

# Up-regulation of protein serine/threonine phosphatase type 2C during $1\alpha,25$ -dihydroxyvitamin $D_3$ -induced monocytic differentiation of leukemic HL-60 cells

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**Abstract** Treatment with 20 nM  $1\alpha,25$ -dihydroxyvitamin  $D_3$  ( $1,25(OH)_2D_3$ ) caused a progressive increase in the activity of  $Mg^{2+}$ -dependent protein serine/threonine phosphatase type 2C (PP2C) in subcellular fractions of HL-60 cells, whereas PP2C activity was relatively constant throughout all-*trans* retinoic acid-induced (1  $\mu$ M) granulocytic differentiation. The increase in PP2C activity appeared to parallel the  $1,25(OH)_2D_3$ -induced phenotypic and functional changes in HL-60 cells. Immunoblot and Northern blot analysis indicated that the increase in PP2C activity corresponded to the increased expression of PP2C protein, which was preceded by an increase in the level of mRNA for PP2C $\beta$ . No mRNA for PP2C $\alpha$  was detected in resting or  $1,25(OH)_2D_3$ -stimulated HL-60 cells. These results suggest that the increased expression of PP2C is related with the  $1,25(OH)_2D_3$ -induced monocytic differentiation of HL-60 cells.

**Key words:** HL-60 cell; Protein phosphatase;  $1\alpha,25$ -Dihydroxyvitamin  $D_3$ ; Differentiation

## 1. Introduction

Reversible phosphorylation of proteins is a key mechanism in signal transduction systems that control various cellular processes, such as metabolism, progression of the cell cycle, and the proliferation and differentiation of cells. Human myeloid leukemia HL-60 cells can be induced to differentiate into monocytes by  $1,25(OH)_2D_3$  or into granulocytes by all-*trans* retinoic acid [1]. Serine/threonine protein phosphatases seem to be involved in the differentiation of HL-60 cells, in addition to protein kinases [2]. The major serine/threonine protein phosphatases in mammalian cells are protein phosphatase type 1 (PP1), protein phosphatase type 2A (PP2A), protein phosphatase type 2B (calcineurin) and protein phosphatase type 2C (PP2C) [3,4]. PP1 and PP2A do not have an absolute requirement for divalent cations, whereas calcineurin and PP2C are  $Ca^{2+}$ /calmodulin- and  $Mg^{2+}$ -dependent, respectively. The PP2C enzyme is a monomeric protein of 43 kDa; it is encoded by a separate gene family that exhibits no sequence similarity to genes for other phosphatases (PP1, PP2A and calcineurin) [3–5]. PP2C is present in many tissues at much lower levels than the other types of protein phosphatases. In mammalian cells, two isoforms of PP2C (PP2C $\alpha$  and PP2C $\beta$ ) have been cloned,

and the two isoforms display an overall homology of 76% [6–10]. However, little is known about their physiological functions, mainly because of the unavailability of specific inhibitors of PP2C. It has been reported that the expression of PP2C gene is enhanced during MyoD1-induced myogenic differentiation of embryonic mesenchymal cells [11]. We reported that okadaic acid and calyculin-A, potent inhibitors of PP1 and PP2A, augmented the granulocytic differentiation of HL-60 cells induced by retinoic acid [12]. In addition, all-*trans* retinoic acid induces down-regulation of PP2A in HL-60 cells, whereas  $1,25(OH)_2D_3$  induces differential redistribution of PP1 isozymes [13–16]. Thus, while it has been shown that PP1 and PP2A are involved in HL-60 cell differentiation, the involvement of calcineurin and PP2C remains to be demonstrated.

To understand the biology of type 2C protein phosphatase, we investigated changes in PP2C activity and expression patterns of PP2C protein and the corresponding mRNA during the monocytic and the granulocytic differentiation of HL-60 cells.

