

Protein kinase C isoforms in murine erythroleukemia cells and their involvement in the differentiation process

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Abstract

In addition to α , δ and ε -protein kinase C, murine erythroleukemia cells contain ζ -PKC and also a c-PKC isoform, named α_1 , which shows cross-reactivity with an anti- α -PKC antipeptide antibody. In a C44 MEL cell clone, characterized by a high rate of differentiation, both c-PKC forms are expressed at a level higher than that of the N23 MEL cell clone which differentiates at a low rate and contains higher levels of ε -PKC and particularly of the δ -PKC isozyme. In the course of MEL cell differentiation, δ -PKC in N23 cells and α_1 -PKC in C44 cells are rapidly down-regulated and the overall process is almost completed before cell commitment. Of the other three PKC isozymes present in both clones, only α -PKC is down-regulated to a significant extent. It is proposed that modulation of the signal delivered by each PKC isozyme is one of the biochemical mechanisms involved in MEL cell differentiation.

Key words: Murine erythroleukemia cell; PKC isozyme; Down-regulation; Cell differentiation

1. Introduction

Protein kinase C (EC 2.7.1.37) belongs to a family of enzymes that are represented in a variety different isoforms in almost all mammalian cells [1]. In MEL cells two isoforms, α and β , of the c-PKC subfamily [2], have been separated and identified [3]. The presence of the α -PKC type was later confirmed whereas the second PKC activity was proved not to belong to the β -PKC isotype [4] thus leaving unsolved the nature of this unknown PKC isoform. Two isozymic forms, ε -PKC [4] and δ -PKC [5], belonging to the n-PKC subfamily [2], were also found to be expressed in these cells.

In terms of biological function the following observations suggested that these different PKCs could play an important role in cell differentiation. (i) In MEL cell clones the sensitivity to the inducer hexamethylenbisacetamide (HMBA) was found to be correlated with the expression of specific PKC isoforms. In fast rate differentiating C44 cells the c-PKC isoforms, and particularly α_1 -PKC, are highly represented [3], whereas in N23, slow rate differentiating cells, high levels of the δ -PKC isozyme are present [5]. (ii) In the early stages of HMBA-induced differentiation, δ -PKC (in N23 cells) [5] and α_1 -PKC (in C44 cells) [3] undergo down-regulation

at the highest rate and to a larger extent. (iii) Introduction of the α_1 -PKC in slow responding N23 cells or of the δ -PKC in fast differentiating C44 cells promotes in the first case a significant increase [6], and in the second case a large decrease [5] in the rate of appearance of benzidine-reactive cells.

In order to extend this information and to better characterize the role of PKC in MEL cell differentiation it was considered of interest to design experiments intended to establish the level and the type of PKC isoforms present in MEL cell clones showing low or high sensitivity to HMBA, as well as the rate and the extent of down-regulation of each PKC isoforms prior to cell commitment.

2. Materials and methods

2.1. Cell culture

MEL cell clones N23 and V3.17.44, from now on named C44, were obtained and cultured as described [3]. Cell differentiation was induced by the addition of 5 mM HMBA to the culture at a cell density of 10^5 /ml. At the indicated times, the proportion of benzidine reactive cells was assayed by the benzidine reaction as described [7].

2.2. Immunoblot analysis of PKC isozymes in MEL cells

MEL cells (4×10^7 cells) were lysed and the soluble cell fraction prepared and submitted to DEAE chromatography as previously described [8]. Two peaks of PKC activity were detected, the first eluted at approximately 70 mM NaCl and containing both c- and n-PKC activities, the second eluted at approximately 120 mM NaCl containing only n-PKC activity. Samples (20 μ L of the eluted fractions) were collected and submitted to 8% SDS-PAGE [9], followed by electroblotting onto Pure Nitrocellulose membrane (Bio-Rad). PKC isotypes were identified by using anti-PKC isotype-specific polyclonal rabbit antisera

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Abbreviations: PKC, protein kinase C; MEL, murine erythroleukemia; HMBA, hexamethylenbisacetamide; HA, hydroxylapatite; α_1 -PKC, PKC isoform detected with anti- α -PKC antibody, showing a lower M_r .

