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Differentiation of HL60 promyelocytic cells is promoted by a 'differentiation enhancing factor' produced by erythroleukemia cells

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A differentiation enhancing factor isolated from murine erythroleukemia cells is also a potent enhancer of the differentiation of HL60 human promyelocytic leukemia cells, induced by retinoic acid and by phorbol ester. This stimulating effect is the result of a large increase in the sensitivity of HL60 cells for retinoic acid and for phorbol 12-myristate 13-acetate (20-fold and 40-fold, respectively). Accelerated differentiation induced by the protein factor, and monitored by the appearance of marker enzymes, is accompanied by a large increase in the fluctuation of the levels of protein kinase C (PKC) isozymes in HL60 cells. These results provide further support for the role of this new protein factor in cell differentiation and indicate that other cell types are susceptible to its biological effect.

Cell differentiation; Protein kinase C; Differentiation enhancing factor; HL60 cells; Erythroleukemia cells

1. INTRODUCTION

The rate of differentiation of murine erythroleukemia (MEL) cells is accelerated by a low M_r protein defined as 'differentiation enhancing factor' (DEF) [1]. Experimental evidence has indicated that DEF is synthesized by MEL cells and upon the addition of the inducer is secreted into the extracellular compartment, where it binds to the external surface of the cell [2]. It has also been shown that the factor is a potent activator of certain Ca^{2+}/phospholipid dependent-PKC isozymes and that this activation is also accomplished by a reduction in the $[Ca^{2+}]$ requirements [3]. The amino acid sequence of tryptic peptides, obtained from purified DEF, has indicated no homology with the primary structure of any other natural protein so far characterized [4]. In order to establish if DEF possesses an enhancing effect on the differentiation of other cell types, we have tested the capacity of this protein to affect the differentiation of HL60 promyelocytic leukemia cells, using as a marker the level of specific enzymes, the increase of which had been previously demonstrated to correlate with the differentiation process [5,6]. It has been established that DEF promotes an acceleration in the differentiation of HL60 cells, accompanied by significant changes in the level of PKC isozymes present in these cells. In terms of mechanism of action the results obtained indicate that DEF induces a profound increase in the sensitivity of the cell for the inducer. These data can be taken as an indication for a broad specificity of DEF activity, not restricted to the type of cell by which it is produced, and are indicative for the presence in DEF of structural domains recognized by different cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture and differentiation*

HL60 human promyelocytic leukemia cells were grown in RPMI-1640 supplemented with 20% fetal calf serum and 2 mM glutamine in a 6% CO_2 humidified incubator. Cell viability was examined by Trypan blue dye exclusion. Unless otherwise indicated, cell differentiation was induced in 30 ml culture medium containing 10^5 cells/ml and 5 μM retinoic acid (RA) or 3×10^5 cells/ml and 20 nM phorbol 12-myristate 13-acetate (PMA). These concentrations of inducers are those found to promote the maximal rate of differentiation. Cells were incubated for four days and differentiation was determined by examination of cell morphology under light microscope, following staining with Wright–Giemsa method [5]. Under these conditions approximately 90–92% of cells reached the differentiated state. To avoid individual discrepancies in the determination of the number of cells showing the morphological changes, we have followed the HL60 cell differentiation by the appearance of marker enzymes typical of the differentiated phenotype. Based on a recent report [5] alkaline phosphatase (AlPase) activity is the marker for the RA-induced differentiation, whereas acid phosphatase (AcPase) is the marker for the PMA-induced differentiation [6]. Under our conditions, in the undifferentiated state, the basal level of AlPase is 0.9 ± 0.1 units/mg of membrane proteins; in the fully differentiated cells, the level increases to 7.5 ± 0.9 units/mg of membrane proteins. Similarly, AcPase has a basal level of 17 ± 2 units/mg membrane proteins in undifferentiated cells and increases to 215 ± 12 units/mg of membrane proteins in the fully differentiated cells. All experiments were done in triplicate; the values reported above represent the means ± S.D. We have also observed that the increase in activity of enzyme markers, which accompanies the differentiation of HL60 cells, proceeds in parallel with a decrease in cell growth rate and with the appearance of morphological changes characteristic of the differentiated state [7].

2.2. *Enzyme assays*

Alkaline phosphatase was assayed as previously described [8], using β-glycerophosphate as a substrate. Acid phosphatase was assayed as

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reported in [9] using β-glycerophosphate as a substrate. The inorganic phosphate released during the reaction was determined as described [10]. One unit of phosphatase activity is defined as the amount that causes the release of 1 nmol of inorganic phosphate per min, under the assay conditions.