## 2. Materials and methods

### 2.1. Reagents

$1,25(OH)_2D_3$  and all-*trans* retinoic acid, purchased from Solvay-Duphar B.V. (Weesp, The Netherlands) and Sigma Chemical Co. (St. Louis, MO), respectively, were dissolved in ethanol ( $10^{-3}$  M) and stored at  $-20^\circ\text{C}$  in darkness. Okadaic acid was purchased from Wako Pure Chemical Ltd. (Osaka, Japan).

### 2.2. Determination of cell differentiation

Procedures of the maintenance of HL-60 cells and the determination of viable cell counts have been described elsewhere [17]. The extent of differentiation was assessed in terms of morphology; the ability to produce superoxide, as monitored by the reduction of nitroblue tetrazolium (NBT; Sigma) and by analysis of the expression of CD11b (OKM1; Ortho Diagnostic System Inc., Raritan, NJ), CD11c (LeuM5; Becton Dickinson, Mountain View, CA) and CD14 (My4; Coulter Immunology, Hialeah, FL) as described previously [14,15]. Cells ( $2 \times 10^5/\text{ml}$ ) were treated with or without 20 nM  $1,25(OH)_2D_3$  and harvested at appropriate times.

### 2.3. Preparation of subcellular fractions

HL-60 cells ( $1 \times 10^8$  cells for each sample) were treated with 20 nM  $1,25(OH)_2D_3$  or with 1  $\mu$ M all-*trans* retinoic acid, harvested at appropriate times and washed three times with ice-cold Tris-HCl-buffered saline (pH 7.4). The cells were disrupted in 1 ml of homogenization buffer, which consisted of 250 mM sucrose in buffer A (20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, and a cocktail of protease inhibitors), by a glass/glass Potter-Elvehjem homogenizer at  $4^\circ\text{C}$  [12,16], and centrifuged at  $1,000 \times g$  for 10 min. The resulting pellet was used as the nuclear fraction, and the supernatant was centrifuged at  $100,000 \times g$  for 1 h to separate the cytosol and membrane fractions. The membrane and nuclear fractions were rinsed

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with the homogenization buffer, and resuspended with the homogenization buffer containing 1% NP-40. After 1 h incubation on ice, these fractions were sonicated and further centrifuged at  $100,000 \times g$  for 1 h to obtain solubilized fractions. We found NP-40 to be most suitable for solubilization of phosphatases since it does not inhibit phosphatase activities at concentrations as high as 1% (v/v).

#### 2.4. Measurement of PP2C activity

[ $^{32}$ P]Phosphorylated myosin light chains were used as the substrate in the assays of phosphatase activity since they are good substrates for PP2C [3,18]. PP2C activity was determined by monitoring the liberation of  $^{32}$ P<sub>i</sub> from the substrate for 5 min at 30°C in a reaction mixture of 50  $\mu$ l with 20 mM Tris-HCl, pH 7.4, 0.1 mg/ml BSA, 50 mM NaCl, 5 mM EGTA, 6 mM MgCl<sub>2</sub>, 0.5  $\mu$ M okadaic acid and 150  $\mu$ M trifluoperazine. Both PP1 and PP2A are completely inhibited by okadaic acid at 0.5  $\mu$ M [19]. Trifluoperazine (150  $\mu$ M) was included to inhibit calcineurin completely, but it is known that PP1, PP2A and PP2C are unaffected by trifluoperazine even at 150  $\mu$ M [20]. Reactions were initiated by addition of extract rather than  $^{32}$ P-labeled substrate and terminated by the addition of 0.1 ml of 25% trichloroacetic acid. Then, samples were centrifuged after the addition of 0.1 ml BSA (5 mg/ml) and radioactivity in the supernatant was quantitated by scintillation counting. The extent of dephosphorylation was restricted to below 10%. Under these conditions, rates of dephosphorylation were linear with respect to time and enzyme dilution. Assays were performed in duplicate, and the radioactivity measured for blank assays without cell extract was subtracted from the total. The phosphatase activity was indicated as nmol of  $^{32}$ P<sub>i</sub> released in 1 min per mg of protein in the extract.