as primary antibodies (Gibco BRL) for α , β , γ , δ , ϵ and ξ -PKC. The sequences of antigen peptides used to produce each antibody were as follow: peptide 313–326 for α -PKC, peptide 313–329 for β -PKC, peptide 306–318 for γ -PKC, peptide 662–673 for δ -PKC, peptide 726–737 for ϵ -PKC, and peptide 577–592 for ξ -PKC [10–12]. The immunoreactive material was detected by the enhanced chemiluminescence method (ECL, Amersham International) [13] and the photographic films were scanned by a Shimadzu CS-9000 densitometer. The relative amount of each PKC isozyme was calculated from the area of the densitometric peaks. The linear range of the signal detected was previously determined to obtain quantitative responses from each experiment. The two peaks were separately collected and the first one utilized for the successive purification step by HA chromatography.

2.3. Separation of *c*-PKC isozymes

The first peak of PKC isozymes eluted from DEAE chromatography was concentrated by ultrafiltration, loaded onto a hydroxylapatite column and chromatographed as previously described [3]. The assay of *c*-PKC activity was performed using samples of the eluted fractions (100 μ l) in the absence or in the presence of Ca^{2+} , phosphatidylserine and diacylglycerol using histone SIII as substrate [8]. One unit of PKC activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of phosphate into the substrate/min in the assay conditions.

3. Results

3.1. Types and levels of PKC isozymes in different MEL cell clones

The PKC content of two MEL cell clones, N23 and C44, the former characterized by a low and the latter by

a high rate of differentiation, was analyzed by the use of antipeptide antibodies specifically raised against α , β , γ , δ , ϵ , and ξ -PKC isoforms. To define the nature of PKC isozymes and to avoid undesired aspecific cross-reactions, the cell cytosolic fractions were first submitted to DEAE chromatography and the eluted fractions analyzed for their content of PKC isozymes by immunoblotting with each antibody (Fig. 1). In both cell clones PKC isozymes separate into two peaks, the first one containing a mixture of α , δ and ξ -PKC; the second one containing only ϵ -PKC. However, the immunoblot obtained with the anti- α -PKC revealed the presence of two reactive proteins, (insert of Fig. 1), one having a molecular mass corresponding to α -PKC (78 kDa), the other one showing a slightly smaller molecular mass of 74–76 kDa. Both proteins interact with the anti- α PKC antibody in a highly specific manner as demonstrated by antigen competition. Due to the fact that both proteins contain the same antigenic site, characteristic of the α -PKC isozyme, we have named the low M_r isoform, identified in Western blot analysis, as α_1 -PKC. At present, no information is available concerning the nature of the process causing the appearance of the α_1 -PKC. At present, no information is available concerning the nature of the process causing the appearance of the α_1 -PKC form in MEL cells. By comparison of the areas under each PKC

