

Augmentation of retinoic acid-induced granulocytic differentiation in HL-60 leukemia cells by serine/threonine protein phosphatase inhibitors

Koichi Morita^b, Masakatsu Nishikawa^b, Kazuhiko Kobayashi^b, Katsumi Deguchi^b, Masaaki Ito^a, Takeshi Nakano^a, Hiroshi Shima^c, Minako Nagao^c, Takayoshi Kuno^d, Chikako Tanaka^d and Shigeru Shirakawa^b

^aFirst and the ^bSecond Departments of Internal Medicine, Mie University School of Medicine, Tsu, Carcinogenesis Division, ^cNational Cancer Research Institute, Tokyo and ^dDepartment of Pharmacology, Kobe University School of Medicine, Kobe, Japan

Received 26 October 1992

To evaluate the involvement of protein phosphatases (PP) in differentiation of human myelogenous leukemia HL-60 cells, we made use of potent inhibitors of PP1 and PP2A, calyculin-A (CAL-A) and okadaic acid (OKA). CAL-A and OKA could augment all-*trans* retinoic acid (ATRA)-induced granulocytic differentiation, whereas the differentiation toward macrophage lineage by 12-*o*-tetradecanoylphorbol acetate (TPA) was unchanged in the presence of CAL-A. CAL-A augmented the phosphorylation of 18K, 23K and 30K proteins induced by ATRA. The PP1 and PP2A were identified and were present mainly in the cytosol of HL-60 cells. These results suggest that either PP1 or PP2A or both may be involved in regulating granulocytic differentiation of HL-60 cells.

HL-60 cell; Differentiation; Phosphatase; Okadaic Acid; Calyculin-A

1. INTRODUCTION

Human myelogenous HL-60 leukemia cells can be induced to differentiate into either monocyte/macrophage-like cells or into granulocytes by various chemical agents [1–4]. These include 1,25-dihydrovitamin D₃ and 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), which mediate monocytic differentiation, and all-*trans* retinoic acid (ATRA) and dimethylsulfoxide for granulocytic differentiation. Some protein kinases have been identified as molecules comprising cellular signal transduction networks and it has become apparent that the regulated expression of protein kinases is crucial as mediator of differentiation signals [5–10]. The regulation of protein function by phosphorylation requires protein phosphatases in addition to protein kinases. It is therefore conceivable that phosphatase activity may influence mechanisms underlying cell differentiation. There appear to be four predominant forms, designated as PP1, PP2A, PP2B and PP2C by the classification system proposed by Ingebristen and Cohen [11]. To clarify the role of PP1 and PP2A, two of four serine/threonine-specific protein phosphatases, in the leukemic cell differentiation, we used two types of potent phosphatase inhibitors, OKA [12] and CAL-A [13,14] as probes for biological processes controlled by phosphorylation. These compounds are cell-permeable, and they inhibit PP1 and PP2A without affecting other

phosphatases or a variety of protein kinases including protein kinase C [12–15]. We also identified and quantitated PP1 and PP2A in HL-60 cells using the procedures [16] based on the use of OKA in conjunction with inhibitor-2 and immunological analysis using specific antibodies to PP1 and PP2A.

2. MATERIALS AND METHODS

2.1. Reagents

CAL-A and OKA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ATRA was purchased from Sigma (St. Louis, MO). Heat-stable phosphatase inhibitor-2 was purified according to the procedure of Yang et al. [17].

2.2. Determination of cell differentiation

The extent of differentiation was examined by the morphological assessment, ability of nitroblue tetrazolium (NBT, Sigma) reduction and surface marker analysis as described [8–10]. Adhesion was examined by quantitating cells that adhered to the culture dish [8].

2.3. *In vivo* phosphorylation of cellular protein

The labelled cells were exposed to reagents and analyzed by two-dimensional isoelectrofocusing/polyacrylamide gels, as described [7].