PKC activity was assayed as previously described, by evaluating the incorporation of ^{32}P into histone type III-S [11]. One unit of PKC activity is defined as the amount that causes the incorporation of 1 nmol of phosphate per min, under the specified assay conditions.

2.3. *Isolation of particulate cell fraction (membranes)*

HL60 or V3.17.44 MEL [12] cells, in an amount of 2×10^7, were collected, washed twice with 10 ml of 20 mM Na-phosphate buffer, pH 7.2, containing 0.14 M NaCl, and suspended in 1 ml of 20 mM Tris-HCl pH 7.2, containing 5 mM 2-mercaptoethanol, 0.34 M sucrose and 1 mM phenylmethylsulphonylfluoride, (buffer A). Cells were lysed by sonication (6 strokes, 10 s each) at 4°C and the suspension was centrifuged at 200,000 × *g* for 10 min. The pellets were collected, washed with 1 ml of buffer A, suspended in 0.25 ml of the same buffer and sonicated three times (10 s each). Finally 0.25 ml of Triton X-100 at a final concentration of 0.5% were added. The resulting solution was used as a source of the particulate cell fraction (membranes) of the two cell lines.

2.4. *Purification of the differentiation enhancing factor (DEF) from MEL cells*

DEF was purified from MEL cells as previously described [2]. DEF activity was assayed by evaluating its enhancing effect on the rate of differentiation of N23 MEL cell clone [2]. One unit of DEF activity is defined as the amount that doubles the proportion of differentiated MEL cells [2], under the specified conditions.

2.5. *Separation of the PKC isoenzymes*

The procedure used was essentially that described by Hashimoto et al. [13]. HL60 cells were lysed and the bulk of PKC activity was collected as a single peak on a DEAE-cellulose column chromatography [13] and submitted to hydroxyapatite (HA) chromatography under the conditions described in [14]. PKC isozymes were identified by their effector requirements [14] and by their position on HA chromatography, using as a standard the PKC isozyme activities present in rat brain [14].

3. RESULTS

3.1. *Effect of DEF on differentiation of HL60 cells induced by RA or PMA*

Human promyelocytic leukemia cell line HL60 can be induced to differentiate to mature myeloid cells by different compounds [7,13,14]. Retinoic acid (RA) induces a preferential differentiation into granulocytic cells, that can be evaluated by the increase in their alkaline phosphatase content [5]; PMA promotes differentiation into macrophagic cells that can be monitored by the accumulation of acid phosphatase [6]. On the basis of this well established information, we have first determined the extent of differentiation after four days of incubation as a function of the concentration of these chemical inducers. As shown in Fig. 1, RA reaches its maximum effect at concentrations around 5 μM (Fig. 1A, bar 4), whereas PMA is maximally effective around 20 nM (Fig. 1B, bar 4). It also appears that RA at 0.1 μM (Fig. 1A, bar 3, full bar) and PMA at 1 nM (Fig. 1B, bar 3, full bar) promote very little changes in the levels of marker enzymes, implying that at these concentrations of the inducers no significant differentiation occurs.

The capability of DEF to enhance differentiation was then investigated by adding aliquots of this factor, purified from V3.17.44 MEL cells [12], to HL60 cells concomitantly exposed to concentrations of RA or PMA almost completely uneffective in inducing cell differenti-

