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1,25-Dihydroxyvitamin D_3-Induced Retardation of the G_2/M Traverse Is Associated With Decreased Levels of $p34^{cdc2}$ in HL60 Cells

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Abstract Cellular differentiation of neoplastic cells after exposure to 1,25-dihydroxyvitamin D_3 (1,25 D_3) is accompanied by altered cell cycle regulation. In previous studies, blocks in both G_1/S and G_2/M checkpoints have been observed in 1,25D_3-treated HL60 cells, but the mechanism of the 1,25D_3-induced G_2/M block has not been previously reported. In this study, we show by cell cycle analysis, using bromodeoxyuridine pulse-chase labeling, that the G_2/M block in 1,25D_3-treated HL60 cells is incomplete. We also demonstrate that although the 1,25D_3-treated cells exhibit elevated levels of cyclin B1, Cdc25C, and Cdk7, which are positive regulators of the G_2/M traverse, these cells have decreased protein levels of $p34^{cdc2}$ and decreased $p34^{cdc2}$ kinase activity. This provides potential mechanisms for the observed accumulation of cells in the G_2 cell cycle compartment and occasional polyploidization following treatment of HL60 cells with 1,25D_3. The data also suggest that the ability of some cells to traverse this block may be the result of cellular compensatory mechanisms responding to decreased $p34^{cdc2}$ activity by increasing the levels of other regulators of the G_2 traverse, such as cyclin B1, Cdc25C, and Cdk7. J. Cell. Biochem. 75:226–234, 1999.

Key words: HL60; 1,25-dihydroxyvitamin D_3; cell cycle checkpoint; $p34^{cdc2}$; cdc25C

Exposure of several types of neoplastic cells in culture to 1,25-dihydroxyvitamin D_3 (1,25 D_3), results in their differentiation, and recent studies have demonstrated that the differentiation-associated anti-proliferative effects are related to cell cycle regulation [Wang et al., 1998]. An extensively studied system for evaluating the effects of 1,25D_3 on differentiation and cell cycle control has been the HL60 human myeloblastic leukemia cell line. Exposure of HL60 cells to 1,25D_3 results in cellular differentiation evidenced by morphologic changes and the acquisition of markers characteristic of mature monocytes/macrophages [Studzinski et al., 1993]. In addition to inducing differentiation, 1,25D_3 affects the cell cycle traverse with the most apparent block during the G_1 phase of the cell cycle. The G_1 block is associated with decreased activity of Cdk2 and reduced levels of cyclin E in the kinase complex. In addition, Cdk6 activity is also inhibited, which is most likely mediated by an up-regulation of p27/Kip1 and increased binding of this protein to the kinase complexes [Wang et al., 1996, 1997], although a role for p21/Cip1 has also been suggested [Liu et al., 1996]. Interestingly, the inhibition of proliferation of HL60 cells by 1,25D_3 is accompanied not only by increased accumulation of cells in the G_1 compartment, but also in the G_2/M compartment. Although the absolute numbers of cells arrested in G_2 are lower than of the cells arrested in G_1, the percentage increases are actually similar in G_1 and in G_2 [Godyn et al., 1994; Zhang et al., 1996]. However, in contrast to several studies addressing the mechanism of the G_1 cell cycle block [Wang et al., 1996, 1997], the mechanisms responsible for the G_2 block have not been reported.

Recently there has been increased interest in the control of the G_2 traverse [e.g., Niculescu et al., 1998]. As reviewed by Nurse [1990], the central regulation of the transition from G_2 to mitosis is through the activation of a complex

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consisting of the $p34^{cdc2}$ protein kinase and its regulatory subunit, cyclin B1. In general, the activity of this complex (initially called maturation or mitosis promoting factor, or MPF) is governed by $p34^{cdc2}$-cyclin B1 association and by the phosphorylation status of $p34^{cdc2}$. Activation of the $p34^{cdc2}$-cyclinB1 complex is controlled by several G_2/M specific kinase/phosphatase cell cycle regulatory proteins. Cdc25C is a protein phosphatase that cleaves the inhibitory phosphate from the tyrosine 15 and threonine 14 of $p34^{cdc2}$. Conversely, wee-1 is a protein kinase that phosphorylates these same sites and thus acts as an inhibitor of G_2/M traverse. An activating kinase that is known to phosphorylate the threonine 161 on $p34^{cdc2}$ is cyclin activating kinase (CAK). CAK is a multi subunit enzyme composed of cyclin H (regulatory subunit) and Cdk7 (catalytic subunit) [Fisher and Morgan, 1994; Nurse, 1990].

