Phospholipid-and Ca\(^{2+}\)-dependent Protein Kinase Activity and Protein Phosphorylation Patterns in the Differentiation of Human Promyelocytic Leukemia Cell Line HL-60

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ABSTRACT

The effects of differentiating agents on the activity and phosphorylation pattern produced by phospholipid- and Ca\(^{2+}\)-dependent protein kinase (PL-Ca-PK) were examined in human promyelocytic leukemia cell line HL-60. Dimethyl sulfoxide (DMSO), retinoic acid (RA), and 1,25-dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\)] increased the appearance of mature myelocytic (DMSO and RA) or monocytic [1,25(OH)\(_2\)D\(_3\)] cells. The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) increased the appearance of adherent macrophage-like cells. Coincident with the appearance of monocytic [1,25(OH)\(_2\)D\(_3\)] cells, as well as PL-Ca-independent phosphorylated proteins indicative of mature myelocytic and monocytic cells, as well as PL-Ca-independent phosphorylated proteins characteristic of the macrophage-like phenotype.

INTRODUCTION

The human promyelocytic leukemia cell line HL-60 constitutes a useful system for the study of the control of cell differentiation by chemical agents. These cells can be induced to terminally differentiate to morphologically mature myeloid cells by a variety of compounds, including DMSO, RA, TPA, and 1,25(OH)\(_2\)D\(_3\) (1-9). DMSO and RA induce differentiation to mature neutrophil-like cells (2-4); TPA can induce a macrophage-like phenotype (5, 6, 9), while 1,25(OH)\(_2\)D\(_3\), the biologically active metabolite of vitamin D\(_3\), induces differentiation of HL-60 cells to a monocyte-macrophage phenotype which is similar to but not identical to that induced by TPA (1, 7, 8).

Although the exact molecular mechanism of the differentiation process in HL-60 cells is still unclear, commitment to a pathway of cellular differentiation may be mediated or associated with the cell surface membrane. Several years ago, Takai et al. (10-12) found that a membrane-bound Ca\(^{2+}\)-activated phospholipid-dependent protein kinase (PL-Ca-PK) is activated by diacylglycerol, one of the products released from inositol phospholipids as a consequence of receptor activation. This protein kinase, originally identified in the brain (10), is distributed widely in mammalian tissues (13), including various types of human acute myeloblastic leukemia cells (14). Castagna et al. (15) have shown that TPA can substitute for unsaturated diacylglycerol to increase the affinity of PL-Ca-PK for Ca\(^{2+}\) and phosphatidylyserine and hence activate the enzyme. Based on this observation, they suggested that PL-Ca-PK may be a membrane target for TPA. Niedel et al. (16) and Vandenbark et al. (17) found that the phorbol diester receptor copurifies with PL-Ca-PK from rat brain or HL-60 cells, which suggests that the two activities reside in the same or closely associated molecules. Whether or not these observations in vitro are relevant to the mechanism whereby phorbol esters modulate cell differentiation or proliferation in intact cells still remains an open question.

Phosphorylation by kinases is a common reaction for controlling the activity of enzymes, as exemplified by the cyclic AMP activation of protein kinase A, which in turn influences other enzyme activities via their phosphorylation (18). PL-Ca-PK may play a similar role and be involved in the control of signal transduction, cell division, and differentiation (19). Kuo et al. (20) have demonstrated that PL-Ca-PK is a major enzyme system capable of phosphorylating numerous endogenous proteins in a variety of tissues.

Therefore, in the present investigation we have assessed PL-Ca-PK activity in HL-60 cells induced to differentiate along two major pathways, as well as the endogenous phosphorylation products of PL-Ca-PK under these conditions.
MATERIALS AND METHODS

**Materials.** Histone H (lysine-rich histone, type V-5 from calf thymus), PMSF, EGTA, phosphatidylserine, NBT, TPA, RA, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO); Triton X-100 was from Research Products International Corp. (Elk Grove Village, IL); DTT was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN); DMSO was purchased from Fisher Scientific Co. (Fairlawn, NJ); and 1,25(OH)2D3 was a gift from Dr. Milan R. Ussokovic, Hoffmann-La Roche Inc. (Nutley, NJ). Rabbit vinculin antiserum was kindly provided by Dr. Susan Craig, Johns Hopkins University (Baltimore, MD). [γ-32P]ATP, tetrathiethylammonium salt (2900 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

**Cell Culture.** Human promyelocytic leukemia cell line HL-60 was obtained from Dr. T. R. Breitman, National Cancer Institute, Bethesda, MD. Cells were grown in plastic tissue culture flasks (Falcon, Oxnard, CA) in RPMI medium 1640 (Quality Biological, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY), 2 mM glutamine, 40 mL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and gentamicin (50 μg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cell counts were determined with a Coulter Counter model ZM (Coulter Electronics Ltd., Luton Beds, England), and morphological examination was made by Cytospin slide preparation of cells stained with Wright-Giemsa.