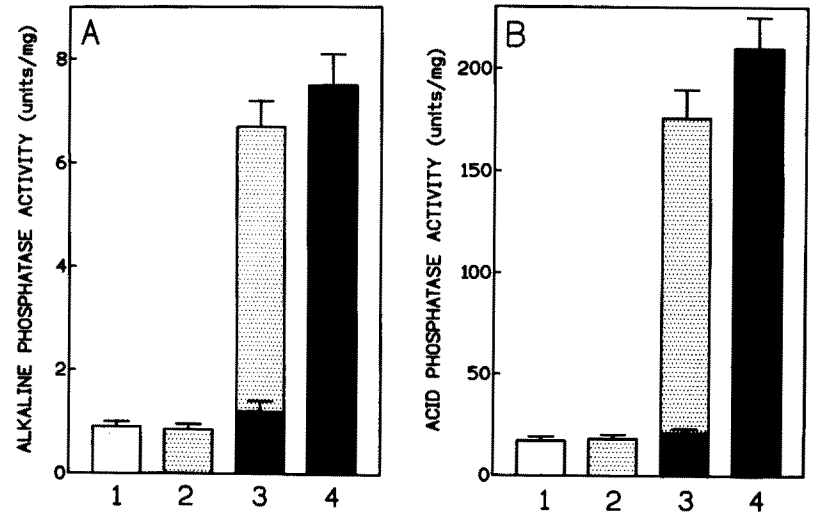

Fig. 1. Differentiation of HL60 cells induced by RA (A) and PMA (B) in the presence or in the absence of DEF isolated from MEL cells. The HL60 cells were incubated as described in section 2 with the indicated concentrations of inducers for 4 days; where indicated, 6 ng/ml of purified (see section 2) DEF were also added to the cell suspension, before incubation. Cells were then collected and the particulate fractions were prepared as reported in section 2. The membrane-associated alkaline or acid phosphatase activities were determined, as described in section 2. The experiments were done in triplicate, the values represent the means ± S.D. (error bars). Bar 1, level of the marker enzyme in unstimulated cells; bar 2, cells treated with DEF alone; bar 3, full part, cells incubated with 0.1 μM RA (A) or 1 nM PMA (B) alone; bar 3, dotted, cells incubated with 0.1 μM RA (A) or 1 nM PMA (B) in the presence of DEF; bar 4, cells incubated with 5 μM RA (A) or 20 nM PMA (B) alone.

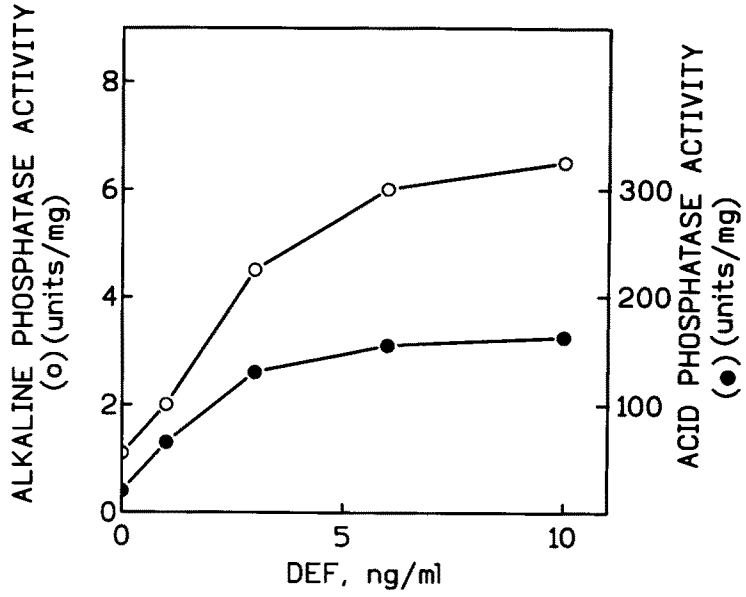

Fig. 2. Effect of DEF on the differentiation of HL60 cells. HL60 cells were incubated as described in section 2 with the indicated amounts of DEF in the presence of 0.1 μM RA (○) or 1 nM PMA (●). After four days of incubation, the cells were collected and the particulate fractions were prepared for the assay of membrane associated alkaline or acid phosphatase activities (see section 2).

ation. Under these conditions, addition of DEF promotes a 6- and 9-fold increase in the levels of AlPase and AcPase activities, respectively, as compared to cells stimulated with the inducers alone (cf. bars 3 and 4 of Fig. 1). The levels of marker enzymes observed under these conditions are similar to those of fully differentiated cells, indicating that addition of DEF causes a 20- to 40-fold increase in the number of differentiated cells. The enhancing effect is dose-dependent as indicated in Fig. 2, reaching an apparent saturation at a concentration of approximately 6 ng of DEF per 10^5 cells, and temptingly suggesting the existence of a rate-limiting receptor on the cell surface.

