Protein kinase C-θ is specifically localized on centrosomes and kinetochores in mitotic cells

Mario PASSALACQUA, Mauro PATRONE, Bianca SPARATORE, Edon MELLONI[1] and Sandro PONTREMOLI
Institute of Biological Chemistry, University of Genoa, Viale Benedetto XV, 1-16132 Genoa, Italy

In this study we provide evidence that the protein kinase C (PKC)-θ isoenzyme is recruited on to the mitotic spindle in dividing murine erythroleukaemia (MEL) cells and associates specifically with centrosome and kinetochore structures. None of the other PKC isoenzymes (-α, -δ, -ϵ, -μ and -ζ) expressed by MEL cells shows this localization on the mitotic spindle. An identical subcellular distribution of PKC-θ is also observed in dividing murine P3 myeloma cells and human LAN-5 neuroblastoma cells, indicating that this PKC isoenzyme interacts with the mitotic apparatus in mammalian cells. In phorbol-ester-treated non-growing MEL cells, a rapid change in the intracellular distribution of PKC-θ occurs. Under these conditions, PKC-θ is translocated from the nuclear to the cytosolic cell compartment, an event that is accompanied by phosphorylation of the PKC-θ molecule and is followed by its down-regulation. The recovery of cell growth capacity results in the concomitant reappearance of PKC-θ. Furthermore, when MEL cells acquire the differentiated non-growing phenotype, the level of PKC-θ is reduced to less than 5%, suggesting that this PKC isoenzyme is no longer required. We propose that, unlike other members of the PKC family, PKC-θ may play a role in cell proliferation.

Key words: cell cycle, mitosis, phosphorylation.

INTRODUCTION

The large protein kinase C (PKC) family is composed of Ca^{2+}-dependent and -independent isoenzymes that have functions in signal transduction in eukaryotic cells [1,2]. The conventional and novel subfamilies of PKC are activated by diacylglycerol generated through different pathways, and represent the major intracellular targets of PMA [3]. In contrast, the isoenzymes belonging to the atypical PKC subfamily are not responsive to Ca^{2+} and diacylglycerol [2]. Murine erythroleukaemia (MEL) cells, which contain five PKC isoenzymes identified as PKC-α, -δ, -ϵ, -θ and -ζ [4], express members of all PKC subfamilies. In addition, the PKC-μ isoform has recently been identified in MEL cells (B. Sparatore, unpublished work), although whether it belongs to the PKC family is still a controversial point [5].

MEL cell clones, characterized by high or low sensitivity to chemical inducer associated with a high or low rate of differentiation respectively [6], also show different levels of expression of PKC isoenzymes [7]. It has been demonstrated previously that some PKC isoenzymes are involved directly in the multiphasic process of MEL cell differentiation. In particular, PKC-δ, which undergoes down-regulation largely before cell commitment, seems to be preferentially involved in the maintenance of an undifferentiated cell phenotype [8,9]. In contrast, PKC-α is translocated into the nucleus and down-regulated in parallel with the onset of cell commitment [10]. Moreover, fluctuations in the intracellular content of PKC-α or PKC-δ, caused by treatment with specific antisense oligonucleotides or by introduction of purified PKC isoenzymes into permeabilized MEL cells, results in a significant change in the rate of differentiation [10,11].

Recent experimental evidence indicates that protein phosphorylation is a ubiquitous mechanism regulating cell-cycle processes [12,13]. Although the molecular mechanisms controlling the initiation and completion of mitosis have not yet been fully elucidated, several protein kinases have been proposed to play important roles in regulating mammalian cell division, following association with mitotic structures in a cell-cycle-dependent manner [14–18]. Mitosis-specific and cell-cycle-regulated phosphoproteins, which participate in the correct assembly and maintenance of the bipolar spindle, have been proposed as targets of a large number of protein kinase activities [12]. Among these, the PKC-θ isoenzyme has been indicated as a modulator of cell-cycle progression [19].

Here we consider the involvement of PKC isoenzymes in the MEL cell cycle. Although different PKC isoenzymes are localized in the nuclear matrix during interphase, PKC-θ acquires a unique distribution in mitosis, being massively translocated on to specific sites of the spindle. Other cell types, also of human origin, show a similar localization of PKC-θ in the mitotic apparatus. In non-proliferating MEL cells, obtained by brief exposure to PMA, PKC-θ at first undergoes a phosphorylation process, concomitant with the rapid removal of the kinase from the nuclear compartment, followed by massive down-regulation. Other experimental evidence from non-growing MEL cells, obtained using different types of treatment, also supports the hypothesis that PKC-θ could be involved in cell-cycle progression.

EXPERIMENTAL

Cell culture

N23 MEL cells were obtained and cultured as specified previously [6]. Human LAN-5 neuroblastoma cells were cultured as reported [20], and murine P3 myeloma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% (v/v) heat-inactivated fetal calf serum.