Cells were grown at a density of 5 to 8 x 10^5/mL and were treated with the various agents dissolved in DMSO. The final concentration of DMSO in the growth medium was 0.05% or less and had no effect on cell growth or morphological differentiation.

**NBT Reduction.** Morphological assessment of cell differentiation in control and treated cells was measured by NBT reduction (4). Cells at a density of 1 x 10^5/mL of medium containing 20% fetal calf serum were incubated for 25 min at 37°C in an atmosphere of 5% CO2 and air with an equal volume of a solution of NBT (1 mg/mL in phosphate-buffered saline) which also contained freshly diluted TPA at a final concentration of 162 nM. The percentage of cells containing intracellular blue-black formazan deposits was determined in Wright-Giemsa-stained Cytospin slide preparations. A minimum of 300 cells were counted by light microscopy for each experimental point.

**Adherent Cells.** Untreated HL-60 cells were grown at a density of 5 x 10^5/mL. After addition of 10^-8 TPA, cells begin to attach to the plastic flask. To count the suspended and adherent cells, the plastic flasks were rotated and gently shaken; the medium was removed, and the flasks were washed with 2 ml of Hanks' solution. The cells in the combined medium and wash are referred to as non-adherent cells. The cells attached to the flask were treated with 0.5 ml of 0.05% trypsin in Hanks' solution deficient in Ca^2+ and Mg^2+ and containing 0.02 M EDTA and incubated for 10 min at 37°C. When cells started to detach, the suspension was transferred to a tube which contained 20% fetal bovine serum, and the procedure was repeated until all of the remaining attached cells were removed. These cells are referred to as adherent cells.

**Preparation of Cell-free Extracts.** HL-60 cells were collected by centrifugation at 400 x g for 8 min. The cells were washed once with 50 volumes of Hanks' solution deficient in Ca^2+ and Mg^2+ and containing 0.02 mM EDTA and twice with 20 volumes of the same solution but without EDTA and were collected by centrifugation at 400 x g for 8 min. The cell pellet was extracted by the procedure of Helfman et al. (14) as follows: the pellet was resuspended in 3 volumes of buffer solution containing 50 mM Tris-HCl (pH 7.5), 1 mM PMSF, 2 mM EGTA, 10 mM DTT, and 0.1% Triton X-100 by shaking on a vortex mixer. The cell suspension was kept on ice for 30 min and then sonicated at 4°C for 1 min and centrifuged for 6 min at 15,000 x g at 4°C in an Eppendorf microcentrifuge. The resulting supernatant was used. Protein concentrations were determined by the method of Bradford (21), with bovine serum albumin as a standard.

**Kinase Assay.** Protein kinase activity was determined by measuring the incorporation of [32P] from [γ-32P]ATP into histone H1 (14). The reaction mixture (200 μl) contained 25 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 40 μg of histone H1, 5 μM [γ-32P]ATP (containing 0.8 to 1.6 x 10^6 cpm), 10 μg of protein of the cell extracts, and either phosphatidylserine (100 μg/ml) and 500 μM CaCl2 or 200 μM EGTA. The reaction was initiated by the addition of [γ-32P]ATP and then incubated for 10 min at 30°C. Assays were stopped by the addition of 25% trichloroacetic acid, and acid-precipitable material was collected on a Millipore HA filter (0.45 μm) which was then washed with 10% trichloroacetic acid-2% sodium pyrophosphate. Dried filters were resuspended in Ready-Seq MP, and the radioactivity was determined using a Searle Mark III liquid scintillation spectrometer. The phosphorylation of endogenous proteins was carried out in the same manner as described above but without the addition of histone and with an [γ-32P]ATP concentration of 10 μM (0.8 to 1.5 x 10^6 cpm). The final concentration of Triton X-100 in the assay was less than 0.005%. This detergent concentration had no effect on the activity of the enzyme. Under the conditions used, the phosphorylation reaction was linear with respect to the amount of enzyme and time of incubation. Assays were run in duplicate and repeated at least four times where the standard error was 7-15% of the mean.