We investigated the effects of 1,25D_3 on the regulatory proteins of the G_2/M traverse in the HL60 cell model. We demonstrate that although 1,25D_3-treated cells contain elevated levels of cyclin B1, Cdc25C, and Cdk 7, which are positive regulators of the G_2/M traverse, these cells have decreased levels of $p34^{cdc2}$ and exhibit a decrease in $p34^{cdc2}$-cyclin B1 kinase activity. This markedly decreased level and activity of $p34^{cdc2}$ provides a potential mechanism for the accumulation of 1,25D_3-treated HL60 cells in the G_2 cell cycle compartment. The incomplete nature of the block at the G_2/M checkpoint may be the result of cellular compensation by increased levels of the positive regulators of $p34^{cdc2}$ activity, which include cyclin B1, Cdc25C, and Cdk 7.

MATERIALS AND METHODS

Materials

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). 1,25D_3 was a kind gift from Dr. Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

HL60 cells were maintained in RPMI 1640 supplemented with 10% complement-inactivated bovine calf serum. Cells were seeded at 3×10^5 cells/ml and treated with 100 nM 1,25 D_3 for 0 (control) or 96 h. Cells were harvested and their viability checked by Trypan blue (0.25%) exclusion.

Flow Cytometry

For DNA content evaluation, 3×10^6 cells were fixed in 75% ethanol at −20°C for 24 h. After washing in ice-cold phosphate-buffered saline (PBS), the cells were incubated with 0.5 ml of propidium iodide stain (10 μg/ml) in the presence of RNase (500 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 (Phoenix Flow Systems, San Diego, CA).

BrdU Incorporation

A total of 10^6 cells were pulsed with bromodeoxyuridine (BrdU) at a final concentration of 10 μM for 45 min in a 4% CO_2 incubator at 37°C, resuspended in fresh medium and cultured for an additional 3, 6, or 24 h. The cells were then centrifuged and fixed in 75% ethanol at −20°C overnight. To produce the single-stranded DNA, the cells were incubated with 2 N HCl/Triton X-100 for 30 min at 25°C, and the cell suspension was neutralized using 0.1 M sodium tetraborate. The cells were incubated with FITC-conjugated anti-BrdU antibody (Becton Dickinson, San Jose, CA) for 30 min, and resuspended in propidium iodide (final concentration: 5 μg/ml). Fluorescence intensity was analyzed on a FACScan flow cytometer (Becton Dickinson).

Immunoblotting

Whole cell extracts were prepared by lysing cell pellets with a Dounce microtip homogenizer in lysis buffer (20 mM Tris-HCl [pH7.5], 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.2 mM NaVO4, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml pepstatin A). Cell debris was removed by centrifugation at 14,000*g* for 20 min at 4°C, and the supernatants were stored at −80°C. Phosphatase digestion was performed by incubating cell lysates in lysis buffer (in the absence of $NaVO_4$) with 2 U of alkaline phosphatase (Sigma) at 37°C for 15 min.

Samples for immunoblotting were prepared by mixing aliquots of the protein extracts with 3× sodium dodecyl sulfate (SDS) sample buffer

(150 mM Tris [pH 6.8], 30% glycerol, 3% SDS, bromophenol blue dye 1.5 µg/dl, 100 mM DTT) and denatured by heating to 100°C for 4 min. Protein samples were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL), and incubated in 5% nonfat milk blocking buffer (Tris-buffered saline, 5% dry milk, and 0.05% Tween-20) for 1 h. The membrane was subjected to immunoblot analysis with an appropriate antibody and proteins were visualized by the enhanced chemiluminescence (ECL) method of detection (Amersham).

Immunoprecipitation

Cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM NaVO4, 1 mM DTT, 1 mM PMSF, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 25 µg/ml pepstatin A) and cell debris removed by centrifugation at 14,000*g* for 20 min at 4°C. A total of 500 µg of total protein was incubated with anti-p34^{cdc2} antibody 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 for 5 min, and resolved by 13% SDS-PAGE.

Kinase Reaction Assays

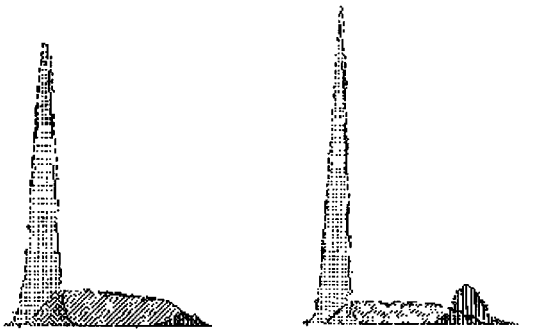
Total cellular lysates were prepared and p34^{cdc2} was immunoprecipitated as described above. As a control to establish specificity, a blocking peptide to the C-terminus of the p34^{cdc2} antibody (Santa Cruz, 954-P) was first incubated the p34^{cdc2} antibody for 1 h before immunoprecipitation. The agarose beads were washed three times with immunoprecipitation buffer and subsequently washed three times with kinase reaction buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, 0.2 mM $NaVO_4$, 1 mM PMSF 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/ml[γ-^{32}P] ATP (spec act: 3,000 Ci/mmol) and 2 µg of histone H1. The reaction was stopped by adding 3× SDS sample buffer. After boiling for 5 min and centrifugation at 3,000*g* for 2 min, the supernatant was resolved on a 13% SDS-PAGE gel. The radioactivity of the ^{32}P-labeled histone was detected by autoradiography.

