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# ROLE OF δ-PKC ON THE DIFFERENTIATION PROCESS OF MURINE ERYTHROLEUKEMIA CELLS

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In murine erythroleukemia (MEL) cells the length of the latent period before the onset of hexamethylenebisacetamide induced terminal erythroid differentiation is inversely correlated to the intracellular level of δ-PKC. This is supported by the following experimental evidence. V3.17[44] MEL cell line, characterized by a very high rate of differentiation, contains an amount of δ-PKC protein one third lower than that present in the N23 MEL cell line, characterized by a very low rate of differentiation. A similar difference in the amount of δ-PKC mRNA is present in the two cell lines. In N23 cells, following addition of HMBA, the amount of δ-PKC protein and δ-PKC mRNA is down-regulated to one third its original value, which now corresponds to that constitutively present in V3.17[44] cells. Furthermore, in these cells the levels of δ-PKC protein and of its specific mRNA are unaffected by treatment with HMBA. Following introduction of homologous purified δ-PKC both MEL cell variants display a longer latent period before the onset of differentiation: from 50 to 75 hours in N23 cell line and from 20 to 40 hours in V3.17[44] cells, respectively.

Taken together, these results suggest that a δ-PKC related signal plays a negative role in the early stages of MEL cell differentiation and that the level of the kinase is controlled through a down-regulation process upon exposure to the chemical inducer. 

HMBA induced MEL cell differentiation is a multi-step process presumably coordinated through the activation-deactivation of a number of metabolic processes, still not precisely identified. Among these, the modulation of specific PKC isoforms appears to be of particular significance (1). Experimental evidences have indicated that a β-like-still unknown PKC form (2), with catalytic and molecular properties typical of the cPKC subfamily (1), is significantly increased in rapidly responsive cells (V3.17[44]). This cPKC isoform introduced in slow responsive N23 cells promotes an increase in the rate of differentiation (3). In the present paper, we have established the presence and evaluated the amount of δ-PKC protein (a component of the nPKC subfamily), and of its mRNA in the two MEL cell

ABBREVIATIONS:
PKC, protein kinase C; MEL, murine erythroleukemia; HMBA, hexamethylenebisacetamide; MBP, myelin basic protein; ECL, enhanced chemioluminescence.

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lines characterized by different responsiveness to HMBA. Modulation of the intracellular content of δ-PKC, following stimulation by HMBA, has also been explored. The results obtained demonstrate that the level of δ-PKC is inversely correlated to the rate of MEL cell differentiation, suggesting a different role for the various PKC isoforms present in these cells as transducers of positive or negative signals.

## MATERIALS

T4 polynucleotide kinase, Moloney murine leukemia virus reverse transcriptase, RNasin (RNase inhibitor) and Taq DNA polymerase were purchased from Promega. Deoxyribonucleotides were from Pharmacia. γ-[$^{32}$P]ATP (>5000 Ci/mmol) was obtained from Amersham. Oligonucleotide probes and primers were prepared on a DNA synthesizer (DNA SM, Beckman). Hexamethylenebisacetamide was from Sigma Chemical Co.

## METHODS

*Cell culture.* MEL cell clones N23 and V3.17[44] were obtained and cultured as described (1). N23 cells were committed to terminal differentiation after 16-22 hours of exposure to HMBA and showed a latent period of approximately 50-55 hours; V3.17[44] cells were committed to terminal differentiation after 4-6 hours of exposure to HMBA and showed a latent period of approximately 16-20 hours. Cell differentiation was induced by addition of 5mM HMBA to the culture at the cell density of $10^5$ cells/ml. After 24 hours for V3.17[44] or 72 hours for N23 MEL cell clones, the proportion of benzidine reactive cells was assayed by the benzidine reaction as previously described (4).

