

# Protein kinase C during differentiation of human promyelocytic leukemia cell line, HL-60

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Protein kinase C (PKC) from human promyelocytic leukemia HL-60 cells can be resolved into three fractions (peak *a*, *b* and *c*) by hydroxyapatite column chromatography. Peak *a* and *c* enzymes are indistinguishable from the brain type II PKC having  $\beta$  ( $\beta$ I and  $\beta$ II)-sequence and type III having  $\alpha$ -sequence, respectively. Peak *b* enzyme is a previously unidentified PKC subspecies that has enzymological properties subtly different from type I (having  $\gamma$ -sequence), type II and type III PKC. Upon treatment of HL-60 cells with 1  $\mu$ M retinoic acid, this peak *b* enzyme is decreased dramatically within 24 h, whilst peak *a* enzyme ( $\beta$ -PKC) is increased, and peak *c* ( $\alpha$ -PKC) enzyme is slightly decreased within 48 h. The result implies that the PKC subspecies in HL-60 cells have distinct functions during cell differentiation.

Protein kinase C; Retinoic acid; Differentiation; (HL-60 cells)

## 1. INTRODUCTION

Protein kinase C (PKC) is a large family of proteins with multiple subspecies that have subtly different enzymological characteristics, and this multiplicity is implicated in the diverse roles of PKC in physiological cellular responses to external signals (for reviews, see [1,2]). Initially, four cDNA clones,  $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ , were isolated, which are highly conserved among various mammalian species. PKC from the brain tissue is normally resolved into three subfractions, types I, II and III, upon hydroxyapatite column chromatography [3], which correspond to the enzymes encoded by  $\gamma$ -,  $\beta$ -( $\beta$ I- plus  $\beta$ II-) and  $\alpha$ -cDNA clone, respectively [4]. Recently, three additional cDNA clones of PKC,  $\delta$ ,  $\epsilon$  and  $\zeta$ , have been isolated from the rat brain cDNA library [5] (also for a review, see [1]).

The human promyelocytic leukemia cell line HL-60 can differentiate to many cell types with the characteristics of mature myeloid cells by various compounds, such as RA, DMSO, 1,25-dihydroxyvitamin

D<sub>3</sub> and TPA (for review, see [6]). Thus, this cell line provides a model system for studying cellular and molecular events involved in proliferation and differentiation. TPA appears to play a critical role in this process through activation of PKC [7,8]. On the other hand, RA does not seem to link directly with PKC, but is shown to increase the PKC activity prior to cell growth and differentiation [9,10]. This communication will describe the identification and properties of PKC subspecies which appear in HL-60 cells subsequent to RA treatment.

## 2. MATERIALS AND METHODS

### 2.1. Materials

The oligopeptide MBP<sub>4-14</sub> (QKRPSQRSKYL), corresponding to the major phosphorylation site of myelin basic protein, was used as a phosphate acceptor specific for PKC [11]. The oligopeptide having a sequence of pseudosubstrate region of PKC (RFARKGALRQKNV), PKC<sub>19-31</sub>, has been reported to be a relatively specific inhibitor of PKC by House and Kemp [12]. These oligopeptides employed in the present studies were synthesized by using an Applied Biosystems peptide synthesizer, Model 430A, followed by purification by gel filtration and reverse-phase column chromatography. PS and DO were purchased from Serdary Research Laboratories. RA (type XX), arachidonic acid, TPA and [ $\gamma$ -<sup>32</sup>P]ATP were obtained from Sigma, Nu-Check-Prep, Chemicals for Cancer Research and DuPont-New England Nuclear, respectively. Packed columns, TSK DEAE-5PW column (0.75  $\times$  7.5 cm), KB hydroxyapatite column (0.78  $\times$  10 cm, Type C) and Superose 12 HR 10/30 column were purchased from Toso, Koken and Pharmacia-LKB Biotechnology, respectively.

### 2.2. Cell culture

HL-60 cells were grown at a cell density between 0.5–2  $\times$  10<sup>6</sup> cell/ml as suspension in RPMI 1640 media (Flow) supplemented with

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*Abbreviations:* PKC, protein kinase C; RA, retinoic acid; DMSO, dimethyl sulfoxide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PS, phosphatidylserine; DO, diolefin; EGTA, [ethylenedibis(oxyethylenenitrilo)]tetraacetic acid

The data are taken in part from the dissertation that will be submitted by K. Hashimoto to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Science

5% fetal bovine serum (Gibco) and 2 mM glutamate at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells in their exponential growth phase were used. The basal PKC activity was not altered by cell density over the range described above. The cell differentiation was initiated by supplementing the media with 1  $\mu$ M RA. RA was solubilized in DMSO. The final concentration of DMSO was less than 0.05%, and it did not affect the differentiation process.

