
Original Articles

D Cyclins in Lymphocytes

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Background: D cyclins are essential for the progression of cells through the G1 phase of the cell cycle. There are three distinct D cyclins. Cyclin D1 has been shown to be expressed by many different types of cells but not by lymphocytes. Cyclins D2 and D3 have been found in lymphocytes.

Methods: We used high-resolution enzymatic amplification staining technology in conjunction with flow cytometry and confocal microscopy and with immunoblotting to reassess the expression of the D cyclins in human lymphocytes.

Results: Using high-resolution technology for flow cytometry, we found all three D cyclins in quiescent human peripheral blood lymphocytes. Cyclin D1 was expressed

in quiescent and activated cells at levels commensurate with those of actively proliferating tumor cell lines. Cyclin D1 was functional inasmuch as it was complexed with CDK4. In the quiescent cells, cyclin D1 was expressed in the cytoplasm but, after activation, was found in the nucleus.

Conclusions: These findings demonstrate that lymphocytes express cyclin D1 and necessitate a reappraisal of the hypothesis that the D cyclins subsume redundant activities with tissue-specific expression. © 2004 Wiley-Liss, Inc.

Key terms: human lymphocytes; cellular proliferation; flow cytometry; D cyclins; subcellular localization; coimmunoprecipitation

D cyclins are expressed during the G1 phase of the cell cycle and bind to the distinct cyclin-dependent kinases, CDK4 or CDK6. The D cyclin/CDK4 and D cyclin/CDK6 complexes effect the phosphorylation of the retinoblastoma protein, Rb, thereby inactivating Rb. The inactivation of Rb allows for the activation of E2F, which regulates the transcription of genes necessary for S-phase induction and the progression of the cell cycle. Thus, D cyclins are essential molecules in one of the most fundamental properties of cells, proliferation (1,2).

There are three distinct D cyclins: D1, D2, and D3. These different molecules share 50% to 60% identity throughout the coding region (3,4) and share functional activity in binding to CDK4 and CDK6 and phosphorylating Rb. The presence of these three related molecules with redundant activity has been explained by unique tissue-specific expression. For instance, cyclin D1 has not been found in lymphocytes; instead, these cells express cyclins D2 and D3 (5-9).

The importance of D cyclin expression has been indicated by several major findings. First, the gene encoding cyclin D1 was found to be the Bcl-1 oncogene in B-lymphocytic malignancies (10,11). Overexpression of cyclin D1 is effected by the Bcl-1 rearrangement, t(11;14)(q13;q32). Moreover, cyclin D1 is overexpressed in

more than 50% of human mammary carcinomas (12,13), and the molecule's overexpression in the mammary glands of transgenic mice results in breast cancers (14). In addition, knockout mice that do not express cyclin D1 are protected against the formation of breast cancers (15). The second major finding is that several γ -herpesviruses, including Kaposi sarcoma-associated herpesvirus, herpesvirus saimiri, and murine γ -herpesvirus 68, encode a viral homolog of D cyclins which may be involved in the capacity of these viruses to transform cells (16-19). It is interesting to note that the complex of viral cyclin with CDK6 demonstrates a unique resistance to inhibition by cellular inhibitors of this pathway (20).

The expression of D cyclins has been difficult to detect by flow cytometric analysis. It is most likely that the sensitivity of this analysis has been inadequate for a defin-

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itive assessment. We have developed a powerful new technology that enhances the resolving power of flow cytometric analysis by as much as 100-fold (21–39). We have applied this technology, enzymatic amplification staining (EAS), to the analysis of D cyclins in quiescent and activated lymphocytes and have found various patterns of expression that belie the conceptualization of tissue-specific expression of the different D cyclins.

MATERIALS AND METHODS

Cells

Peripheral blood was obtained from healthy volunteers with approval from the institutional review board of Case School of Medicine, and mononuclear cells were isolated by Ficoll Hypaque discontinuous gradient centrifugation. These cells were cultured in RPMI 1640 with 10% fetal bovine serum at 37°C in a humidified incubator with an atmosphere of 7% CO₂. Phytohemagglutinin (PHA; Difco Laboratories, Detroit, MI, USA) at 1 µg/ml was added to some cultures. The K562, U937, JY(LCL), and HeLa cell lines were maintained in RPMI 1640 with 5% to 10% fetal bovine serum. HeLa cells were treated with Accutase (Innovative Cell Technologies, San Diego, CA) to obtain a single-cell suspension.

Antibodies

Fluoresceinated murine monoclonal antibodies specific for cyclin D1, clones DCS-6 and G124-326, and fluoresceinated isotype/subtype-matched control immunoglobulin were obtained from BD Bioscience (San Jose, CA, USA). The manufacturer states that these antibodies do not cross-react with cyclin D2 or cyclin D3. Immunoblotting experiments showing the reactivity of the DCS-6 clone with human cyclin D1 but not with cyclin D2 or cyclin D3 have been published (40). An unconjugated rat monoclonal antibody to cyclin D1, clone Ab-1, was obtained from Oncogene Research Products (Manhasset, NY, USA). The manufacturer documents that this antibody does not cross-react with other cyclins or with cyclin D2 or cyclin D3. Specificity was determined by western blotting with purified recombinant cyclins, cyclin D1, cyclin D2, and cyclin D3 proteins. A murine monoclonal antibody to cyclin D2, clone DCS-3.1 was obtained from Biosource International (Camarillo, CA). The manufacturer documents that this antibody is specific for cyclin D2 and does not react with cyclin D1 or cyclin D3. A fluoresceinated murine monoclonal antibody to cyclin D3, clone G107-565, was obtained from BD Bioscience in addition to a fluoresceinated isotype/subtype-matched control immunoglobulin. The manufacturer documents that the antibody does not cross-react with cyclin D1 or cyclin D2 and that specificity has been determined by western analysis and enzyme-linked immunosorbent assay. Murine monoclonal antibodies specific for human CD3 and human CD19 and conjugated with allophycocyanin were obtained from BD Bioscience.

