

# Cyclin-dependent Kinase 6 Is the Principal Target of p27/Kip1 Regulation of the G<sub>1</sub>-phase Traverse in 1,25-Dihydroxyvitamin D<sub>3</sub>-treated HL60 Cells<sup>1</sup>

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## Abstract

Control of cell proliferation remains of intense interest in cancer research. In the 1,25-dihydroxyvitamin D<sub>3</sub> HL60 cell system, G<sub>1</sub> arrest has been shown to be mediated by elevated levels of p27/Kip1 protein. We show here that the main target of the elevated p27/Kip1 in this system is cyclin-dependent kinase (Cdk) 6. The activity of Cdk2 is also down-regulated, and this is associated with altered and reduced levels of cyclin E in the kinase complex. Paradoxically, the kinase activity of Cdk4 is elevated, in spite of an almost complete G<sub>1</sub> block. These data show that the functions of Cdk4 and Cdk6 are not redundant and that Cdk6 and Cdk2 activities are regulated by 1,25-dihydroxyvitamin D<sub>3</sub>.

## Introduction

The antiproliferative actions of 1,25D<sub>3</sub><sup>3</sup> and its structural analogues are being used for the treatment of hyperproliferative diseases such as psoriasis (1), and epidemiological evidence suggests that sunlight-generated 1,25D<sub>3</sub> plays a role in reducing the incidence of common human malignancies, such as carcinoma of the breast, prostate, and colon (reviewed in Ref. 2). *In vitro*, 1,25D<sub>3</sub> arrests the growth of cell lines derived from these tumors (2), but the most extensively used system for such studies is provided by cultured HL60 human promyelocytic leukemia cells HL60 (see, e.g., Ref. 3). Exposure of HL60 cells to 1,25D<sub>3</sub> results in the acquisition of several markers of mature monocytes/macrophages, and this is accompanied by the cessation of cell proliferation (reviewed in Ref. 4). However, there is no general agreement on how the antiproliferative effects of 1,25D<sub>3</sub> are mediated in these cells. The principal 1,25D<sub>3</sub>-induced block to cell cycle progression in HL60 cells is in the G<sub>1</sub> phase of the cell cycle, although a less marked block in the G<sub>2</sub> phase has also been reported (5). One potential mediator of this G<sub>1</sub> block is the CKI p21/WAF1 protein, and increased expression of this CKI has been reported in HL60 (6, 7) and U937 (8) cells exposed to differentiating agents. However, the expression of p21/WAF1 in 1,25D<sub>3</sub>-treated HL60 (9) cells was transient and did not correlate temporally with the onset of the G<sub>1</sub> block (8, 9). Conversely, the appearance of a related CKI protein, p27/Kip1 (10), does occur at the onset of the 1,25D<sub>3</sub>-induced block (9). Although this suggests that p27/Kip1 plays a major role in the 1,25D<sub>3</sub>-induced inhibition of the G<sub>1</sub> traverse by HL60 cells, the target or targets of its action have not been identified.

The recent development of sublines of HL60 cells resistant to the antiproliferative effects of 1,25D<sub>3</sub> (11) has offered an additional tool for deciphering the mechanisms responsible for 1,25D<sub>3</sub>-induced growth arrest. We have used this system to show that there is a

specific increase in Cdk6-associated p27/Kip1 protein in 1,25D<sub>3</sub>-treated wild-type HL60 (HL60-G) cells but not in a 1,25D<sub>3</sub>-resistant subline of HL60 (40AF) cells. This provides a mechanism for 1,25D<sub>3</sub>-mediated G<sub>1</sub> arrest in wild-type HL60 cells.

## Materials and Methods

**Cell Culture.** HL60-G cells, a subclone of HL60 promyelocytic leukemia cells, were maintained in RPMI 1640 supplemented with 1% glutamine and 10% complement-inactivated bovine calf serum. The 1,25D<sub>3</sub>-resistant HL60 subline (40AF) was derived as described (4).

**Flow Cytometry.** For determining DNA content, 3 × 10<sup>6</sup> cells were fixed in 80% ethanol at -20°C for 24 h, washed once in PBS, resuspended in PBS, and incubated with 1.0 μl RNase (500 μg/ml) for 1 h at 37°C. Cells were then washed again in PBS and incubated with 0.5 ml of propidium iodide stain (10 μg/ml) for 2 h at 4°C. The DNA content was determined using the Epics Profile II Flow cytometer (Coulter, Hialeah, FL), and cell cycle distribution was analyzed by the Multicycle software package.