**SDS-Polyacrylamide Gel Electrophoresis.** Endogenous protein phosphorylation was analyzed by slab gel electrophoresis in a 10% polyacrylamide gel as described by Laemmli (22). The reaction was carried out as described above and terminated by the addition of 2 volumes of 25% trichloroacetic acid. The suspension was centrifuged at 4°C at 15,000 x g, and the pellet was washed twice with 10% trichloroacetic acid-2% pyrophosphate and three times with cold acetone. The dried pellet was dissolved in sample buffer containing 0.083 M Tris-HCl (pH 6.8), 5 mM DTT, 2% SDS, 0.001% bromophenol blue, and 20% sucrose. The proteins were completely dissociated by immersing the samples for 2 min in boiling water. Prestained protein molecular weight standards (BRL, Inc., Gaithersburg, MD) were used as markers. The dried gels were exposed to XK-1 Kodak film using a Kodak X-Omatic intensifying screen.

**Immunoprecipitation.** PL-Ca-PK assays were performed with cell extracts from TPA-treated cells (48 h) as described above. Assays were stopped with 6 volumes of ice-cold acetone, and proteins were precipitated overnight at -20°C. The samples were centrifuged for 1 min in an Eppendorf microcentrifuge and dissolved in 50 μl of 1% (v/v) Nonidet P-40, 1% (v/v) sodium deoxycholate, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), and 1 mM PMSF (RIPA buffer). Forty-five μl of lysate were incubated with 2 μl of vinculin antiserum or nonimmune serum at 4°C for 1 h and for a further 30 min with 0.5 ml of 10% fixed Staphylococcus aureus (IGG-80; New England Enzyme Center, Boston, MA). The sample was centrifuged for 10 min at 2000 x g, and the pellet was washed three times with 1 ml of RIPA buffer. The washed pellet was dissolved in Laemmli sample buffer and processed for gel electrophoresis as described above.

RESULTS

**Cell Growth and Differentiation.** Induction of differentiation in HL-60 cells by DMSO, RA, 1,25(OH)2D3, and TPA and their effects on cell growth are shown in Chart 1. Untreated cells grew as a single cell suspension which contained 70-80% promyelocytes. Treatment with 10^-8 M TPA reduced cell growth (Chart 1B) and induced the appearance of an adherent macrophage-like cell type (Chart 1D). After 48 h of treatment with TPA, approximately 75% of the cells became adherent, and both adherent and nonadherent cells were viable, as determined by trypan blue exclusion. Virtually none of the adherent TPA-treated cells reduced NBT, while less than 10% of the nonadherent cells were NBT positive. Following exposure of cells to 2.5 x 10^-8 M 1,25(OH)2D3, there was inhibition of cell growth which was
PHOSPHOLIPID- AND Ca²⁺-DEPENDENT PROTEIN KINASE ACTIVITY

Chart 1. Cell growth and differentiation of HL-60 cells at various times after treatment with DMSO, RA, 1,25(OH)₂D₃, and TPA. Cells became loosely adherent, and by day 7, 80% of the cells were NBT positive (Chart 1C) and morphologically resembled monocytes. Cells treated with 1.2% DMSO began to cease growth by day 5, whereas those treated with 10⁻⁶ M RA ceased growth by day 4 (Chart 1A). The reduced growth pattern was inversely related to the appearance of neutrophil-like cells which were NBT positive (Chart 1C).

Protein Kinase Activity. The temporal relationship between differentiation and PL-Ca-PK activity was measured following treatment with the four differentiating agents (Chart 2). Treatment with DMSO, RA, or 1,25(OH)₂D₃ resulted in a marked elevation in PL-Ca-PK activity assayed with histone H₁ as substrate at 4 to 7 days after continuous exposure to these agents, while PL-Ca-independent protein kinase activity remained unchanged (Chart 2A). An elevation in PL-Ca-PK activity was also observed when activity was measured with endogenous substrates contained in the cell extract (Chart 2C). Increasing the length of exposure to DMSO, RA, and 1,25(OH)₂D₃ resulted in increased PL-Ca-PK wherein 1,25(OH)₂D₃ produced a greater effect than RA, which in turn showed a slightly greater effect than DMSO. Again, endogenous PL-Ca-independent protein kinase activity was unchanged following treatment with these compounds.