Immunofluorescence microscopy

Cells were fixed and permeabilized by the Triton/paraformaldehyde method, as described in [21]. Non-specific protein binding to cells was blocked by a 30 min incubation with 5% (v/v) fetal calf serum diluted in PBS. Cells were then treated with 2 μg/ml

Abbreviations used: MEL, murine erythroleukaemia; HMBA, hexamethylenebisacetamide; PKC, protein kinase C.

[1] To whom correspondence should be addressed (e-mail melloni@csita.unige.it).

anti-(PKC isoenzyme) antibody (Santa Cruz Biotechnology; antibodies were raised against the C-terminal peptide of each PKC isoenzyme), followed by a FITC-conjugated anti-(rabbit Ig) secondary antibody (Amersham Pharmacia Biotech) at a concentration of 2.5 μg/ml for 1 h. Pretreatment of the anti-(PKC isoenzyme) antibodies with their immunogen peptide resulted in the complete disappearance of cell immunostaining. Staining of chromatin was carried out by incubating fixed cells with 2 μg/ml ethidium bromide for 5 min [22]. Cells were mounted on coverslips with FluoroGuard anti-fade reagent (Bio-Rad Laboratories) before analysis. Images of the samples were collected the same day by confocal microscopy using a Bio-Rad MRC1024 instrument (krypton/argon laser) on a Nikon Diaphot 200, using a planapochromat $\times 60$ oil-immersion objective with normal aperture 1.4 (Figures 1, 3 and 7 below) or a planapochromat $\times 40$ objective with normal aperture 0.75 (Figure 2). The excitation/emission wavelengths were 488/522 nm for fluorescein-labelled antibodies, and 488/605 nm for ethidium bromide-stained chromatin.

Evaluation of MEL cell growth and induction of differentiation

To measure cell growth, aliquots of MEL cell cultures were collected at various times and the cells counted in triplicate. For control conditions, cells were cultured in complete culture medium starting with a density of 10^5 cells/ml. The percentage cell growth under different experimental conditions was calculated by dividing the actual cell density by the value obtained under control conditions, and multiplying by 100. MEL cell differentiation was induced by the addition of 5 mM hexamethylenebisacetamide (HMBA) to a culture containing 10^5 cells/ml. The proportion of differentiated cells was assayed by evaluating the percentage of benzidine-reactive cells [23].

Immunoprecipitation of PKC-θ isoenzyme

N23 MEL cells (5×10^6 cells) were suspended in 1 ml of 20 mM Tris/HCl buffer, pH 7.6, containing 0.5 % Nonidet P40, 2.5 mM EDTA, 2.5 mM EGTA, 0.15 M NaCl, 0.1 mg/ml leupeptin, 1 mM PMSF and 20 μg/ml aprotinin (buffer A) and lysed by sonication (six bursts of 10 s each at 0 °C). After centrifugation at 12000 ***g*** for 5 min, the supernatant was collected, precleared by incubation with 20 μl of Protein A–Sepharose for 15 min at 4 °C, then incubated with 1 μg of the anti-PKC-θ antibody for 1 h at 4 °C. Protein A–Sepharose (20 μl) was then added to the mixture, which was incubated for a further 1 h at 4 °C. The Sepharose was pelleted, washed with 5×1 ml of buffer A and suspended in 50 μl of SDS/PAGE loading buffer. The samples were immediately heated at 95 °C for 2 min and submitted to SDS/PAGE on an 8 % (w/v) polyacrylamide slab gel [24].

Quantitative analysis of PKC-θ by immunoblotting

MEL cells were washed three times with PBS, counted and lysed directly in the sample buffer for electrophoresis. Samples corresponding to 10^5 cells were submitted to SDS/PAGE, then the proteins were transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. Membranes were probed with a specific anti-(PKC isoenzyme) antibody followed by a peroxidase-conjugated secondary antibody as described [25], and developed with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Blots were quantified by scanning analysis with a Shimadzu CS-9000 densitometer. The linear range of the detected signal had been determined previously, and the relative amount of each immunoreactive band was calculated by measuring the area of the densitometric peaks.

Labelling of MEL cells with [^{32}P]P_i

MEL cells (2×10^7 cells) were suspended in 4 ml of α-minimal essential medium containing 10 % (v/v) fetal calf serum and 1.6 mCi of $H_3{}^{32}PO_4$. After 30 min at 37 °C, the cell suspension was divided into aliquots of 1 ml, which were incubated further at 37 °C in the presence or absence of 160 nM PMA. All the cell samples were finally collected by centrifugation (600 ***g***; 5 min) and suspended in 1 ml of buffer A containing 2 μM okadaic acid. Cell lysis and PKC-θ immunoprecipitation were carried out as described above.