RESULTS

Effects of 1,25D$_3$ on G$_2$/M Traverse of HL60 Cells

The effects of 1, 25D$_3$ on cell cycle traverse were analyzed by flow cytometry of propidium iodide-stained HL60 cells (Fig. 1). As previously reported [Godyn et al., 1994], a marked G_1/S block was apparent, with an increase of the proportion of cells in the G_1 phase and a decrease in the proportion of cells in the S phase compartment in cells treated with 100 mM 1,25D$_3$ for 96 h. An increase of cells in the G_2/M compartment was also detected in the 1,25D$_3$-treated group, and was proportionally greater than in the G_1 compartment (Fig. 1).

To investigate whether this G_2/M block was partial or complete, pulse-chase labeling with BrdU was performed. In Figure 2, the cell populations in panels A, B, and C at 0 h represent BrdU-positive cells in early S, mid S, and late S phases, respectively; panel D represents cells in G_2/M; and panel E, cells in G_1. After chasing BrdU- labeled control cell populations for 3 h in medium not containing BrdU, cell density in panel 2A decreased with increases in panels 2B,C, indicating continued cell cycle traverse. As cells continued through the cell cycle, repopulation of panel 2A with BrdU-labeled cells was



	G_1	S	G_2-M
Control	59%	38%	3%
1,25D$_3$	79%	11%	10%

Fig. 1. Effect of 1,25D$_3$ on cell cycle distribution of HL60 cells. Example of the effect of 1,25D$_3$ on cell cycle distribution of HL60 cells as determined by DNA content. HL60 cells were treated with 10^{-7} M 1,25D$_3$ for 96 h **(Right panel)**. G_1/S and G_2/M blocks are evident after treatment. This experiment was repeated 3 times with essentially the same results.