#### 2.5. Immunoblot analysis

Antiserum against PP2C was obtained by immunizing a rabbit with the full-length recombinant rat PP2C $\alpha$  that had been produced in *E. coli* [21]. HL-60 cells were incubated with 20 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> and subcellular fractions, obtained as described above, were subjected to SDS-PAGE on a 12.5% polyacrylamide running gel. Proteins on the gel were transferred electrophoretically onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) [13–16]. The membrane was reacted with the PP2C-specific antibodies, and immune complexes were stained by the avidin–biotin peroxidase complex method (Vectastain; Vector Laboratories, Burlingame, CA). Prestained SDS-PAGE standards (Bio-Rad, Richmond, CA) were used as molecular mass markers. Estimations of the level of PP2C in subcellular fractions were made densitometrically with a Molecular Dynamics scanning densitometer (Sunnyvale, CA) in conjunction with the ImageQuant program run on a Dell personal computer (Austin, TX). The areas of individual peaks above background on densitometric tracings were expressed in arbitrary units.

#### 2.6. RNA isolation and northern blot analysis

Total cellular RNA was extracted by the guanidinium isothiocyanate technique [22]. Northern blot procedure was performed as described elsewhere [13–15]. The cDNA probes used were the 0.8-kb *Eco*RI fragment of rat PP2C $\alpha$  and the 0.9-kb *Eco*RI-*Sac*I fragment of mouse PP2C $\beta$  [8–10]. Hybridization conditions with the  $^{32}$ P-labeled probe were as described elsewhere [13,14], and hybridized filters were exposed to Kodak O-mat film at  $-80^\circ\text{C}$  or exposed to the imaging plate of an Bio Imaging Analyzer (BAS2000; Fuji Photo Film Co., Kanagawa, Japan). For quantification of relative amounts of mRNA, the intensity of images was calculated directly by the BAS2000.

### 3. Results and discussion

The Mg<sup>2+</sup>-dependent phosphatase activity of PP2C was assayed in cytosol, nuclear and membrane fractions of HL-60 cells before and after treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> or all-*trans* retinoic acid. The activities in cytosol, nuclear and membrane fractions of untreated HL-60 cells were  $1.20 \pm 0.15$ ,  $0.55 \pm 0.09$  and  $0.25 \pm 0.03$  nmol/mg/min (mean  $\pm$  S.E.,  $n = 4$ ), respectively; approximately 55–60% of the total activity of PP2C was present in the cytosol fraction. Cytosol PP2C activity appeared to be equivalent to approximately 10% of cytosol PP1 or PP2A