RESULTS

In order to establish the possible involvement of PKC isoenzymes in cell-cycle progression in MEL cells, we evaluated the subcellular localization of these isoenzymes by immunofluorescence labelling. Because PKC-θ has been suggested to be involved directly in the cell cycle [19], we first investigated its distribution in dividing MEL cells. The micrograph images shown in Figures 1(A) and 1(B) clearly demonstrate the localization of PKC-θ at the level of the spindle poles, identified as two large fluorescent spots, and also on the kinetochores, distinguishable as pairs of points on each chromosome. Pretreatment of the anti-PKC-θ antibody with its immunogen peptide completely abolished the appearance of all fluorescent spots, indicating the specificity of this immunodetection (Figures 1C and 1D). None of the other PKC isoenzymes present in MEL cells showed a similar distribution. As shown in Figure 2, PKC-α was located preferentially in the cytosolic matrix, both in interphase and during mitosis. PKC-δ was distributed in the cytosol and in the nucleus during interphase, whereas, in dividing cells, it became concentrated in the middle of the mitotic spindle. PKC-μ localization was similar to that of PKC-α, and its distribution was not modified in mitotic cells. PKC-θ was almost entirely located in the nucleus during interphase and, as also shown in Figure 1, was recruited on to the mitotic apparatus during cell division. Finally, PKC-ζ was associated with the nucleus in interphase and became almost completely undetectable during mitosis. PKC-ϵ, another novel PKC isotype identified previously in MEL cells, was not detectable with this technique.

The specific interaction of PKC-θ with the mitotic spindle also occurs in human LAN-5 neuroblastoma cells and murine P3 myeloma cells (Figure 3), and persists during the different mitotic phases (results not shown). Taken together, these findings suggest that the interaction of PKC-θ with centrosomes and kinetochores is a specific property of eukaryotic cells.

In order to define the relevance of PKC-θ in mitotic events, we analysed the level and distribution of this kinase in non-growing MEL cells. Following exposure to the chemical inducer HMBA, these cells undergo spontaneous growth arrest, and display the fully differentiated erythroid phenotype [23]. We measured the level of PKC-θ and the growth capacity of MEL cells treated with HMBA for different times. The level of PKC-θ did not change during the first 40 h of incubation (Figure 4), but after this a rapid decrease of over 90 % was observed. After longer incubation periods, only 2–5 % of the original PKC-θ was detectable, which probably arose from the few undifferentiated cells still present in the culture. Identical kinetics were observed for cell growth, which decreased in parallel with the disappearance of PKC-θ. For comparison, the level of PKC-δ, the novel PKC isoform having the highest structural similarity with PKC-θ [5], is also shown in Figure 4. Exposure of MEL cells to HMBA promoted a very rapid down-regulation of the PKC-δ isoenzyme, a process that was completed largely before the onset of cell commitment, which occurs 14–16 h after the addition of