Flow cytometry

Cells were fixed, permeabilized, and stained with 20 µl of the fluoresceinated anti-cyclin D1 or 20 µl of the fluoresceinated matched control immunoglobulin (as recommended by the manufacturer; with 15 µg/ml of anti-cyclin D2 or 15 µg/ml of the isotype/subtype-matched control immunoglobulin, 20 µl of the fluoresceinated anti-cyclin D3, or 20 µl of the fluoresceinated matched control immunoglobulin, as recommended by the manufacturer). Cells were incubated in a reaction volume of 50 µl for 10 min at room temperature. After washing cells were processed for signal enhancement with an EAS kit from FlowAmp Systems (Cleveland, OH) (21,22). Kit instructions were followed for this procedure. EAS has been described in detail elsewhere (21–23,29,30). Amplification of the specific fluorescent signal by EAS is based on peroxidase catalyzed deposition of fluorescent label in a proximity-regulated fashion. Fluorescence data were acquired on a FACScan (BD Biosciences) and analyzed with CellQuest software. If histograms that were obtained were unimodal (single peaks), data could be presented as a mean fluorescence ratio, which was defined as the ratio of the mean fluorescence channel number for the specific monoclonal antibody to the mean fluorescence channel number for the isotype/subtype-matched control.

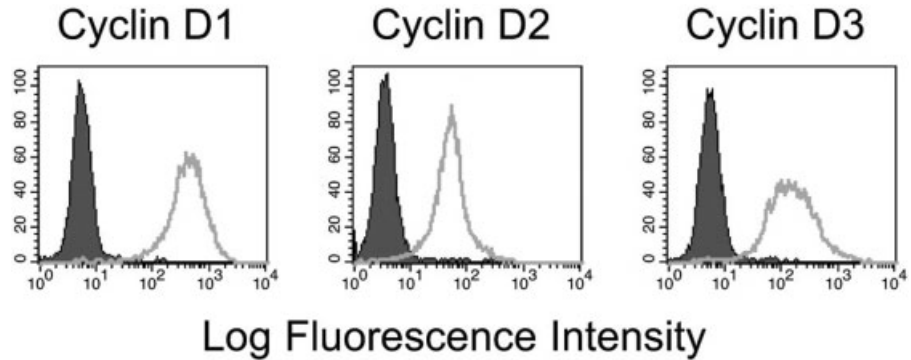
Fluorescence confocal microscopy

4',6-Diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA) was added to cells stained for flow cytometry. Cells were visualized on an LSM510 laser scanning confocal microscope (Zeiss, Thornwood, NY, USA) with an argon laser for excitation at 488 nm and a tunable titanium:sapphire laser for two-photon imaging.

Western analysis

Ten million cells were lysed for 15 min at 37°C in 0.2 ml of lysis buffer (0.5% NP-40, 5 mM Tris at pH 7.0, 120 mM NaCl, 10 µl/ml DNase, 2.5 µl/ml RNase, 70 µg/ml phenyl methyl sulfonyl fluoride, and 10 µl/ml protease inhibitor cocktail; Sigma Chemical Co., St. Louis, MO, USA). An equivalent volume of sodium dodecylsulfate (SDS) buffer (1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris base, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 70 µg/ml phenyl methyl sulfonyl fluoride, and 10 µl/ml protease inhibitor cocktail) was added, and the samples were run on SDS polyacrylamide gel electrophoresis (PAGE). The gel used was a pre-made 4% to 20% gradient gel (Bio-Rad, Richmond, CA, USA). After electrophoresis, proteins were transferred from the gel to a membrane. The membrane was blocked with 5% milk proteins in Tris-buffered saline for 1 h at room temperature. The membrane was washed and incubated with rat anti-cyclin D1 monoclonal antibody for 1 h at room temperature. The membrane was washed and incubated for 1 h at room temperature with goat anti-rat conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA). The membrane was washed and incubated with an enhanced chemoluminescent reagent

FIG. 1. Expression of D cyclins in K562 Cells. K562 cells were fixed, permeabilized, and stained with EAS processing for expressions of cyclins D1, D2, and D3 (open histograms). Cells were also stained with isotype-/subtype-matched control immunoglobulin (shaded histograms). Cells were analyzed by flow cytometry on a FACScan.



(Amersham, Arlington Heights, IL, USA). The membrane was placed in a cassette with film (Eastman-Kodak, Rochester, NY, USA) and exposure proceeded for 2 min. Molecular weight standards were purchased from Sigma Chemical Co.