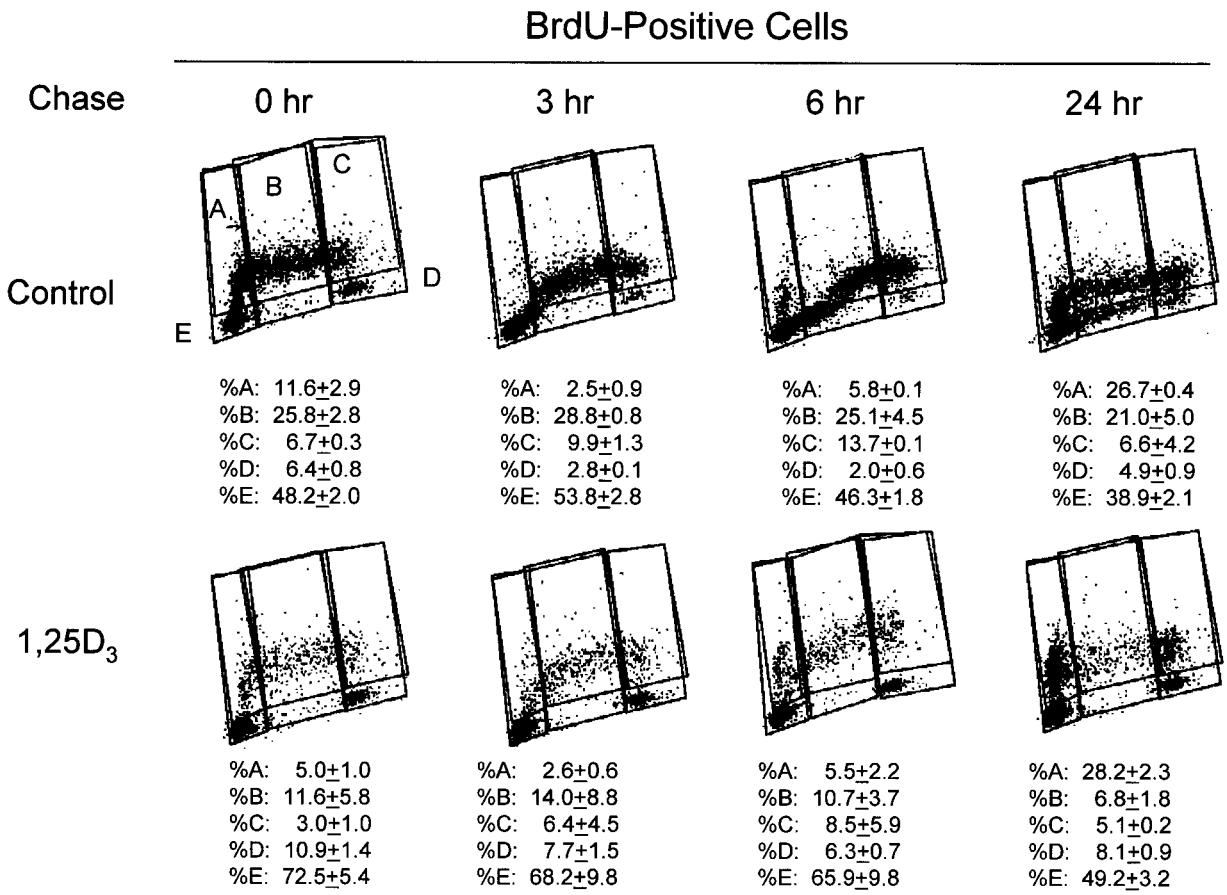


Fig. 2. Pulse-chase BrdU labeling: HL60 cells treated for 96 h with 10^{-7} M 1,25D$_3$ or equivalent vehicle were pulsed with bromodeoxyuridine (BrdU), washed, and cultured for an additional 3, 6, or 24 h (chase). **A,B,C:** Cell populations represent BrdU-positive cells in early S, mid S, and late S phases, respectively. **D,E:** Cells in G$_2$/M and G$_1$, respectively. As cells continue through the cell cycle, re-population of **A** with BrdU-labeled cells is seen as early as 6 h and peaks at 24 h, representing cellular traverse of G$_2$/M and re-entry into the next cell cycle. Despite a G$_2$/M block with 1,25D$_3$, re-population of **A** at 6 and 24 h after pulse suggests an incomplete G$_2$/M arrest. Mean values and standard deviations are shown (n = 3).

seen as early as 6 h and increased further at 24 h. This represents cellular traverse of G$_2$/M and re-entry into the next cell cycle. After treatment for 96 h with 10^{-7} M 1,25D$_3$, the total number of cells that incorporated BrdU was decreased (percentage S phase, 0 h: 44.1 ± 6.8 control cells vs 19.6 ± 3.8 1,25D$_3$-treated cells, $P < 0.001$). While the proportion of S phase cells in the 1,25D$_3$-treated group was greatly reduced, the proportion of cells in the G$_2$/M compartment was significantly increased (percentage G$_2$/M, 0 h: 6.4 ± 0.8 control cells vs 10.9 ± 1.4 1,25D$_3$-treated cells, $P < 0.05$), showing that the rate of exit from G$_2$/M was reduced in the treated cells. Despite this 1,25D$_3$-induced G$_2$/M block, we observed a re-population of panel A at 6 and 24 h after the BrdU pulse, indicating that the G$_2$/M arrest is incomplete (Fig. 2).

Effects of 1,25D$_3$ on p34^{cdc2}

To initiate a study of the mechanisms of the partial G$_1$/M block, the protein levels and kinase activity of p34^{cdc2} were examined. Western blot analysis for p34^{cdc2} of cell lysates from untreated cells revealed a major band at 34kD, and a minor band at 38 kD (Fig. 3A). This more slowly migrating band has been reported to represent the phosphorylated form of p34^{cdc2} [Draetta et al., 1989]; this was confirmed by alkaline phosphatase treatment of the lysates [data not shown). Treatment of HL60 cells with 10^{-7} M 1,25D$_3$ for 96 h resulted in decreased protein levels of both bands of p34^{cdc2}, but particularly of the upper band (Fig. 3A). Reprobing the same membrane with the antibody for calreticulin, a protein unaffected by 1,25D$_3$, showed

