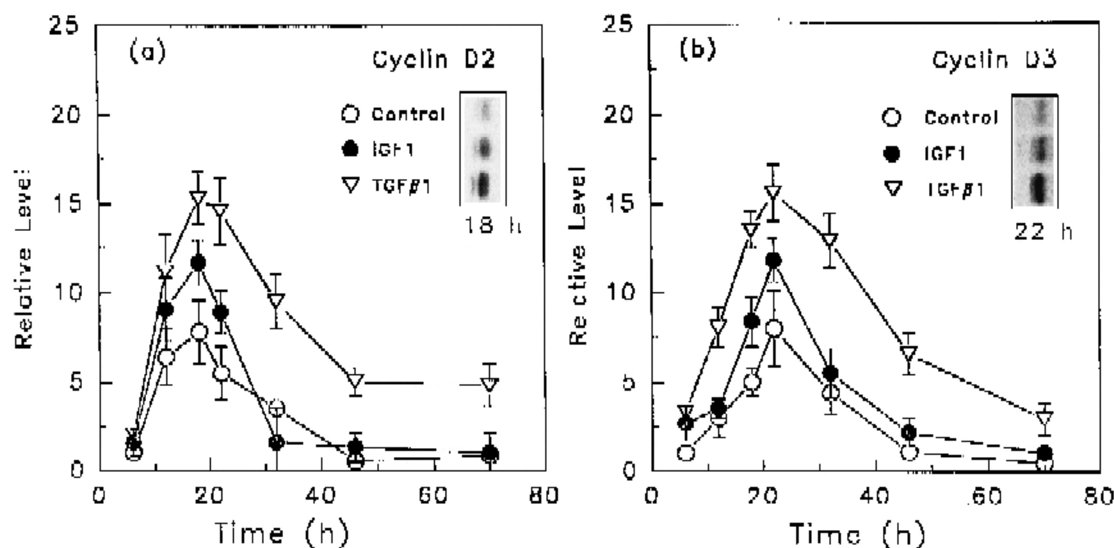


Fig. 6. Cyclin D2 and D3 levels present in nuclear extracts from synchronized ML-1 cells, cultured in RPMI 1640 medium plus 0.5% FBS in the presence of 40 ng/ml of the growth-factor IGF-1 or 60 ng/ml of the differentiation factor TGF-$\beta$1. The extracts were immunoblotted and the blots probed with monoclonal or with polyclonal antibodies to the cyclins D2 and D3. Cells incubated in the absence of either cytokine served as control. All values obtained were normalized to the 6 h control value. Each experimental point represents the mean $\pm$ SD derived from three separate experiments.

and d). This result implies that, in the proliferation-stimulated cells, these two proteins may have undergone some modification that promotes complex formation (e.g. phosphorylation), or indicates that auxiliary binding proteins required for complexation were present.

Analogously, complexation of cdk5 with cyclin D1 occurred predominantly in nuclear extracts from differentiation-induced cells (Fig. 4B, b and c), even though small concentrations of these proteins were present in control and in proliferation-stimulated cells (Fig. 4B a and d). No significant complex formation between cdk5 and the D2 or D3 cyclins was observed (data not shown).

In contrast to cdk2, expressed at a relatively high concentration in growth, and cdk5, expressed at an elevated level in differentiation, the cdks 4 and 6 were present at similar levels in the nuclei of control as well as of proliferation- and differentiation-induced cells (Fig. 5). This similiarity may mean that they affect cellular activities that are common to control as well as to proliferating and differentiating cells. Under conditions where cyclin D1 was bound to cdk5, no significant binding of cyclin D1 to cdk4 or cdk6 was observed (data not shown).

Similarly, as shown in Fig. 6, the D2 and D3 cyclins were expressed in both growth and differentiation-induced cells relatively early in G1. Cyclin D2 reached its peak level after approximately 18 h, whereas cyclin D3 reached that level after approx. 22 h of incubation. Both cyclins were expressed at significantly greater levels in differentiation- than in proliferation-induced cells or in control, but the time point at which they were maximally expressed was the same in all instances.

## 4. Discussion

The orderly progression of a cell through its proliferation cycle is linked to the sequential activation of several cyclin/cdk complexes that assure the proper integration of the multitude of cellular activities required for error-free cell division [1]. By their ability to phosphorylate specific substrates, these complexes regulate the expression of cycle-specific genes. Different cyclin/cdk pairs are utilized for regulating specific stages of the cell cycle, and their level of expression, the extent of their phosphorylation and the presence of negative regulatory proteins, all deter-

mine the kinase activity that results. The determinant role this activity plays in cell proliferation has been documented in an enormous number of publications, and only a very few of the most recent reviews can be referenced here [3,27–29].

Unlike the massive amount of data pertaining to the role cyclin/cdk complexes play in regulating growth, relatively little information has been accrued concerning the functions they may serve in differentiation.

The study reported here indicates that at least one specific cyclin/cdk pair is involved in cellular events related to the transit from growth to differentiation. While the regulatory action of the cyclin E/cdk2 pair in the G1/S phase transition has been well documented [30], the differentiation-associated expression of the cyclin D1/cdk5 pair has not previously been described.