Immunoprecipitation

Lysates of peripheral blood mononuclear cells *ex vivo* and peripheral blood mononuclear cells stimulated with PHA for 3 days were made as described above. Immunoprecipitation was accomplished by the addition of 0.2 mg/ml of anti-cyclin D1 monoclonal antibody (clone HD11, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to the lysates. After incubation for 2 h on ice, 200 μ l of protein-G-agarose beads (Zymed, San Francisco, CA, USA) was added and the suspension was incubated overnight at 4°C. The suspension was centrifuged and the beads were washed three times in ice-cold lysis buffer. After washing beads were suspended in sample buffer and boiled. Supernatants were run on SDS-PAGE and transferred to a membrane. The membrane was blocked with 0.5% bovine serum albumin for 30 min at room temperature and then probed with goat anti-CDK4 conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at 2 μ g/ml for 1 h at room temperature. The membrane was washed and incubated with an enhanced chemoluminescent reagent (Amersham). The membrane was placed in a cassette with film (Eastman-Kodak) and exposure proceeded for 30 min. Molecular weight standards (Sigma Chemical Co.) were included to ascertain the molecular weight corresponding to the band observed.

RESULTS

Flow Cytometric Analysis of the D Cyclins

K562, an erythroleukemia cell line, expresses messages for cyclin D1, cyclin D2, and cyclin D3 (41,42). We used EAS to stain these cells for the D cyclins, and fluorescence was analyzed by flow cytometry (Fig. 1). As expected, the results demonstrated the expression of all three D cyclins in these cells. For a first level of validation of our staining, we assessed expression of the D cyclins in HeLa cells that had been cultured overnight at 100% confluency and HeLa cells that had been cultured at low density overnight. Previously, it has been shown by western analysis that

100% confluency results in downregulation of cyclin D1 expression (43). The results demonstrated that the D cyclin expression, as assessed by EAS, was modulated as expected (Fig. 2). Histograms were unimodal, so data could be presented as mean fluorescence ratios.

Cyclin D1 Expression in Lymphocytes

Initial studies of human peripheral blood mononuclear cells showed no cyclin D1 mRNA by northern analysis, and subsequently, the protein was not found in these cells (5-9). A consensus that cyclin D2 and cyclin D3, but not cyclin D1, are expressed in lymphocytes was reached. However, recently one group of investigators used reverse transcriptase polymerase chain reaction and found message for cyclin D1 in human peripheral blood lymphocytes (44). To see whether EAS would provide a more complete picture of cyclin D1 expression in human lymphocytes, we stained peripheral blood mononuclear cells for cyclin D1 with two

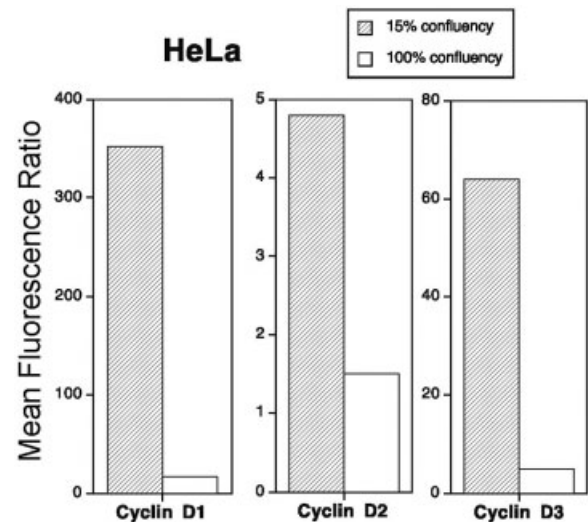


FIG. 2. Expression of D cyclins in HeLa cells. HeLa cells were grown at 100% confluency (open columns) or at 15% confluency (shaded columns). Cells were fixed, permeabilized, and stained with EAS processing for expressions of cyclins D1, D2, and D3. Cells were analyzed by flow cytometry. The histograms obtained were monophasic; hence, the mean fluorescence ratio was calculated.

Cyclin D1 Staining

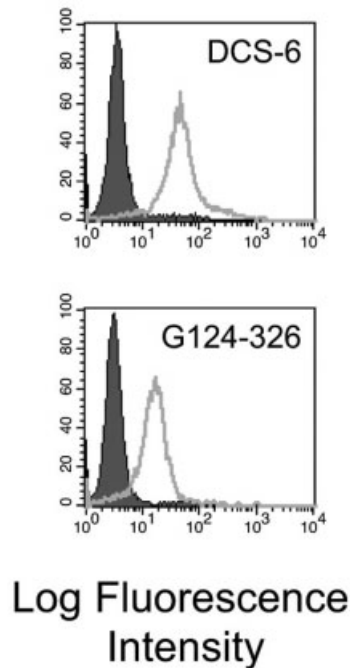


FIG. 3. Expression of cyclin D1 in human lymphocytes. Human peripheral blood mononuclear cells from a healthy volunteer were fixed, permeabilized, and stained with EAS processing with isotype/subtype-matched control immunoglobulin (shaded histograms), with anti-cyclin D1 monoclonal antibody DCS-6 (open histogram, top), or with anti-cyclin D1 monoclonal antibody G124-326 (open histogram, bottom). Events within the characteristic lymphocytic gates on forward scatter and side scatter analysis were gated for this determination.

different, independent murine monoclonal antibodies (Fig. 3). The results showed that both monoclonal antibodies demonstrated positive staining of quiescent human peripheral blood lymphocytes. Double staining to identify B and T lymphocytes showed equivalent levels of cyclin D1 expression in both populations (Fig. 4).