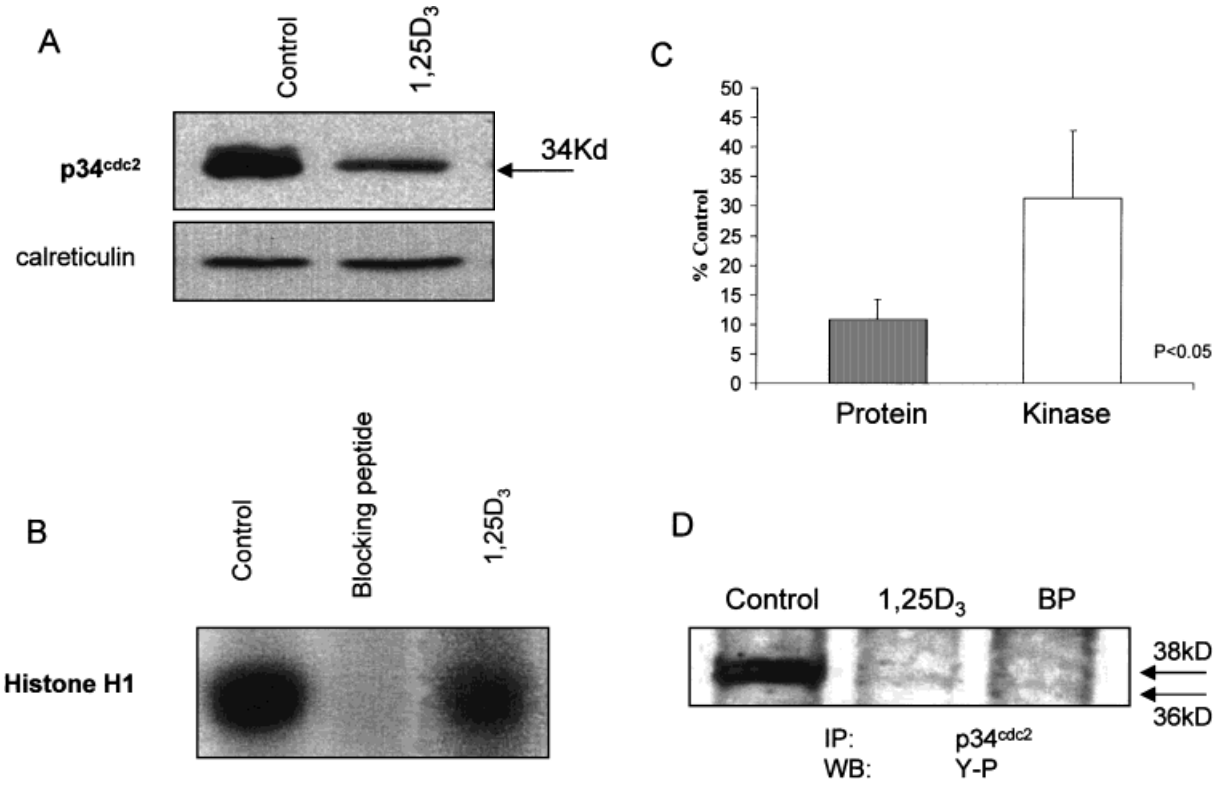


Fig. 3. Effect of $1,25D_3$ on $p34^{cdc2}$. **A:** Immunoblot analysis of HL60 cells for $p34^{cdc2}$ demonstrates a major band at 34 kD and minor band at 38 kD. Treatment of HL60 cells with 10^{-7} M $1,25D_3$ for 96 h resulted in approximately a 10-fold decrease in $p34^{cdc2}$ protein level. Reprobing the membrane with the antibody for calreticulin, a protein unaffected by $1,25D_3$, shows equal loading. **B:** Using histone H1 as substrate, $p34^{cdc2}$ kinase activity was significantly decreased after exposure to $1,25D_3$ ($n = 3$, $P < 0.05$). To demonstrate the specificity of the kinase reaction assay, the anti $p34^{cdc2}$ antibody was preincubated with blocking peptide to $p34^{cdc2}$ **(lane 2)**. **C:** Densitometric quantitation of $p34^{cdc2}$ protein levels and kinase activity in three experiments were performed and expressed as percentage of control. The mean decrease in protein level of $p34^{cdc2}$ after exposure to $1,25D_3$ was significantly greater than the mean decrease in $p34^{cdc2}$ kinase activity ($P < 0.05$). **D:** Cell lysates from control and $1,25D_3$-treated cells were immunoprecipitated for $p34^{cdc2}$, with subsequent immunoblotting with a phosphotyrosine-specific antibody. While control cells demonstrated a baseline level of tyrosine phosphorylation of $p34^{cdc2}$, exposure to $1,25D_3$ markedly decreased the levels of tyrosine-phosphorylated $p34^{cdc2}$.

equal loading. Immunoprecipitated $p34^{cdc2}$ was analyzed for kinase activity using histone H1 as a substrate. As expected, $1,25D_3$-treated cells exhibited markedly decreased kinase activity compared to untreated controls, though to a lesser extent than the observed decrease in total protein levels (Fig. 3B,C).

Because tyrosine phosphorylation is an important inhibitory mechanism for regulation of $p34^{cdc2}$ activity, increased tyrosine phosphorylation could explain the reduced $p34^{cdc2}$ kinase activity. Therefore, immunoblot analysis with anti-phosphotyrosine antibody of $p34^{cdc2}$ immunoprecipitates of HL60 cells was performed. This experiment demonstrated that $p34^{cdc2}$ from control cell lysates manifested a baseline level of tyrosine phosphorylation, which rather surprisingly became undetectable after $1,25D_3$ treatment (Fig. 3D). These data indicate that the decrease in $p34^{cdc2}$ activity is not the result of increased inhibitory tyrosine phosphorylation, but most likely due to the observed decrease in total $p34^{cdc2}$ protein levels.