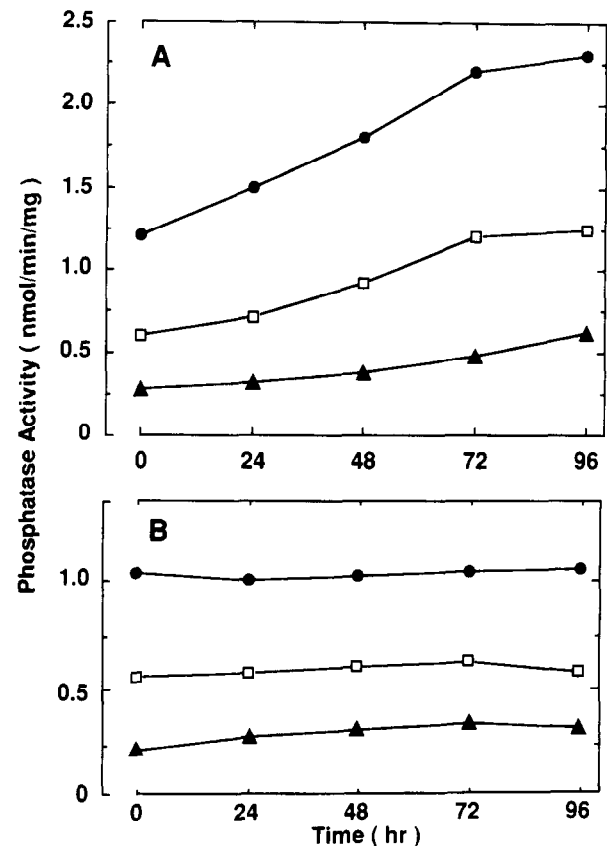


Fig. 1. Time course of changes in PP2C activity in subcellular fractions of HL-60 cells stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> or all-*trans* retinoic acid. HL-60 cells ( $1 \times 10^8$  cells) were treated with 20 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> (A) or 1  $\mu$ M all-*trans* retinoic acid (B), and cytosolic (●), nuclear (□), and membrane (▲) fractions of HL-60 cells were prepared at indicated times. Mg<sup>2+</sup>-dependent phosphatase activity was measured as described in section 2. Means of results from three separate experiments are shown; S.D. were less than 10% in all cases.

activity in untreated HL-60 cells [14–16]. As shown in Fig. 1, exposure of exponentially growing cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> resulted in a gradual increase in the activity of cytosolic PP2C and the activity reached its highest level (approx. 2.1-fold increase over that in untreated HL-60 cells) after 4 days. Concomitantly, there were significant increases (2.0- and 2.2-fold) in PP2C activity in both the nuclear and membrane fractions (Fig. 1). In contrast, PP2C activity in each subcellular fraction was unaltered during the course of all-*trans* retinoic acid-induced granulocytic differentiation. The treatment of HL-60 cells with 1,25(OH)<sub>2</sub>D<sub>3</sub> resulted in monocytic differentiation, as shown in Fig. 2, and withdrawal from the cell cycle (Data not shown). Morphologic examination of May-Giemsa-stained preparations showed that control, untreated HL-60 cells retained a blast-like phenotype throughout all the experiments, whereas 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated cells gradually acquired a differentiated phenotype, as indicated by positivities of CD11b, CD11c and CD14, and the ability to reduce NBT. The increase in phosphatase activity of PP2C appeared to coincide with increases in the positivity of the surface marker CD11c and the extent of reduction of NBT. These data suggest that an increase in PP2C

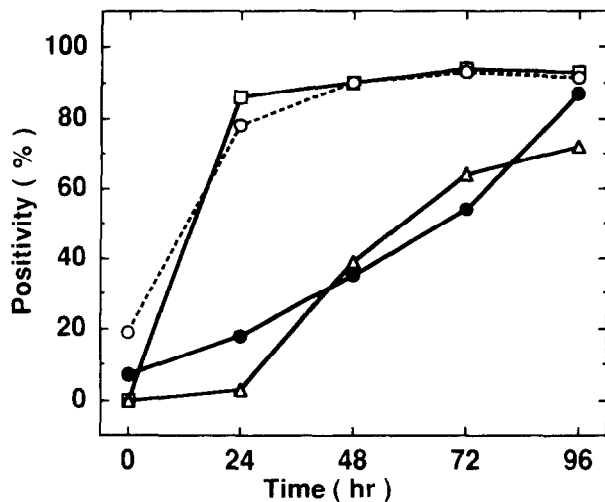


Fig. 2. Time course of changes in positivity of surface markers and the ability to reduce NBT positivity of HL-60 cells treated with  $1,25(\text{OH})_2\text{D}_3$ . HL-60 cells were incubated with 20 nM  $1,25(\text{OH})_2\text{D}_3$  and surface markers CD11b ( $\circ$ ), CD11c ( $\Delta$ ) and CD14 ( $\square$ ), as well as the ability to reduce NBT ( $\bullet$ ) were analyzed as described in section 2. Means of the results from three separate experiments are shown; S.D. were less than 10% in all cases.

activity is associated with the  $1,25(\text{OH})_2\text{D}_3$ -induced monocytic differentiation of HL-60 cells.