**Immunoprecipitation.** Cells were washed in PBS and lysed in the immunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM NaVO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 25 μg/ml pepstatin A). Cell debris was removed by centrifugation at 14,000 × g for 20 min at 4°C. Five hundred μg of total proteins were incubated with anti-Cdk2 (UBI, Lake Placid, NY), anti-Cdk4 (UBI), and anti-Cdk6 (Pharmingen, San Diego, CA) antibodies for 2 h at 4°C, followed by incubation with protein A-agarose beads for 1 h. The protein complexes were washed three times with immunoprecipitation buffer and released from the beads by boiling in 3× SDS sample buffer (150 mM Tris, 30% glycerol, 3% SDS, 1.5 mg/100 ml bromophenol blue dye, and 100 mM DTT) for 5 min and resolved in a 13% SDS-PAGE gel. The proteins in the gel were transferred to a nitrocellulose membrane and incubated with 5% nonfat milk blocking buffer (Tris-buffered saline, 5% dry milk, and 0.05% Tween-20) for 1 h. Subsequently, the membranes were blotted with anti-p27 (UBI) antibody and then with an antirabbit (Amersham Corp., Arlington Heights, IL) secondary antibody coupled to horseradish peroxidase. The proteins were visualized using an enhanced chemiluminescence detection kit (Amersham).

**Kinase Reaction Assay.** Total lysate was prepared and immunoprecipitated with the antibody as described above. The beads were washed three times in immunoprecipitation buffer and then three times in kinase buffer (50 mM HEPES, 10 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 0.2 mM NaVO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 50 mM NaF). The kinase reaction was carried out at 37°C for 30 min in 40 μl of kinase reaction buffer containing 10 μM ATP, 0.4 mCi [ $\gamma$ -<sup>32</sup>P]ATP, and 2 μg of histone H1 (BMB; Boehringer Mannheim Indianapolis, IN) or glutathione S-transferase-pRb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) substrates. The reaction was stopped by adding 3× SDS sample buffer. After boiling for 5 min and centrifugation at 3000 × g for 2 min, the supernatant was resolved on a 13% SDS-PAGE gel. The radioactivity was detected by autoradiography.

**<sup>35</sup>S-Metabolic Labeling.** Cells were incubated for 30 min at 37°C with methionine and cysteine-free 1640 RPMI containing 10% dialyzed fetal bovine serum, and then for 4 h at 37°C in the presence of 200 μCi/ml of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (specific activity, 1175 Ci/mmol). Immunoprecipitation with anti-Cdk2, anti-Cdk4, and Cdk-6 antibodies were performed as described above. Protein complexes collected on the beads were separated on 13% SDS-PAGE gel. The <sup>35</sup>S-labeled protein bands were visualized using autoradiography.

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<sup>3</sup> The abbreviations used are: 1,25D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; UBI, Upstate Biotechnology, Inc.; Rb, retinoblastoma.

## Results

We have initiated this study by asking whether the p27/Kip1 protein can be demonstrated in complexes with the three principal Cdk's active in the G<sub>1</sub> phase of leukemia cells, Cdk2, Cdk4, and Cdk6. Fig. 1, A and B, shows that 1,25D<sub>3</sub> had different effects on the amount of p27/Kip1 protein associated with each of these complexes. The complexes immunoprecipitated with an antibody to Cdk2 showed no significant difference between the levels of p27/Kip1 protein in untreated HL60-G cells, HL60-G cells treated for 96 h with 100 nM 1,25D<sub>3</sub> ( $n = 6$ ;  $P > 0.05$ ), 89% of which were arrested in G<sub>1</sub> phase