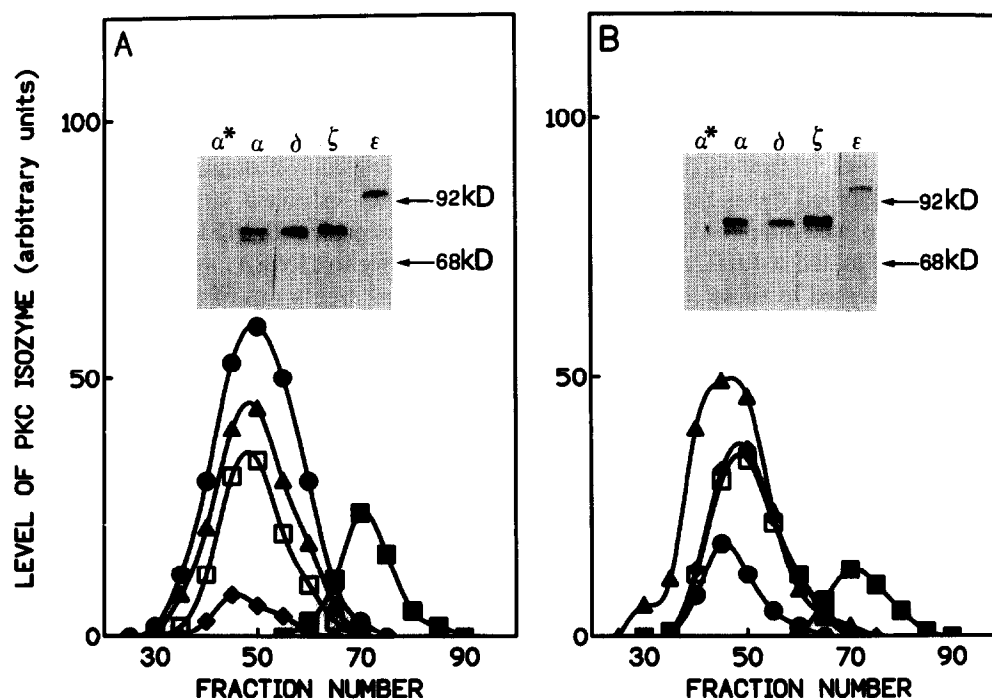


Fig. 1. Identification of PKC isozymes from MEL cell clones. N23 (A) and C44 (B) cells were lysed and submitted to DEAE-chromatography as described in section 2. The eluted fractions were assayed for reactivity with anti-PKC antipeptide antibodies (see section 2) following SDS-PAGE and immunoblotting. The immunocomplex was revealed with the ECL method, the amount of each PKC isozyme was calculated by the area of the peaks obtained from densitometric scanning of the photographic film and reported as arbitrary units. ($\blacktriangle$) α -PKC; ($\blacklozenge$) α_1 -PKC; ($\bullet$) δ -PKC; ($\blacksquare$) ϵ -PKC; ($\square$) ξ -PKC. β - and γ -PKC isozymes were undetectable (data not shown). The insert shows the immunostaining obtained with fraction number 50 and 70 (for ϵ -PKC). α^* represents the immunoblot obtained with anti- α -PKC antibody incubated in the presence of 10 μ g of the peptide used to prepare the anti- α -PKC antibody.