A markedly different effect was observed after treatment of cells with TPA. PL-Ca-PK activity diminished rapidly in both the adherent and nonadherent cells when assayed with either histone H₁ (Chart 2B) or endogenous substrates (Chart 2D). On the other hand, PL-Ca-independent PK activity increased in adherent cells after a 17-h lag period, while this activity in nonadherent cells fell from an initial elevation in activity 6 h after TPA exposure to a level equal to or slightly less than control activity (Chart 2, B and D). Overall, there was an inverse relationship between phosphorylation activity and the appearance of macrophage-like cells following TPA treatment, while DMSO, RA, and 1,25(OH)₂D₃ treatment produced a positive correlation between PL-Ca-PK activity and the appearance of the differentiated phenotype.

Gel Electrophoretic Patterns. The phosphorylation pattern of endogenous proteins assayed under PL-Ca-dependent and -independent conditions were assessed by SDS-gel electrophoresis (Figs. 1–5). Following treatment with DMSO, pp21, pp37, and pp38 increased with the length of exposure to DMSO and were dependent on PL-Ca (Fig. 1). In contrast, the PL-Ca-dependent pp19 did not change, while pp31, which was not totally PL-Ca-dependent, increased following DMSO treatment. A similar phosphorylation pattern was noted following treatment with RA (Fig. 2) or 1,25(OH)₂D₃ (Fig. 3), except that pp31 was less intense in RA-treated cells and virtually absent after 1,25(OH)₂D₃ treatment. The phosphorylation pattern from TPA-treated cells showed a universal lack of PL-Ca-dependent phosphorylation (Figs. 4 and 5). The distinguishing features of the phosphorylation pattern in these cells were (a) the disappearance of PL-Ca-dependent pp19 and pp21, and (b) the appearance of pp130, which is antigenically related to vinculin (Fig. 6). In common with the phosphorylation pattern from cells treated with DMSO, RA, and 1,25(OH)₂D₃ was the appearance of pp31, pp37, and pp38, except for their PL-Ca independence. The phosphorylation pattern in nonadherent cells (Fig. 4) was similar to but less intense than that in adherent cells (Fig. 5).
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DAYS AFTER DMSO TREATMENT

Fig. 1. Autoradiograph of phosphorylated endogenous proteins in extracts of HL-60 cells after treatment with 1.2% (v/v) DMSO. The concentration of Ca²⁺ was 500 μM and of phosphatidylserine was 100 μg/ml. Experimental details are described under "Materials and Methods." Numbers on left ordinate indicate the molecular weight of myosin heavy chain (200 kd), phosphorylase b (97 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), α-chymotrypsinogen (26 kd), and β-lactoglobulin (18 kd). Numbers on the right ordinate identify the molecular weight of phosphorylated proteins.

DAYS AFTER RA TREATMENT

Fig. 2. Autoradiograph of phosphorylated endogenous proteins in extracts of HL-60 cells after treatment with 10⁻⁶ M RA. Experimental details are described in Fig. 1.

DISCUSSION

The promyelocytic leukemia cell line HL-60 responds to a variety of differentiating agents by acquiring many of the characteristics of either myelocytes or monocytes-macrophages (1-9). This cell line also contains PL-Ca-PK (14, 17), and thus we have explored the relationship between PL-Ca-PK activity and...

DAYS AFTER 1,25-(OH)₂D₃ TREATMENT

Fig. 3. Autoradiograph of phosphorylated endogenous proteins in extracts of HL-60 cells after treatment with 2.5 x 10⁻⁸ M 1,25(OH)₂D₃. Experimental details are described in Fig. 1.