### 2.3. Separation and assay of PKC

All operations were performed at 0–4°C. HL-60 cells ( $2 \times 10^8$  cells) were suspended in 4 ml of 20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.4 mM leupeptin. The cells were lysed by sonication using three 15-s bursts, and centrifuged for 30 min at 100000  $\times g$ . The supernatant was applied to a DEAE-5PW column, which was connected to an FPLC system (Pharmacia LKB Biotechnology) and equilibrated beforehand with Buffer A (20 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA and 10 mM 2-mercaptoethanol). After washing the column with 20 ml Buffer A, PKC was eluted by increasing the concentration of NaCl linearly from 0 to 0.3 M in 20 ml Buffer A. The enzyme fraction was applied to a packed hydroxyapatite column, which was connected to an FPLC system and equilibrated beforehand with Buffer B (20 mM potassium phosphate, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10% (v/v) glycerol and 10 mM 2-mercaptoethanol). The column was washed with 28 ml Buffer B, and then, the PKC subspecies were eluted by increasing the concentration of potassium phosphate linearly from 20 to 200 mM in 84 ml Buffer B at a flow rate of 0.4 ml/min. Fractions of 1 ml each were collected. PKC was assayed with a synthetic oligopeptide, MBP<sub>4–14</sub>.

### 2.4. Antibodies

The polyclonal antibodies, designated CKpC1 $\beta$ -a, CKpV3 $\alpha$ -a, CKpV5 $\beta$ I-a, CKpV5 $\beta$ II-a and CKpV3 $\gamma$ -a, were prepared against the sequence-specific oligopeptides FARKGALRQKNVHEVKNHKF (type II ( $\beta$ ) PKC, residue 20–39), LGPAGNKVISPSDR (type III ( $\alpha$ ) PKC, residue 310–324), SYTNPEFVINV (type II ( $\beta$ I) PKC, residue 661–671), SFVNSEFLKPEVKS (type II ( $\beta$ II) PKC, residue 660–673) and SPIPSPSPSPSTDSK (type I ( $\gamma$ ) PKC, residue 322–335), respectively [13]. These antibodies were purified by column chromatography on goat anti-rabbit IgG antibody-Sepharose.

## 3. RESULTS

### 3.1. Isolation of PKC subspecies

The soluble cytoplasmic fraction of HL-60 cells was subjected to DEAE-5PW column chromatography as described above. A major peak with some shoulders of PKC was eluted from the column at 0.12–0.15 M NaCl. The enzyme fraction was subjected to hydroxyapatite column chromatography using an FPLC system. Three peaks of PKC, peak *a*, *b* and *c*, were detected (Fig. 1A). Peak *a* and *c* enzymes were eluted at the position corresponding to the rat brain type II and type III PKC, respectively, whereas peak *b* enzyme did not correspond to any of the three PKC subfractions from the brain tissue (Fig. 1C). Co-chromatography of the HL-60 and brain enzymes gave four peaks.

The peak *a*, *b* and *c* enzymes all showed an approximate molecular mass of 80 kDa as estimated by gel filtration analysis. Immunoblot analysis with several PKC subspecies-specific antibodies listed in section 2 indicated that the peak *a* enzyme reacted with an-