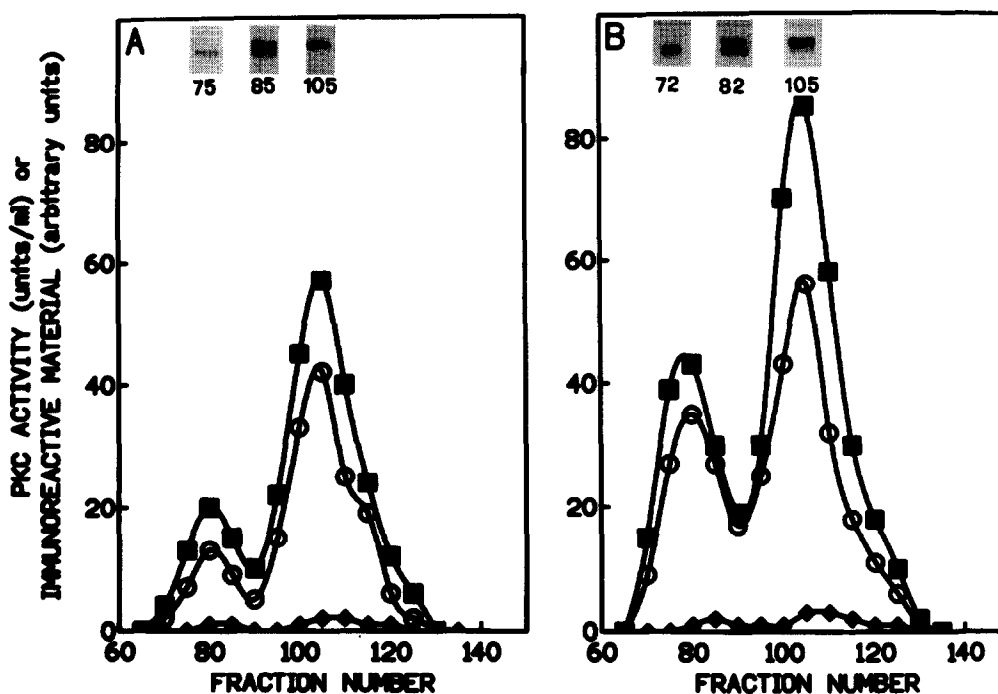


Fig. 2. Separation and identification of c-PKC isozymes from MEL cells. The peaks containing the cPKC isozymes, eluted from the DEAE-cellulose chromatography (see Fig. 1) and obtained from N23 (A) or C44 cells (B), were submitted to HA-chromatography (see section 2), in conditions known to separate c-PKC isozymes. Aliquots (100 μl) of the eluted fractions were assayed for PKC activity in the absence ($\diamond$) or in the presence ($\circ$) of Ca^{2+} , phosphatidylserine and diacylglycerol. Samples of 100 μl were also used for the evaluation of the cross-reactivity with anti- α -PKC antibody ($\blacksquare$), as described in the legend to Fig. 1. The insert shows the immunostaining with anti- α -PKC antipeptide antibody of the indicated fractions submitted to SDS-PAGE.

isozyme peak, it has been established that, with respect to N23 (Fig. 1A), C44 cells (Fig. 1B) contain a higher amount of α -PKC and an approximately 5-times higher level of α_1 -PKC; in contrast ϵ - and δ -PKC levels were found to be expressed at 1.6- and 4-fold lower level, respectively. The level of ξ -PKC was found to be almost identical in the two clones. No cross-reactive material, using anti- β -PKC and anti- γ -PKC antibodies, was detected.

In order to better characterize the two PKC forms recognized by the anti- α -PKC antibodies, the first peak eluted from the DEAE-cellulose chromatography and containing both cross-reacting proteins, was submitted to chromatography on HA column. The eluted fractions were then analyzed for their catalytic properties, as well as for their reactivity against anti- α -PKC antibody. As shown in Fig. 2, two peaks of PKC activity were separated from N23 (Fig. 2A) and C44 cells (Fig. 2B), fully active on histone S-III in the presence of Ca^{2+} , phosphatidylserine and diacylglycerol, confirming that both kinases belong to the c-PKC family. The immunoblotting of the eluted fractions revealed that the first minor peak contained almost exclusively the low M_r protein, and that the second major peak contained the α -PKC isozyme. These results also confirmed a higher expression of α -PKC and especially of α_1 -PKC isoform in C44 cells.

3.2. Different down-regulation of PKC isozymes during MEL cell differentiation

In N23 cells exposed to HMBA (Fig. 3A), δ -PKC was rapidly degraded and, in approximately 4–6 h, 70% of the original protein was lost. Down-regulation of α_1 -PKC was almost completed in 14 h and accounted for the disappearance of approximately 60% of the protein, whereas the decay of α -PKC proceeded at a slower rate and only after 24 h, 25% of the protein disappeared. In these conditions only a slight decay (10%) of ϵ -PKC isoforms was observed, whereas the level of ξ -PKC remained almost unchanged. A resistance of this atypical PKC isozyme [1] to down-regulation was also reported for activated glioma cells [14].

In C44 cells, the overall down-regulation process of PKC isoforms is more rapid (Fig. 3B) and involves especially the α_1 -PKC isozyme, which is reduced up to 80% in 2–4 h, and the α -PKC which decreases by 60% in 6–8 h. The level of δ -PKC, by contrast, is only 25% reduced in the first 2 h, and also the level of ϵ -PKC is slightly affected, while that of ξ -PKC remains unmodified throughout incubation. These results provide clear indication that down-regulation of each PKC isozyme is significantly different in these two cell lines and that the rate of the overall process is accelerated in the C44 cell clone.