*Purification of δ-PKC.* N23 MELC ($10^9$ cells) were collected at a cell density of $10^6$ cells/ml and δ-PKC was isolated as described (3) and assayed using the oligopeptide MBP 4-14 as a substrate (6). Purified δ-PKC showed a specific activity of 128,000 units/mg and was immunoreactive with a rabbit policlonal antiserum against δ-PKC peptide 662-673 (Research and Diagnostic Antibodies) while was unreactive with policlonal antisera specific for the other cPKC and nPKC isozymes . One unit of δ-PKC activity is the amount of enzyme that incorporates 1 pmol of $^{32}$P/min into MBP 4-14 under the assay conditions.

*Incorporation of exogenous δ-PKC into MEL cells.* MEL cells ($1.5x10^6$ cells) were permeabilized by treatment with glycerol and lysolecithin as specified by Nomura et al. (7) and successively incubated with 200 units of purified δ-PKC following the procedure previously reported (3).

*Immunoblot analysis.* MEL cells ($5x10^6$ cells) were lysed by sonication and the cell soluble fraction was prepared as described (3). Samples of cytosoluble proteins (20 μg) were subjected to SDS/PAGE on 8% polyacrylamide slab gel (8) followed by electroblotting onto Pure Nitrocellulose membrane (Bio-Rad). Immunodetection was carried out using a rabbit policlonal antiserum (see above), as isozyme specific primary antibody, and an Anti-Rabbit Ig, Horseradish peroxidase linked antibody (Amersham International) as secondary antibody. Positive immunoreactive signals were detected by enhanced chemioluminescence method (ECL; Amersham International) and the autoradiograms were scanned by a Shimadzu CS-9000 densitometer. The relative δ-PKC protein amount was calculated from the area of the densitometric peaks.

*Extraction of total RNA from MEL cells.* Total cellular RNA was isolated by guanidinium thiocyanate lysis of cells followed by centrifugation through a cesium chloride gradient (9).

*Quantification of δ-PKC mRNA in MEL cells.* A quantitative estimate of δ-PKC transcript was obtained by in vitro co-amplification of the products of δ-PKC gene and a reporter gene, β-actin, by means of the polymerase chain reaction. The procedure described by Chelly et al. (10) was essentially followed. Briefly, total RNA (1 μg) was reverse transcribed into cDNA by extension of 5'-[$^{32}$P] labeled antisense primers specific for either δ-PKC or β-actin (10 pmoles each, $0.5x10^5$ cpm/pmol). One hundred units of reverse transcriptase were used for each reaction, which was carried out at 37°C for 1 hour and stopped by heating at 95°C for 5 min. Second strand cDNA synthesis and co-amplification of

both transcripts were performed by adding sense primers for δ-PKC and β-actin (10 pmoles each), and Taq DNA polymerase (2.5 units) to a final reaction volume of 100 μl. Twenty cycles of co-amplification were carried out with the following pattern: denaturation at 95°C for 30 sec.; annealing at 60°C for 30 sec.; extension at 72°C for 30 sec. Linear ramps of 1 min. each were interposed between the segments of each cycle. Ten μl of each reaction mixture were resolved by electrophoresis on a 10% polyacrylamide/7M urea gel, which was subsequently subjected to autoradiography. The radioactive bands were excised from the gel and radioactivity quantified in a scintillation counter. The sequences of the primers used in this step were: δ-PKC sn: 5'TATCCCCGTTGGATCACCAAGG and δ-PKC asn: 5'TGTCACTGAAGGAAAGCTGAGG, respectively corresponding to nucleotides 1925-1946, and complementary to nucleotides 2150-2171 of mouse δ-PKC cDNA (11); β-actin sn: 5'GAATGGGCTAGAAGGACTCC and β-actin asn: 5'CCATCACAATGCCTGTGGTACG, respectively corresponding to nucleotides 217-236 and complementary to nucleotides 519-540 of mouse β-actin cDNA (12). Another two oligonucleotides were synthesized to serve as specific hybridization probes for the amplification products, being their sequences located between the respective amplification primers. Their sequences were: δ-PKC intercalated: 5' TTACTCCCAGCCTCTTGTCAGGGT, complementary to nucleotides 1983-2006 of δ-PKC cDNA, and β-actin intercalated: 5'GGTGTTGAAGGTCTCAAACATG, complementary to nucleotides 446-467 of β-actin cDNA.