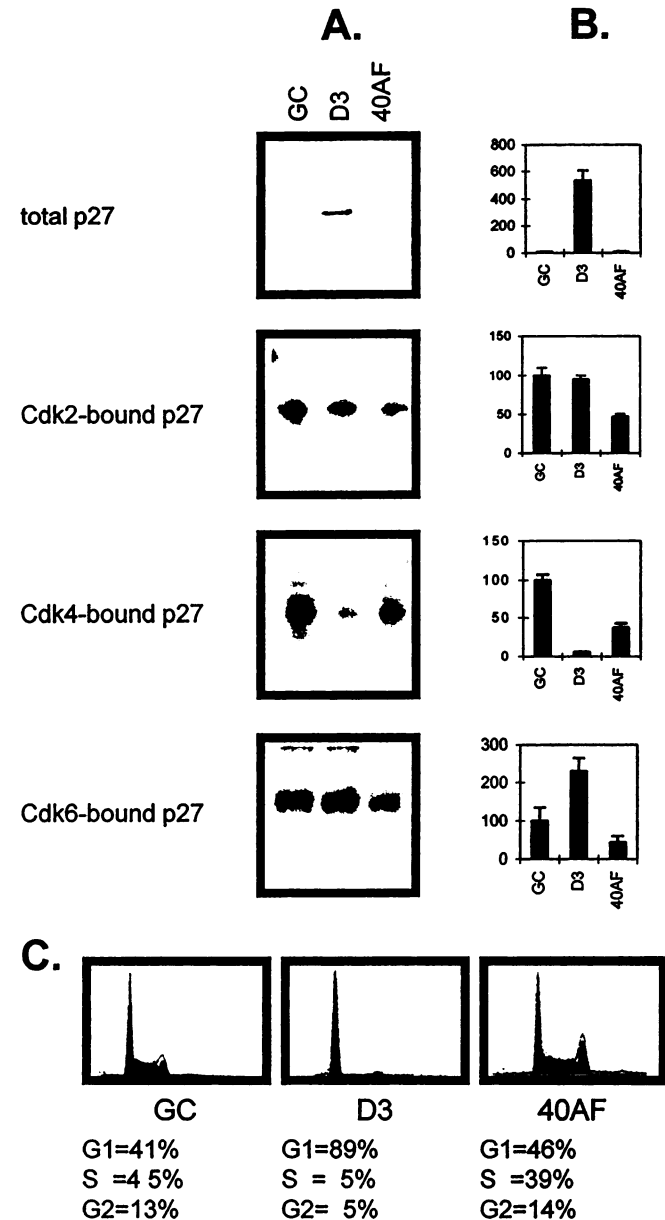


Fig. 1. Association of p27/Kip1 with Cdk2, Cdk4, and Cdk6. A, total-cell extracts from untreated HL60-G cells (GC); HL60-G cells treated with 100 nM 1,25D<sub>3</sub> for 96 h (D3); and 40AF cells (40AF), a 1,25D<sub>3</sub>-resistant HL60 subline growing continually in the presence of 40 nM 1,25D<sub>3</sub>, were immunoprecipitated with anti-Cdk2, anti-Cdk4, or anti-Cdk6 antibodies. The bound p27 in each immunocomplex and the amount of p27 in total lysate were then determined by Western blotting. B, densitometric quantitation of p27 protein levels in arbitrary units. The values in these and subsequent experiments are means of four to six determinations, as indicated in the text. Error bars are not shown if the SE is smaller than the value. C, cell cycle distribution in exponentially growing HL60-G cells (GC), HL60-G treated for 96 h with 100 nM 1,25D<sub>3</sub> (D3), and 40AF cells growing in 40 nM 1,25D<sub>3</sub> (40AF).