NON-ADHERENT CELLS: HOURS AFTER TPA TREATMENT

Fig. 4. Autoradiograph of phosphorylated endogenous proteins in non-adherent HL-60 cells after treatment with 10⁻⁶ M TPA. Experimental details are described in Fig. 1 and in "Materials and Methods."
PL-Ca-dependent phosphorylation of endogenous proteins and the induction of differentiation along the myelocyte-macrophage pathway by DMSO, RA, 1,25(OH)₂D₃, and TPA. Our study establishes a significant correlation between the appearance of the differentiated phenotype induced by DMSO, RA, and 1,25(OH)₂D₃ and the elevation in PL-Ca-PK activity. Previous studies by Kraft and Anderson (23) also demonstrated the elevation of PL-Ca-PK in F9 teratocarcinoma cells induced to differentiate with RA. In contrast, exposure of HL-60 cells to TPA resulted in the rapid disappearance of PL-Ca-PK and PL-Ca-dependent phosphorylated proteins which accompanied the appearance of a macrophage-like phenotype in both adherent and nonadherent cells. This effect is similar to that reported for TPA-treated mouse 3T3 fibroblasts, where a rapid and complete disappearance of PL-Ca-PK activity occurred within 24 h of exposure to TPA (24). Kraft et al. (25) also reported the rapid disappearance (within 1 min) of PL-Ca-PK from TPA-treated mouse EL4 thymoma cells, but their enzyme preparation differed in that it was cytosolic and not a detergent-solubilized extract. On the other hand, our data do not provide confirmation of unchanged total (cytosolic plus detergent-solubilized particulate) PL-Ca-PK activity following TPA treatment (26). In the latter study, a rapid decrease in cytosolic activity with a concomitant increase in membrane-bound activity accompanied phorbol ester treatment, which could explain the reduction in cytosolic PL-Ca-PK activity observed by Kraft et al. (25) but not the reduction in total enzyme activity reported by Rodriguez-Peña and Rozengurt (24) or in our study. Since PL-Ca-PK is activated in vitro by TPA (15-17), our results and those of others (24) imply that this phenomenon is not pertinent to the effects of TPA upon long-term treatment of cells.

The PL-Ca-dependent phosphorylation pattern in extracts from DMSO-, RA-, or 1,25(OH)₂D₃-treated cells was consistent with the progressive elevation of PL-Ca-PK activity accompanying the differentiated phenotype, and these results are summarized in Table 1. The major PL-Ca-dependent phosphorylated proteins which increased with differentiation to either myelocytic or monocytic cells were pp37 and pp38. The latter phosphorylated protein also appeared in untreated HL-60 cells as reported...
Our studies indicate the slow appearance of pp130 during treatment (48 h after treatment) strongly indicates that the phosphorylation is not mediated by PL-Ca-PK. In fact, the total absence of this phosphorylated protein noted in cells treated with DMSO, RA, or 1,25(OH)2D3, except that they lacked dependence on phospholipid and calcium. This effect was not unexpected, since PL-Ca-PK activity was reduced rapidly, while PL-Ca-dependent PK activity increased. Thus, the increase in phosphorylation of the common proteins, pp31, pp37, and pp38 in adherent macrophage-like cells indicates that the phosphorylation of these endogenous substrates is not necessarily restricted to PL-Ca-PK. Accompanying the disappearance of PL-Ca-PK was a corresponding reduction in pp19 and pp21. This effect is in contrast to the increase in situ of pp17–20 and pp27 in TPA-treated HL-60 cells (27, 28). However, since the latter phenomenon reflects pulse-labeling of cells with 32P, as well as short-term (30–60 min) exposure to TPA, it is difficult to assess the relevance of this effect to cell differentiation.

One of the most distinctive changes noted upon exposure of HL-60 cells to TPA was the appearance of pp130. This effect reached a peak when cell adherence was maximal and the cells were fully differentiated. The immunoprecipitation of pp130 with vinculin antiserum indicates that these proteins are antigenically related. Vinculin has been reported previously to serve as a specific substrate for PL-Ca-PK (29) and to be rapidly phosphorylated in chick embryo fibroblasts and 3T3 cells treated with TPA (30). Our studies indicate the slow appearance of pp130 during differentiation induced by TPA and the probability that this effect is not mediated by PL-Ca-PK. In fact, the total absence of this enzyme at a time when the intensity of pp130 is at a maximum (48 h after treatment) strongly indicates that the phosphorylation of vinculin is not dependent on PL-Ca-PK but rather on the appearance of the macrophage phenotype. The importance of vinculin in cell shape and adhesion (31, 32) implies that the phosphorylation of this protein may be more closely related to the macrophage phenotype rather than to the monocytic pathway in general.
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*Cancer Res* 1985;45:5159-5164.

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