Because cyclin D1 expression has not been previously detected in lymphocytes (5-9), we sought to validate the staining by western blotting. Lysates from resting peripheral blood mononuclear cells obtained from four healthy volunteers and from K562 cells were analyzed (Fig. 5). It is important to note that the filters were blotted with Ab-1, a third independent monoclonal antibody specific for human cyclin D1 (Fig. 5). With the identification of a band running at the appropriate molecular weight and coincident with the band from K562 cells, the results verified that cyclin D1 is expressed by lymphocytes. Peripheral blood mononuclear cells comprise lymphocytes and monocytes. Although monocytes represent approximately 10% of the total cells in this population and showed lower amounts of cyclin D1 expression than did lymphocytes (data not shown), we removed the monocytes by two rounds of adherence, which produced a population of enriched lymphocytes (<95%). A lysate of this lympho-

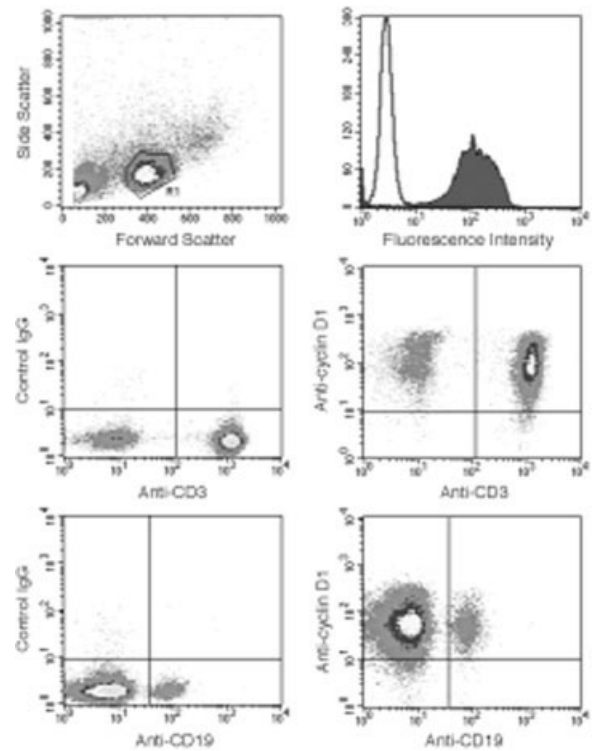


FIG. 4. Cyclin D1 in T and B lymphocytes. Human peripheral blood mononuclear cells from a healthy volunteer were fixed, permeabilized, and stained with EAS processing with isotype/subtype-matched control immunoglobulin (Ig; upper right panel, open histogram) or with anti-cyclin D1 monoclonal antibody DCS-6 (upper right panel, solid histogram). The forward scatter and side scatter analysis is shown, and the characteristic lymphocytic gates are outlined (upper left panel). Cells were also stained with control Ig (middle and lower left panels) or anti-cyclin D1 monoclonal antibody DCS-6 (middle and lower right panels) and doubly stained with anti-CD3 allophycocyanin (middle panels) to identify T lymphocytes or with anti-CD19- allophycocyanin (lower panels) to identify B lymphocytes.

cyte-enriched population of cells was immunoblotted for cyclin D1 expression, and it also produced the characteristic cyclin D1 band (data not shown).

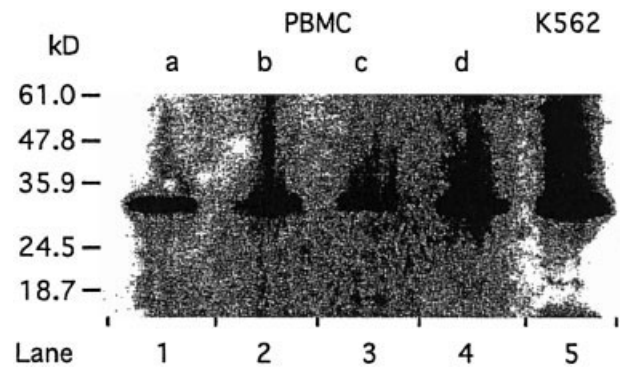


FIG. 5. Cyclin D1 in human lymphocytes. Human peripheral blood mononuclear cells (PBMC) from four healthy volunteers (a-d) were lysed. Lysates were analyzed by SDS-PAGE and western analysis with anti-cyclin D1 monoclonal antibody. A lysate from K562, a cell line known to express cyclin D1, was included as a control.

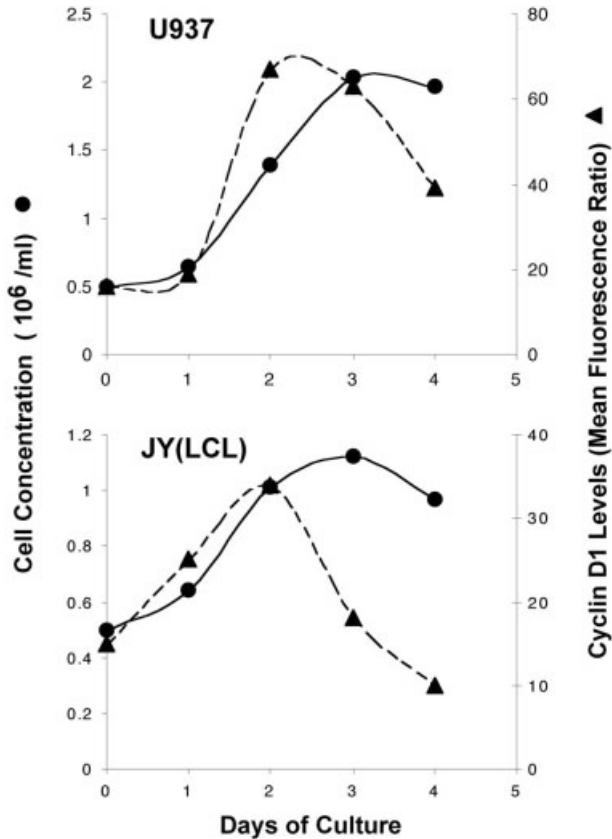


FIG. 6. Variations in cyclin D1 levels in immortalized cell lines. U937 cells and JY(LCL) cells were counted after 3 days of growth and re-plated at 0.5×10^6 cell/ml in culture medium. Cultures were counted in triplicate on a hemocytometer (circles) and simultaneously stained for cyclin D1 expression (triangles) on a daily basis for 4 days. Mean fluorescent ratios are shown to indicate cyclin D1 expression because the histograms were monophasic.