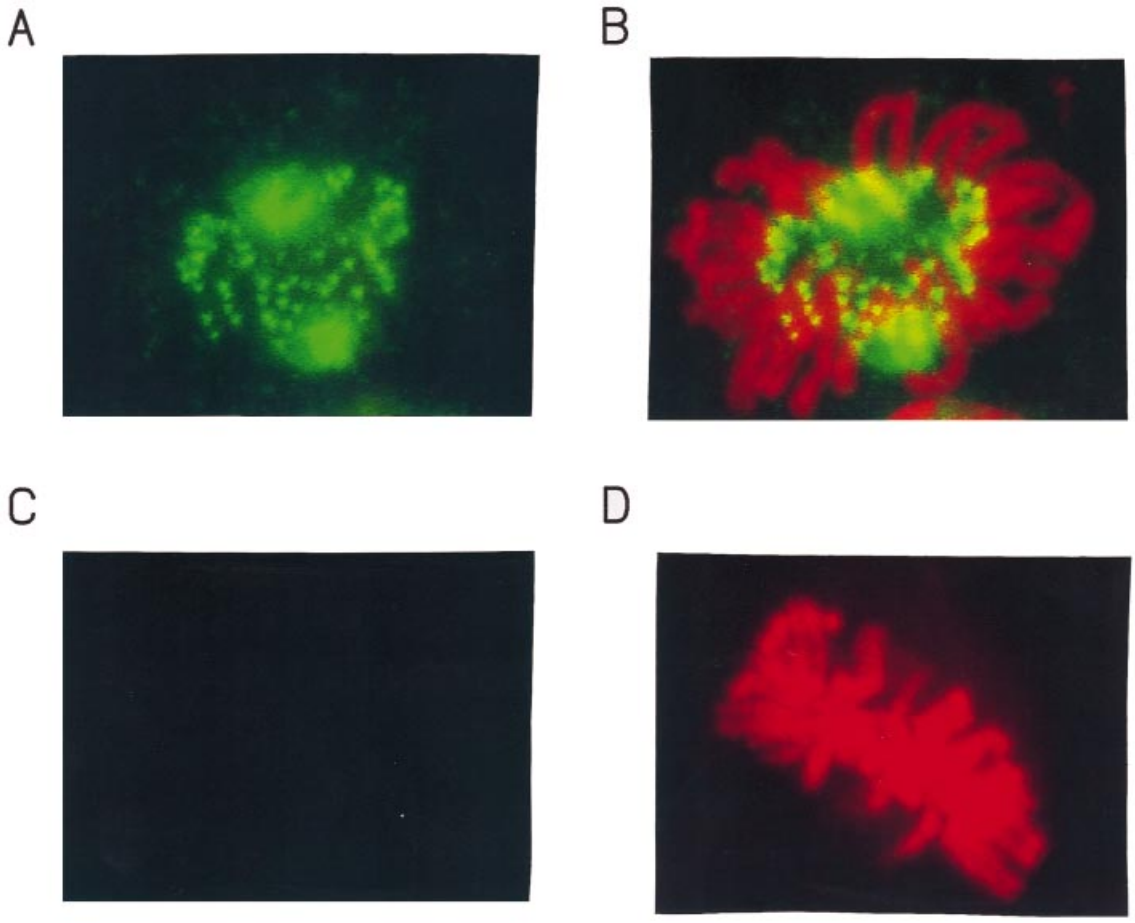

Figure 1 Localization of the PKC-θ isoenzyme in dividing MEL cells

In (**A**) and (**B**), cells were fixed and double-stained with anti-PKC-θ antibody (green fluorescence) and ethidium bromide (red fluorescence), as described in the Experimental section. In (**C**) and (**D**), cells were stained as in (**A**) and (**B**), using anti-PKC-θ antibody that had been pre-incubated with the immunogen peptide. (**A**) and (**C**), images obtained at 488 nm excitation/522 nm emission; (**B**) and (**D**), confocal overlay pictures obtained at 488 nm excitation/522 and 605 nm emission. Using three separate batches of MEL cells, at least 50 mitotic MEL cells were analysed for each condition, and showed the same localization of PKC-θ.

the chemical inducer [6]. Since PKC-θ is down-regulated at longer times, corresponding to the cessation of cell growth, we can postulate that this kinase is involved in cell proliferation rather than in erythroid differentiation.

Similar results were obtained with MEL cells cultured for sufficient time to reach the density-induced growth arrest [6], corresponding to a density of about 3×10^6 cells/ml. Under these conditions, we observed that PKC-θ undergoes down-regulation, resulting in a decrease of more than 70% compared with its original level (Figure 5). Dilution of the culture to 10^5 cells/ml with fresh medium restored MEL cell growth, which was accompanied by a progressive increase in the intracellular amount of PKC-θ. Following 48 h of incubation after cell dilution, both the level of PKC-θ and the rate of cell growth were comparable with those of control growing cells.

We have observed previously that exposure of MEL cells to PMA for a few hours promotes growth arrest together with a loss of PKC activity [7]. Due to the high efficiency of PMA in producing non-growing cells, we considered this experimental approach suitable to better define the relationship between the intracellular level of PKC-θ and cell growth.

Overnight exposure of MEL cells to 160 nM PMA produced a greater than 70% decrease in the PKC-θ cell content (Figure 6). Following removal of PMA from the culture medium, both recovery of cell growth and increasing expression of PKC-θ were detectable. After 48 h of incubation, more than 90% of PKC-θ levels had been restored, and the growth rate was similar to that of control cells.

In order to analyse the early changes in PKC-θ caused by PMA, we exposed MEL cells to a 15 min treatment with the phorbol ester and evaluated the intracellular distribution of the kinase. The micrograph image shown in Figure 7(A) demonstrates that, under these conditions, PKC-θ was translocated outside the nucleus (cf. Figure 2) and accumulated in the soluble cell fraction. These data are consistent with the hypothesis that a change in the intracellular localization of PKC-θ is required for MEL cell growth arrest. To clarify the molecular processes accompanying the export of PKC-θ outside the nucleus, we also analysed the level and molecular properties of the kinase before and after a brief cell exposure to PMA. As shown in Figure 7(B), the 76 kDa band representing native PKC-θ in untreated cells was progressively replaced by a new kinase form having a lower electrophoretic mobility, corresponding to an apparent molecular mass of 78–79 kDa. This conversion was completed within