To confirm the increase in PP2C phosphatase activity during  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation, protein expression of PP2C in subcellular fractions was investigated by Western blot analysis. As shown in Fig. 3, the polyclonal antibodies against PP2C detected a major immuno-reactive protein of 43 kDa in each cellular fraction of untreated HL-60 cells. We were unable to estimate the concentration of PP2C accurately because purified isozymes were not available, but we were able to compare changes in the levels of PP2C during monocytic differentiation. As shown in Fig. 3, the addition of  $1,25(\text{OH})_2\text{D}_3$  led to a dramatic increase in the levels of PP2C protein in each subcellular fraction, with a peak reached at 96 h. The increases in levels of PP2C protein reflected the increases in PP2C phosphatase activity shown in Fig. 1.

PP2C exists as two enzymatically indistinguishable isozymes, PP2C $\alpha$  and PP2C $\beta$ , in mammalian cells [6–10], and the amino acid sequences of the two isoforms display an overall homology of 76% [7,10]. However, no information is available about possible functional differences. To determine whether the  $1,25(\text{OH})_2\text{D}_3$ -induced increase in levels of PP2C protein in HL-60 cells would be reflected at the level of mRNA expression, Northern blot analysis was performed with total cellular RNA from HL-60 cells, before and after treatment with  $1,25(\text{OH})_2\text{D}_3$  (Fig. 4). The probe for PP2C $\beta$  mRNA detected a major band of 2.8 kb. Treatment of HL-60 cells with  $1,25(\text{OH})_2\text{D}_3$  resulted in a dramatic increase in the level of PP2C $\beta$  mRNA. At 12 h after treatment with  $1,25(\text{OH})_2\text{D}_3$ , PP2C $\beta$  mRNA levels were 5-fold higher than the basal level in untreated HL-60 cells, when normalized to GAPDH mRNA expression. The up-regulation of PP2C $\beta$  mRNA expression clearly preceded the  $1,25(\text{OH})_2\text{D}_3$ -induced monocytic differentiation. However, no PP2C $\alpha$  mRNA was detected in resting or  $1,25(\text{OH})_2\text{D}_3$ -stimulated HL-60 cells. These results are presented graphically, as the ratio of

intensities of PP2C mRNA-specific signals to those of GAPDH mRNA-specific signals at various times after the addition of  $1,25(\text{OH})_2\text{D}_3$  to HL-60 cells (Fig. 4B). PP2C $\alpha$  and PP2C $\beta$  are encoded by two different genes [7]. The present data suggest that expression of the two isotypes of PP2C might be differently regulated during the  $1,25(\text{OH})_2\text{D}_3$ -induced monocytic differentiation of HL-60 cells. The up-regulation of PP2C may be