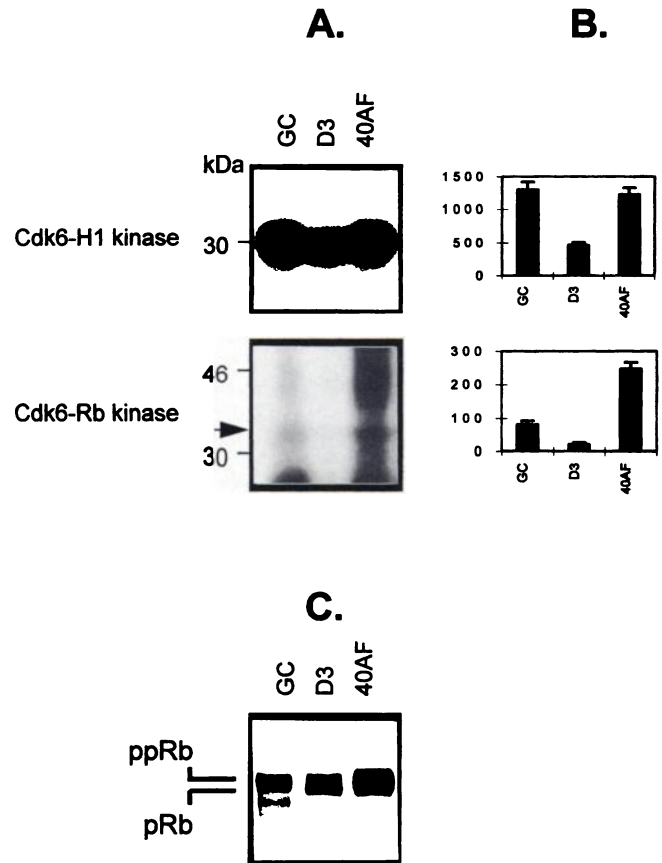


Fig. 2. Cdk6-associated histone H1 and glutathione S-transferase-pRb kinase activities and phosphorylation of the Rb protein. A, total lysate prepared from untreated HL60-G cells (GC), HL60-G cells treated with 100 nM 1,25D<sub>3</sub> (D3), and 40AF cells (40AF) were immunoprecipitated with the anti-Cdk6 antibody (Pharmingen). Histone H1 (BMB) and the recombinant protein GST-pRb (Santa Cruz Biotechnology) containing the C-terminal residues of the Rb protein were used as the *in vitro* phosphorylation substrates. B, densitometric quantitation of A (arbitrary units). C, phosphorylation of the Rb protein. Thirty  $\mu$ g of total lysate proteins, prepared from HL60-G, 1,25D<sub>3</sub>, and 40AF cells, were resolved on a 8% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunoblotted with an anti-Rb antibody (Oncogene Science). The hyperphosphorylated Rb (ppRb) has lower electrophoretic mobility than the hypophosphorylated Rb (pRb).

(Fig. 1C), and HL60-40AF cells ( $n = 6$ ;  $P > 0.05$ ), which were proliferating in the presence of 40 nM 1,25D<sub>3</sub>. In contrast, complexes immunoprecipitated from 1,25D<sub>3</sub>-treated HL60-G cells with anti-Cdk4 antibody had a reduced p27/Kip1 content ( $n = 4$ ;  $P = 0.04$ ), whereas complexes precipitated with anti-Cdk6 antibody contained approximately 2-fold higher amounts of immunodetectable p27/Kip1 protein compared to untreated cells ( $N = 4$ ;  $P = 0.04$ ; Fig. 1A). Determination of kinase activity of these complexes showed that Cdk6-associated complexes from 1,25D<sub>3</sub>-treated HL60-G cells had very low kinase activity on either histone H1 or Rb protein substrate, consistent with their increased association with p27/Kip1, but complexes from untreated or 1,25D<sub>3</sub>-resistant HL60 cells showed easily detected levels of this kinase activity (Fig. 2, A and B). Also consistent with this result was the low Rb phosphorylation level of Rb protein in 1,25D<sub>3</sub>-treated cells (Fig. 2C). Surprisingly, however, the effect of 1,25D<sub>3</sub> treatment of HL60 cells on Cdk4 complexes was to increase their kinase activity, as demonstrated using either the Rb protein or histone H1 protein as the substrate (Fig. 3, A and B). This is consistent with the decreased association of p27/Kip1 with the Cdk4 complex and may also be related to its continued stimulation by the D-type cyclins responsive to serum-derived growth factors in the mid-G<sub>1</sub> phase (12). Although we were not able to detect D-type cyclins in Cdk4 immunoprecipitates using antibodies available to use, whole-