2.4. Preparation of protein phosphatases, and enzyme assay

The HL-60 cells (1×10^6 cells) were homogenized in buffer A (20 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 2 mM EGTA, 2 mM EDTA, and a cocktail of protease inhibitors) containing 0.25 M sucrose by a glass-to-glass Potter-Elvehjem homogenizer. The homogenate was immediately centrifuged at $1,000 \times g$ for 10 min to separate the crude nuclear fraction. The supernatant was then ultracentrifuged at $100,000 \times g$ for 1 h to separate the cytosol and plasma membrane fraction. The cytosolic fraction of HL-60 cells was treated by freezing and thawing in the presence of 0.2 M 2-mercaptoethanol, and was applied to a column (1.5 $\times$ 5.7 cm) of heparin-Sepharose CL-6B equilibrated with buffer A [8,18]. The column was extensively washed with

Correspondence address: M. Nishikawa, 2nd Department of Internal Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514, Japan. Fax: (81) (592) 31 5200.

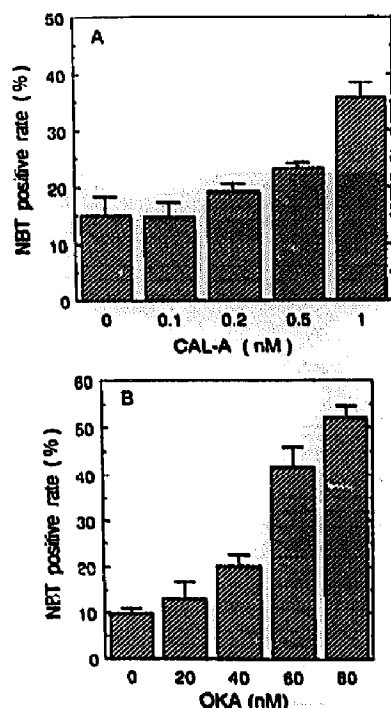


Fig. 1. Effects of CAL-A or OKA on differentiation of HL-60 cells induced by ATRA. Dose-dependent effects of CAL-A (A) or OKA (B) on the differentiation of HL-60 cells induced by 1 μ M ATRA. Phosphatase inhibitors were added 1 h prior to stimulation with differentiating inducing agents. Differentiation was determined by NBT reduction test after a 72 h incubation. Data represent the mean $\pm$ S.D. from three separate experiments.

buffer A and eluted batchwise with buffer A containing 0.5 M NaCl. Phosphatase activity was determined by the liberation of 32 P_i from the substrate (32 P-labelled myosin light chain) at 30°C according to the methods of Pato and Kerc [19].

2.5. Preparation of antibodies against PP1 and PP2A, and immunoblot analysis

Protein from the fraction with a peak phosphatase activity was analyzed by immunoblot [10] using rabbit polyclonal antibodies specific for PP1 [20] and PP2A [21].

Table I

Morphological changes of HL-60 cells after incubation with 1 μ M ATRA in the presence or absence of 1 nM CAL-A for 4 days

	Myeloid cell, % of total cells					
	Mybl.	Promy.	Myelo.	Meta.	Band.	Seg.
Control	2.0	88.5	8.5	1.0	0	0
ATRA (1 μ M)	0.5	1.5	19.5	62.0	13.0	3.5
ATRA (1 μ M) + CAL-A (1 nM)	0	0	4.5	46.0	42.5	7.0

HL-60 cells were incubated in conditions indicated. Differential counts were performed on May-Grünwald-Giemsa stained cytospin preparations of the cell suspensions after 4 days incubation. Results from a representative determination are shown. In each case, a minimum of 200 cells were scored. Mybl., myeloblast; Promy., promyelocyte; Myelo., myelocyte; Meta., metamyelocyte; Band., banded neutrophil; Seg., segmented neutrophil.