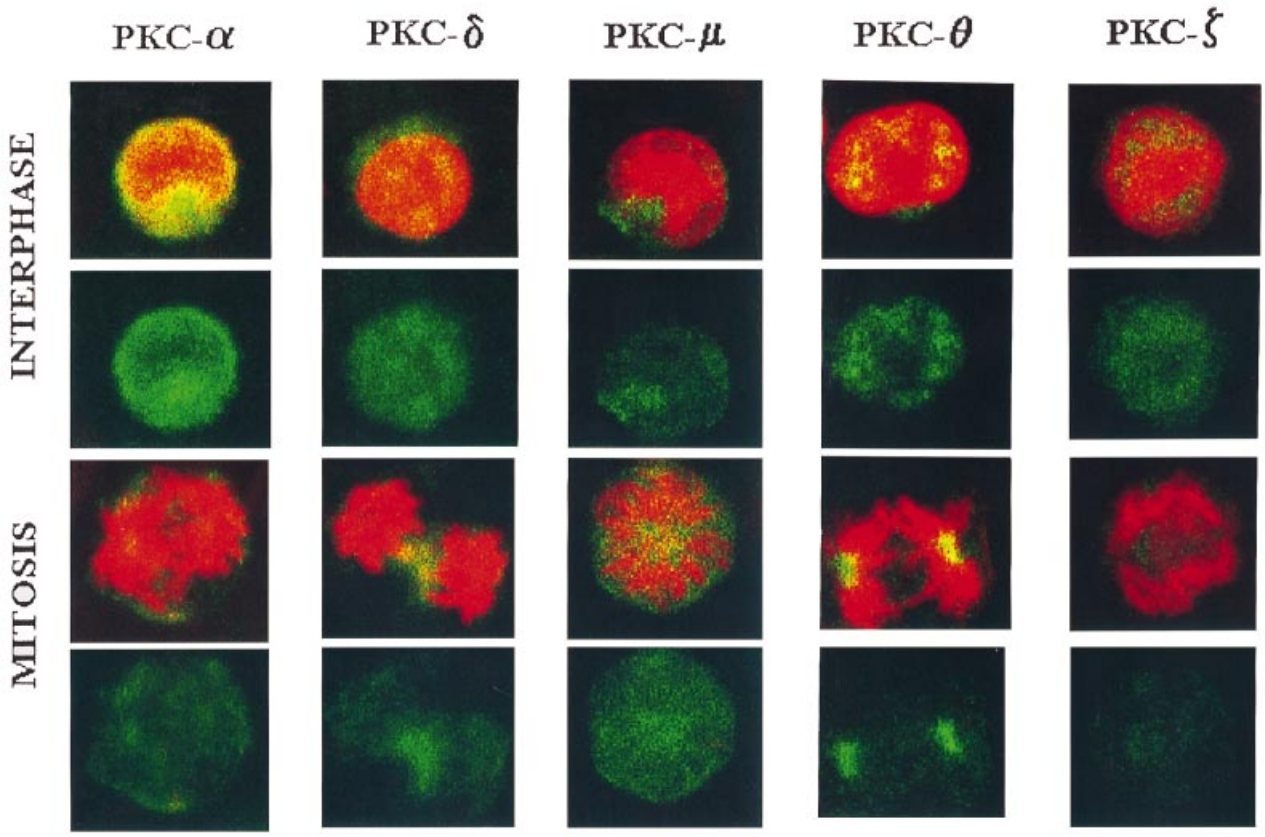

Figure 2 Localization of PKC isoenzymes during interphase and mitosis

MEL cells from three separate batches (10^6 total cells in each batch), grown to a cell density of 3×10^5 cells/ml under control conditions, were collected, fixed and stained both with the anti-(PKC isoenzyme) antibody indicated and with ethidium bromide, as specified in the Experimental section. Rows 2 and 4, images obtained at 488 nm excitation/522 nm emission; rows 1 and 3, confocal overlay pictures obtained at 488 nm excitation/522 and 605 nm emission Each individual cell shows the typical distribution of the indicated PKC isoenzyme that was observed in at least 50 cells analysed for each condition.

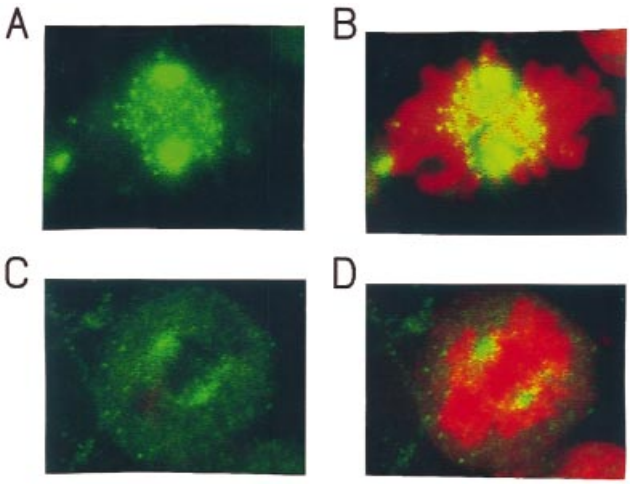

Figure 3 Localization of PKC-θ in dividing murine P3 myeloma cells (A and B) and human LAN-5 neuroblastoma cells (C and D)

Samples of 10^6 cells, from two separate batches, were collected for each cell line, fixed and stained with anti-PKC-θ antibody and with ethidium bromide, as described in the legend to Figure 1. (**A**) and (**C**), images obtained at 488 nm excitation/522 nm emission; (**B**) and (**D**), confocal overlay pictures obtained at 488 nm excitation/522 and 605 nm emission. At least 50 mitotic cells were analysed for each condition, and showed an identical localization of PKC-θ.