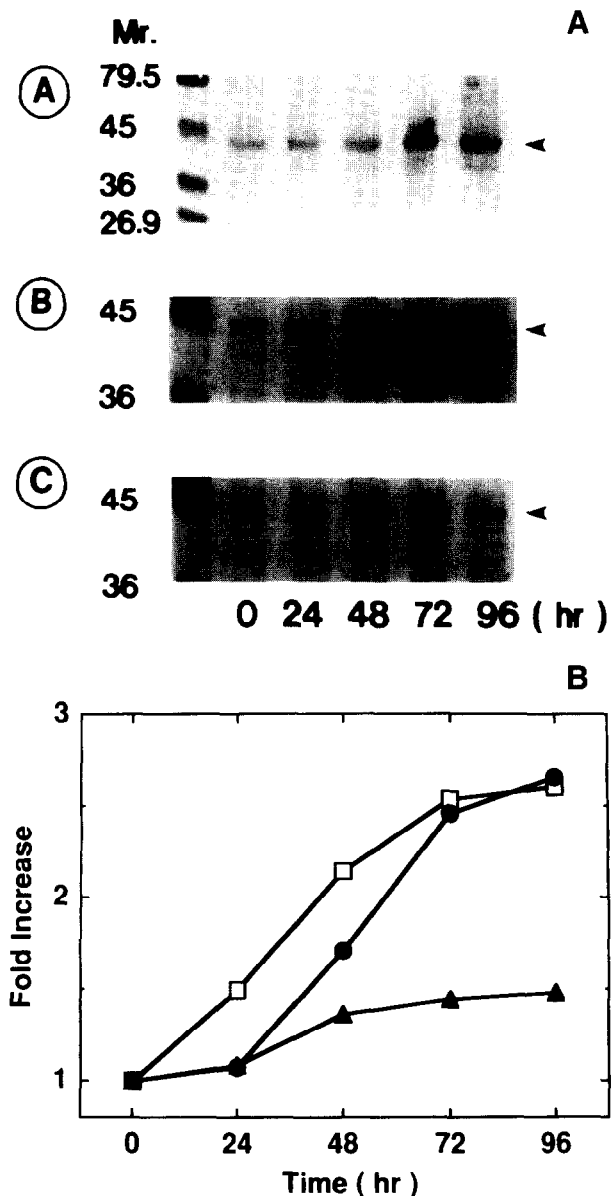


Fig. 3. Time course of changes in levels of immunoreactive PP2C in subcellular fractions of HL-60 cells during  $1,25(\text{OH})_2\text{D}_3$ -induced monocytic differentiation. HL-60 cells ( $1 \times 10^8$  cells) were treated with 20 nM  $1,25(\text{OH})_2\text{D}_3$ , and each subcellular fraction was obtained as described in section 2. (A) Immunoblots stained with anti-PP2C antibody of subcellular fractions of HL-60 cells. 10  $\mu\text{g}$  of cytosol protein (A), and 20  $\mu\text{g}$  of nuclear (B) and membrane (C) proteins were analyzed with antiserum specific for PP2C as described in section 2. Molecular weight mass ( $M_r \times 10^{-3}$ ) are shown on the left. These data are from one representative experiment, but similar results were obtained in two other experiments. (B) Graphic display of levels of PP2C protein and as quantitated by densitometry for cytosol ( $\bullet$ ), nuclear ( $\square$ ), and membrane ( $\blacktriangle$ ) fractions. Results are expressed as the extent (-fold) of the increase in PP2C of 43 kDa relative to the level at 0 h.