3. RESULTS

3.1. Effect of CAL-A and OKA on ATRA-induced granulocytic and TPA-induced macrophage lineage differentiation in HL-60 cells

CAL-A at concentrations of 1 nM or lower had no effect on proliferation. CAL-A alone induced no differentiation. ATRA suppressed the proliferation of HL-60 cells and induced HL-60 cells to differentiate terminally into granulocytes. The addition of CAL-A in combination with ATRA led to further maturation, when compared to findings with ATRA alone (Table I). The reduction of NBT provides a functional marker for granulocytic differentiation of HL-60 cells. The combination of CAL-A and ATRA resulted in a further increase in the number of NBT-positive cells (Fig. 1A). CAL-A alone induced no increase in the number of NBT-positive cells. The enhancement of ATRA-induced differentiation monitored by NBT reduction occurred in a dose-dependent manner in concentrations from 0.1 nM to 1.0 nM of CAL-A (Fig. 1A). OKA, another phosphatase

Table II

Adhesion rate and expression of surface membrane antigens of HL-60 cells during differentiation induced by ATRA or TPA in the presence or absence of 1 nM CAL-A

	Control	ATRA (1 μ M)	ATRA + CAL-A (1 μ M) (1 nM)	TPA (2 nM)	TPA + CAL-A (2 nM) (1 nM)
Adhesion (%)	0	0	0	43.8 $\pm$ 2.4	44.1 $\pm$ 3.1
CD11b	0.1	78.2	90.3	48.5	50.4
CD11c	0	28.4	60.8	49.6	49.4
CD54	0	95.4	96.8	98.7	99.0
CD71	97.6	13.0	6.1	83.0	81.2

In ATRA-induced differentiation, HL-60 cells were cultured for 84 h under the condition indicated and analyzed for antibody binding by flow cytometry. In TPA induced differentiation, to assess the adherence rate, HL-60 cells were pretreated with or without 1 nM CAL-A for 6 h and then cultured with 5 nM TPA for 24 h. Results represent the mean $\pm$ S.D. from three experiments. In surface marker analysis, the cells were cultured with 2 nM TPA for 15 h after pretreatment with or without 1 nM CAL-A for 6 h. Values represent the means of duplicate experiment and are expressed as the percentage of positive cells. Standard deviations were less than 10%.