## RESULTS

*Presence of δ-PKC in MEL cell clones.* The amounts of δ-PKC protein and mRNA have been evaluated in two MEL cell clones defined as N23 and V3.17[44] (1). The first one shows a low rate of commitment to terminal erythroid differentiation induced by HMBA with a latent period of 50-55 hours before the onset of differentiation; the second one is 100 times more rapidly induced with a latent period of 16-20 hours (1). As shown in Fig.1A, the immunoblot analysis reveals that both MEL cell clones contain significant amounts of δ-PKC protein. However in N23, slow responsive cells, the level of δ-PKC protein results to be approximately three times higher as compared to that present in highly responsive V3.17[44] cells. Also the amount of δ-PKC mRNA differs significantly in these two cell types (Fig. 1B), being much higher (60 copies/cell) in N23 and lower (20 copies/cell) in V3.17[44]. Thus, both δ-PKC protein and mRNA appear to be expressed in significantly higher amounts in slow responsive cells.

*Modulation of δ-PKC level during HMBA induced MEL cell differentiation.* Changes in the level of δ-PKC protein and mRNA have been evaluated in the two MEL cell clones as a function of the time of exposure to the inducer. As shown in Fig. 2, in N23 cells the level of δ-PKC protein undergoes a rapid decline within the first four hours, down to approximately one third its original value, which now corresponds to that constitutively present in the rapidly responsive V3.17[44] cells. This down regulation process, occurring only in N23 cells, precedes cell commitment and suggests the need of a critical level of δ-PKC for commitment of MEL cells to terminal differentiation. Following addition of HMBA, also δ-PKC mRNA declines in N23 (Fig. 3A), but remains unmodified in V3.17[44] cells (Fig.

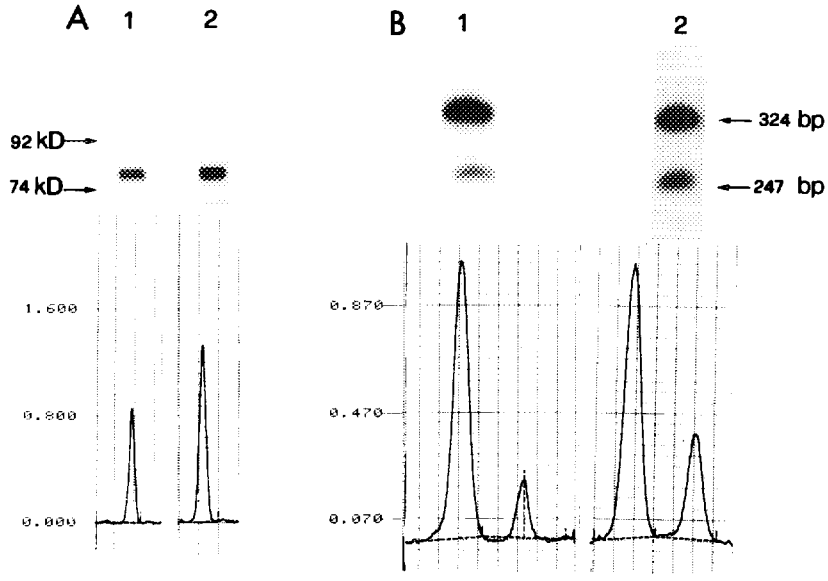


Fig. 1. *Levels of δ-PKC in MEL cell clones.* A, Autoradiograms of western blots for δ-PKC, using 20 μg of soluble fractions of V3.17[44] (lane 1) and N23 (lane 2) cell clone, were performed as reported under Methods. The molecular weights of standard proteins are reported on the left. The lower part of the figure shows the relative amounts of δ-PKC obtained by densitometric scanning of the films. B, Autoradiograms of polyacrylamide/urea gel electrophoresis performed on co-amplified products of δ-PKC and β-actin genes. The quantitation of δ-PKC mRNA of V3.17[44] clone (lane 1) and N23 clone (lane 2) was carried out following the experimental procedure specified in Methods. The relative amounts of β-actin (324 bp) and δ-PKC (247 bp) amplification products are reported in the lower part of the figure as obtained by densitometric scanning of the exposed films. The same ratios between β-actin and δ-PKC were obtained by excision of the radioactive bands from the gel and evaluation of [$^{32}$P] content by a scintillation counter.