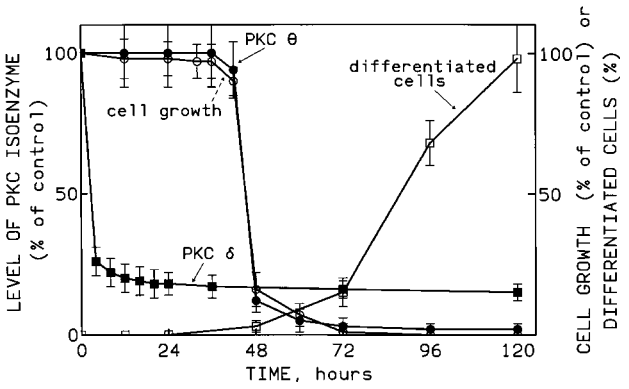

Figure 4 Levels of PKC-θ and -δ and rate of cell growth in differentiating MEL cells

MEL cell differentiation was induced by adding 5 mM HMBA to a culture containing 10^5 cells/ml. At the indicated times, cell growth (○), the proportion of differentiated cells (□) and the levels of PKC-θ (●) and PKC-δ (■) isoenzymes were evaluated as specified in the Experimental section. Results are means ± S.D. for determinations carried out using three separate batches of cells.

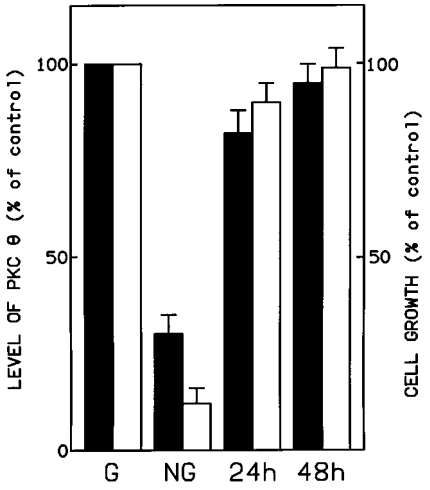

Figure 5 Levels of PKC-θ in proliferating and density-growth-arrested cells

MEL cells were collected at a density of 3×10^5 cells/ml (exponential growth; G) or 3×10^6 cells/ml (density growth arrest; NG), or 24 h or 48 h after dilution to 10^5 cells/ml. The level of PKC-θ was quantified by immunoblotting, as described in the Experimental section. The levels of PKC-θ (closed bars) and cell growth (open bars) are means ± S.D. of values obtained using three separate batches of cells.

10 min. The high-molecular-mass form of PKC-θ was then down-regulated, and a decrease of 10–15% was detectable after 1 h (results not shown). A similar molecular modification has been described previously for other PKC isoenzymes, and was ascribed to an enhanced phosphorylation of the protein kinase molecule [25].

To assess whether PMA causes a change in the phosphorylation status of PKC-θ, we immunoprecipitated this kinase from ^{32}P-labelled MEL cells (Figure 7C). No radioactive band was present in ^{32}P-labelled control cells, whereas a 78 kDa radioactive band was detectable in cells treated for 10 or 15 min with PMA. The amount of PKC-θ recovered in the immunoprecipitates was similar for any conditions used (results not shown). In cells

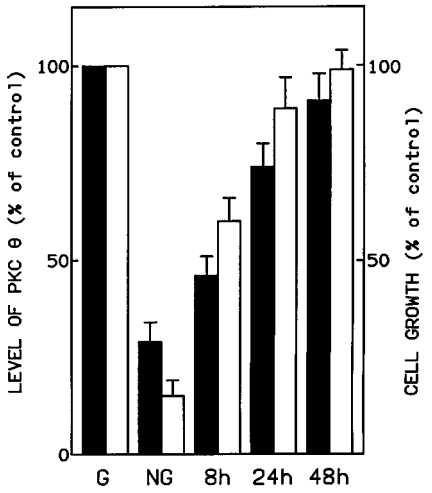

Figure 6 Levels of PKC-θ in proliferating and growth-arrested cells following exposure to PMA

MEL cells were collected during exponential growth (G), after 18 h of exposure to 160 nM PMA (NG), or 8, 24 or 48 h after the removal of PMA, as indicated. The levels of PKC-θ (closed bars) and cell growth (open bars) are means ± S.D. of values obtained using three separate batches of cells.

exposed for 10 min to PMA, but immunoprecipitated with anti-PKC-θ antibody pretreated with the immunogen peptide, both the radioactive band and the corresponding PKC-θ immunoreactive band were undetectable (results not shown). These findings indicate that PMA induces the rapid and complete conversion of native PKC-θ into a phosphorylated form, and that such a modification might be involved in the concomitant translocation of the enzyme outside the nucleus.

DISCUSSION

In this paper, we provide for the first time evidence indicating a specific localization of the PKC-θ isoenzyme in MEL cells during mitosis. Although several PKC isoenzymes are located in the nucleus of these cells, only PKC-θ is accumulated in mitotic spindle centrosomes and in chromosome kinetochores. This peculiar distribution seems to be a characteristic of other mammalian cells of human and murine origin. From these observations, it can be postulated that PKC-θ may play a specific role in cell growth.

