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Activation of Extracellular Signal-Regulated Kinases (ERKs) Defines the First Phase of 1,25-Dihydroxyvitamin D_3-Induced Differentiation of HL60 Cells

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Abstract Activation of ERK1 and ERK2 protein kinases has been implicated in diverse cellular processes, including the control of cell proliferation and cell differentiation (Marshall [1995] Cell 80:179). In human myeloblastoid leukemia HL60 cells rapid (ca. 15 min) but transient activation of ERK1/2 has been reported following induction of macrophage/monocyte differentiation by phorbol esters, or by very high (10^{-6} M) concentrations of 1,25-dihydroxyvitamin D_3 ($1,25D_3$), while retinoic acid-induced granulocytic differentiation was accompanied by sustained activation of ERK1/2. We report here that monocytic differentiation of HL60 cells induced by moderate (10^{-9} to 10^{-7} M) concentrations of $1,25D_3$ could be divided into at least two stages. In the first phase, which lasts 24–48 h, the cells continued in the normal cell cycle while expressing markers of monocytic phenotype, such as CD14. In the next phase the onset of G1 cell cycle block became apparent and expression of CD11b was prominent, indicating a more mature myeloid phenotype. The first phase was characterized by high levels of ERKs activated by phosphorylation, and these decreased as the cells entered the second phase, while the levels of p27/Kip1 increased at that time. Serum-starved or PD98059-treated HL60 cells had reduced growth rate and slower differentiation, but the G1 block also coincided with decreased levels of activated ERK1/2. The data suggest that the MEK/ERK pathway maintains cell proliferation during $1,25D_3$-induced monocytic differentiation of HL60 cells, but that ERK1/2 activity becomes suppressed during the later stages of differentiation, and the consequent G1 block leads to ''terminal'' differentiation. J. Cell. Biochem. 80:471–482, 2001.

Key words: differentiation; signal transduction; vitamin D; leukemic cells; cell cycle; p27/Kip1

Cancer cells characteristically are poorly differentiated, but have growth advantages over untransformed cells. It seems therefore reasonable to assume that an understanding of the mechanisms that regulate the delicate balance between proliferation and differentiation should provide insights which can guide the development of new strategies for cancer chemotherapy. The MEK/ERK module offers a potential mechanism for such regulation, since it has been shown to be important for control of both proliferation and differentiation. For instance, constitutively active MEK has been shown to reduce growth factor requirement and transform fibroblasts [Brunet et al., 1994; Mansour et al., 1994], and indeed its activation was found to be both necessary and sufficient for fibroblast transformation [Cowley et al., 1994]. Also, extracellular signal-regulated kinases (ERKs) function and its translocation to the nucleus have been clearly implicated in the activation of growth factor-responsive transcription factors [Hill et al., 1993; Blenis, 1993; Frost et al., 1994].

On the other hand, numerous studies demonstrated the dependence of mammalian cell differentiation on the ERK pathway. Activation of MEK is necessary and sufficient for neuronal differentiation of rat pheochromocytoma PC12 cells [Cowley et al., 1994], and interference with its enzyme activity by the chemical inhibitor PD98059 blocks this differentiation [Pang et al., 1995]. Other models of cell differentiation in which the requirement for ERK

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pathway activation appears to have been well established include lineage commitment in thymocytes [Alberola-Ila et al., 1995; Crompton et al., 1996], and differentiation of hematopoietic stem cells towards the megakaryocytic lineage where constitutive expression of activated MEK needs to be sustained [Racke et al., 1997], but is sufficient to initiate this differentiation program [Whalen et al., 1997]. It has been suggested that the duration of ERK activation determines whether proliferation or differentiation is the outcome of the signal provided to the cell, with sustained activation resulting in differentiation [Traverse et al., 1992; Marshall, 1995].

The proliferation–differentiation switch can be studied with relative ease using leukemia cells in suspension culture. For instance, HL60 cells, derived from human acute myeloblastic leukemia with promyelocytic features [Gallagher et al., 1979], can be induced to the macrophage phenotype using TPA [Rovera et al., 1982], to the monocytic phenotype using $1,25D_3$ [McCarthy et al., 1983], and to granulocyte-like cells by retinoic acid [Breitman and He, 1990]. In all these instances changes in MEK/ERK pathway have been reported, but a consistent pattern of activation has not emerged. A myelin basic protein and c-jun Y peptide phosphorylating activity ("MAP kinase") was found to be rapidly increased following exposure of HL60 cells to TPA [Kharbanda et al., 1994]. Similarly, exposure of HL60 cells to very high (10^{-6} M) concentrations of $1,25D_3$ [Marcinkowska et al., 1997], and of human promyelocytic leukemia cells NB4 to moderate (10^{-8} M) concentrations of $1,25D_3$, also produced rapid stimulation of ERK activity [Song et al., 1998]. However, in contrast to other differentiation systems, in these three differentiation systems the activation of ERK was reported to be transient, declining to basal levels in 15–30 min. On the other hand, studies of HL60 cell differentiation by Yen et al. [1998] showed that retinoic acid-induced granulocytic differentiation is accompanied by sustained ERK2 activation.

To establish if the duration of ERK activation characterizes the lineage of differentiation, monocyte/macrophage vs granulocyte, we investigated the relationship of the MEK/ERK pathway to monocytic differentiation and to the cell cycle block using moderate concentrations (10^{-9} to 10^{-7} M) of $1,25D_3$, and detected ERK activation by reactivity with antibodies specific for phosphorylated ERKs. The results show that in this system ERK activation is prolonged, approximately 24–48 h in duration depending on the concentration of $1,25D_3$, but is not sustained, since levels of activated ERKs decline when the onset of a cell cycle block and later stages of maturation become apparent.