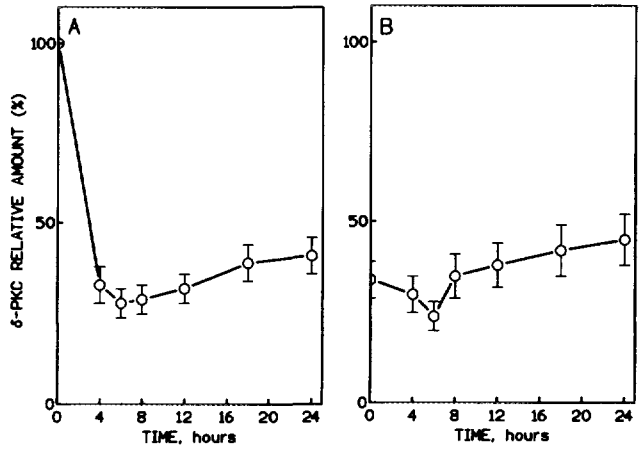


Fig. 2. *Changes in the level of δ-PKC protein in MEL cell clones induced by the addition of HMBA.* N23 (A) and V3.17[44] (B) MEL cell clones ($5x10^5$ cells/ml) were induced with 5 mM HMBA. At the indicated times, $5x10^6$ cells were collected, lysed and western blot was carried out as reported in Methods. The relative amounts of δ-PKC are calculated from the area of the peaks obtained by densitometric analysis of the ECL autoradiograms. Each value is reported as percentage of the δ-PKC protein recovered in uninduced N23 cells and represents the mean ± SD of three separate experiments.

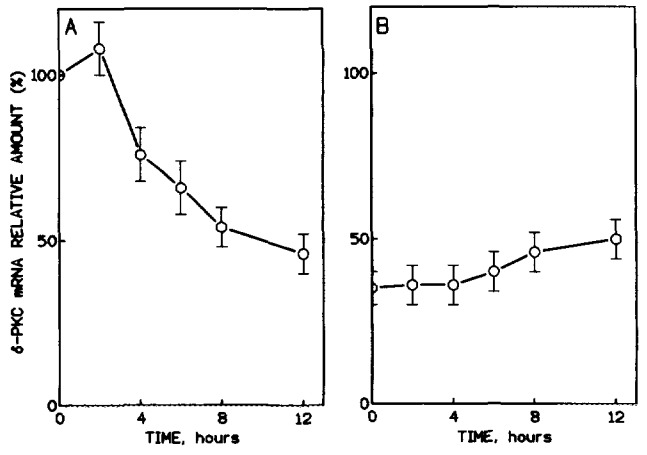


Fig.3. *Changes in the level of δ-PKC mRNA in MEL cell clones induced by addition of HMBA.* MEL cells were induced as in the legend to Fig. 2. At the indicated time, $5x10^6$ cells were collected, total RNA was prepared and δ-PKC/β-actin mRNA co-amplification was performed as specified in Methods. Electrophoresis and δ-PKC mRNA quantitation were performed as described under Methods. Each value is reported as percentage of δ-PKC mRNA recovered in uninduced N23 cells and represents the mean ± SD of three separate experiments.

3B). The kinetics of the decline reveals that it occurs before cell commitment and the value reached at the equilibrium corresponds to that constitutively present in rapidly responding cell lines. Taken together these data suggest that in MEL cells adjustment of δ-PKC mRNA and δ-PKC protein levels represent a necessary requirement for cell commitment.