This assumption is supported by the fact that non-growing MEL cells are characterized by the almost complete absence of PKC-θ. Differentiated MEL cells, having acquired a non-growing red-cell-like phenotype, express less than 5% of the original PKC-θ content (see Figure 4); in addition, in the non-growing phenotype obtained by reaching density growth arrest or by treatment with PMA, the kinase is down-regulated. To study the processes accompanying the disappearance of PKC-θ from non-growing cells, we have induced very rapid growth arrest by exposure of MEL cells to PMA. This tumour promoter has been found to induce, at first, a rapid phosphorylation of PKC-θ, which probably affects the intracellular distribution of the enzyme. In fact, the phosphorylated kinase molecule leaves the nucleus and accumulates in the cytosolic compartment. These events, occurring within a few minutes, are followed by a slower down-regulation, which is reversed after restoration of cell growth capability.

Altogether, these findings open a new field of research on the

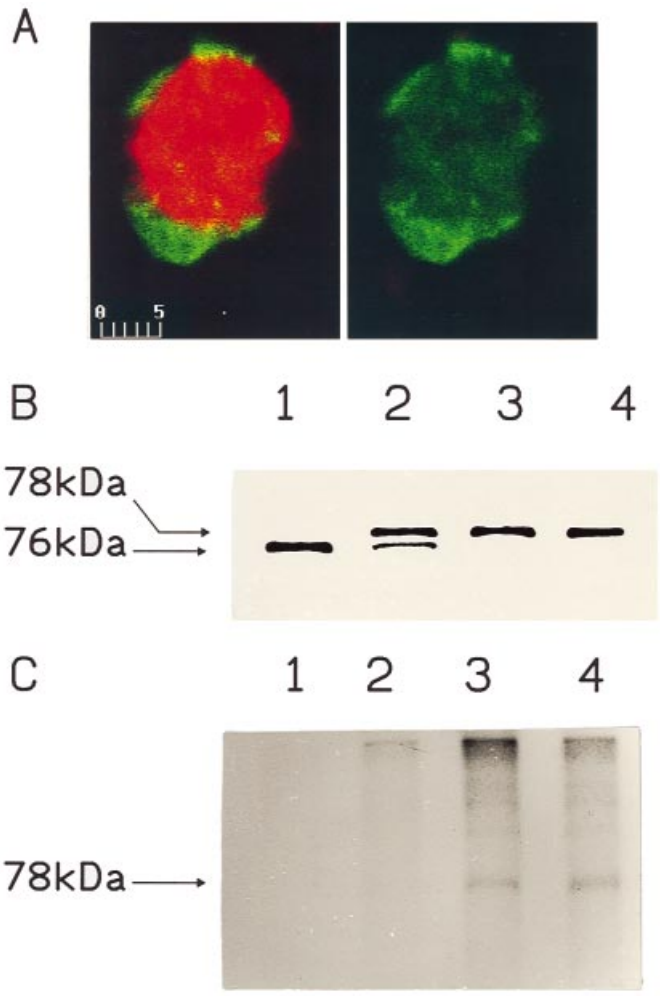

Figure 7 Cell localization and molecular properties of PKC-θ in MEL cells after a brief exposure to PMA

(**A**) MEL cells were treated with 160 nM PMA for 15 min, then fixed and stained with anti-PKC-θ antibody and with ethidium bromide, as described in the legend to Figure 1. Left panel, confocal overlay image obtained at 488 nm excitation/522 and 605 nm emission; right panel, image obtained at 488 nm excitation/522 nm emission. The scale bar is 5 μm. (**B**) A Western blot of PKC-θ was carried out with untreated cells (lane 1) and with cells exposed to 160 nM PMA for 5 min (lane 2), 10 min (lane 3) or 15 min (lane 4). The arrows indicate the positions of marker proteins. (**C**) MEL cells were labelled with ^{32}P, as described in the Experimental section, then incubated in the absence of PMA for 10 min (lane 1) or in the presence of 160 nM PMA for 10 min (lanes 2 and 3) or 15 min (lane 4). PKC-θ was immunoprecipitated using the anti-PKC-θ antibody and submitted to SDS/PAGE followed by autoradiography. Pretreatment of the antibody with the immunogen peptide abolished the immunoprecipitation of radioactive PKC-θ (lane 2).

involvement of this kinase in cell growth. In contrast with other PKC isoenzymes, previously shown to be involved in the early phases of the MEL cell differentiation process [4,9,26], PKC-θ seems to be required preferentially by proliferating cells. In other mammalian cell models, some PKC isoenzymes have been suggested to participate in cell-cycle progression, whereas other forms undergo changes in their level of expression as a function of the cell-cycle phase [27–29]. More recently, PKC-θ has been suggested to be the isoenzyme involved in cell-cycle progression, mediating the angiogenic effects exerted by phorbol esters in rat capillary endothelial cells [19]. In addition, a function for PKC-θ in T-cell activation, through interaction of this kinase with the microtubule-organizing centre, has also been proposed [30,31]. Despite similarities with regard to primary structure, cell localization and enzymic properties, PKC-θ plays specialized intracellular roles distinct from those of all other novel PKC forms, when expressed concomitantly in the same cell type. Specific subcellular locations, together with the presence of peculiar regulatory domains modulating catalytic activation and interactions with target proteins, are the most likely elements responsible for well defined functions of individual members of the PKC family [5]. Further experiments are in progress to identify the targets of PKC-θ activity on the mitotic spindle.