MATERIALS AND METHODS

Chemicals and Antibodies

$1,25D_3$ was provided by Dr. Milan Uskokovic, Hoffmann-LaRoche, Nutley, NJ. It was dissolved in absolute ethanol at 2.5×10^{-4} M. PD98059 was purchased from New England BioLabs (Beverly, MA). It was dissolved in DMSO as stock at 50 mM. The antibodies used in the experiments were MEK-1 (c-18, rabbit polyclonal antibody), ERK1/2 (k-23, rabbit polyclonal antibody), cyclin D1 (A-12, mouse monoclonal antibody), and p90RSK (c-21, rabbit polyclonal antibody, Santa Cruz, Santa Cruz, CA). The antibodies used to detect phosphorylated MEK1/2 and ERK1/2 (rabbit polyclonal antibodies) were purchased from New England BioLabs. The antibody used to detect p27/kip1 (FL, mouse monoclonal) was purchased from Pharmingen, San Diego, CA, and anti-calreticulin rabbit polyclonal antibody from Affinity Bioreagents, Golden, CO, was used as a loading control for Western blotting.

Tissue Culture

HL60-G cells [Studzinski et al., 1991], a subclone of human promyelocytic leukemia cells [Gallagher et al., 1979], were grown in the suspension culture at 37°C in RPMI 1640 medium (Mediatech, Washington, DC) with 1% glutamine and 10% heat-inactivated, defined, iron-supplemented bovine calf serum (Hyclone, Logan, UT). The HL60-G cells were passaged and fed 2–3 times weekly to maintain log phase growth. The vitamin D resistant HL60-40AF cells [Studzinski et al., 1996] were split and fed with medium containing 4×10^{-8} M $1,25D_3$ every two days. For all experiments, the HL60 cells were suspended at 3×10^5 cells/ml of fresh medium, at which time $1,25D_3$ was added. The MEK1 inhibitor PD98059 was added for various times at a final concentration of 20 μM for 1 h before exposure to the stated concentrations of $1,25D_3$. In the serum starvation experiment, HL60 cells were

deprived of serum for 24 h before setting up the experiments. Cell viability was determined by trypan blue (0.25%) using a Neubauer hemocytometer. Each experiment was repeated at least three times.

Determination of Markers of Differentiation

Aliquots of 1×10^6 cells were harvested at indicated times, washed twice with PBS, then incubated for 45 min with 0.5 μl MY4-RD1 and 0.5 μl MOl-FITC (Coulter, Miami, FL) each containing 0.5 μg of the antibody, at room temperature to analyze the expression of surface cell markers CD14 and CD11b, respectively. After the incubation, the cells were washed three times with ice-cold $1 \times$ PBS. The cells were then resuspended in 0.5 ml $1 \times$ PBS and two parameter analysis was performed using an Epics Profile II instrument (Coulter Electronics, Hialeah, FL). Isotypic mouse Ig G1 was used to set threshold parameters.

Cell Cycle Distribution

The details of the procedures have been described before [Studzinski et al., 1996]. Briefly, aliquots of 1×10^6 cells were washed twice with ice-cold $1 \times$ PBS. The cell fixed in 75% ethanol at −20°C overnight, then washed twice with PBS, and incubated with 100 U/ml of RNase (BMB, Indianapolis, IN) at 37°C for 1 h. The cell pellet was again washed twice with PBS and resuspended in 1 ml propidium iodide solution (PI, 10 μg/ml, Sigma). The cells were analyzed using an Epics Profile II instrument (Coulter) and the raw data of cell cycle distribution were analyzed by the Multicycle Software Program (Phoenix Flow Systems, San Diego, CA). Debris, doublets, and aggregates were gated out using a bit map surrounding the dispersed cell population.

Cell Extracts and Western Blotting

Western blotting was performed using either whole cell, cytoplasmic or nuclear extracts, as indicated. Whole cell extracts for Western blotting were prepared by homogenizing frozen cell pellets with a lysis buffer (20 mM Tris–HCl, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA (pH 7.5), 1 mM PMSF and 0.02% leupeptin) and equal amount of $3 \times$ SDS sample buffer (150 mM Tris, 30% glycerol, 3% SDS, 1.5 mg/ml bromophenol blue dye, 100 mM dithiothreitol) was added to each sample. Nuclear extracts were prepared by the procedure described before [Andrews and Faller, 1991]. Briefly, 2×10^7 cells were harvested and washed twice with ice-cold PBS, and resuspended in 0.2 ml ice-cold cell extract buffer (10 mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethysulfonyl fluoride (PMSF). The cells were kept on ice for 10 min to allow them to swell, vortexed for 10 s, and microfuged at 4°C at 16,000*g* for 30 s. The supernatant was saved as the cytoplasmic extract, and the pellet was resuspended in 30 μl of nuclear extraction buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF), placed on ice for 20 min, and centrifuged at 4°C at 16,000*g* for 2 min. The supernatant was saved as the nuclear extract and stored at −80°C.

The proteins in 40 μg of whole cell extracts were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked with 5% milk in TBS/0.1% Tween-20 for 1 h, subsequently blotted with primary antibodies, then the membranes were blotted with a horseradish-linked secondary antibody for 1 h. The protein bands were visualized with a chemiluminescence assay system (Amersham). The protein loading of the gel and efficiency of the transfer were controlled by stripping the membrane and reprobing for calreticulin, a constitutively expressed protein in these cells. The optical density (OD) of each band was quantitated using an image quantitator (Molecular Dynamics, Sunnyvale, CA).

Statistical Methods

All experiments were repeated for a minimum of three times. The effects of PD98059 on $1,25D_3$-induced expression of CD14 and CD11b were assessed by one-way analysis of variance. Significance of differences between mean values was assessed by two-tailed student's *t* test. All computations were performed with an IBM-compatible personal computer using Microsoft EXCEL.