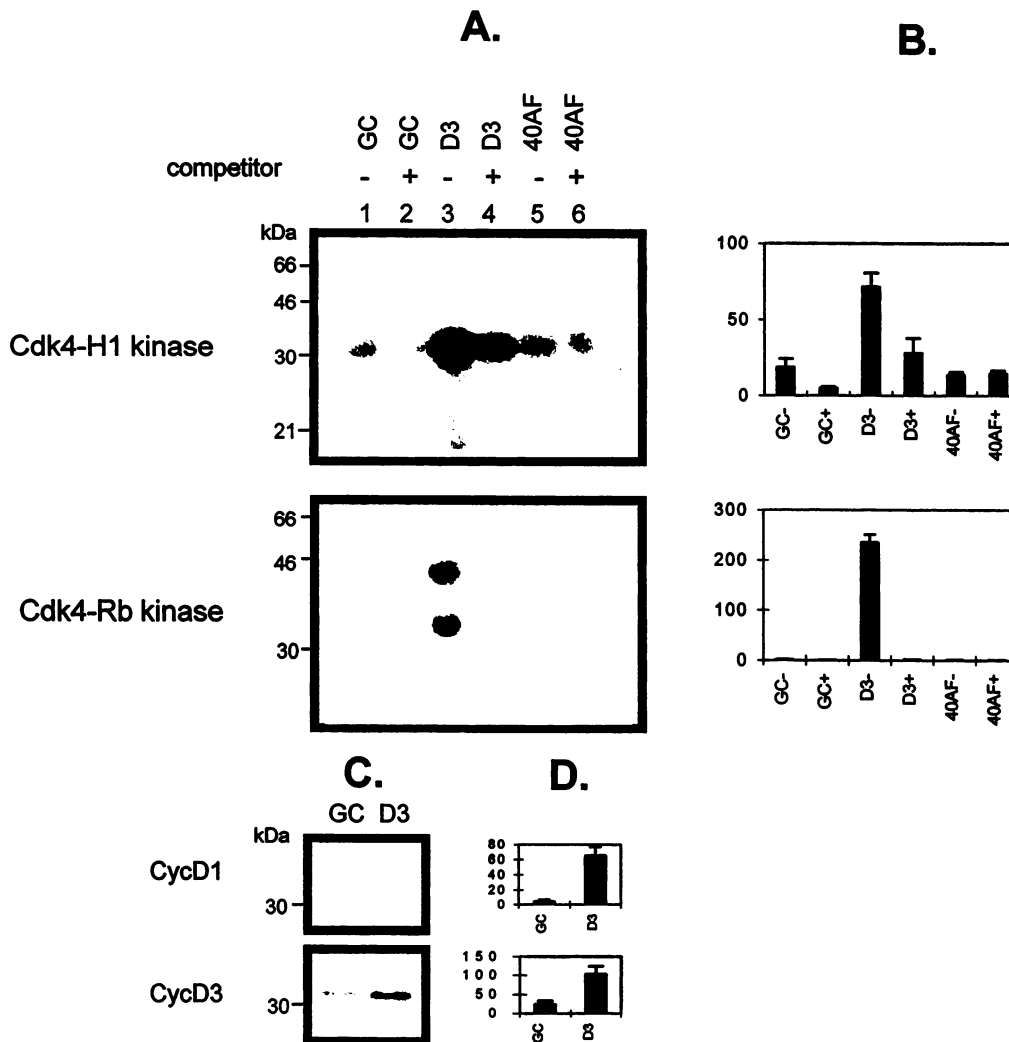


Fig. 3. Cdk4-associated histone H1 and pRb kinase activities and cyclin D content of cell lysates. *A*, aliquots of 500  $\mu$ g of total lysate extracted from HL60-G (GC), 1,25D<sub>3</sub> (D3), and 40AF cells were immunoprecipitated with the anti-Cdk4 antibody (UB1) (Lanes 1, 3, and 5). To demonstrate the specificity of the kinase reaction assay, the anti-Cdk4 antibody was preincubated with the peptide that was used to immunize the animals to produce the antibody (Lanes 2, 4, and 6). Histone H1 and GST-pRb were used as the phosphorylation substrates. *B*, densitometric quantitation of the autoradiograms in arbitrary units. *C*, Western blot for cyclin D1 and cyclin D3 in total lysates of untreated HL60-G cells (GC) or HL60-G cells exposed to 100 nM 1,25D<sub>3</sub> (D3) for 96 h. *D*, densitometric quantitation of autoradiograms shown in *C*, in arbitrary units.

