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Correlation between Levels of δ Protein Kinase C and Resistance to Differentiation in Murine Erythroleukemia Cells

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It has been demonstrated that the level of δ protein kinase C is inversely correlated to the responsiveness of murine erythroleukemia cells to chemical induction to terminal erythroid differentiation. In these cells, δPKC is largely present in a membrane associated form, and thus in a constitutively active state, a condition which characterizes the undifferentiated phenotype. Accordingly, commitment to cell differentiation has been shown to be preceded by down regulation of δPKC, a process significantly accelerated and induced to almost completion by the differentiation enhancing factor (DEF) in a dose dependent manner. The present results provide a better understanding of the role of δPKC in characterizing the undifferentiated MEL cell phenotype, and suggest a relationship between the acceleration in the rate of differentiation induced by DEF and the down regulation of this kinase form.

The chemical induced differentiation of murine erythroleukemia (MEL) cells is a multistep process including early events occurring during the lag period that precedes cell commitment (1,2). We have shown that in this lag phase two crucial steps occur, one mediated by a protein factor called DEF and identified as a protein of the HMG1 family (3), the other by protein kinase C (PKC) isozymes (4). In unstimulated cells, DEF is retained intracellularly. Upon n exposure to the chemical inducer, hexamethylenebisacetamide (HMBA), a large fraction of this protein factor is secreted and accumulated in the extracellular medium where it becomes active in increasing the rate of MEL cell differentiation (5). In previous experiments, at least two distinct PKC isoforms were shown to play a specific role in MEL cell differentiation, being involved in the acceleration of the process or in the preservation of the undifferentiated phenotype. It has been thus established that one of the earliest events occurring following HMBA treatment and largely preceding cell commitment, is a rapid down regulation of δPKC (6). The resulting hypothesis attributing to δPKC a stabilizing role on the undifferentiated phenotype subsequently received the following experimental supports: a) the expression of δPKC is much higher in MEL cells characterized by a high resistance to differentiation (7,8); b) loading permeabilized MEL cells with purified homologous δPKC increases significantly the resistance to differentiation (6); c) the increase or the decrease in the intracellular amount of δPKC, obtained by treatment with verapamil or with A23187 Ca^{2+} ionophore, produces either an increase or a decrease in the sensitivity to the inducer (9); d) repression by an antisense oligonucleotide (directed against the translation initiation region of δPKC mRNA) of the expression of the corresponding PKC isoform, induces an increased responsiveness to HMBA in MEL cells (10).

In order to further explore and define the role of δPKC in chemical induced MEL cell differentiation, we have now investigated first its intracellular distribution and subsequently the correlation among different locations of the kinase, the kinetics of its down regulation and the rate of cell commitment induced by HMBA, in the presence or absence of DEF. The data presented in this

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Abbreviations: PKC, protein kinase C; MEL, murine erythroleukemia; HMBA, hexamethylenebisacetamide; DEF, differentiation enhancing factor; HMG1, high mobility group 1; MEM, minimal essential medium.

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paper indicate that a large fraction of δPKC is localized on the plasma membrane in an amount that is directly related to that present in the soluble fraction. The rate of degradation of δPKC depends on the amount of the kinase form associated to the membranes and the overall process is significantly enhanced by DEF. The correlation between the level of δPKC and the rate of differentiation has also been analyzed in MEL cell clones characterized by constitutively different or experimentally modified with respect to their content in this kinase form.

MATERIALS AND METHODS

Cell culture. MEL cell clones R1 (11), N23, and V3.17.44, named C44 in this paper (4), were cultured in α minimal essential medium supplemented with 10% fetal calf serum, as previously reported (4). Cells were induced to terminal erythroid differentiation by addition of 5 mM HMBA at a density of 10^5 cells/ml. At the indicated times the amount of differentiated cells was determined by adding to cell suspensions equal volumes of 0.5 M acetic acid containing 0.2% benzidine and 1% oxygen peroxide. After 1 min of incubation cells were scored for the presence of hemoglobin (blue color) (12). When indicated MEL cells were incubated for 24 hours in the presence of A23187 Ca^{2+} ionophore or verapamil and then induced by addition of 5 mM HMBA.