The cyclin D1 bands from quiescent lymphocytes were similar in intensity to the cyclin D1 band from the K562 cells (Fig. 5). Hence, we sought to determine how the level in the nonproliferating human lymphocytes relates to immortalized cells that continuously cycle. To achieve this goal, we analyzed U937 cells, a monoblastic tumor cell line, and an Epstein-Barr virus transformed B-lymphocyte line, JY(LCL), for cyclin D1 expression as the cells proliferated in culture and compared the mean fluorescent ratios with the different cell concentrations (Fig. 6). The results showed that cyclin D1 levels were low when cells were initially seeded at a low concentration. As the cells progressed through a rapid growth phase, the cyclin D1 levels increased to a maximum. Subsequently, when the cultures reached a plateau stationary phase, the cyclin D1 level decreased. For JY(LCL) the mean fluorescent ratios were in the range 20–40 for cyclin D1 during the growth phase and in the range 40–80 for U937. Interestingly, these values reflected the degree of proliferation for the two cell lines with JY(LCL) cells accumulating to approximately half of the cell concentration of the U937 cells over the same period.

For comparison, mean fluorescent ratios for cyclin D1 in peripheral blood lymphocytes from eight different healthy donors varied from 11 to 83. Although the relation of protein expression with fluorescence intensity was not directly correlated with this staining method, the range of mean fluorescent ratios for quiescent peripheral blood lymphocytes was similar to that for continuously proliferating immortalized cells in culture.

D Cyclins in Resting and Activated Lymphocytes

Using EAS we proceeded to assess the expression of all three D cyclins in resting and activated lymphocytes (Fig. 7). The staining pattern showed that resting and stimulated lymphocytes contain all three D cyclins. Interestingly, the patterns for the three D cyclins diverged. Cyclin D1 was expressed abundantly in resting cells and increased only slightly after PHA stimulation for 3 days, at a time when cells are large and proliferating. Cyclin D2 was expressed in resting cells at a low level and showed no major change with PHA stimulation. In contrast, cyclin D3 was expressed in low levels in resting cells but markedly increased after PHA stimulation. Comparison of eight consecutive determinations of cyclin D3 levels in unstimulated versus PHA-stimulated cells from six different donors showed a statistically significant increase in cyclin D3 levels with PHA stimulation.

With these results we wondered why resting lymphocytes were not proliferating because they expressed abundant amounts of cyclin D1. As an initial experiment to address this issue, we examined cells stained for the D cyclins by fluorescence confocal microscopy. DAPI was added to the cells as a counterstain to visualize nuclei (Fig. 8). The results demonstrated that the cyclin D1 in resting lymphocytes was expressed primarily in the cytoplasm. In most of the cells examined, there was a clear-cut shadow that coincided with the location of the nucleus identified by DAPI staining. After activation for 3 days with PHA, there was a significant proportion of larger cells that demonstrated a uniform distribution of cyclin D1 staining that coincided with the nucleus. In these cultures there were also some small cells displaying the cytoplasmic pattern of staining.

Cyclin D3 exhibited a different pattern. In the unstimulated lymphocytes, the level of staining was not visible when using the same laser power settings as for cells stained for cyclin D1. By increasing the laser power 10-fold, we found that the small amount of cyclin D3 in these cells was primarily nuclear. After 3 days of PHA stimulation, the large activated lymphocytes also showed a nuclear staining pattern, but the level of expression was greatly enhanced (Fig. 8). Thus, cyclin D1 is regulated in lymphocytes by subcellular localization, and cyclin D3 is regulated by the induction of higher levels of expression.

To determine whether cyclin D1 in unstimulated peripheral blood lymphocytes and in PHA-stimulated lymphocytes was biologically functional, we immunoprecipitated cyclin D1 from lysates of these cells and probed the immunoprecipitates for CDK4 (Fig. 9). The data showed that cytoplasmic cyclin D1 in the lymphocytes *ex vivo* and the nuclear cyclin D1 in the PHA-stimulated cells were complexed with CDK4.