RESULTS

Rapid Activation of ERK1/2 by 10^{-7} M $1,25D_3$ is Prolonged, but not Sustained in HL60 Cells

Time course of activation of ERK1 and ERK2 (ERK1/2) was determined using an antibody

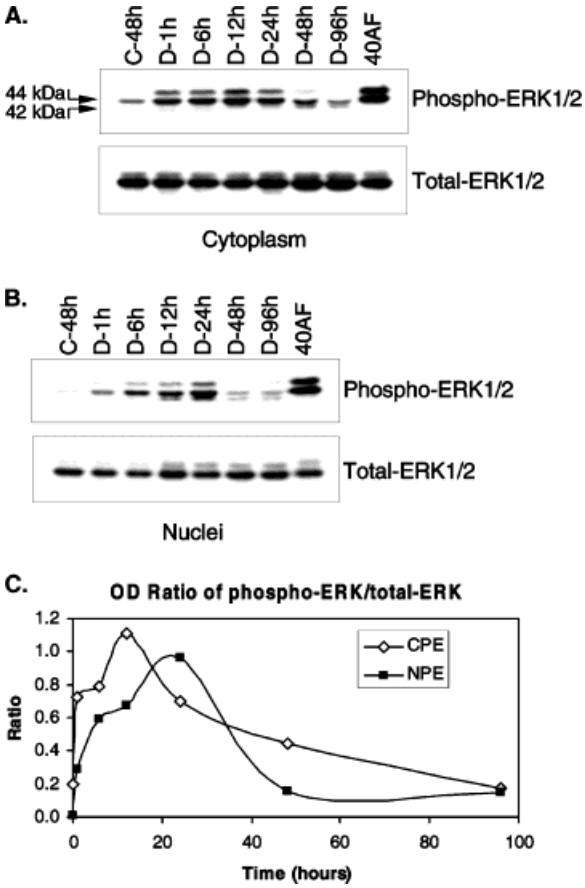

Fig. 1. Immunoblotting analysis of protein levels of phospho-ERK1/2 and total-ERK1/2 in cytoplasmic (**A**) and nuclear (**B**) fractions following exposure of HL60 cells to $1,25D_3$ (10^{-7} M) for the indicated times. 40 AF is a $1,25D_3$-resistant subline of HL60 cells, and was continuously exposed to 40 nM $1,25D_3$. Optical density (OD) of the protein signals was determined by Fluorimage with Image Quant software (Molecular Dynamics, Sunnyvale, CA). The ratios of optical densities of phospho-ERK to total ERK were plotted versus time in the presence of $1,25D_3$ (**C**). (◇) CPE = OD ratio of cytoplasmic phospho-ERK1/2 to total ERK1/2, (■) NPE = OD ratio of nuclear phospho-ERK1/2 to total ERK1/2. This experiment was repeated twice more, with similar results.

specific for phosphorylated ERK1/2 [Yung et al., 1997]. In the initial experiments the levels of phospho-ERK1/2 were studied separately in the cytoplasmic and nuclear fractions, and compared with the total protein levels of ERK1/2 (Fig. 1). It is apparent that ERK2 is the predominant member of the ERK family expressed in HL60 cells, and is more abundant in the cytoplasm than in the nucleus. In the absence of $1,25D_3$ little activated ERK1 or ERK2 was detected in the nuclear faction but some was evident in the cytosol. Within 1 h of the addition of 10^{-7} M $1,25D_3$ the levels of phospho-ERK1/2 increased, and the increase continued in both the cytosol and the nuclei, but with kinetics which suggested cytosol to nucleus translocation, characteristic of ERK activation [Kharbanda et al., 1994; Marcinkowska et al., 1997; Song et al., 1995]. Unlike the previously described prolonged activation of ERKs during differentiation of several other cell systems [e.g., Racke et al., 1997; Yen et al., 1998; Qui and Green 1992], after 24 h in the presence of 10^{-7} M $1,25D_3$ the levels of phospho-ERK1/2 began to decrease and to approach basal levels (Fig. 1C). The increase and waning of phospho-ERK levels followed similar kinetics in the subsequent two experiments, though the magnitude of the increases and the precise timing showed some variability.

Monocytic differentiation in this series of experiments was followed by determining the presence of two surface markers of myeloid differentiation. The surface receptor CD14, characteristic of the monocytic phenotype, showed marked up-regulation at 12 h after addition of $1,25D_3$, when phospho-ERK 1/2 levels were still high, while CD11b, a more general myeloid cell marker expressed by both granulocytic and monocytic lineages, showed a major increase at 48 h, when nuclear phospho-ERK1/2 levels were near basal levels (Table I). Determination of cell cycle phase transitions by flow cytometry showed that $1,25D_3$-induced G1 block also became pronounced at 48 h, indicating that in this system phospho-ERK1/2 is present only during the phase of differentiation in which there is no or only a minimal G1 block. This was further supported by the presence of high levels of phospho-ERK1/2 in extracts of $1,25D_3$-resistant 40 AF cells, which continue to proliferate in the presence of high concentrations of $1,25D_3$ [Wajchman et al., 1996], and show no G1 block (Table I).

These effects of $1,25D_3$ were compared with the effects of 10^{-6} M all-trans retinoic acid (RA). As previously reported [Yen et al., 1998], RA induced an increase in the levels of phospho-ERK1/2 which was sustained during the 96-h period of observation in this experiment, while in $1,25D_3$-treated cells phospho-ERK1/2 levels decreased below control levels at 48 h (Fig. 2). Interestingly, the levels of cyclin D1, one of the down-stream targets of the ERK cascade [Weber et al., 1997; Baldin et al., 1993], increased early and continued to increase in $1,25D_3$-treated but not in RA-treated cells (Fig.