*Incorporation of exogenous δ-PKC into MEL cells.* Incorporation of purified δ-PKC into permeabilized MEL cell clones affects the rate of HMBA induced differentiation as indicated by a large decrease in the accumulation of benzidine-reactive cells (Table I). On a kinetics basis, it appears that the decreased

Table I
Effect of exogenous δ-PKC on the rate of HMBA induced differentiation

| Clone | Benzidine-reactive cells (%) | |
|---|---|---|
| | - δ-PKC | + δ-PKC |
| N23 | 12 | 0 |
| V3.17[44] | 42 | 6 |

N23 and V3.17[44] cells were permeabilized as descibed in Methods and enriched with δ-PKC using the procedure previously reported. The percentage of benzidine-reactive cells, determined following 72 hours or 48 hours of incubation with HMBA for N23 and V3.17[44], respectively, is evaluated as indicated in Methods.

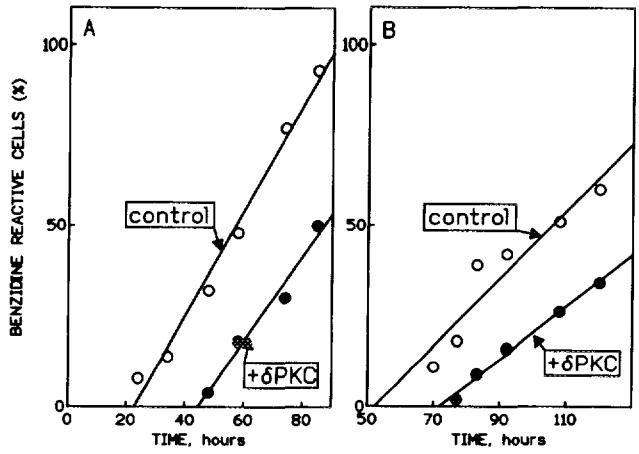


Fig. 4. *Effect of exogenous δ-PKC on the kinetics of HMBA induced differentiation.* MEL cells ($3x10^5$ cells) were permeabilized by treatment with lysolecithin (see Methods) and incubated with purified δ-PKC (200 units). The cells were then incubated for 24 hours without lysolecithin and finally induced with 5 mM HMBA. At the indicated time, samples were collected and stained with benzidine (see Methods). Benzidine-reactive cells are expressed as percentage of total. A, V3.17[44] MEL cell clone; B, N23 MEL cell clone.

proportion in the amount of benzidine reactive cells, is largely accounted for by an extension of approximately 20 hours of the latent period in both N23 and V3.17[44] cell lines. In fact, as shown in Fig. 4, the rate of accumulation of benzidine reactive cells is poorly affected by the permeabilization treatment, whereas the latent period is increased of approximately 20 hours in both clones.

Thus, introduction of δ-PKC modifies the rate of differentiation, suggesting that the intracellular level of the kinase is inversely correlated to the lenght of the latent period before the onset of MEL cell differentiation.

## DISCUSSION

Protein kinase C is expressed in mammalian cells in the form of a variety of isoenzymes that can be related to two sub-families: a first one containing the so called cPKC isoforms, characterized by a strict dependency to $Ca^{2+}$, and a second one, containing the nPKC isoenzymes, characterized by lipid dependency (13). In almost all mammalian cells, so far examined, different patterns in terms of amount and type of each PKC isozyme have been found and interpreted as an indication for the existence of selective PKC phenotypes each one related to a specific cell function (13). It has been previously shown that in V3.17[44] rapidly inducing MEL cell line the level of a β-like PKC isozyme is constitutively higher than in N23 slow responsive MEL cell variant (1). This observation suggested the existence of a relationship between the concentration of the β-like PKC isoform and the rate of MEL cell