Immunoblot analysis of PKC isozymes. The appropriate amounts of MEL cell fractions were submitted to 8% SDS-polyacrylamide gel electrophoresis (13) followed by electroblotting onto nitrocellulose membrane as previously described (10). The bands corresponding to PKC isozymes were identified using isotype-specific polyclonal rabbit antibodies (Santa Cruz Biotechnology) and the immunoreactive material detected by using [^{125}I]-Protein A (Amersham) (10). The radioactive bands were revealed by autoradiography and then excised and quantified in a γ-counter.

Preparation of cell fractions. To obtain the cell soluble and membrane fractions, MEL cells were collected, washed with PBS, pH 7.4 and suspended in the amount of lysis buffer indicated elsewhere, containing 0.25 M sucrose, 10 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 2 mM phenylmethylsulfonilfluoride, and 0.1 mg/ml leupeptin. Cells were lysed by sonication (six strokes of 10 seconds each) and centrifuged at 200,000×g for 10 min. The clear supernatant (cell

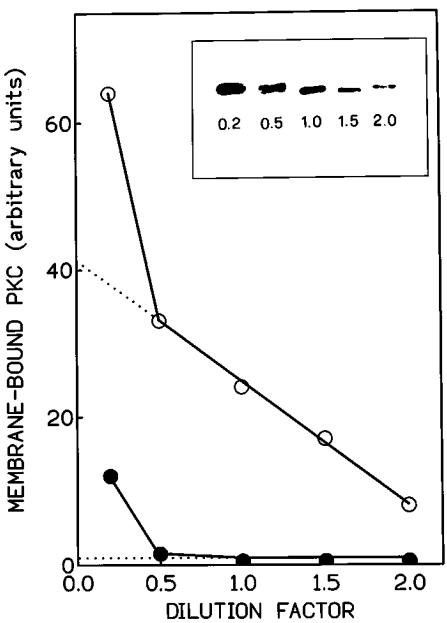

FIG. 1. *Quantification of membrane-bound αPKC and δPKC.* N23 MEL cells (10^7 cells) were diluted with the indicated volume (ml) of lysis buffer and then lysed by sonication. The membrane fractions were prepared and submitted to SDS-polyacrylamide gel electrophoresis as reported in Materials and Methods. Immunoblotting was performed using an anti αPKC antipeptide antibody (●) or alternatively anti δPKC antipeptide antibody (○). Immunoreactivity was revealed and quantified as described in Materials and Methods. The amount of membrane bound PKC isozyme was calculated as a percentage of total cellular amount. The dotted line shows the calculated amount of membrane bound PKC isozyme in undiluted cells. The inset shows the immunoreactive bands obtained with δPKC antibody.

soluble fraction) was collected and the pellet (cell membrane fraction) was suspended in the loading buffer for SDS-polyacrylamide gel electrophoresis.

Purification of DEF. The differentiation enhancing factor (DEF) was purified from the cytosolic fraction of C44 MEL cells following the procedure previously described (3).

RESULTS

Distribution of δPKC in MEL cells. Since small changes in the volume of the lysis buffer were found to produce significant modifications in the distribution of δPKC between the soluble and the membrane associated form, a great difficulty was encountered in establishing the real percentage of δPKC bound to the cell membrane. To quantify the real fraction of total δPKC present in a membrane bound form (Fig. 1), the same number and volume of cells were diluted with increasing volumes of buffer containing EDTA, and cells were lysed by sonication. The particulated fraction was then collected and the amount of membrane-bound δPKC was determined by immunoblotting (Fig. 1 inset). The plot of the level of δPKC as a function of the dilution of the soluble fraction of the cells resulted a straight line (Fig. 1) that intercepts the ordinate axis at a value corresponding to 40%. As control, also the levels of αPKC were determined on the same samples used for the quantification of δPKC. Only at the highest cell concentration small amounts of αPKC were detectable on the cell membranes, whereas, at increasing dilutions, this kinase isoform becomes undetectable; indicating that its association to membrane is not a function of the mass action. Due to the fact that the lysing buffer contained large excess of chelating agents (1 mM EDTA), it can