TABLE I. Effects of 1,25D_3 on Differentiation Markers CD14 and CD11b, and the Cell Cycle Traverse[a]

	Differentiation markers (%)		Cell cycle distribution (%)		
Treatment	CD14	CD11b	G1	S	G2
Control	3.2 ± 2.6	0.3 ± 0.2	52.8 ± 2.1	37.7 ± 1.9	9.5 ± 0.5
D-1H	6.1 ± 3.1	4.8 ± 2.5	53.2 ± 1.9	37.0 ± 1.5	9.8 ± 1.1
D-6H	9.5 ± 1.9	7.1 ± 3.9	53.9 ± 3.6	37.5 ± 2.0	8.6 ± 1.6
D-12H	52.0 ± 11.2	13.5 ± 2.9	54.3 ± 2.2	37.3 ± 1.1	8.4 ± 1.1
D-24H	79.0 ± 6.8	20.5 ± 6.1	57.0 ± 3.5	34.5 ± 2.0	8.5 ± 1.5
D-48H	98.1 ± 2.6	52.9 ± 7.7	64.1 ± 4.0	23.8 ± 2.5	12.1 ± 1.9
D-96H	98.7 ± 1.9	58.6 ± 10.1	77.4 ± 4.9	12.9 ± 2.0	9.7 ± 2.1
40 AF	1.4 ± 1.2	2.3 ± 0.8	45.6 ± 3.5	40.3 ± 1.9	4.1 ± 1.5

[a]HL60 cells were treated with 1,25D_3 (D, 10^{-7} M) at indicated times, followed by staining with anti-CD11b-FITC and anti-CD14-RD1 or with propidium iodide, then the percentages of CD11b and CD14 positive cells, and the cell cycle distribution were determined by flow cytometry. The 40 AF subclone was grown continuously in the presence of 40 nM 1,25D_3, to which it is resistant. Mean values ± standard deviation were obtained from three experiments.

2). Thus, although there are some similarities, there are also major differences in regulatory gene expression in these two models of differentiation.

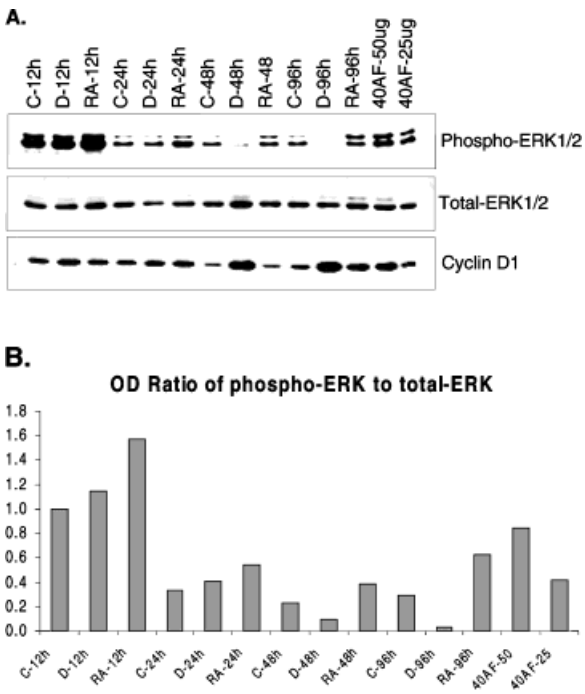

Fig. 2. Comparison of the effects of 1,25D_3 and retinoic acid on activation by phosphorylation of ERK1/2, and on protein levels of the ERKs and cyclin D1. HL60 cells were treated with either 1,25D_3 (10^{-7} M) or retinoic acid (RA, 10^{-6} M) for the indicated times. (**A**) Western blots of whole cell extracts are shown. Different amounts of 40 AF lysates were loaded in the gel in order to check the linearity of the detection procedure. (**B**) The ratios of OD of phospho-ERK to total ERK were plotted versus time in the presence of either 1,25D_3 or retinoic acid. The experiment illustrates three similar results.

Similar to the experiments illustrated in Figure 1, in the early phase of 1,25D_3-induced differentiation (the first 24 h) the CD14 marker showed high levels of expression, but CD11b expression and G1 block were noted only after 48 h of exposure to 1,25D_3 (Table II), when phospho-ERK 1/2 levels were low (Fig. 2). In contrast, RA-induced differentiation markers and the G1 block became appreciable only at 96 h, when phospho-ERK1/2 levels were well above the control values. Thus, only in the 1,25D_3 differentiation system does activated ERK1/2 help to define the early proliferative phase of differentiation, while the absence of phospho-ERK1/2 indicates onset of "terminal" 1,25D_3-induced differentiation.

1,25D_3 Induces Reduced Levels of Phospho-ERK1/2 and G1 Block in Serum-Starved HL60 Cells with Normal Kinetics, but the Appearance of Differentiation Markers is Delayed

To facilitate the interpretation of studies of ERK activation these experiments are usually performed on serum-starved cells, which have low levels of phospho-ERKs as the result of the starvation. We have therefore repeated the experiments on HL60 cells grown in serum-free medium for 24 h. The viability of the cells was not altered after this period of starvation (data not shown). Surprisingly, the basal levels of phospho-ERK1/2 were increased in the starved cells, as was the abundance of p90/RSK, a reported downstream target of ERKs in some cells [Frodin and Gammeltoft, 1999]. This is shown in Figure 3, where equal loading of the gel is demonstrated by a non-specific band in

TABLE II. Comparison of the Effects of 1,25D$_3$ and RA on Differentiation Markers CD11b and CD14, and on the Cell Cycle Traverse[a]