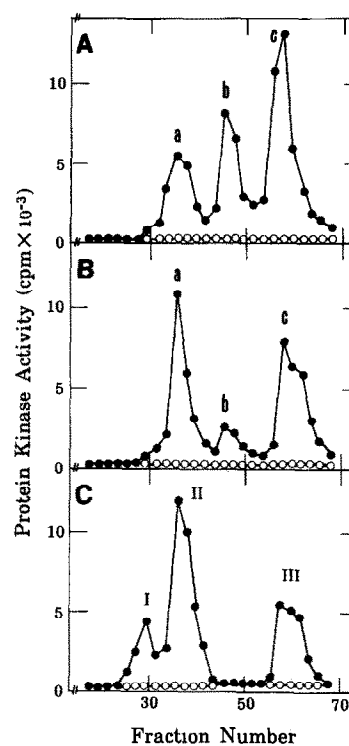


Fig. 1. Resolution of PKC subspecies of undifferentiated and differentiated HL-60 cells upon hydroxyapatite column chromatography. The PKC preparation of DEAE-5PW column fraction was subjected to hydroxyapatite column chromatography as described in section 2. An 8- $\mu$ l aliquot of each fraction (1 ml each) was assayed for PKC activity with MBP<sub>4–14</sub> as substrate. The DEAE-cellulose fraction of rat brain cytoplasm was prepared as described [14] and subjected to hydroxyapatite column chromatography under the same conditions. (A, B and C) Cytoplasmic fractions of undifferentiated and differentiated (4-days RA-treatment) HL-60 cells and rat brain, respectively. PKC activity in the presence of 0.3 mM CaCl<sub>2</sub>, 8  $\mu$ g/ml PS and 0.8  $\mu$ g/ml DO (●) or in the presence of 0.5 mM EGTA instead of CaCl<sub>2</sub>, PS and DO (○). The letters *a*, *b*, *c*, I, II and III designate the PKC fractions.

tibodies specific to type II PKC having the  $\beta$  ( $\beta$ I plus  $\beta$ II)-sequence, and the peak *c* enzyme reacted with antibodies specific to type III PKC having the  $\alpha$ -sequence. On the other hand, the peak *b* enzyme reacted with none of the antibodies which recognize type I, II and III PKC, indicating that this enzyme is a new entity of PKC subspecies. The enzyme did not appear to be  $\delta$ -,  $\epsilon$ - or  $\zeta$ -PKC because of its unique kinetic and enzymological properties (see below).

### 3.2. Characterization of peak *b* PKC

The PKC subspecies, peak *b* enzyme, which was eluted between type II and III PKC from the hydroxyapatite column, showed typical characteristics of PKC. The enzyme responded well to both PS and DO over a wide range of Ca<sup>2+</sup> concentrations (Fig. 2). The enzyme was sensitive to Ca<sup>2+</sup>, and TPA could replace DO. Free arachidonic acid was much less active for the peak *b* enzyme (Fig. 3A). Pseudosubstrate peptide,

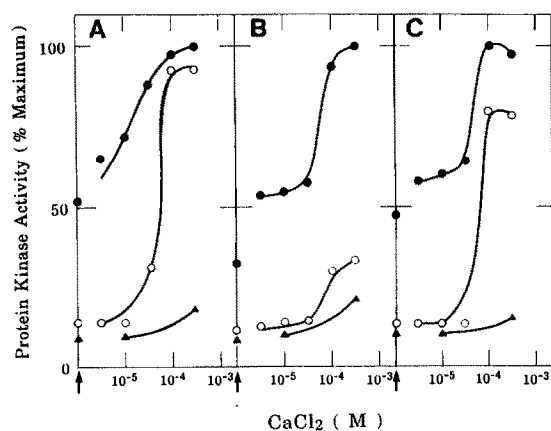


Fig. 2. Activation of PKC fractions by  $\text{Ca}^{2+}$ , PS and DO. (A, B and C) The fractions of peak *a*, peak *b* and peak *c*, respectively. Each fraction of enzyme was assayed with MBP<sub>4-14</sub> as substrate at various  $\text{CaCl}_2$  concentrations in the presence of  $8 \mu\text{g/ml}$  PS plus  $0.8 \mu\text{g/ml}$  DO (●),  $8 \mu\text{g/ml}$  PS alone (○) or  $0.8 \mu\text{g/ml}$  DO alone (▲). Where indicated by the arrow, EGTA ( $0.5 \text{ mM}$ ) was added instead of  $\text{CaCl}_2$ . Results are normalized to the maximal activation obtained in the presence of  $\text{CaCl}_2$ , PS and DO.

PKC<sub>19-31</sub>, was less effective to this enzyme (Fig. 3B). Several lines of evidence indicate that the peak *b* enzyme is most likely a previously unidentified PKC subspecies. It does not appear to represent a proteolytic or other post-translational modification of the previously known PKC subspecies, since none of the antibodies could recognize this enzyme. The PKC subspecies having  $\delta$ -,  $\epsilon$ - or  $\zeta$ -sequence were not sensitive to  $\text{Ca}^{2+}$  [15,16].