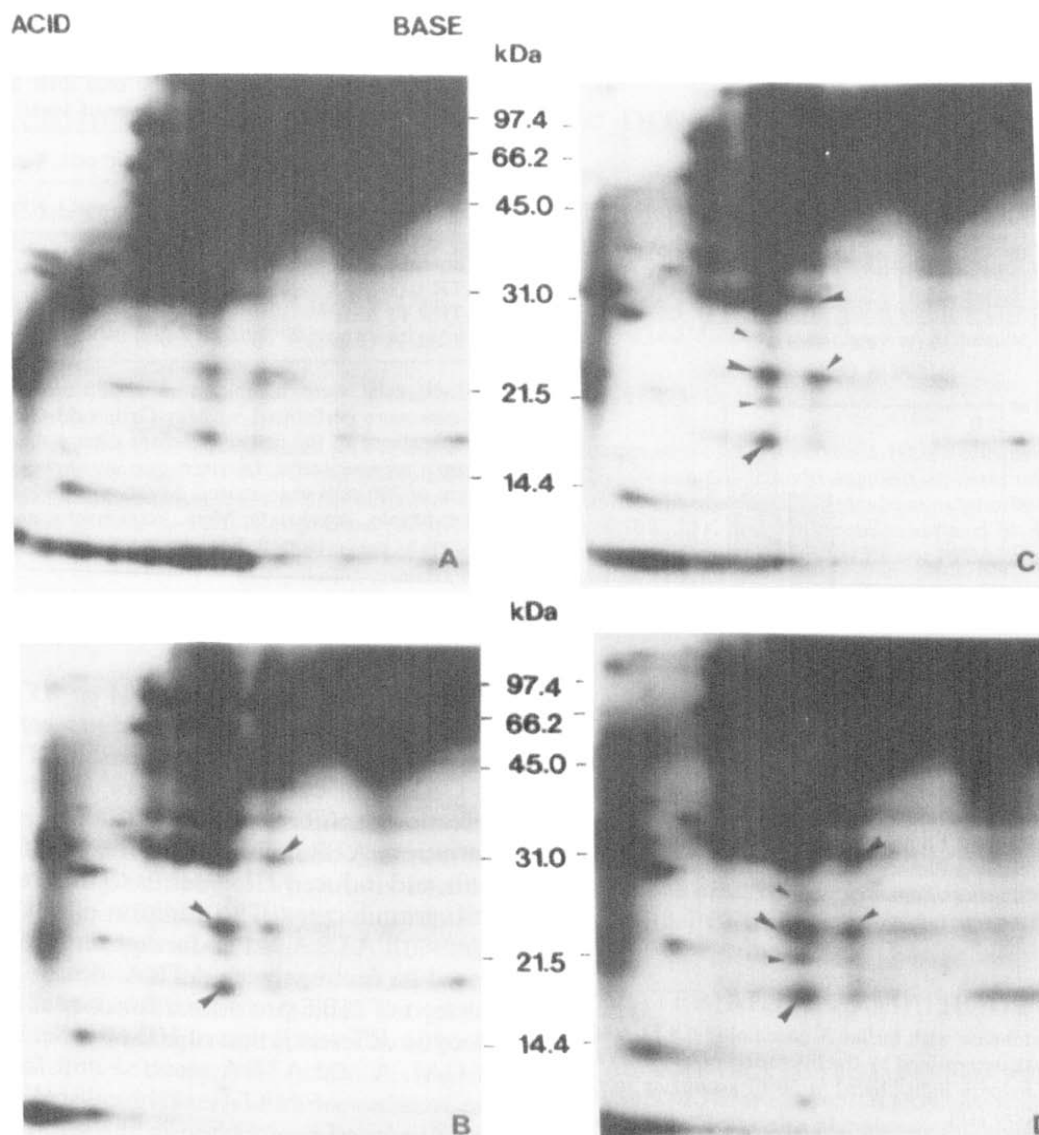


Fig. 2. Effects of CAL-A on phosphorylation of cellular proteins in HL-60 cells. HL-60 cells were incubated with ^{32}P for 1 h at 37°C and then exposed to $10\text{ }\mu\text{M}$ ATRA or/and 10 nM CAL-A for an additional 30 min. The cells were lysed and the whole cell proteins analyzed by two-dimensional gel electrophoresis and autoradiography. Designations of proteins (indicated by arrows) with increased phosphorylation were made after consistent observation in two or more experiments. A representative experiment is shown. A, control; B, $10\text{ }\mu\text{M}$ ATRA; C, 10 nM CAL-A; D, $10\text{ }\mu\text{M}$ ATRA plus 10 nM CAL-A; kDa, molecular weight in thousands.

inhibitor, also enhanced ATRA-induced differentiation dose-dependently in the concentration range of $20\text{--}80\text{ nM}$ (Fig. 1B). Table II shows the flow cytometric assessment of enhanced ATRA-induced granulocytic differentiation in the presence of CAL-A. The percentage of surface antigens detected by monoclonal antibodies CD11b (OKM1), CD11c (LeuM5) and CD54 (anti-ICAM) increased and that of CD71 (OKT9) decreased together with the maturation by $1\text{ }\mu\text{M}$ ATRA. These changes in surface markers were consistent with differentiation to more mature granulocytes. This modulation of surface antigen expression was significantly enhanced in the presence of CAL-A. Effects of CAL-A on

TPA-induced differentiation of HL-60 cells into macrophage-like cells were also examined by differentiation parameters such as adhesion rate and surface markers (Table II). CAL-A did not affect the differentiation into macrophages.

3.2. Stimulation of protein phosphorylation in intact HL-60 cells by ATRA and/or CAL-A

ATRA treatment increased the phosphorylation of 30K, 23K and 18K proteins compared with the control (Fig. 2). CAL-A by itself enhanced the phosphorylation of several distinct proteins (molecular mass = 30K, 26K, 23K, 20K, 18K). The extent of increased phospho-