cell lysates showed increased levels of cyclin D1 and cyclin D3 after a 96-h exposure to 100 nM 1,25D<sub>3</sub> (Fig. 3, *C* and *D*), and metabolic labeling with [<sup>35</sup>S]methionine/cysteine demonstrated that protein with electrophoretic mobility of D-type cyclins could be detected in the Cdk4 complex but not in the Cdk6 complex, and treatment with 1,25D<sub>3</sub> increased this association (Fig. 4). Cdk4 and Cdk6 kinase activities are also known to be negatively regulated by p16/INK4A (13). As shown in Fig. 4, p16/INK4A was increased equally in Cdk4 and Cdk6 complexes after treatment with 1,25D<sub>3</sub>, so p16/INK4A cannot be the CKI that causes the opposite changes in Cdk4 and Cdk6 activities. Also, the only *M<sub>r</sub>* 27,000 band detected by this analysis was apparent in the Cdk6 complex immunoprecipitated from 1,25D<sub>3</sub>-treated HL60-G cells, thus confirming further that p27/Kip1 mainly targets Cdk6 in this system (Fig. 4).

Cdk2 is believed to function downstream from Cdk4 and Cdk6 kinases, near the G<sub>1</sub>-to-S phase transition (14). Although the association with p27/Kip1 in extracts of 1,25D<sub>3</sub>-treated 1,25D<sub>3</sub>-sensitive cells was not significantly increased (Fig. 1), the Cdk2 complexes had markedly reduced histone H1 and pRb kinase activities (Fig. 5, *A* and *B*). The reason for this observation was sought by an evaluation of the effects of 1,25D<sub>3</sub> on the levels of cyclin E, which activates the kinase

activities of Cdk2 (15). Fig. 5, *C* and *D*, shows that 1,25D<sub>3</sub> treatment of HL60-G cells resulted in the loss of the *M<sub>r</sub>* 37,000 form of cyclin E, whereas the abundance of the *M<sub>r</sub>* 45,000 form of cyclin E was reduced by the 96-h exposure to 1,25D<sub>3</sub>, which results in G<sub>1</sub> arrest, but not in 40AF cells, which are able to proliferate in the presence of 1,25D<sub>3</sub> (Fig. 1C). This correlated with the reduced histone H1 kinase and pRb kinase activities of Cdk2 complexes (Fig. 5, *A* and *B*) and low phosphorylation level of Rb (Fig. 2C) in cells treated for 96 h with 1,25D<sub>3</sub> and suggested that cyclin E is the limiting factor for Cdk2 activity in 1,25D<sub>3</sub>-treated HL60 cells, although a contribution by p27/Kip1 to this effect cannot be at present excluded. Cyclin A is unlikely to be involved in the 1,25D<sub>3</sub>-induced block in HL60 cells, because its presence is limited to the late S and G<sub>2</sub> phases in these cells.<sup>4</sup>

#### Discussion

The available data suggest the following sequence of events when 1,25D<sub>3</sub> is added to HL60-G cells (Fig. 6). (*a*) Differentiation-related

<sup>4</sup> Q. M. Wang, X. Luo, A. Kheir, F. D. Coffman, and G. P. Studzinski, manuscript in preparation.

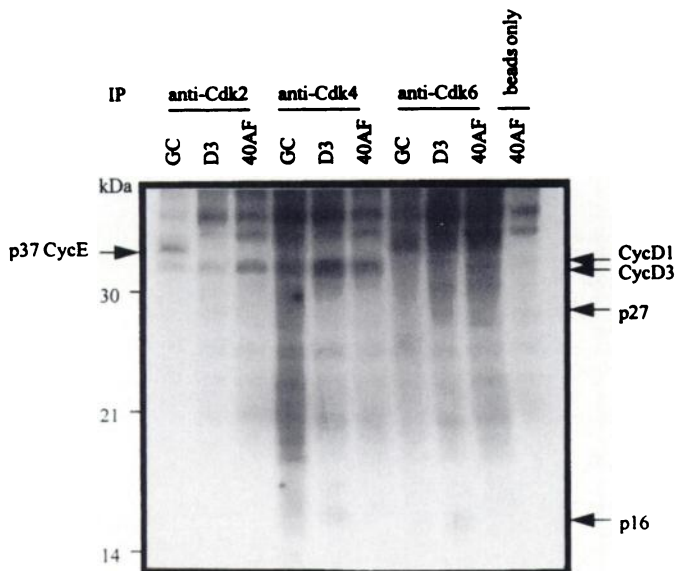


Fig. 4. Metabolic labeling. HL60-G (GC), 1,25D<sub>3</sub> (D3), and 40AF (40AF) cells were labeled by incubation for 4 h with 200  $\mu$ Ci/ml [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine in methionine/cysteine-free RPMI 1640. Total protein lysates prepared from each group were immunoprecipitated with anti-Cdk2, anti-Cdk4, and anti-Cdk6 antibodies. Proteins associated with each antibody were determined using autoradiography. For a negative control, total lysate extracted from 40AF cells was incubated with beads not coated with the antibody.