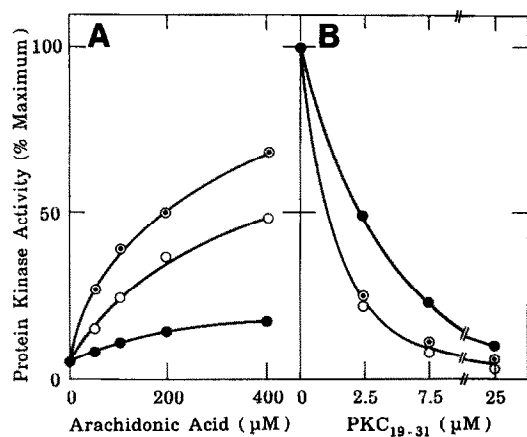


Fig. 3. Effects of arachidonic acid and pseudosubstrate peptide, PKC<sub>19-31</sub>, on PKC fractions of HL-60 cells. (A) Activation of PKC fractions of HL-60 cells by arachidonic acid. Each fraction of the enzyme was assayed with MBP<sub>4-14</sub> as substrate at various concentrations of arachidonic acid in the presence of  $0.3 \text{ mM}$   $\text{CaCl}_2$ . (B) Inhibition of PKC fractions of HL-60 cells by PKC<sub>19-31</sub>. PKC was assayed with MBP<sub>4-14</sub> as substrate at various concentrations of PKC<sub>19-31</sub> in the presence of  $0.3 \text{ mM}$   $\text{CaCl}_2$ ,  $8 \mu\text{g/ml}$  PS and  $0.8 \mu\text{g/ml}$  DO. (○), (●) and (◐), peak *a*, *b* and *c*, respectively. Results are normalized to the maximal activation obtained in the presence of  $\text{CaCl}_2$  ( $0.3 \text{ mM}$ ), PS ( $8 \mu\text{g/ml}$ ) and DO ( $0.8 \mu\text{g/ml}$ ).

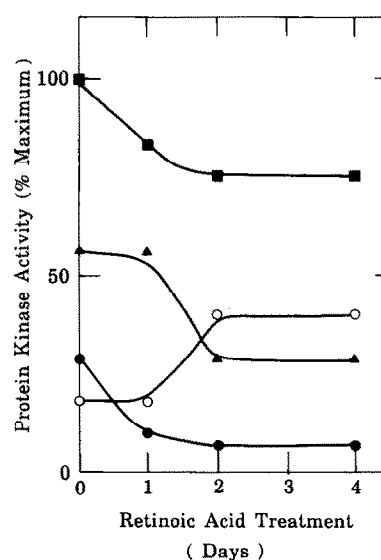


Fig. 4. PKC subspecies of HL-60 cells after RA treatment. HL-60 cells were cultured with  $1 \mu\text{M}$  RA as described in section 2 for various days as indicated. Then, the cytoplasmic PKC fractions of HL-60 cells were quantitated through DEAE-5PW and hydroxyapatite column chromatography as described in section 2. (●), (○), (◐) and (▲), total PKC activity (peak *a* + peak *b* + peak *c*), peak *a* activity, peak *b* activity and peak *c* activity, respectively. Results are normalized to the total PKC activity found at 0 day.

### 3.3. PKC subspecies during differentiation

RA is well known to induce differentiation of HL-60 cells along the myeloid pathway [6]. Under the culture conditions presented herein, the growth rate was decreased by about 50%, and more than 30% of the cells showed a granulocyte-like segmented nucleus 5 days after the supplement of  $1 \mu\text{M}$  RA to the culture medium. The HL-60 cells treated for 4 days with RA were harvested, and PKC subspecies were fractionated as described above. The elution profile of PKC given in Fig. 1B indicates that the peak *b* enzyme was dramatically decreased within 24 h, whilst peak *a* enzyme increased over a longer time-period at 1–2 days, concomitantly with a slight decrease in peak *c* enzyme. At all stages of differentiation PKC activity in the particulate fraction was negligible, unless TPA was added. Fig. 4 summarizes the analysis of the PKC subspecies, peak *a*, *b* and *c*, during differentiation induced by RA.

## 4. DISCUSSION

Makowske, et al. [17] previously reported that PKC in HL-60 cells was resolved into three subfractions, which were all induced in parallel during differentiation after treatment with either RA or DMSO. Although these authors thought that the three PKC subfractions were type I, II and III PKC, the present biochemical and immunoblot analysis indicates that HL-60 cells have type II ( $\beta$ ) and III ( $\alpha$ ) PKC and an additional structurally unknown PKC subspecies (peak *b* enzyme). The studies of our and other laboratories have shown

that type I ( $\gamma$ ) PKC is expressed only in the central nervous tissues and spinal cord [14,18].

In contrast to the RA-induced differentiation, exposure of HL-60 cells to TPA results in the macrophage-like differentiation, accompanying phosphorylation of several endogenous proteins [7,8]. TPA, however, causes rapid translocation to membranes and subsequent disappearance, down-regulation, of PKC. Several mutant cell lines, which do not translocate or down-regulate PKC, do not appear to differentiate to macrophage-like cells in response to TPA [19,20]. It is suggestive that the members of the PKC family in HL-60 cells play different functions and are distinctly regulated during cell differentiation.

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