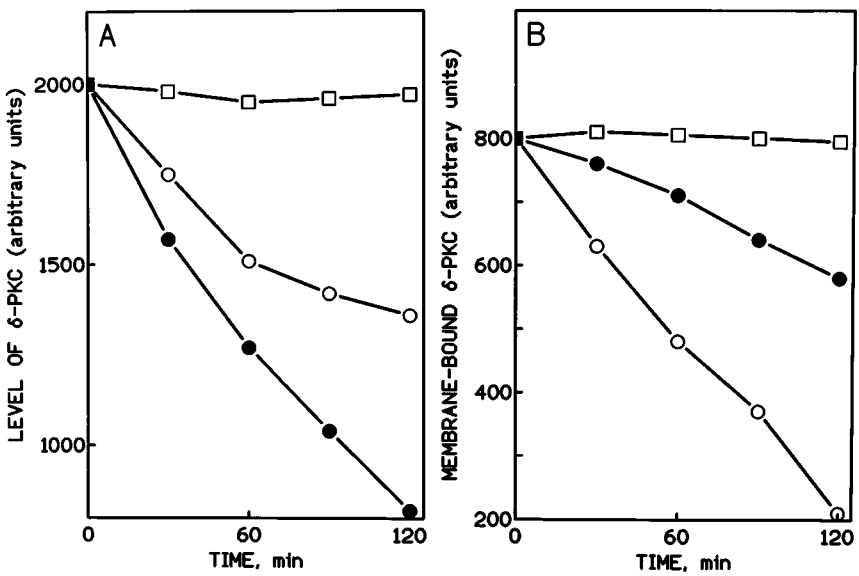

FIG. 2. (A) *Effect of DEF on the rate of down-regulation of δPKC during MEL cell differentiation.* N23 MEL cells (5×10^6 cells, 20 ml) were stimulated by addition of 5 mM HMBA to the culture medium in the absence (○) or in the presence (●) of 4 pmol of purified DEF. Control cells were incubated with 4 pmol of DEF alone (□). At the indicated times 4 ml was removed from each cell suspension, cells were collected by centrifugation and aliquots, corresponding to 5×10^5 cells, submitted to electrophoresis followed by immunoblotting to quantify the intracellular amount of δPKC (see Materials and Methods). The immunoreactive bands identified by autoradiography were excised from the nitrocellulose membrane and counted in a γ-counter. δPKC units correspond to cpm/10^6 cells. **(B)** *Effect of DEF on the level of membrane bound δPKC during MEL cell differentiation.* N23 MEL cells (5×10^6 cells in 20 ml) were stimulated with 5 mM HMBA in the absence (○) or in the presence (●) of DEF as described in the legend to Fig. 1. Control cells were cultured in the presence of DEF alone (□). At the indicated times 4 ml of cell suspension was collected by centrifugation and the cytosolic and membrane fractions were separated as described in Materials and Methods. Aliquots of cell membranes, corresponding to 5×10^5 cells, were subjected to SDS-polyacrylamide gel electrophoresis and membrane bound δPKC was quantified by immunoblot analysis as specified in Materials and Methods. δPKC units correspond to cpm/10^6 cells.

TABLE 1
Correlation between Level of δPKC and Resistance to Differentiation

Clone[a]	Pretreatment[b]	Level of δPKC (arbitrary units)[c]	Requirement for HMBA (mM)[d]	Time of appearance of differentiated cells (hours)[e]
N23	Verapamil	2680	5	74
N23	None	2080	5	60
N23	A23187	1720	4	51
C44	Verapamil	1550	3	40
C44	None	750	2.5	23
C44	A23187	380	2	15

The values reported refer to the arithmetic means of three different experiments.