changes in translation and/or protein degradation machinery (16, 17) result in increased levels of p27/Kip1 protein, which binds to Cdk6 complexes and inhibits phosphorylation of Cdk6-specific sites on the Rb family of proteins. (b) Although the Rb proteins continue to be phosphorylated by Cdk4 (18), this is insufficient to adequately phosphorylate all sites known to be present on the Rb proteins (19), as was confirmed in this system by gel mobility of the Rb protein (Fig. 2C). Consequently, the E2F family transcription factors remain sequestered by the Rb proteins. This does not allow the cell to progress past the cell cycle restriction point believed to represent the up-regulation of cyclin E synthesis (20) probably by the released E2F transcription factors (21). Cyclin E activates Cdk2 to phosphorylate histones in preparation for the S phase and to further phosphorylate the Rb proteins, so that the traverse of the late G<sub>1</sub> phase and the S phase by the cells can continue (22). Therefore, the unavailability of cyclin E in 1,25D<sub>3</sub>-treated HL60-G cells, and possibly somewhat increased association with p27/Kip1, results in low Cdk2 activity and a mid-G<sub>1</sub>-

phase arrest. (c) Because in mid-G<sub>1</sub> phase cells cyclin D genes appear to be responsive to serum-derived growth factors in the culture medium (12), cyclins D1 and D3 continue to be synthesized and activate Cdk4. However, as mentioned above, Cdk4 phosphorylation of Rb appears unable to drive the cells past the restriction point in these cells. This is consistent with the complex role of pRb phosphoryla-

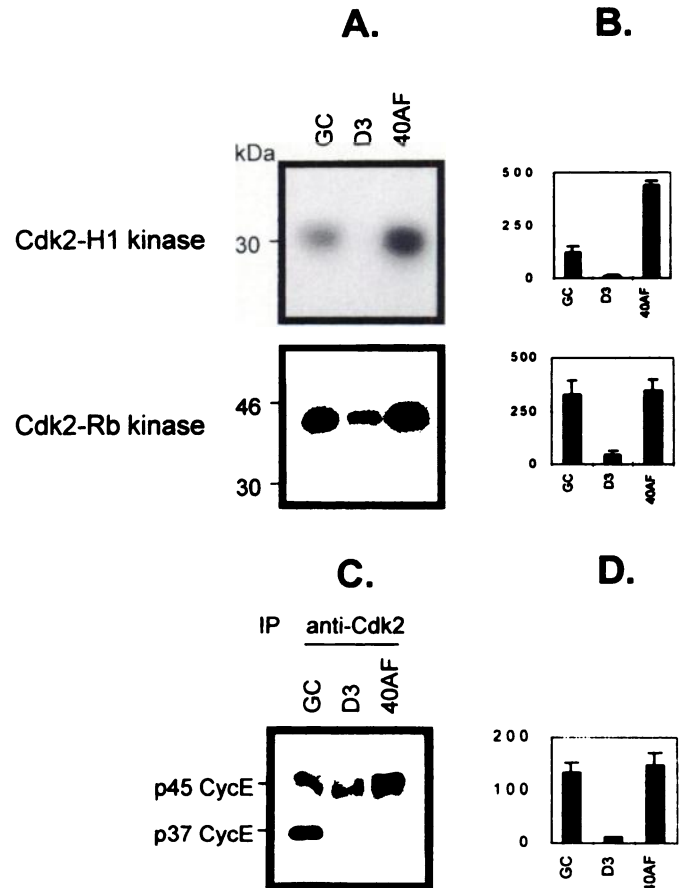
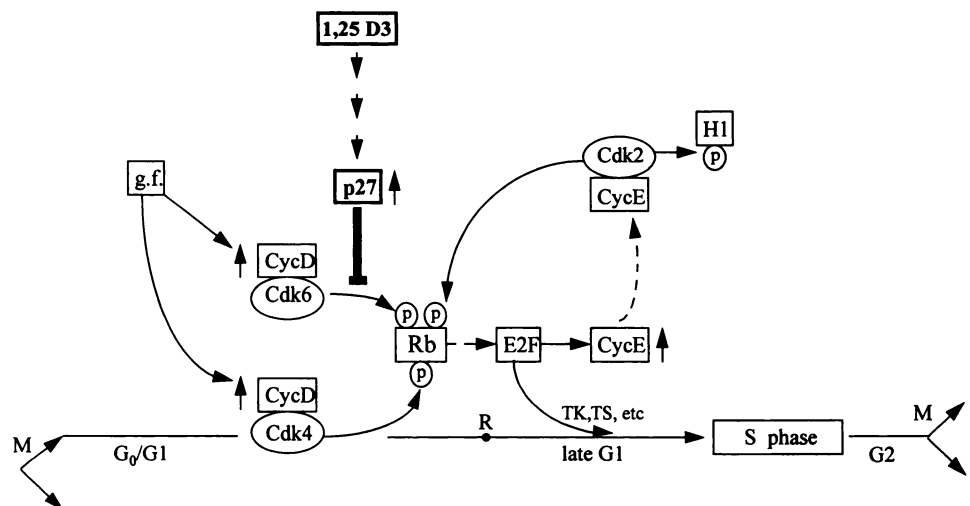