	Differentiation markers (%)		Cell cycle distribution (%)		
Treatment	CD14	CD11b	G1	S	G2
C-12h	1.3 ± 0.5	0.3 ± 0.1	58.0 ± 2.5	32.8 ± 1.6	9.2 ± 0.9
D-12h	69.3 ± 9.5	3.8 ± 1.4	58.5 ± 2.0	31.8 ± 1.5	9.7 ± 0.8
RA-12h	0.5 ± 0.2	0.7 ± 0.4	55.7 ± 2.1	34.0 ± 2.0	10.3 ± 1.3
C-24h	1.0 ± 0.6	0.7 ± 0.5	53.6 ± 3.2	38.2 ± 2.8	8.2 ± 1.4
D-24h	78.0 ± 12.4	15.1 ± 3.1	54.6 ± 2.4	35.3 ± 1.4	10.1 ± 1.0
RA-24h	0.9 ± 0.6	3.3 ± 2.0	56.1 ± 1.8	35.5 ± 1.0	8.4 ± 0.8
C-48h	2.5 ± 1.6	0.3 ± 0.2	56.7 ± 4.1	35.1 ± 2.9	8.2 ± 1.2
D-48h	96.3 ± 3.4	37.4 ± 7.3	70.4 ± 4.2	18.5 ± 2.1	11.1 ± 2.0
RA-48h	6.5 ± 2.1	5.9 ± 4.1	61.1 ± 1.9	29.5 ± 1.2	9.4 ± 0.7
C-96h	2.4 ± 1.8	0.4 ± 0.2	53.6 ± 1.6	38.3 ± 0.9	8.1 ± 0.7
D-96h	96.5 ± 3.3	40.7 ± 10.9	80.5 ± 4.4	9.8 ± 3.1	9.7 ± 1.4
RA-96h	9.5 ± 3.8	9.7 ± 4.4	68.5 ± 2.8	21.8 ± 2.0	9.7 ± 1.3
40 AF	2.3 ± 1.1	0.6 ± 0.3	48.4 ± 3.2	45.1 ± 1.9	6.5 ± 1.3

[a]HL60 cells were treated with either 1,25D$_3$ (D, 10^{-7} M) or retinoic acid (RA, 10^{-6} M) at indicated times, followed by staining with anti-CD11b-FITC and anti-CD14-ReD1 or with propidium iodide, then percentage of CD11b and CD14 positive cells, and the cell cycle distribution were determined by flow cytometry. Mean values ± standard deviation were obtained from three experiments. C = control, untreated cells.

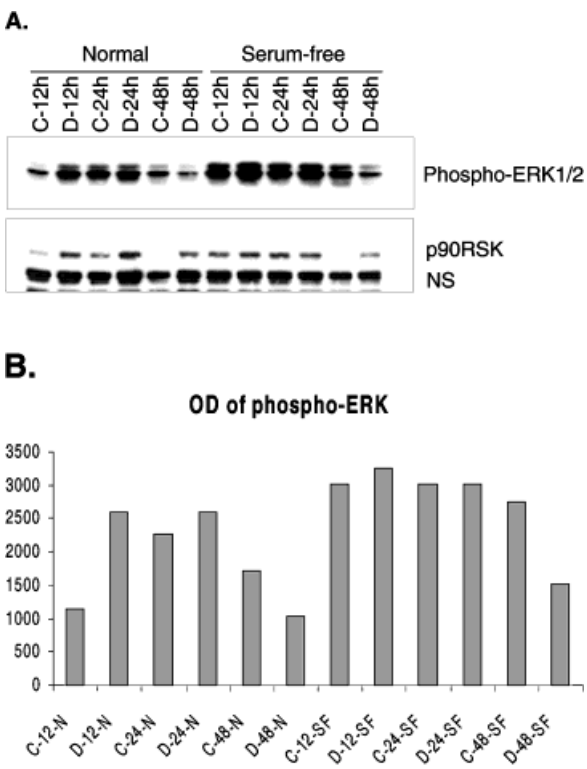

Fig. 3. Effects of 1,25D$_3$ on phospho-ERK1/2 and p90RSK levels under different culture conditions. HL60 cells were cultured either under normal conditions or in serum-free for medium for 24 h then treated with 1,25D$_3$ (10^{-7} M) for the indicated times. (**A**) Western blotting of whole cell extracts was performed. NS: A non-specific band demonstrating similar loading of the gel. (**B**) The optical densities of phospho-ERK shown in arbitrary units. Similar results were observed in subsequent two experiments.

the lower panel. Addition of 10^{-7} M 1,25D$_3$ to these cells produced a slight increase in phospho-ERK1/2 levels, followed by a decrease at 48 h, i.e., the kinetics of the 1,25D$_3$ effect on ERK1/2 activation were not altered by the absence of serum.

The proliferation of serum starved cells was reduced, as shown by the higher, though constant, proportion of G1 cells in the starved cultures—there were 50% G1 cells in the control, but approximately 68% G1 cells in the starved cultures (Table III). Nonetheless, the G1 block (79% in G1) induced by 1,25D$_3$, which in longer experiments was progressive, was observed in the starved cultures, and again coincided with the decrease in phospho-ERK1/2 levels at 48 h of exposure to 1,25D$_3$. In contrast, serum starvation blocked the expression of CD11b during this period of observation and markedly retarded the expression of CD14 (Table III) serum-starved cultures. Thus, ERK1/2 activities inversely correlate with the anti-proliferative effects of 1,25D$_3$, but do not correlate with the effect of 1,25D$_3$ on differentiation.

MEK Inhibitor PD98059 Produces a G1 Block and Retards the Appearance of Differentiation Markers

The kinase inhibitor PD98059 is considered to be specific for MEK, and as a consequence to selectively inhibit ERK activation [Pang et al.,

TABLE III. Comparison of the Effects of 1,25D_3 on Monocytic Differentiation Markers and Cell Cycle Traverse of HL60 Cells Under Different Cell Culture Conditions