Effects of $1,25D_3$ on the Regulators of $p34^{cdc2}$ Activity and the G_2/M Traverse

The effects of $1,25D_3$ on protein levels of the regulators of $p34^{cdc2}$ activity, and therefore the G_2/M traverse, were also studied. Figure 4 shows that cyclin B1 protein levels were increased after treatment of HL60 cells with 10^{-7} M $1,25D_3$ for 96 h. A similar evaluation by immunoblot analysis of the G_2/M-specific kinase/phosphatase cell cycle regulatory proteins, wee-1 and Cdc25C, demonstrated no change in wee-1 protein levels after treatment with

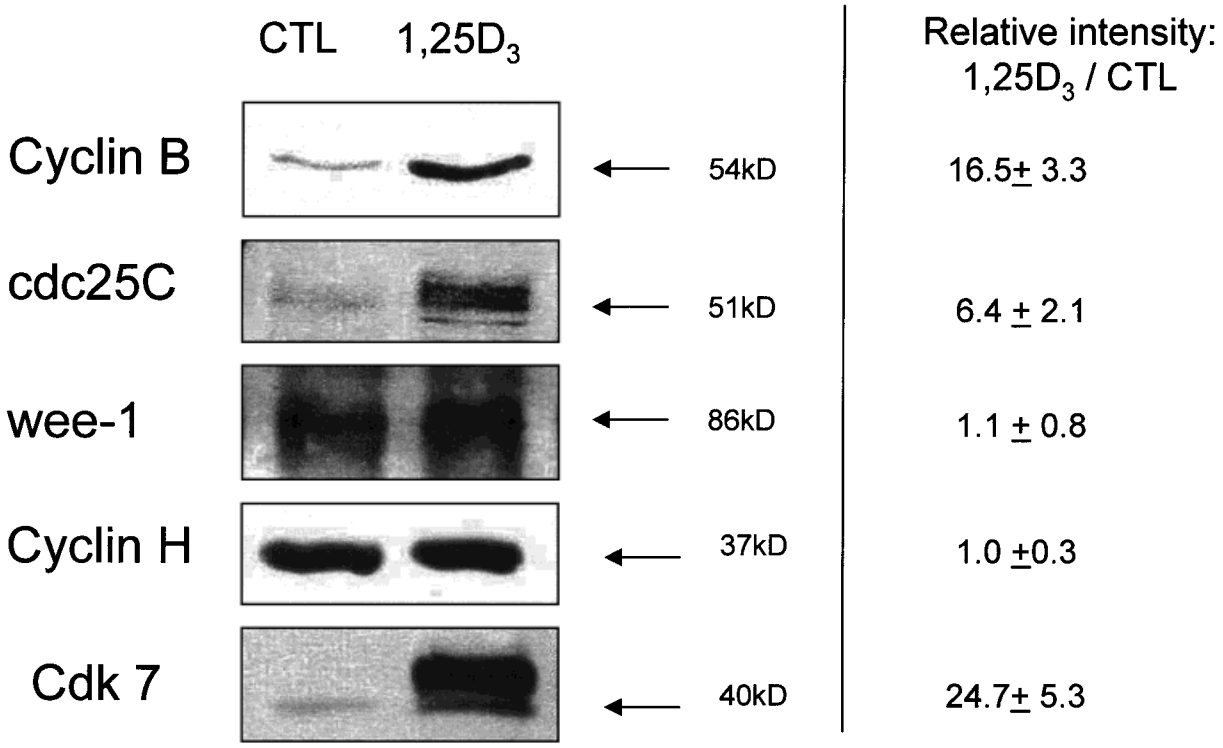


Fig. 4. Immunoblot blot analysis of the regulatory proteins of G_2/M traverse. HL60 cells were treated with 10^{-7} M 1,25D_3 for 96 h. 1,25D_3 increased cyclin B1, Cdc25C, and Cdk 7 protein levels, but protein levels of wee-1 and cyclin H were unaffected by 1,25D_3 treatment.

1,25D_3, but protein levels of Cdc25C, an activating phosphatase, were elevated in response to 1,25D_3 treatment.

An activating kinase known to phosphorylate threonine 161 in $p34^{cdc2}$ is CAK, a multisubunit enzyme composed of cyclin H and Cdk7. While cyclin H level was unchanged after 1,25D_3 exposure, the protein levels of Cdk7 were dramatically increased (Fig. 4).