The effect of DEF on the differentiation of HL60 cells was then investigated in the presence of increasing concentrations of either inducers. The data obtained (Fig. 3) indicate that DEF promotes a maximal rate of differentiation even when RA and PMA are present in amounts of 0.1 μM and 1 nM, respectively, concentrations at which the differentiation in the presence of the

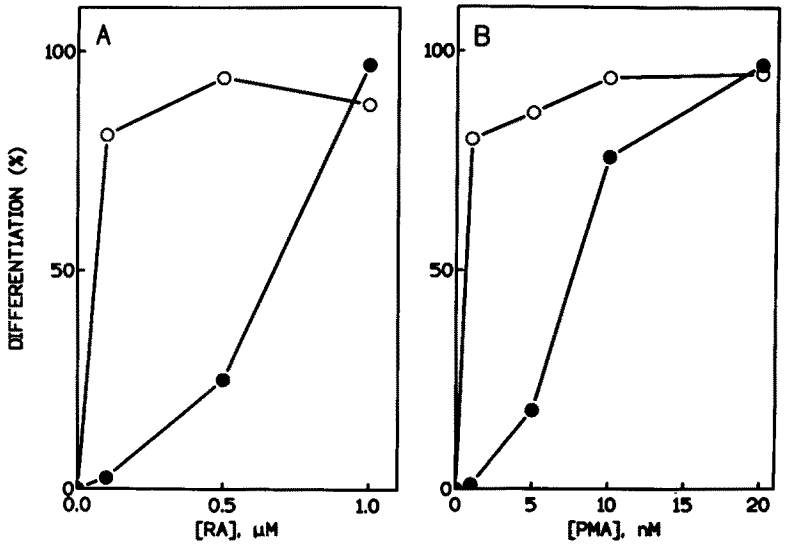

Fig. 3. Effect of DEF on the concentrations of RA (A) or PMA (B) required for the differentiation of HL60 cells. Cells were incubated as described in section 2 with the indicated concentrations of inducers in the absence (●) or in the presence (○) of 6 ng/ml of purified DEF. After four days cells were collected, the particulate fractions were prepared and the membrane associated alkaline and acid phosphatase activities were assayed as reported in section 2. The proportion of differentiated cells was calculated from the increase in the expression of marker enzymes as described in section 2.

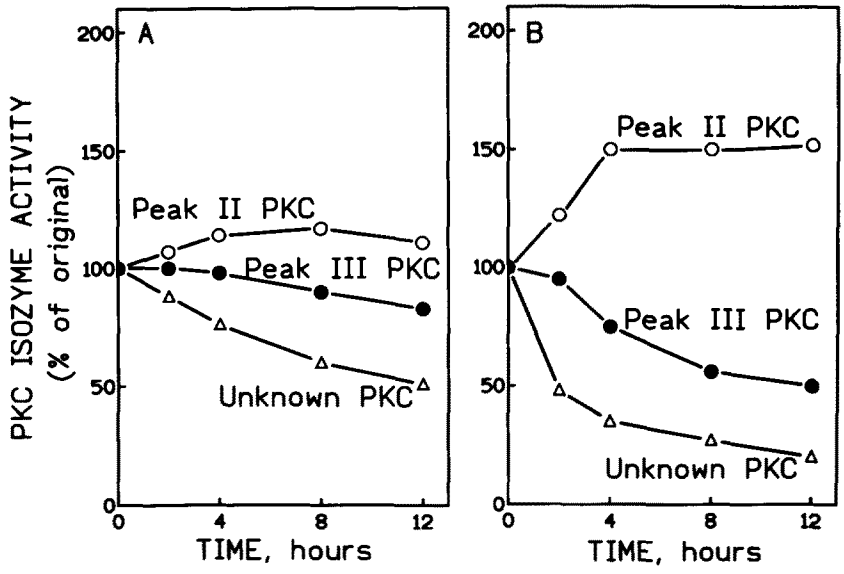

Fig. 4. Effect of DEF on the level of PKC isozymes in HL60 cells during differentiation. Cells (2×10^7) were incubated as described in section 2 with 0.1 μM RA in the absence (A) or in the presence (B) of 6 ng/ml of purified DEF. At the indicated times, cells were collected lysed and the soluble fractions utilized as a source of PKC isozymes as reported in section 2. The various PKC isoforms were identified in the HA-chromatography by the elution positions, using the rat brain α- and β-PKC isoforms as standards [13]. The level of each PKC isozyme was calculated from the area of the eluted peaks and plotted as the percentage of the starting values (% of original).

inducers is almost undetectable. At higher concentrations of either RA or PMA and when differentiation has reached its maximal rate, DEF has no longer an enhancing effect (Fig. 3), suggesting that the factor induces a dramatic increase in the sensibility of the cell for the inducer, thereby accelerating the transition between a pre-inducible to a fully inducible state. The possibility has been evaluated that HL60 cells synthesize DEF-like protein, by assaying the presence of DEF-like biological activity in both the soluble and particulate fractions of these cells. As shown in Table I, a DEF-like activity was undetectable in both cell compartments and therefore, we can conclude, that HL60 cells do not contain this protein factor.