This work was supported by grants from the Italian Consiglio Nazionale delle Ricerche, Progetto Finalizzato Biotecnologie, from the Associazione Italiana per la Ricerca sul Cancro and from the Fondazione Cassa di Risparmio di Genova e Imperia.

REFERENCES

1 Nishizuka, Y. (1995) FASEB J. **9**, 484–496
2 Dekker, L. V. and Parker, P. J. (1994) Trends Biochem. Sci. **19**, 73–77
3 Nishizuka, Y. (1984) Nature (London) **308**, 693–695
4 Pessino, A., Sparatore, B., Patrone, M., Passalacqua, M., Melloni, E. and Pontremoli, S. (1994) Biochem. Biophys. Res. Commun. **204**, 461–467
5 Mellor, H. and Parker, P. J. (1998) Biochem. J. **332**, 281–292
6 Melloni, E., Pontremoli, S., Damiani, G., Viotti, P., Weich, N., Rifkind, R. A. and Marks, P. A. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3835–3839
7 Melloni, E., Pontremoli, S., Viotti, P. L., Patrone, M., Marks, P. A. and Rifkind, R. A. (1989) J. Biol. Chem. **264**, 18414–18418
8 Patrone, M., Pessino, A., Passalacqua, M., Sparatore, B., Melloni, E. and Pontremoli, S. (1994) FEBS Lett. **344**, 91–95
9 Sparatore, B., Pessino, A., Patrone, M., Passalacqua, M., Melloni, E. and Pontremoli, S. (1993) Biochem. Biophys. Res. Commun. **193**, 220–227
10 Sparatore, B., Patrone, M., Passalacqua, M., Melloni, E. and Pontremoli, S. (1991) Biochem. Biophys. Res. Commun. **179**, 153–160
11 Pessino, A., Passalacqua, M., Sparatore, B., M., Patrone, M., Melloni, E. and Pontremoli, S. (1995) Biochem. J. **312**, 549–554
12 Renzi, L., Gersch, M. S., Campbell, M. S., Wu, L., Osmani, S. A. and Gorbsky, G. J. (1997) J. Cell Sci. **110**, 2013–2025
13 Roghi, C., Giet, R., Uzbekov, R., Morin, N., Chartrain, I., Le Guellec, R., Couturier, A., Dorée, M., Philippe, M. and Prigent, C. (1998) J. Cell Sci. **111**, 557–572
14 Bailly, E., Dorée, M., Nurse, P. and Bornens, M. (1989) EMBO J. **8**, 3985–3995
15 Golsteyn, R. M., Mundt, K. E., Fry, A. M. and Nigg, E. A. (1995) J. Cell Biol. **129**, 1617–1628
16 Nigg, E. A., Schafer, G., Hilz, H. and Eppenberger, H. M. (1985) Cell **41**, 1039–1051
17 Ohta, Y., Ohba, T. and Miyamoto, E. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5341–5345
18 Pietromonaco, S. F., Seluja, G. A. and Elias, L. (1995) Blood Cell Mol. Dis. **21**, 34–41
19 Tang, S., Morgan, K. G., Parker, C. and Ware, J. A. (1997) J. Biol. Chem. **272**, 28704–28711
20 Passalacqua, M., Patrone, M., Picotti, G. B., Del Rio, M., Sparatore, B. M., Melloni, E. and Pontremoli, S. (1995) Neuroscience **82**, 1021–1028
21 Higgins, D. G. and Sharp, P. M. (1988) Gene **73**, 237–244
22 Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (eds.) (1991) Current Protocols in Immunology, Unit 3.17, Wiley, New York
23 Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. A. and Marks, P. A. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 862–866
24 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
25 Palejwala, S. and Goldsmith, L. T. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4202–4206
26 Edwards, A. S. and Newton, A. C. (1997) J. Biol. Chem. **272**, 18382–18390
27 Patrone, M., Pessino, A., Passalacqua, M., Sparatore, B., Melloni, E. and Pontremoli, S. (1996) Biochem. Biophys. Res. Commun. **220**, 26–30
28 Thompson, L. J. and Fields, A. P. (1996) J. Biol. Chem. **271**, 15045–15053
29 Nakaigawa, N., Hirai, S.-I., Mizuno, K., Shuin, T., Hosaka, M. and Ohno, S. (1996) Biochem. Biophys. Res. Commun. **222**, 95–100
30 Romanova, L. Y., Alexandrov, I. A., Schwab, G., Hilbert, D. M., Mushinski, J. F. and Nordan, R. P. (1996) Biochemistry **35**, 9900–9906
31 Monks, C. R. F., Kupfer, H., Tamir, I., Barlow, A. and Kupfer, A. (1997) Nature (London) **385**, 83–86

Received 31 July 1998/24 September 1998; accepted 28 October 1998