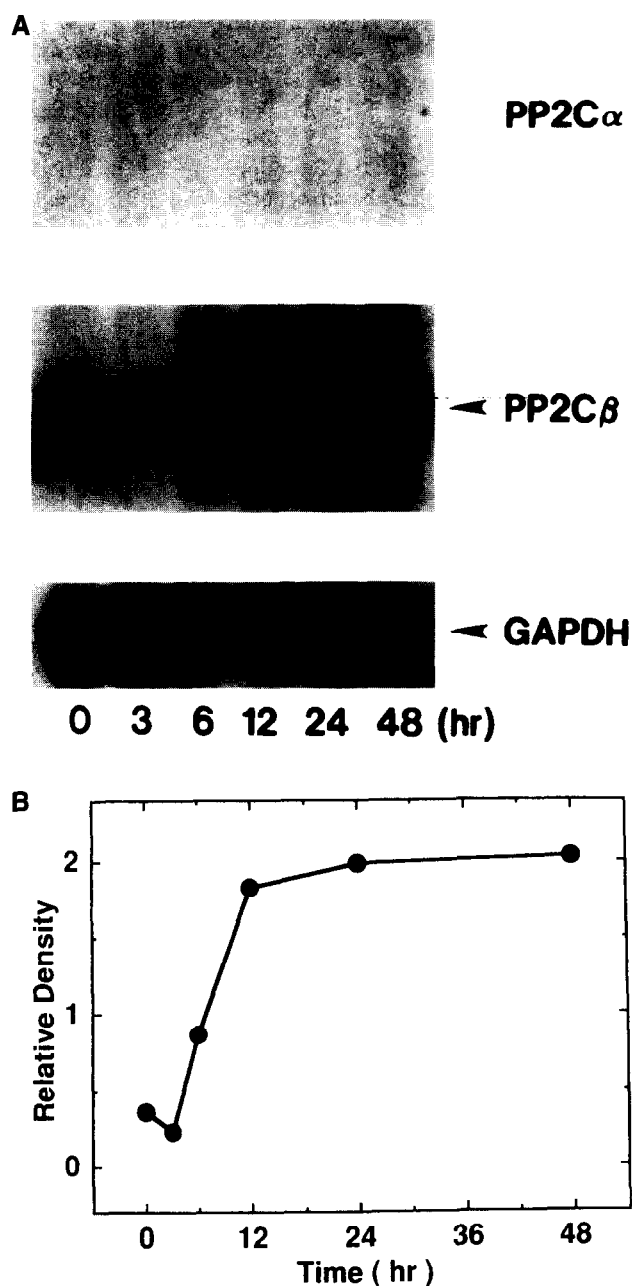


Fig. 4. Time course of changes in the expression of PP2C mRNA in HL-60 cells stimulated with  $1,25(\text{OH})_2\text{D}_3$ . HL-60 cells ( $1 \times 10^6$  cells) were treated with 20 nM  $1,25(\text{OH})_2\text{D}_3$ . Total RNA (20  $\mu\text{g}$ ) was isolated from HL-60 cells at the times indicated below the corresponding lanes. (A) Northern blots of total RNA were hybridized with  $^{32}\text{P}$ -labeled cDNA probes for PP2C $\alpha$  and PP2C $\beta$ . The same RNA blots were hybridized to the GAPDH cDNA probe that detected a 1.5 kb mRNA species, for normalization of levels of RNA in each lane. (B) Graphic representation of the relative intensities of hybridization signals due to PP2C $\beta$  mRNA (●) normalized to the expression of GAPDH mRNA obtained by densitometric scanning of the autoradiogram shown in panel A. The ratios of signals were determined for each time point following  $1,25(\text{OH})_2\text{D}_3$  addition. Similar results were obtained in two independent experiments.

associated with the expression of certain differentiation markers (CD11c positivity and the ability to reduce NBT) related to monocytes, although the direct target of PP2C that is important for the HL-60 cell differentiation is unknown at present. The

identification of a specific inhibitor of the PP2C enzyme would help us to clarify the physiological roles of PP2C in HL-60 cell proliferation and differentiation.

$1,25(\text{OH})_2\text{D}_3$  is capable of regulating the expression of many genes that are critical for development and cellular differentiation. The biologic responses are mediated by the binding to nuclear receptors for  $1,25(\text{OH})_2\text{D}_3$  which belong to the steroid/thyroid receptor superfamily. These receptors act as ligand-dependent transcription factors that bind to specific DNA sequences [23]. Ligand-bound receptors interact with specific enhancer or suppressor DNA elements to modulate gene expression [24].  $1,25(\text{OH})_2\text{D}_3$  modulates the expression of genes for a number of proteins. Our results demonstrate that the gene for PP2C $\beta$  is an additional gene subject to up-regulation by  $1,25(\text{OH})_2\text{D}_3$ . Although protein kinase C is up-regulated by treatment with either all-*trans* retinoic acid or  $1,25(\text{OH})_2\text{D}_3$  [2], the dynamics of protein phosphatases seem to differ in association with different pathways of differentiation. Together with our previous findings [12–16], the present data suggest that the modulation of various protein serine/threonine phosphatases, in addition to the activation of protein kinases, might play an important role in regulating the net phosphorylation of critical substrate(s) that subsequently mediate the proliferation and/or differentiation of HL-60 cells.

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