3.2. *Effect of DEF on the level of PKC subspecies in HL60 cells during differentiation*

It has been suggested that the large variations in the levels of intracellular PKC isozymes, shown to occur during RA-induced HL60 cell differentiation [13], are important molecular events critically involved in the overall differentiating process. In order to establish if the increased rate of HL60 cell differentiation induced by DEF was accompanied by a change in the rate and extent of fluctuation in the levels of PKC isozymes present in these cells, HL60 cells were incubated with or without DEF in the presence of suboptimal concentrations of RA. As shown in Fig. 4A and in agreement with previous observations [13], within 12 h after the addition of RA rapid changes in the activity of PKC subspecies occur. Peak II (PKC II) activity (corresponding to the β-PKC isozyme) slightly increases, whereas peak III (PKC III) activity (corresponding to the α-PKC isozyme), as well as a third peak, corresponding to a still structurally unknown PKC subspecies, undergo a progressive decrease. Following the addition of DEF all these fluctuations in the levels of each PKC isozyme are significantly amplified and accelerated (Fig. 4B) and become identical to those observed in the presence of optimal concentrations of RA (data not shown). In particular, within the first four hours all the modifications are completed with the result that the structurally unknown PKC isoform is down-regulated to almost complete disappearance, whereas the level of peak II PKC activity increases by approximately 50%.

Table I

Level and distribution of DEF-like activity in MEL and HL60 cell lines

Cell line	DEF-like activity (units/10^6 cells)	
	Soluble fraction	Particulate fraction
MEL cells	68 ± 5	0.2 ± 0.1
HL60 cells	< 0.01	n.d.

V3.17.44 MEL cells [12] and HL60 cells (10^7 cells) were collected at a cell density of 10^6 cells/ml, and the particulate fraction was prepared for each cell line as described in section 2, except that the particulate fraction was dispersed by sonication and not solubilized in Triton X-100. DEF activity was measured as described in section 2. The values are the means ± S.D. of three different experiments. n.d. = not detectable.

4. DISCUSSION

It has been previously reported that MEL cells synthesize and accumulate intracellularly a protein factor that is secreted into the extracellular compartment during the earlier stages of the differentiation process induced by examethylen bisacetamide [2]. This factor, called differentiation enhancing factor (DEF), once added to the culture medium or spontaneously secreted by the MEL cells, induces a very large increase in the rate of differentiation of MEL cells to the mature erythroid phenotype [1]. 'In vitro' experiments have shown that DEF is also a potent activator of the Ca^{2+}/phospholipid dependent-PKC isoforms [3], whereas addition of DEF to intact cells was followed by an acceleration in the rate of down-regulation of PKC activity [1] occurring during the cell differentiation process and before cell commitment.

In order to establish if DEF was equally effective in stimulating the rate of differentiation of other cell types and particularly cells, we have studied its effect on the HL60 promyelocytic leukemia cell line. We have observed that following the addition of DEF, HL60 cells exposed to suboptimal concentrations of either RA or PMA undergo an acceleration in the rate of differentiation that accounts for a 20- to 40-fold increase over that observed in the presence of the inducer alone. Differentiation was monitored by the increase in the levels of marker enzymes [5,6] and by the appearance of characteristic morphological changes in the cells [6,7]. Furthermore, the results obtained have indicated that, in the early stages of cell differentiation, DEF also induces an increase in the rate and in the extent of variations in the levels of PKC isoforms present in HL60 cells.

It is relevant for the purpose of the present paper to recall that, based on preliminary molecular analysis [4], including the amino acid sequence of tryptic peptides, it has been established that the protein factor DEF is distinguishable from any other proteins, including all cytokines which have been shown to promote HL60 cell differentiation [15]. This conclusion is also supported by the fact that DEF with both HL60 and MEL cells does not behave as an inducer since its biological function is expressed through the promotion of an increase in the rate of cell differentiation.

Taken together, the present observations strongly indicate that the effect of DEF is not restricted to a single cell line, but can be extended to a different cell type apparently not involved in the synthesis of this protein factor. Whether this response reflects a specific type of interaction, is the purpose of additional experiments.

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