DISCUSSION

Entry into the M phase of all eukaryotic cells depends on activation of the universal MPF, a complex of the protein kinase $p34^{cdc2}$ and its regulatory subunit, cyclin B1. While activation of this complex requires binding of the two subunits, the activity is also regulated by activating and inhibitory phosphorylations and activating dephosphorylations [Nurse, 1990]. For instance, an important aspect of regulation of the activity of the mitotic complex is through the activating phosphatase Cdc25C and the inhibitory kinase, wee-1. Cdc25C promotes traverse through G_2/M by cleaving the phosphate groups on the tyrosine 15 and threonine 14 residues of $p34^{cdc2}$, while the activity of wee-1 is inhibitory by phosphorylation of these same sites [Heald et al., 1993]. In addition, activation of this complex requires phosphorylation of the threonine 161 residue of $p34^{cdc2}$ by CAK [Fisher and Morgan, 1994].

Our data demonstrate that the G_2/M block observed with 1,25D_3 treatment of HL60 cells is incomplete and is associated with decreased kinase activity of $p34^{cdc2}$, presumably due to a decrease in the total protein levels of $p34^{cdc2}$ (Fig. 3). While a similar decrease of $p34^{cdc2}$ kinase activity has been associated with other models of chemical or radiation-induced G_2 arrest, the decreased activity in those studies has been attributed to the accumulation of tyrosine-phosphorylated $p34^{cdc2}$ [Lock and Ross, 1990]. After exposure of HL60 cells to 1,25D_3, we did not observe an increase in tyrosine phosphorylation, but rather a substantial decrease. We postulate that this decrease in tyrosine phosphorylation of the remaining $p34^{cdc2}$ represents cellular compensation as a result of a negative feedback loop, and may explain the incomplete nature of the G_2/M block in this system. This is supported by the observation that the decrease in $p34^{cdc2}$ protein levels was approximately 10-

fold after $1,25D_3$ exposure, whereas the decrease in kinase activity was only about threefold (Fig. 3C).

The 96-h treatment with $1,25D_3$ resulted in redistribution of HL60 cells within the cell cycle compartments (Figs. 1, 2). There was an increase in the proportion of cells in the both G_1 and G_2 compartments, and a marked decrease in the S phase compartment. Thus, it is formally possible to argue that the reduced $p34^{cdc2}$ protein content, or its activity, could be a result of this redistribution. This is unlikely for several reasons. First, only the S phase compartment is reduced following exposure to $1,25D_3$ (Figs. 1, 2), a cell cycle phase with which $p34^{cdc2}$ has no known association. Second, the $1,25D_3$-treated cells exhibit elevated content of G_2-specific components, such as cyclin B1 (Fig. 4), indicating that the biochemical changes characteristic of the G_2 block are detectable in this system.

In an attempt to determine the mechanism of the observed decrease in $p34^{cdc2}$ tyrosine phosphorylation after $1,25D_3$ exposure, protein levels of wee-1 and Cdc25C were measured. The decreased tyrosine phosphorylation of $p34^{cdc2}$ in our system could not be explained by decreased protein levels of wee-1, since immunoblot analysis demonstrated no change in these levels after treatment with $1,25D_3$. Since wee-1 activity is regulated by both protein amount and the level of inactivating phosphorylations [Watanabe et al., 1995], it is possible that that the activity of wee-1 was decreased following $1,25D_3$ exposure in the absence of overall protein changes. While this is possible, we did not observe any obvious phosphorylation bands related to wee-1 during immunoblot analysis of $1,25D_3$-treated cellular lysates. Conversely, we did observe that the protein levels of Cdc25C were markedly increased after $1,25D_3$ treatment. These findings are in contrast to studies in which G_2 arrest was evaluated following radiation or treatment with epidermal growth factor and TPA, where $p34^{cdc2}$ kinase inhibition was primarily associated with a decrease in the phosphatase activity of Cdc25C [Barth et al., 1996a,b]. The increase in Cdc25C in our experiments may represent a mechanism by which the depleted pool of $p34^{cdc2}$ is activated in an attempt to overcome the $1,25D_3$-induced G_2/M block, and this permits an eventual escape of some of the cells from this block (Fig. 5).

For cells to traverse the G_2/M, activated $p34^{cdc2}$ and cyclin B1 need to form a complex. Normally, cyclin B1 begins to accumulate in the S phase, reaches a maximum at mitosis, and is rapidly degraded at the metaphase-anaphase transition [Nurse, 1990]. We observed that protein levels of cyclin B1 were increased following $1,25D_3$ treatment. Similar increases in cyclin B1 have been reported after drug-induced cell cycle arrest [Datta et al., 1996]. While this may also represent an attempt to overcome G_2/M block, other factors may be responsible for the increased cyclin B1 protein levels. It has been reported that exposure of HL60 cells to $1,25D_3$ increases the proportion of binucleated cells and increases the proportion of cells in the G_2/M compartment [Godyn et al., 1994; Zhang et al., 1996] and, since G_2/M cells have high cyclin B1 content [Steinman et al., 1991], this may in part explain the increased cyclin B1 protein levels observed after $1,25D_3$ treatment. Other evidence is derived from $1,25D_3$-resistant HL60 cell lines, which have a tetraploid karyotype [Wajchman et al., 1996] and express high levels of cyclin B1 protein (L.E. Harrison and G.P. Studzinski, unpublished observation).