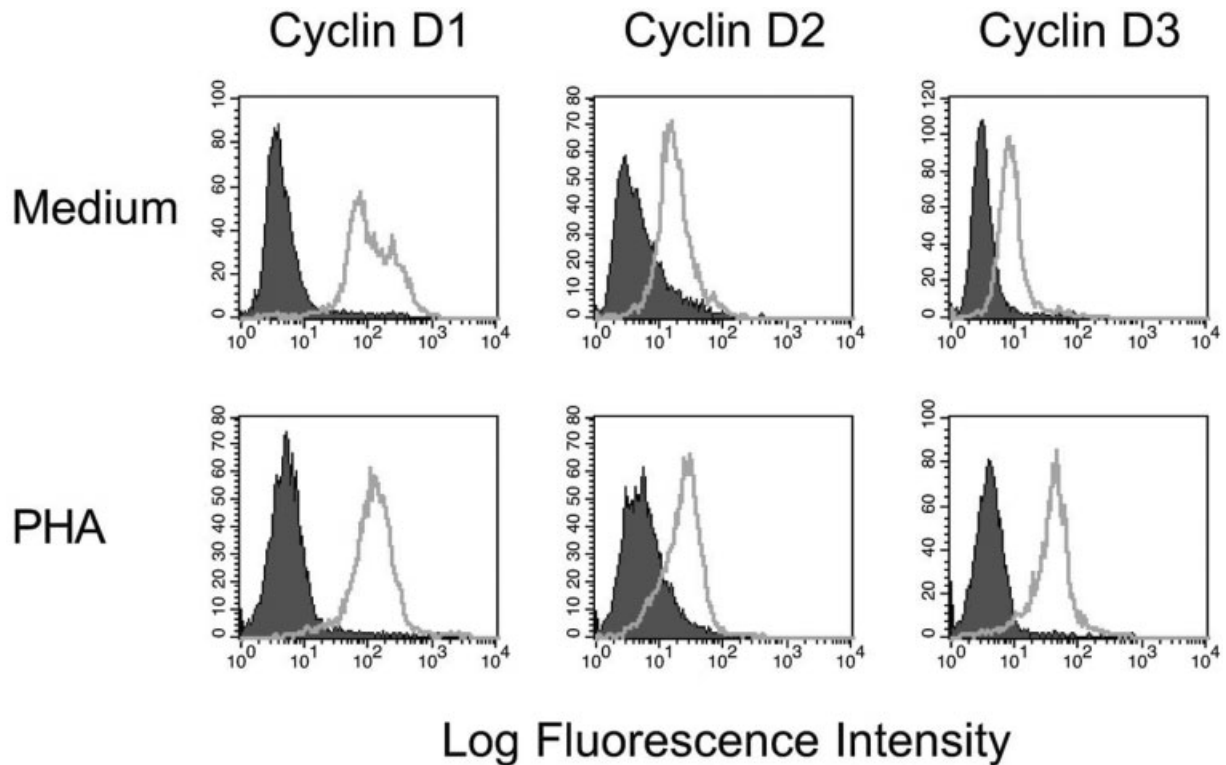


FIG. 7. D cyclins in human lymphocytes. Human peripheral blood mononuclear cells were cultured for 3 days in medium alone or with PHA. Cells were harvested from cultures, fixed, permeabilized, and stained with EAS processing. Cells were stained with specific anti-D cyclin antibodies for cyclin D1, D2, or D3 (open histograms) or with isotype-/subtype-matched control immunoglobulin (solid histograms).

DISCUSSION

We analyzed human peripheral blood mononuclear cells for the expression of the three D cyclins. In contrast

to previous studies (5-9), we found that resting lymphocytes express all three D cyclins. The finding of cyclin D1 expression in these cells was unexpected because others

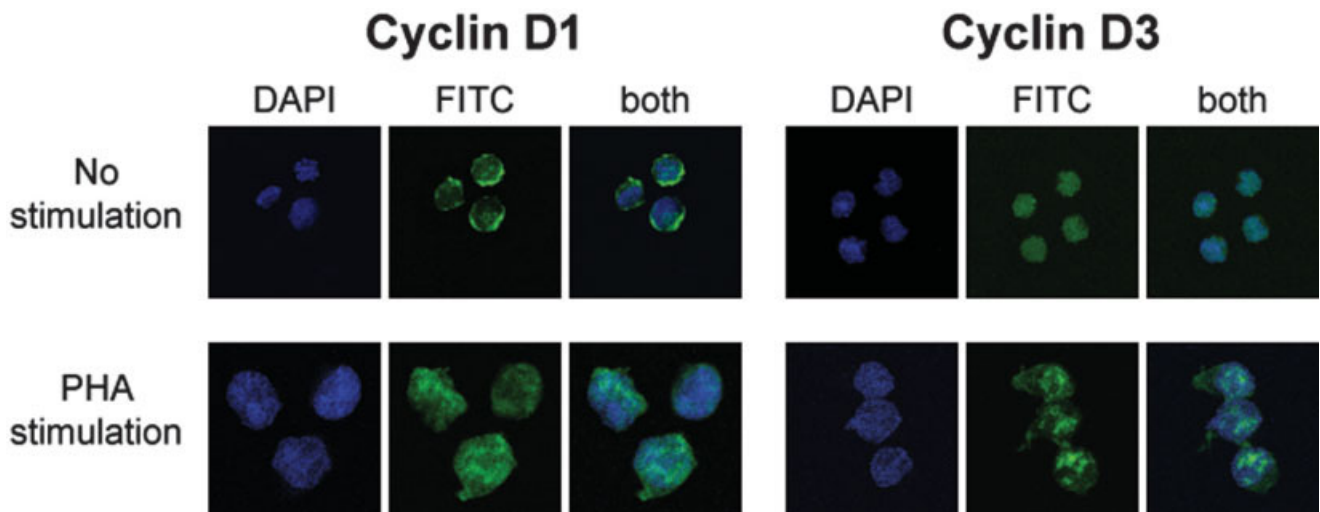


FIG. 8. Subcellular localization of D cyclins in human lymphocytes. Human peripheral blood mononuclear cells were cultured for 3 days in medium alone or with PHA. Cells were harvested from the cultures, fixed, permeabilized, and stained with EAS processing. Cells were stained with specific anti-cyclin D1 or with specific anti-cyclin D3 (green). Cells were counterstained with DAPI (blue), cytocentrifuged, and observed on a fluorescence confocal microscope. Overlays of the D cyclin fluorescein stain and the DAPI stain are shown with coincident staining appearing light blue or green-blue. For cyclin D3 staining of the unstimulated cells, the laser power was increased 10-fold to visualize the dim fluorescence in these cells. FITC, fluorescein isothiocyanate.