	Differentiation markers (%)		Cell cycle distribution (%)		
Treatment	CD14	CD11b	G1	S	G2
C-12-N[a]	1.8 ± 0.2	0.4 ± 0.3	50.2 ± 3.1	37.9 ± 1.1	11.9 ± 2.0
D-12-N	44.3 ± 9.8	4.5 ± 3.2	49.8 ± 4.2	37.8 ± 2.9	12.4 ± 1.3
C-24-N	2.1 ± 1.5	1.1 ± 0.5	50.9 ± 3.8	38.1 ± 2.8	11.0 ± 1.0
D-24-N	56.5 ± 12.9	13.7 ± 6.2	52.7 ± 1.8	38.5 ± 0.9	8.7 ± 1.1
C-48-N	1.7 ± 1.0	0.6 ± 0.3	51.7 ± 2.4	37.4 ± 1.4	10.9 ± 1.0
D-48-N	80.5 ± 12.6	35.6 ± 4.9	61.1 ± 4.2	28.7 ± 3.1	10.2 ± 1.1
C-12-SF[b]	1.5 ± 1.0	0.3 ± 0.1	67.9 ± 1.2	24.0 ± 0.8	8.1 ± 0.5
D-12-SF	12.2 ± 6.2	0.8 ± 0.4	68.5 ± 2.8	21.5 ± 1.8	10.0 ± 1.2
C-24-SF	2.2 ± 2.0	0.8 ± 0.5	68.5 ± 3.2	23.1 ± 2.0	8.4 ± 1.2
D-24-SF	24.5 ± 6.9	0.9 ± 0.6	71.6 ± 4.9	19.5 ± 2.9	8.9 ± 2.0
C-48-SF	2.1 ± 1.2	0.4 ± 0.2	67.9 ± 3.3	24.2 ± 1.9	7.9 ± 1.4
D-48-SF	57.3 ± 8.7	1.1 ± 0.8	79.4 ± 5.6	12.5 ± 2.8	8.1 ± 3.0

[a]HL60 cells were treated with 1,25D_3 (D, 10^{-7} M) at the indicated times under normal culture conditions, followed by staining with anti-CD11b-FITC and anti-CD14-RD1 or PI, then the monocytic differentiation markers and cell cycle distribution were determined by flow cytometry. N = normal tissue culture conditions, 10% bovine calf serum in RPMI 1640 medium.
[b]HL60 cells were cultured in serum-free RPMI 1640 medium starting 24 h before setting up the serum-starvation experiments. SF = serum-free conditions. C = control, untreated cells. Mean values ± standard deviation were obtained from three experiments.

1995; Alessi et al., 1995]. As expected, the addition of PD98059 1 h prior to exposure to 1,25D_3 markedly reduced the levels of phospho-ERK1/2 and their down-stream targets cyclin D1 and p90/RSK (Fig. 4). The inhibitor administered alone also reduced the ambient levels of ERK1/2, and of their targets (Fig. 4), and perhaps as a consequence produced increased protein levels of p27/Kip1 (Fig. 4) and a prolongation of the G1 phase, evidenced by an increased proportion of cells in the G1 compartment (Table IV). While PD98059 alone did not induce differentiation markers, it did retard the appearance of 1,25D_3-induced CD14 and CD11b positive cells (Table IV), as well as the intensity of the expression of these markers (data not shown). Thus, both serum starvation and inhibition of MEK activity reduce the rates of cell proliferation and inhibit differentiation. In contrast, ERK1/2 is highly activated by phosphorylation in 1,25D_3-resistant 40 AF cells (Figs. 1 and 4), consistent with their rapid rate of proliferation [Studzinski et al., 1997]. Together, these data strongly suggest that ERK1/2 activation is associated with proliferation but not with differentiation of HL60 cells.

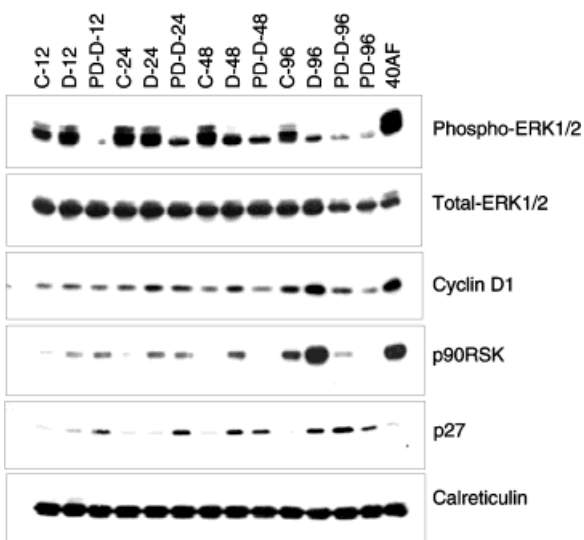

Fig. 4. The effects of MEK inhibitor PD98059 on 1,25D_3-induced activation of ERK1/2 and its downstream targets. HL60 cells were pretreated with 20 μM PD98059 for 1 h before exposure to 1,25D_3 (10^{-7} M). Western blotting was performed on whole cell extracts. An extract of 40 AF cells, which have an active MAPK pathway, was used as a positive control and to allow comparison of signal intensity between different blots. Loading was controlled by reprobing the membrane for calreticulin.

In all experiments presented here the decrease in 1,25D_3-induced ERK activation coincided with the 1,25D_3-induced prolongation of G1 phase. To determine if this relationship is maintained when the induction of the differentiation process is prolonged by using a low concentration of 1,25D_3, we performed another series of experiments using 10^{-9} M 1,25D_3. As shown in Figure 5, activation of ERK pathway

TABLE IV. Effects of MEK Inhibitor PD98059 on 1,25D$_3$-Induced Expression of Differentiation Markers CD11b and CD14 and the Cell Cycle Traverse[a]

	Differentiation markers (%)		Cell cycle distribution (%)		
Treatment	CD14	CD11b	G1	S	G2
C-12	1.7 ± 0.4	0.4 ± 0.2	56.7 ± 3.1	34.1 ± 2.8	9.2 ± 1.0
D-12	40.7 ± 4.4	2.5 ± 0.8	56.2 ± 3.5	33.8 ± 2.0	10.0 ± 1.5
PD-D-12	20.8 ± 3.2	0.8 ± 0.7	69.5 ± 4.2	20.8 ± 3.2	9.7 ± 1.1
C-24	2.3 ± 0.3	0.4 ± 0.2	56.3 ± 1.9	33.3 ± 1.7	10.4 ± 1.1
D-24	55.8 ± 6.7	10.8 ± 6.8	58.5 ± 3.3	32.9 ± 2.8	8.6 ± 0.6
PD-D-24	33.6 ± 3.6	4.5 ± 2.9	73.5 ± 4.8	18.7 ± 2.8	7.8 ± 2.0
C-48	1.6 ± 0.5	0.6 ± 0.2	54.5 ± 2.3	36.8 ± 1.1	8.7 ± 2.0
D-48	86.4 ± 6.9	31.5 ± 3.6	59.8 ± 3.5	31.5 ± 2.0	8.7 ± 1.5
PD-D-48	63.5 ± 4.5	13.9 ± 5.6	79.6 ± 5.4	15.4 ± 2.4	5.0 ± 3.0
C-96	4.0 ± 2.1	0.4 ± 0.3	53.2 ± 2.6	37.6 ± 1.4	9.2 ± 1.2
D-96	95.1 ± 3.2	64.0 ± 7.2	68.3 ± 5.1	22.7 ± 3.8	9.0 ± 1.4
PD-D-96	69.0 ± 4.3	27.7 ± 6.3	72.9 ± 4.8	16.5 ± 2.7	10.6 ± 2.1
PD-96	1.3 ± 0.4	0.3 ± 0.2	68.5 ± 3.1	25.5 ± 1.4	6.0 ± 2.1