Cyclin E/cdk2 complexes have been observed to associate with transcription factors such as E2F, which act at promoters of genes encoding proteins required for DNA replication and growth [15,31–33]. By analogy, the cyclin D1/cdk 5 complex may interact with factors which promote the expression of the stable messages suggested to be associated with differentiation-related gene expression [34]. Whatever the case, the simultaneous expression of these two complexes appears incompatible with progression towards growth or differentiation. Thus, down-regulation of the cdk2 or cyclin E levels appears crucial for PC12 cells to undergo neuronal differentiation [35], whereas down-regulation of cyclin D1 is required for human fibroblasts to initiate DNA replication and repair [36].

Since initiation of ML-1 cell differentiation by TGF-$\beta$1 is accompanied by the down-regulation of cdk2/cyclin E levels (Fig. 2a, b) followed by growth arrest (Fig. 1), it was of interest to note that TGF-$\beta$1 can affect cdk2-cyclin E activity in other cell types as well. Thus, TGF-$\beta$1 was shown to block cyclin E expression in human keratinocytes [37] and, in Mv1Lu cells, this cytokine inhibited the assembly of the cyclin E/cdk2 complex [38]. The growth inhibitory effect exerted by TGF-$\beta$1 on keratinocytes involved the enhanced binding of p21/Cip1/Waf1 to cdk2 [39], whereas the inhibition of complex formation in Mv1Lu cells [38] was mediated through the increased binding of p27Kip1 to cdk2. A TGF-$\beta$1-induced increase in the level of p21/Cip1/Waf1 was observed in two colon cancer cell lines, where binding of this protein to cyclin E resulted in reduced cyclin E activity [40]. Additionally, TGF-$\beta$1 was found to interfere with cdk2 phosphorylation in late G1, preventing, thereby, the activation of its histone kinase activity [41].

Thus, TGF-$\beta$1 interferes with cyclin E/cdk2 formation and growth not only in ML-1, but also in diverse other cell types, and it remains to be established whether in such cells -as is the case in ML-1- the cyclin D1/cdk5 pair is expressed preliminary to the induction of differentiation. Indeed, the level of cdk5 as well as of cyclin D1 was found to be increased during the differentiation of various other cell types. Thus, cdk5 levels and activity increased in embryonic mouse forebrain as increasing numbers of cells exited proliferation, whereas cdk2 expression and activity was maximal when the majority of cells were proliferating and fell to barely detectable levels when mitotic activity ceased [16]. These observations were interpreted to mean that cdk5 has a role in neuronal differentiation but not in cell division.

The level of cyclin D1, was similarly observed to increase during differentiation of diverse cell types. For example, treatment of three leukemic cell lines with the differentiation-inducer TPA, led to a marked increase in cyclin D1 mRNA, to an extensive decrease in cyclin E and cyclin A mRNA, and to cell growth arrest [42]. Similarly, following treatment of HL-60 cells with TPA, expression of cyclin D1 mRNA was increased, whereas cyclin A and E expression was markedly inhibited [43]. Cyclin D1 expression was also upregulated in megakaryocytes induced to differentiate with TPA [44], and increasing the level of endogenously or ectopically expressed cyclin D1 led to inhibition of the growth of normal human diploid fibroblasts. [45]. Finally, cyclin D1 expression was enhanced during the neuronal differentiation of PC 12 cells induced by nerve growth factor (NGF) [46], and constitutive overexpression of cdk2 prevented the neuronal differentiation of rat pheochromocytoma PC12 cells [35]. Inversely, the SV40 large T antigen-mediated immortalization of human fibroblasts led to a decrease in cyclin D1 expression, accompanied by an increase in the expression of both the cyclins E and A [47]. Thus, in a variety of cell systems, the expression of cyclin D1, like that of cdk5, is associated with growth arrest and

cell differentiation, and it will be of interest to establish whether in these cells, as they do in ML-1, the two proteins interact, likely to regulate differentiation-induction.

Our observation that, in nuclear extracts from growth-stimulated ML-1 cells, cdk2 forms a complex only with cyclin E, whereas in differentiation-induced cells cdk5 combines only with cyclin D1, differs somewhat from results obtained in total extracts from diverse other cell systems. Thus, in a squamous carcinoma cell line, cyclin D1 was found to associate with cdk4 and cdk6 [48], whereas in diploid human fibroblasts this cyclin coprecipitated with cdk2, cdk4 and cdk5 [48,49]. Additionally, quarternary complexes were observed to have formed between diverse cyclin/cdk combinations and the 21 kDa Cip1 and the 36 kDa PCNA proteins [18,49]. It may be that the formation of some of these complexes involves both the nuclear as well as the cytoplasmic cell fraction.

Our finding that in control as well as in proliferation- and differentiation-induced ML-1 cells the D2 and D3 cyclins are expressed relatively early in G1 (Fig. 6) implies that they participate in events common to both growth and differentiation, possibly involving the transit from G0 to G1. As in ML-1 cells, cyclin D2 was expressed earlier than cyclin D3 in proliferation-stimulated T-lymphocytes [50], and in these -proliferating- cells, cyclin D1 expression was not detected. Similarly, in proliferating hematopoietic 32D cells, both the D2 and D3 cyclins were expressed, while cyclin D1 expression was absent [51]. Expression of D2 and D3 cyclins was also induced in mitogen-stimulated normal lymphocytes and in MOLT-4 lymphoblastic leukemia cells, where their expression peaked between 8–24 h [52].

## Acknowledgements

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