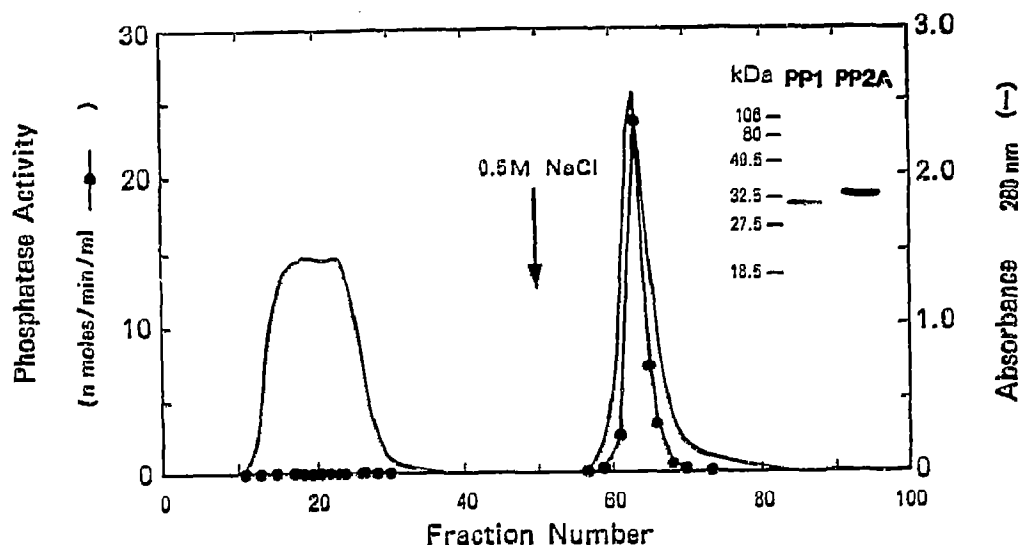


Fig. 3. Chromatography of the phosphatase activity of the cytosol from HL-60 cells on heparin-Sepharose CL-6B. Proteins from cytosol fractions of HL-60 cells were applied to a heparin-Sepharose column equilibrated with buffer A. Column was eluted with buffer A switching to buffer A containing 0.5 M NaCl where indicated. Phosphatase activity (●) was measured using phosphorylated myosin light chain as substrate, and absorbance (—) was measured at 280 nm. Immunoblot analysis of phosphatase in the cytosol of HL-60 cells is shown in the insert. A sample (10 μ l) of peak phosphatase fractions from heparin-Sepharose chromatography was subjected to immunoblot analysis using antibodies specific for PP1 and PP2A.

rylation in response to the combination of CAL-A and ATRA indicated that the effect of both ATRA and CAL-A may be additive.

3.3. Activity of protein serine/threonine phosphatases in HL-60 cells

We employed 32 P-phosphorylated myosin light chain as a substrate of phosphatase assay, because it is a good substrate for both mammalian PP1 and PP2A. Analysis of subcellular distribution of phosphatase activity from crude extracts of HL-60 cells revealed that phosphatase is distributed in various locations with the largest activity present in the cytosol. Relative activities of cytosol, plasma membrane and nuclear fractions were approximately 59%, 16% and 25%, respectively. After the HL-60 cytosolic fraction had been treated by freezing and thawing in the presence of 0.2 M 2-mercaptoethanol to convert from holoenzyme to a free catalytic subunit, it was adsorbed onto a heparin-Sepharose column (bed volume = 10 cm³) and then eluted batch-wise with 0.5 M NaCl (Fig. 3). Immunoblot analysis (Inset of Fig. 3) revealed that peak fractions eluted at 0.5 M NaCl contained both PP1 and PP2A. OKA and CAL-A inhibited this peak activity dose-dependently with IC₅₀ values of 2.5 nM and 4.2 nM, respectively. The use of a combination of OKA and inhibitor-2 allows PP1 and PP2A to be quantitated in two independent ways [16]. PP2A is inhibited completely by 2 nM OKA; in contrast, PP1 is hardly affected at this concentration, complete inhibition requiring 1 μ M. On the other hand, more than 90% of PP1 is inhibited by inhibitor-2 after 15 min preincubation. PP1 can be taken as the activity which is sensi-

tive to the inhibitor-2 and PP2A can be taken as the activity which is blocked by 2 nM OKA. Based on this simple procedure, the proportion of PP1 relative to PP2A was estimated to be almost its equivalent (Fig. 4).