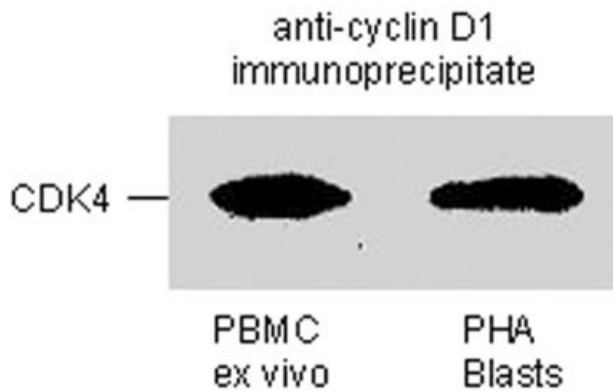


FIG. 9. Coimmunoprecipitation of CDK4 with cyclin D1. Lysates from peripheral blood mononuclear cells (PBMCs) *ex vivo* and PBMCs stimulated with PHA and cultured for 3 days were made, and cyclin D1 was immunoprecipitated from these lysates with a specific monoclonal antibody. Immunoprecipitates were run on SDS-PAGE, transferred to a membrane, and probed with anti-CDK4 antibodies. Molecular weight markers were also included to identify the appropriate size of CDK4 (33 kDa).

have not found cyclin D1 in resting or activated lymphocytes, but it is in accordance with the recent demonstration of cyclin D1 message by reverse transcriptase polymerase chain reaction in these cells (44). It should be noted that these investigators did not look for cyclin D1 protein in the lymphocyte samples. They interpreted their results to indicate that cyclin D1 message occurs in the absence of the protein. In our study we found the expression of cyclin D1 protein in resting lymphocytes, and our findings were validated by the use of two different techniques and three different monoclonal antibodies.

The failure of other investigators to detect cyclin D1 in resting or activated lymphocytes by immunoblotting is paradoxical in view of our findings (6,8,9). We found that cyclin D1 was particularly susceptible to protease degradation. Fastidious use of protease inhibitors was essential for detecting this molecule. We postulate that lymphocytes express a specific protease that readily cleaves cyclin D1 and that this protease is responsible for the difficulty in detecting this molecule in these cells.

Although cells within the characteristic forward scatter and side scatter lymphocytic gates are heterogeneous, our results demonstrated that these cells express similar levels of the various D cyclins. Double staining experiments showed that B and T cells express similar levels of cyclin D1. From these results there was no suggestion of any heterogeneity in the set point of expression of the D cyclins among quiescent CD4⁺ T cells or CD8⁺ T cells, natural killer cells, and B-lymphocyte subsets. It is interesting to note that monocytes *ex vivo* had a different set point for the D cyclins. They expressed less cyclin D1, equivalent levels of cyclin D2, and more cyclin D3 than did lymphocytic cells (data not shown). The level of D cyclin expression may reflect the proclivity of these various cells to respond to activation with proliferation.

The finding that all of the D cyclins are expressed in lymphocytes suggests that they may encompass unique

functions and redundant activity. The D cyclins are similar but not identical (3,4). They all bind to cyclin-dependent kinases and phosphorylate Rb, thereby preventing Rb from inhibiting transactivation by E2F. However, human D cyclins share greater similarity with their specific murine homologs than with other human members of their family (3,4). This result suggests that the D cyclins have differential functional properties that may be important for determining the cellular phenotype. Additional support for this perspective has been obtained in a study of the enzymatic activity of the D cyclins, which showed that murine cyclin D3 possesses a unique kinase activity (45). Although a correlation between this enzymatic activity and the cellular phenotype was not assessed, it is reasonable to speculate that differential enzymatic activity among the D cyclins may have a profound influence on cellular proliferation and/or differentiation.

D cyclins are regulated in two ways. First, Rb is a nuclear protein; as a consequence, for D cyclins to be active they must localize to the nucleus. Cyclin D1 localizes to the nucleus during G1 but resides in the cytoplasm for the remainder of interphase (46,47). The significance of nuclear expression of D cyclin in terms of proliferation has been demonstrated in an investigation of neonatal cardiomyocytes. These cells were transfected with a vector encoding for production of cyclin D1, but the cyclin D1 accumulated in the cytoplasm and the cells did not proliferate. Transfection with a vector encoding for the production of cyclin D1 directly linked to a nuclear localization signal sequence resulted in nuclear localization and subsequent cellular proliferation (48). The importance of D cyclin subcellular localization has been especially well demonstrated in a study that demonstrated the presence of cytoplasmic cyclin D1 in cells of healthy thyroids and thyroid adenomas but localization of the molecule to the nucleus in thyroid papillary carcinoma cells (49).