[a] Clones were grown in α-MEM supplemented with 10% fetal calf serum, as described in Materials and Methods. N23, a slow responding clone, and C44, a more sensitive clone, have been obtained as previously reported (4).

[b] Before induction the MEL cells were incubated in complete α-MEM supplemented with 10% fetal calf serum for 48 hours with 5 nM A23187 ionophore or 5 μM verapamil, as described in Materials and Methods. The cells were then collected and induced with HMBA.

[c] Before induction aliquots of growing cells (5×10^5 cells) were collected and total amount of δPKC was measured by immunoblotting, as described in Materials and Methods.

[d] MEL cells were incubated with increasing concentrations of HMBA, from 0 to 5 mM. Higher concentrations were not used for the cell toxicity of the inducer. The values refer to the HMBA concentration promoting the highest fraction of differentiated cells.

[e] The values refer to times at which the differentiated cells appear in the incubation mixtures.

be concluded that the binding of δPKC to the plasmamembranes is calcium independent and is related to a structural property of the kinase. The extent of the binding appears to be a function of the concentration of δPKC in the soluble fraction.

Degradation of δPKC during MEL cell differentiation. We have previously shown that DEF promotes increase in the rate of MEL cell differentiation, accompanied by an increase of down-regulation of PKC (14). This effect has now been analyzed in details particularly regarding the δPKC isozyme. As shown in Fig. 2 treatment of MEL cells with HMBA causes the disappearance of δPKC at a rate higher in the first 60 minutes and then significantly slower. When cells are exposed to HMBA in the presence of DEF, the rate of δPKC degradation is accelerated and remains constant for all the time of the experiment. DEF alone has no effect on the level of δPKC (Fig. 2A).

We have now analyzed if the different rates of δPKC down regulation are due to changes in its intracellular distribution. As shown in Fig. 2B, following treatment of cells with HMBA, the amount of δPKC associated to membranes progressively and linearly decreased in 2 hours from the original value of 40% to approximately 10%. In the presence also of DEF, the amounts of membrane associated δPKC were maintained at higher levels and, following 2 hours of incubation, only 13% of membrane bound δPKC was disappeared. These findings can explain the more rapid degradation observed in cells induced in the presence of DEF, since association of PKC to membrane is known as the first event triggering degradation of the kinase by calpain (15). These data indicate that an efficient degradation of δPKC is a prerequisite for the enhancement of the differentiation process.

Correlation between the level of δPKC and the rate of differentiation of MEL cells. To define the role of δPKC in MEL cell resistance to differentiation, we have compared the level of this kinase with the rate of differentiation and the inducer requirement in two MEL cell clones both untreated or treated with A23187 Ca^{2+} ionophore or with verapamil, conditions which cause changes in Ca^{2+} influx and degradation or accumulation of δPKC respectively (9). As shown in Table I, cells having the higher levels of δPKC showed also the maximal requirement for the inducer as well as the longer time to reach the differentiated state. These data indicate that δPKC

is involved in the maintenance of an undifferentiated cell phenotype with also high resistance to the chemical inducer and low rate of differentiation.

DISCUSSION

The results reported in the present paper indicate that in MEL cells a large fraction of δPKC is associated with the cell membrane, probably in a constitutive active form. αPKC, on the contrary, is almost exclusively present in the soluble fraction of the cell, thus in an inactive form requiring translocation to membranes for its activation (16). Accordingly, upon addition of the inducer, δPKC undergoes a rapid down regulation at the membrane site where, in these conditions, calpain, the main proteinase involved in PKC degradation, is also translocated and activated (7, 9). DEF, a protein factor which positively affects MEL cell differentiation, promotes an increased rate of δPKC degradation favouring the association of the kinase to the membrane. Finally, evidences have been produced by means of a careful and extensive analysis, that a reverse correlation exists between the level of δPKC and the time required to MEL cell to reach the differentiated phenotype as well as the requirement for the inducer.

Further experiments are in progress to explore additional mechanisms concerning the action of DEF in MEL cell differentiation and thereby to better characterize the biological properties of this natural protein factor.

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