4. DISCUSSION

Serine/threonine protein phosphatase inhibitor, CAL-A did augment the differentiation toward granulocytes by ATRA of HL-60 cells as evidenced by altered morphology, expression of differential surface markers and NBT reduction ability. CAL-A by itself, however, did not directly affect the differentiation. In contrast, CAL-A had no effect on differentiation into macrophages by TPA. CAL-A enhanced the protein phosphorylation of HL-60 cells induced by ATRA. CAL-A by itself stimulated the phosphorylation of some proteins (30K, 26K, 23K, 20K, 18K) in HL-60 cells. Moreover, CAL-A augmented ATRA-induced phosphorylation of 30K, 23K and 18K proteins. These results imply that in addition to basal protein kinase activities, protein phosphatases are active and prevent the accumulation of phosphorylated proteins in unstimulated HL-60 cells. One or more of these increased phosphoproteins may be the target factor(s) responsible for augmented differentiation toward granulocytes. The 23K protein phosphorylation seemed to be specific to granulocytic differentiation since it was not detected in TPA-induced differentiation [7], although the nature and subcellular localization of this protein are unknown. The precise mechanism by which ATRA exerts its biologic effects is unclear. ATRA is thought to elicit

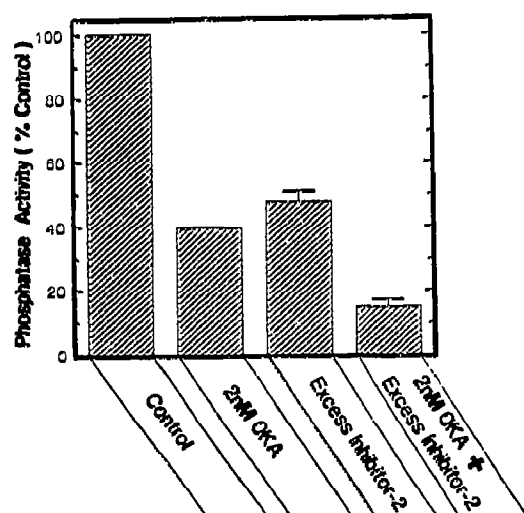


Fig. 4. Inhibition of phosphatase activity from a cytosolic fraction of HL-60 cells by OKA (2 nM) and excess inhibitor-2 in vitro. Phosphatase which was partially purified by heparin-Sepharose chromatography was also used. The enzyme mixture was preincubated with the indicated compounds 15 min prior to adding the substrate. Data are expressed as percent of control activity and represent the mean $\pm$ S.D. from three experiments.

its effects by binding to nuclear RA receptor (RAR) belonging to an *erbA*-related steroid/thyroid receptor family and the ATRA-RAR complex is thought to regulate the transcription of target genes [22]. Our results suggest that protein phosphorylation is partly involved in intracellular events that may initiate the process leading to cessation of proliferation and to terminal differentiation of the HL-60 cells subsequent to ATRA-RAR interaction. The identification of these enhanced phosphoproteins will be of a great importance in understanding the cellular differentiation mechanism. Thus, CAL-A-sensitive protein phosphatases may be, at least in part, involved as negative regulators of the signal transduction in ATRA-induced granulocytic differentiation.

We also identified and quantitated CAL-A-sensitive protein phosphatases, PP1 and PP2A, in the cytosol of HL-60 cells by the procedure based on the use of OKA and inhibitor-2 and immunological analysis using specific antibodies to PP1 and PP2A. The varying IC_{50} values for PP1 and PP2A allow OKA to be used to distinguish between these activities in cell-free systems, but in whole cells complication can arise in differentiating between the effect of OKA on PP1 and PP2A.

Therefore, it is not possible to conclude whether PP1 or PP2A or both are relevant to signal transduction of ATRA-induced differentiation processes at present. Further studies are necessary to characterize these phosphatases of HL-60 cells in more detail, including the purification, substrate specificities and regulatory mechanisms.

Acknowledgements: We thank M. Ohara for critical comments. This work was supported in part by grants for research from the Ministry of Education, Science and Culture of Japan.

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