Lymphocytic activation induces the movement of cyclin D1 from the cytoplasm to the nucleus. This result indicates that the activation signal includes a trigger for the mechanism responsible for relocation of the molecule. It is known that nuclear localization is lost in cyclin D1 mutated to express alanine instead of threonine at position 156 (50). This mutant can bind to CDK4 but the complex is not phosphorylated by CDK-activating kinases nor can it bind to other factors that are required for nuclear localization. Intracellular migration of cyclin D1 from the nucleus to the cytoplasm at the end of G1 is triggered by GSK-3 β -dependent phosphorylation of cyclin D1 at threonine 286. This phosphorylation promotes the association of the molecule with the nuclear exportin, CRM1 (51).

Regulation of cyclin D1 by subcellular localization suggests that cyclin D1 is singularly important in the initial steps of cellular activation, possibly explaining why it is sustained in the cytoplasm of the quiescent lymphocyte in levels commensurate with actively proliferating hematopoietic tumor cells in culture. Moreover, at least a proportion of the cyclin D1 is complexed with CDK4. In that

sense, lymphocytes are maintained poised to begin proliferation as soon as an appropriate signal is received. Although cyclin D1 is expressed in the cytoplasm of quiescent lymphocytes, there is a constitutive, but low, expression of cyclin D3 in the nucleus. The concentration of nuclear cyclin D3 is inadequate to initiate progression through the G1 phase, an inhibitor inactivates the nuclear cyclin D3, or cyclin D3 activity is not by itself sufficient to induce proliferation of the cells. After activation cyclin D3 is significantly upregulated. The increased level of cyclin D3 occurs 2 days after stimulation (data not shown), suggesting a role for this D cyclin later in the proliferative response. It is possible that cyclins D1 and D3 play important and distinct roles at the peak of cellular proliferation to maximize the progression of the cell through the G1 phase.

Second, D cyclins are transcriptionally regulated. For instance, the level of cyclin D1 and cyclin D3 mRNA is decreased in human diploid fibroblasts by serum starvation or contact inhibition. They reappear with serum addition and culture at low cellular density (52). We were able to assess the protein expression levels of the D cyclins in lymphocytes with and without activation. Cyclin D1 was expressed in the quiescent lymphocytes and the level was not appreciably changed after activation. In contrast, cyclin D3 was expressed at low levels in the resting lymphocytes and expression was markedly enhanced by activation. This finding is in concert with previous investigations of cyclin D3 expression in human lymphocytes that have shown activation-dependent regulation of cyclin D3 (9,53,54). Cyclin D2 levels were low and did not change with activation; however, an important role of the molecule in lymphocytic proliferation cannot be ruled out. Differential regulation of the 3 D cyclins is consistent with the possibility that these molecules possess unique functional attributes.

Others have found that cyclin D2 is increased in human immunoglobulin D/low B cells after stimulation with anti-immunoglobulin M, anti-CD40, and interleukin-4 (9). This increase occurred over 1 to 2 days, but by 3 days after stimulation cyclin D2 expression had dropped to constitutive levels. Similarly, PHA-stimulated peripheral blood mononuclear cells demonstrated a peak of cyclin D2 expression between 8 and 24 h (54). Stimulation of Jurkat cells with PHA and anti-CD28 has been shown to produce an increase in cyclin D2 within 15 min that lasts at least 2 h (55). We did not find any significant change in cyclin D2 of PHA-stimulated peripheral blood mononuclear cells 3 days after activation. It is possible that cyclin D2 may have been induced quickly and returned to constitutive levels by 3 days. Moreover, induction of cyclin D2 is likely dependent on the specific stimulatory regimen and on the specific cellular subsets analyzed.

We found that cyclin D1 in the quiescent peripheral blood lymphocytes and in the stimulated and actively proliferating lymphocytes is complexed with CDK4; as a consequence, the cyclin D1 in these cells is functional. The complex is responsible for the inactivation of Rb by phosphorylation (1,2). It is important to note that there

may be a proportion of cyclin D1 in these cells that is not bound to CDK4. The D cyclins are known to mediate activities beyond regulation of the cell cycle. For instance, cyclin D1 in murine macrophages has been shown to control cellular adhesion and motility (56).

The finding of a D cyclin complexed to a CDK molecule in the cytoplasm of a quiescent lymphocyte has recently been reported (57). In this case murine CD8⁺ memory cells expressed cyclin D3 bound to CDK6 in the cytoplasm. The investigators demonstrated that neutralization of these complexes blocks rapid division of the cells. These results are similar to the findings we have presented here. It is also interesting to note that the investigators found cyclin D1 expression in naive and memory murine CD8⁺ cells by western blotting (57). Although they did not pursue these findings, they are compatible with our results involving human cells and suggest the importance of cyclin D1 expression in lymphocytic cells.

Expression of nuclear cyclin D1 has been used in the diagnosis of mantle cell lymphoma, a B-lymphoid neoplasm (58). Our finding that quiescent, peripheral blood B lymphocytes express abundant cyclin D1 in the cytoplasm suggests that the pathogenesis of lymphoma involves not only deregulation of the molecule's expression but also a deregulation in its subcellular localization. The molecular mechanism that might affect subcellular localization of cyclin D1 in lymphoma cells has not been elucidated.

EAS is a powerful technology that has helped us understand the role of the D cyclins in lymphocytic proliferation. We previously demonstrated the capabilities of this new technology in enhancing the diagnosis of lymphocytic leukemia (23,27), in understanding B-cell subsets (22), and in providing a high-resolution immunophenotype of platelets (28). Others have successfully used EAS for the detection of molecules that could not be detected otherwise (31-39). EAS provides significantly greater sensitivity for flow cytometric analysis.

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