Fig. 5. Cdk2-associated histone H1 and pRb kinase activities and cyclin E content of the Cdk2 complex. A, total protein lysates prepared from HL60-G (GC), 1,25D<sub>3</sub> (D3), and 40AF (40AF) cells were immunoprecipitated with the anti-Cdk2 antibody. Histone H1 and GST-pRb proteins were used as phosphorylation substrates. B, densitometric quantitation of autoradiograms shown in A, in arbitrary units. C, Western blot for Cdk2-associated cyclin E. D, densitometric quantitation of C, in arbitrary units.

Fig. 6. Schematic representation of the suggested effects of 1,25D<sub>3</sub> on the control of the cell cycle in wild-type HL60-G cells.



tions in pRb function (19) and may leave the cell poised to renew the cell cycle traverse when the growth-inhibitory signal is removed (23).

Our experiments suggest a different target for p27/Kip1 mediation of G<sub>1</sub> arrest in HL60 cells than that proposed for the G<sub>1</sub> arrest in the Mv1Lu mink lung epithelial cell-transforming growth factor  $\beta$  system (24). In cycling Mv1Lu cells, p27/Kip1 is sequestered in cyclin D-Cdk4/Cdk6 complexes, allowing the formation of an active cyclin E-Cdk2 complex, but following transforming growth factor  $\beta$  addition, p27/Kip1 is transferred from the cyclin D-Cdk4 complexes to the cyclin E-Cdk2 complexes, thus inhibiting cyclin E-Cdk2 activity (24). Also, p27/Kip1 is known to associate with and/or inactivate cyclin E-Cdk2 complex in quiescent but not proliferating murine fibroblasts (25), murine T lymphocytes (26), human vascular smooth muscle cells (27), and human pre-B lymphoblasts (28). In contrast, p27/Kip1 was increased in a human gastric carcinoma cell line treated with IFN- $\beta$  and was found to be bound preferentially to Cdk6 (29). It will be interesting to find out whether there are agents other than 1,25D<sub>3</sub> and IFN- $\beta$  that preferentially target p27/Kip1 to Cdk6.

Our results show that Cdk4 and Cdk6 functions are not redundant in HL60 cells and add weight to the previous evidence that p27/Kip1 is an important regulator of transitions between quiescence and the cell cycle traverse (25). They also imply that in HL60 cells, Cdk6 is a major target for the 1,25D<sub>3</sub>-induced p27/Kip1 protein and that an elevated Cdk4 activity is insufficient to allow the completion of the G<sub>1</sub> phase traverse. This specificity in the signaling pathway for growth arrest should aid the design of more effective antileukemic agents.

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## Cyclin-dependent Kinase 6 Is the Principal Target of p27/Kip1 Regulation of the G<sub>1</sub>-phase Traverse in 1,25-Dihydroxyvitamin D<sub>3</sub>-treated HL60 Cells

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