[a]HL60 cells were treated with 1,25D$_3$ (D, 10^{-7} M) in the presence or absence of 20 μM PD98059 (PD) at the indicated times. The differentiation markers and cell cycle distribution were determined by flow cytometry. C = control, untreated cells. Mean values ± standard deviation were obtained from three experiments. One way analysis of variance showed that PD98059 significantly retarded 1,25D$_3$-induced expression of CD14 and CD11b.

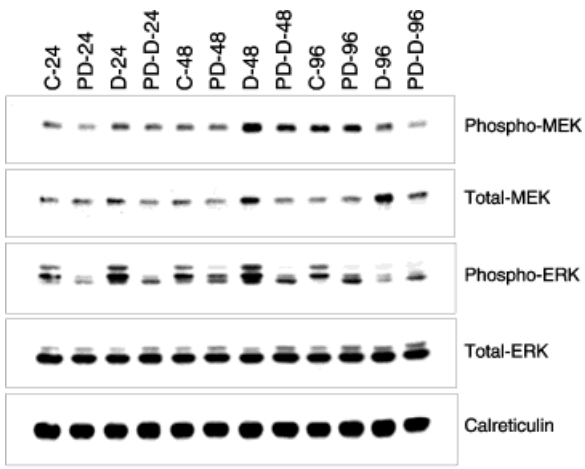

Fig. 5. The effects of PD98059 on activation of ERK pathway by low concentrations of 1,25D$_3$. HL60 cells were pretreated with 20 μM PD98059 for 1 h before their exposure to 10^{-9} M 1,25D$_3$. Western blotting of whole cell extracts was performed to detect activation and protein levels of MEK and ERKs, and the results were correlated with the onset of 1,25D$_3$-induced partial G1 block shown in Figure 6. Loading was controlled by reprobing the membrane for calreticulin.

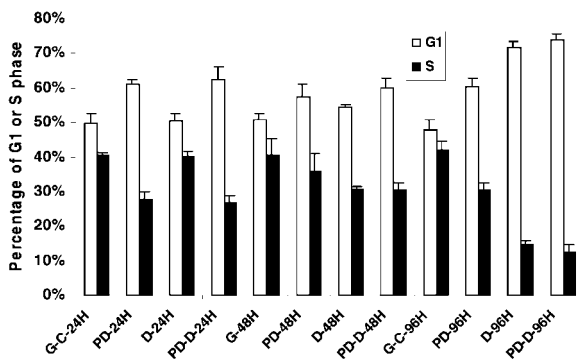

Fig. 6. Cell cycle distribution at the indicated times following exposure of HL60 cells to a low concentration of 1,25D$_3$ (10^{-9} M) in the presence or absence of PD98059. Mean values of three determinations ± SE are shown. G1 = G1 phase, S = S phase, PD = PD98059, D = 1,25D$_3$.

was indeed prolonged, with both phospho-MEK and phospho-ERK1/2 still higher than control levels at 48 h after the addition of 1,25D$_3$. However, at 96 h phospho-MEK and phospho-ERK1/2 were below the control levels, and a 1,25D$_3$-induced prolongation of the G1 phase was evident (Fig. 6). Thus, reduced activity of the MEK/ERK pathway appears to be a prerequisite for the emergence of the 1,25D$_3$-induced G1 block in HL60 cells.

DISCUSSION

We describe here a pattern of ERK activation that seems to be unique in models of cell differentiation. In previous reports ERK activation has been described either as "transient," usually associated with stimulation of growth or neoplastic transformation [Mansour et al., 1994; Cowley et al., 1994; Pang et al., 1995], although reported as resulting from an expo-

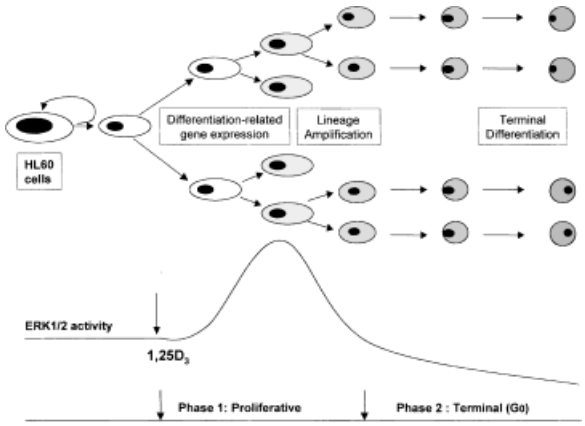

Fig. 7. Schematic representation of the relationship of ERK1/2 activation and proliferative activity of HL60 cell cultures induced to differentiate by $1,25D_3$. Following exposure to $1,25D_3$ HL60 cell express markers of differentiation but continue to proliferate for 1 or 2 cell generations. ERK1/2 are activated by phosphorylation during this "proliferative" phase. The second phase is characterized by proliferative quiescence (G0 phase) and reduced levels of activated ERK1/2.

sure to some differentiation-inducing agents [Kharbanda et al., 1994; Marcinkowska et al., 1997; Song et al., 1995], or as "sustained" during induction of differentiation in several well-studied systems [Racke et al., 1997; Whalen et al., 1992; Traverse et al., 1992; Yen et al., 1998]. In contrast, addition of $1,25D_3$ to HL60 cells resulted in rapid activation of both ERK1 and ERK2 which was not transient in the experiments described here, Since it persisted for 24–48 h under conditions studied here, yet it was not sustained either, as ERK1/2 activation was no longer present when the cells were entering the period of proliferative quiescence (Figs. 1–5). This establishes an "intermediate" form of ERK activation, and suggests a role for the ERK pathway that involves its participation in an early phase of differentiation, as well as providing a trigger that activates self-perpetuating transcriptional changes in the cell nucleus.