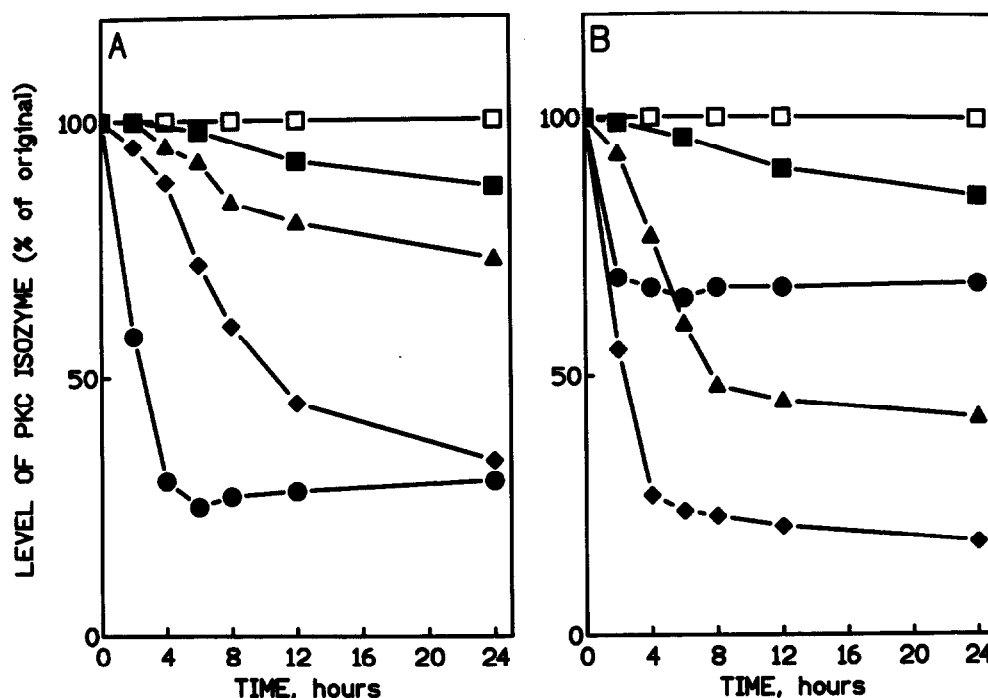


Fig. 3. Changes in the levels of PKC isozymes during MEL cell differentiation. MEL cells N23 (A) or C44 (B) were induced with 5 mM HMBA and, at the indicated times, 5×10^4 cells were collected and submitted to SDS-PAGE as reported in section 2. After electrophoresis, immunoblot analysis was performed as specified in section 2 using the various antipeptide antibodies. The amount of each PKC isoform was calculated from the area of the densitometric peaks. ($\blacktriangle$) α -PKC; ($\blacklozenge$) α_1 -PKC; ($\bullet$) δ -PKC; ($\blacksquare$) ϵ -PKC; ($\square$) ξ -PKC.

4. Discussion

The data presented in this paper extend the characterization of the various PKC isoforms that are expressed in different amounts in MEL cell clones showing slow (N23) or fast (C44) rates of differentiation.

In both cell clones, the presence of α , δ and ϵ -PKC was confirmed. An additional c-PKC, different in M_r from α -PKC, but cross-reacting with anti- α -PKC antibody, named α_1 , and ξ -PKC have also been demonstrated to be present. In C44 cells α -PKC and α_1 -PKC are highly represented, whereas in N23 the amounts of ϵ - and particularly that of δ -PKC are consistently higher. Thus an increased expression of c-PKC isoforms seems to be a characteristic property of the fast differentiating clone and, vice versa, a high level of expression of n-PKC isoforms represents a constitutive property of the slow responding clone.

These conclusions are supported not only by previous findings indicating that introduction of the α_1 -PKC isoform in N23 [6], or of δ -PKC in C44 [5] cells induces, respectively, an increase or a decrease in the rate of differentiation; but also by the kinetics of down-regulation of each PKC isoform induced by HMBA in slow or fast responding MEL cells. In fact, the data reported in the present paper have demonstrated that the decay of the two PKC isoforms (α_1 -PKC and δ -PKC), largely expressed in the two MEL cell clones, occurs at a very

rapid rate and is almost completed largely before cell commitment [3]. The rapid degradation of δ -PKC, that precedes also that of α_1 -PKC in N23 cells, may be interpreted as the removal of a negative signal on cell commitment, delivered by the former kinase. In C44 cells, characterized by a constitutively low level of δ -PKC, the utilization of α_1 -PKC form is much more rapid and is correlated with the onset of cell commitment. Further experiments are in progress to characterize the biochemical mechanism responsible for modulation of the level of PKC isoforms in MEL cell differentiation.

Recent results, obtained with PCR amplification experiments, carried out on cDNA from MEL cells have indicated that the θ -PKC [15,16] isoform is also expressed in this cell type (unpublished observations). The levels and the possible role of this kinase isoform in MEL cells will be investigated in additional experiments.

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