A third potential mechanism of overcoming the $1,25D_3$-induced G_2/M block may be related to the markedly increased levels of Cdk7 (the catalytic subunit of CAK) after $1,25D_3$ exposure. CAK is an activating kinase, which functions in both G_1/S and G_2/M by phosphorylating the $p34^{cdc2}$ threonine 161 and the Cdk2 threonine 160 [Fisher and Morgan, 1994].

The proposed cellular adaptations after the onset of G_2/M block induced by $1,25D_3$ exposure (Fig. 5) may result in one of three alternative outcomes (Fig. 6). One possible outcome is that the inability of the cell to overcome the $1,25D_3$-induced G_2/M block results in the cellular demise. A second outcome may be a complete rescue of the block through the increased activity of the remaining $p34^{cdc2}$ because of elevated levels of Cdc25C, cyclin B, and Cdk7. Finally, an additional outcome may be that a rescue pathway allows a cell to bypass mitosis and to reenter the cell cycle at a higher ploidy level. In this scenario, the presence of elevated cyclin D1 protein levels and Cdk4 activity following $1,25D_3$ exposure [Wang et al., 1996] permits DNA replication in $1,25D_3$-treated cells. Although eventually some of these cells become arrested in late G_1 due to elevated levels of p27Kip1 [Wang et al., 1997, 1998], some cells

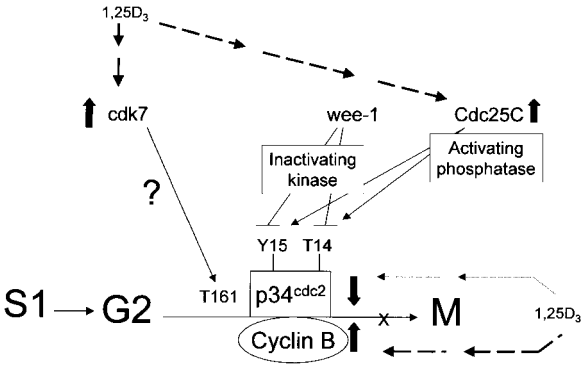


Fig. 5. Schematic representation of the suggested effects of 1,25D_3 on the G_2/M traverse. 1,25D_3 exposure resulted in a decrease in $p34^{cdc2}$ protein level. In an attempt to overcome the decreased pool of $p34^{cdc2}$, increased levels of cdk7, cyclin B1, and Cdc25C may activate the remaining $p34^{cdc2}$.

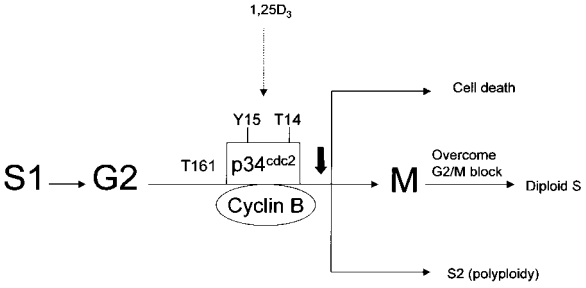


Fig. 6. Alternative outcomes. (1) Inability of cells to compensate results in cell death. (2) Rescue mechanisms include activation of remaining $p34^{cdc2}$ through cellular adaptation with completion of mitosis and re-entry into the cell cycle. (3) DNA re-replication is a possible third pathway, leading to polyploidization.

may continue to proliferate. This third pathway may offer a rationale for the increased polyploidy seen after long-term exposure of HL60 cells to 1,25D_3 [Wajchman et al., 1996]. The acquisition of polyploid DNA content [Godyn et al., 1994; Zhang et al., 1996] and the marked reduction in $p34^{cdc2}$ protein levels and activity noted in our system have also been observed in megakaryocytic differentiation of HEL cells [Yokoe et al., 1997]. This suggests that a potential mechanism for the observed accumulation of HL60 cells in the G_2 cell cycle compartment after exposure to 1,25D_3 is through decreased protein levels of $p34^{cdc2}$. The incomplete nature of this block appears to be due to an upregulation of the activating regulatory proteins of $p34^{cdc2}$. A rescue pathway through DNA re-replication in some cells offers a rationale for the observed polyploidization of HL60 cells after 1,25D_3 exposure.

In conclusion, this report demonstrates the novel and unexpected finding that a partial G_2/M block occurs in the presence of upregulation by 1,25D_3 of several activators of the G_2/M traverse. This partial G_2/M block can be explained by markedly reduced levels of the kinase that controls this phase of the cell cycle, $p34^{cdc2}$.

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