There have been previous proposals to subdivide differentiation into several stages. Perhaps the most relevant to our studies is the suggestion by Yen et al. [1985, 1992] that there is an "early" segment of myeloid or monocytic differentiation during which the cells become primed for differentiation but do not express lineage-specific phenotype. The ERK activation described in the present studies serves to define this early, proliferative phase, but it should be noted that CD14 surface marker which characterizes monocytic lineage [Ziegler-Heitbrock and Ulevitch, 1993; Zhang et al., 1994] is highly expressed during this phase (Tables I–IV), so lineage selection is already evident, and thus cannot be described as "precommitment" [Yen et al., 1987]. In the differentiation system studied here the early phase may be considered to serve as the period for amplification, by cell proliferation, of the cohort of cells in the initial stages of differentiation (Fig. 7). Thus, levels of phospho-ERK1/2 relative to the control levels at that time can be used to determine if the cells are present in this phase.

The onset of $1,25D_3$-induced G1 block further helps to define the end of the early cell amplification stage. We invariably found that this block became evident in a significant proportion of cells only when the levels of phospho-ERK1/2 fell below the control levels, as illustrated by the prolongation from 24 h to 48 h of both the elevated phospho-ERK1/2 levels and the appearance of the G1 block when ambient concentration of $1,25D_3$ was reduced from 10^{-7} to 10^{-9} M (Figs. 5 and 6). This is consistent with the reports which indicate that the ERK pathway plays a direct role in degradation of p27/Kip1 [Kawada et al., 1997; Treinies et al., 1999], a cell cycle inhibitory protein up-regulated by $1,25D_3$ in HL60 cells at the onset of the G1 block [Wang et al., 1996, 1997], with kinetics similar to those observed in the experiments presented here. Also, this can explain the continuous growth of the 40 AF subclone of HL60 cells in the presence of high concentrations of $1,25D_3$, since 40 AF cells have high levels of phospho-ERK1/2 (Figs. 1 and 3). We hypothesize therefore that in $1,25D_3$-induced differentiation of HL60 cells the up-regulation of p27/Kip1 and therefore the onset of the G1 block are delayed by the activity of ERK1/2 in the early phase of differentiation, and in 40 AF cells do not occur at all.

The G1 block induced by $1,25D_3$ was manifested by progressively increasing numbers of cells in the G1 compartment of the cell cycle when the culture was examined at successive intervals. For instance, there was little increase in G1 cells following exposure to 10^{-7} M $1,25D_3$ for periods up to 12 h, but from 24 h on the proportion of G1 cells progressively increased (Table I). Although serum starvation for 24 h or an exposure to the MEK inhibitor

PD98059 also resulted in an increased proportion in G1 compartment, this increase was not progressive (Tables III and IV, Fig. 6), indicating that there was a slower traverse of G1 relative to the other phases of the cell cycle, rather than a block to the cell cycle progression with a gradually increasing accumulation of cells in G1/G0 phase.

In serum-starved cells the levels of phospho-ERK1/2 were consistently increased (e.g. Fig. 3), perhaps as a compensatory mechanism to maintain residual cell growth by responding to autocrine and paracrine factors, but these levels were reduced by $1,25D_3$ with similar kinetics to the ERK1/2 reduction in cells cultured in serum-supplemented medium, and the emergence of the G1 block also showed similar kinetics. The appearance of the differentiation markers, however, was markedly retarded, consistent with the previous reports that differentiation and cell cycle arrest are not strictly coupled in $1,25D_3$-treated HL60 cells [Studzinski et al., 1997]. Thus, suboptimal growth conditions elicited here by either serum starvation or by an exposure to PD98059 retarded, but did not prevent, differentiation, and had no apparent effect on the onset of G1 block. This demonstrates that the mechanisms responsible for the $1,25D_3$-induced G1 block, such as upregulation of p27/Kip1 [Wang et al., 1996, 1997] are not strictly coupled to phenotypic differentiation.

Interestingly, two regulatory molecules reported to be downstream in the ERK pathway, cyclin D1 [Weber et al., 1997] and p90/RSK [Frodin and Gammeltoft, 1999] showed sustained increases during $1,25D_3$-induced differentiation (Figs. 2–4). This argues that there is either no relationship to the ERK pathway in this system, or that the ERKs initiate cyclin D1 and p90/RSK upregulation which is then maintained by another pathway. Why this upregulation is maintained is puzzling, because both cyclin D1 and p90/RSK participate in growth processes [Baldin et al., 1993; Frodin and Gammeltoft, 1999], yet in $1,25D_3$-treated HL60 cells their protein levels increase while the cells are becoming proliferatively quiescent. Perhaps the explanation is that normal human monocytes/macrophages as well as quiescent, differentiated HL60 cells retain the ability to proliferate after an appropriate stimulus [Studzinski and Harrison, 1999], because some of the components of their growth and proliferation machineries are dormant but available for reactivation.

This demonstration of an "intermediate" pattern of ERK activation has two practical consequences. ERK activation serves to define an early phase of monocytic differentiation of HL60 cells which should facilitate further analysis of this process, and the limited duration of ERK activation suggests that agents which inhibit the ERK pathway will have only a limited anti-leukemic effect when combined with $1,25D_3$. These studies are currently underway.

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