differentiation. This hypothesis was also supported by the observation that the introduction of this purified β-like cPKC isoform in N23 cells resulted in an increase in the rate of HMBA induced differentiation, thus indicating an enhancing role played by the β-like cPKC isoenzyme during the cell differentiating process (3). The present results provide evidence for the existence of another important difference in the pattern of expression of PKC isoenzymes in the two MEL cell clones previously considered. This is indicated by the finding that in the low responsive N23 cells the amounts of δ-PKC protein and mRNA are three times higher than in highly responsive V3.17[44] MEL cells. In N23 cells, following addition of the inducer and largely before their commitment to terminal differentiation, δ-PKC protein and mRNA undergo a rapid and significant decrease down to a concentration close to that constitutively present in V3.17[44] cells. In these cells the addition of the inducer is not accompanied by variations in the level of δ-PKC protein or δ-PKC mRNA. These data suggest that a δ-PKC related signal negatively affects induction of MEL cell differentiation. Direct evidence for this hypothesis is provided by the observation that introduction of purified δ-PKC in N23 and in V3.17[44] cell results in an inhibitory effect on the process of differentiation as indicated by a significant delay in the onset of accumulation of benzidine reactive cells (from 50 to 70 hours in N23 and from 20 to 40 hours in V3.17[44] cells). Quantitative determinations revealed that the δ-PKC mRNA is present in approximately 60 copies/cell in the N23 clone and in 20 copies /cell in theV3.17[44] clone. These data are of significant interest because they indicate that δ-PKC isoform is largely predominant among all others PKC isoforms (14) and that the large amount of this kinase, probably in association with other factors, could be related to the undifferentiated state of the cell. In conclusion, MEL cells appear to be present in two functionally distinct phenotypes: one containing large amount of a β-like cPKC isoform, related to an increased sensitivity to the inducer and to an acceleration of the rate of differentiation; the second one containing large amount of δ-PKC, involved in the mechanism controlling the onset of the differentiated state.

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REFERENCES

1. Melloni,E., Pontremoli,S., Viotti,P.L., Patrone,M., Marks, P.A., and Rifkind, R.A. (1989) J. Biol. Chem. 264, 18414-18418.
2. Aihara, H., Asaoka, Y., Yoshida, K., and Nishizuka, Y. (1991) Proc. Natl. Acad. Sci. USA 88, 11062-11066.

3. Melloni, E., Pontremoli, S., Sparatore, B., Patrone, M., Grossi, F., Marks, P.A., and Rifkind, R.A., (1990) Proc. Natl. Acad. Sci. USA 87, 4417-4420.
4. Singer,D., Cooper,M., Maniatis,G.M., Marks, P.A., and Rifkind,R.A. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2668-2670.
5. Ogita, K., Miyamoto, S.-I., Yamaguchi, K., Koide, H., Fugjisawa, K., Kikkawa, U., Sahara, S., Fukami, Y., and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. USA 89, 1592-1596.
6. Hashimoto, K., Kishimoto, A., Aihara, H., Yasude, I., Mikawa, K., and Nishizuka, Y. (1990) FEB Lett,. 263, 31-34.
7. Nomura, S., Kamiya, T., and Oishi, M., (1986) Exp. Cell Res. 163, 434-444.
8. Laemmli,U.K. (1970) Nature (London) 227, 680-685.
9. Sambrook,J., Fritsch,E.F., Maniatis,T. (1989) in: "Molecular cloning: a laboratory manual" Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
10. Chelly,J., Kaplan, J.C., Maire, P., Gautron,S., and Kahn,A. (1988) Nature 333, 858-860.
11. Mischak,H., Bodenteich, A., Kolch,W., Goodnight,J., Hofer,F., and Mushinski, J.F. (1991) Biochemistry 30, 7925-7931.
12. Tokunaga,K., Taniguchi,H., Yoda,K., Shimizu,M., and Sakiyama,S. (1986) Nucl. Acid Res. 14, 2829-2832.
13. Asaoka,Y., Nakamura,S., Yoshida,K., and Nishizuka,Y. (1992) TIBS 17, 414-417.
14. Powell, C.T., Leng, L., Dong, L., Kiyokawa, H., Busquets, X., O'Driscoll, K., Marks, P.A., and Rifkind, R.A. (1992) Proc. Natl. Acad. Sci. USA 89, 147-151.