Protein kinase C-θ is specifically activated in murine erythroleukaemia cells during mitosis

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Abstract Protein kinase C-θ is a member of the n-protein kinase C subfamily that in mitotic cells translocates to centrosomes and kinetochores. Although this kinase is expressed in comparable amounts in murine erythroleukaemia cells during the interphase or metaphase, when localized in the mitotic structures, it selectively phosphorylates a 66 kDa protein, also associated to chromosomes. Moreover, protein kinase C-θ immunoprecipitated from cells at the metaphase results four times more active in the absence of lipid cofactors as compared with the kinase obtained from cells in the interphase. This activation is accomplished by interaction of protein kinase C-θ with a protein factor which also promotes an increased autophosphorylation of the kinase. These findings indicate that in the mitotic phase of the cell cycle, protein kinase C-θ recognizes a protein factor which operates as a positive modulator of the kinase activity in the absence lipids.

Key words: Protein kinase C-θ; Protein kinase C activation; Murine erythroleukaemia cell cycle; Enzymology

1. Introduction

Individual protein kinase C (PKC) isoenzymes are involved in specific signal transduction pathways [1–4] as well as in the regulation of multiple basal cell functions, including the control of intracellular pH, maintenance of the cytoskeleton and progression of the cell cycle [5–8]. The different functions attributed to single members of the PKC family expressed in eukaryotic cells cannot be explained only on the basis of differences observed in their catalytic properties, such as substrate specificity or sensitivity to activators [9–12]. It has been hypothesized that the subcellular localization, the presence of characteristic domains, capable to promote or prevent association of PKCs with specific sites and interaction with activating or inhibiting protein factors could play a relevant role for the intracellular modulation of PKC isoenzyme activities. In fact, it has been recently reported that isoenzymes belonging to the conventional, novel or atypical PKC subfamilies [13] interact with specific proteins both under in vivo and in vitro conditions [9,14,15]. Several RACK proteins have been yet identified as sites of binding for activated PKC isoenzymes [16]. Moreover, it has been proposed that protein receptors specific for the inactive form of the kinases (RICKs) are capable to anchor single PKC isoenzymes to different subcellular sites. A number of molecular motifs, identified both in the regulatory and catalytic domain of PKC isoenzymes, seem to be involved in these associations [17].

We reported previously that PKC plays a fundamental role in murine erythroleukaemia (MEL) cell differentiation [18]. However, the level of PKC-α, that in these cells is present in two forms, is positively correlated with the sensitivity to the chemical inducer and the decrease in the latent period preceding cell commitment [19]. An opposite role is played by PKC-δ, an isoenzyme highly expressed in inducer-resistant MEL cell clones and rapidly down-regulated following induction [20]. More recently, we observed that PKC-θ, an almost completely nuclear PKC isoenzyme in MEL cells, seems not to be involved in MEL cell commitment and disappears late during the multistep differentiation process, at a time corresponding to the cell acquirement of the ungrowing phenotype [21]. PKC-θ, a member of the novel PKC subfamily, has been indicated as a mediator of many specific functions in different cell types. This PKC isoenzyme is required in the process of apoptosis in thymocytes [22], during mitosis and formation of F-actin stress fibers in endothelial cells [6] and in the process of activation of T-cells [23]. Moreover, the 14-3-3 tau protein has been proposed as a specific modulator of PKC-θ functions preventing its translocation to the plasma membrane in Jurkat T-cells [24]. A specific PKC-θ protein substrate, moesin, has also been identified in human leucocytes, among the membrane/cytoskeletal linkage proteins [25]. In MEL cells, as well as in other murine and human cells, PKC-θ associates to centrosomes and kinetochores during mitosis [21], suggesting an active role for this kinase in the control of protein functions linked to the cell cycle progression. To establish the biochemical relevance of the interaction of PKC-θ to mitotic spindle and chromosomal structures, here, we have investigated the expression of phosphorylating activity of the kinase recovered from mitotic MEL cells, searching also for protein substrates localized in the same structures.

2. Materials and methods

2.1. Cell culture, synchronization and induction of differentiation

N23 MEL cells were obtained and cultured as specified previously [26]. Cells were arrested at the metaphase by thymidine/nocodazole (Sigma Chemical) treatment as described [27] except that the concentration of nocodazole was lowered to 50 ng/ml. This procedure resulted in a mitotic index of 80% as judged by fluorescence labelling of DNA [28]. MEL cell differentiation was induced by addition of 5 mM hexamethylenebisacetamide (HMBA) (Sigma Chemical) to a culture containing 10^5 cells/ml. At the indicated times, the percentage of differentiated cells was determined by the benzidine reaction method [29].

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Abbreviations: PKC, protein kinase C; MEL, murine erythroleukaemia; HMBA, hexamethylenebisacetamide; PMA, phorbol-12-myristate-13-acetate; PS, phosphatidylserine

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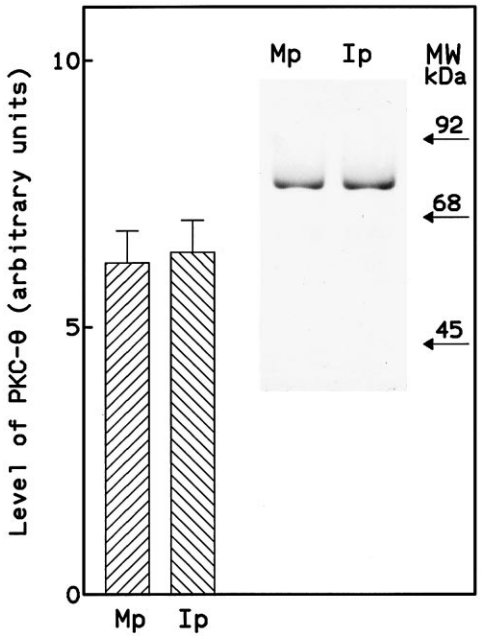

Fig. 1. The level of PKC-θ in MEL cells during the interphase or metaphase. MEL cells (10^5 cells) in the interphase (Ip) or from a culture synchronized at the metaphase (Mp) were submitted to SDS-PAGE followed by a Western blot for PKC-θ as described in Section 2. The hatched bars represent a quantification of PKC-θ by densitometric analysis of the 76 kDa band detected by the chemiluminescence method, as specified in Section 2. The data are reported as mean ± S.D. for triplicate measurements. Arrows on the right of the Western blot indicate the migration of molecular weight markers.

2.2. Immunoprecipitation of PKC-θ and assay of catalytic activity

N23 MEL cells (10^7 cells) were immunoprecipitated with an affinity-purified polyclonal antibody raised against the C-terminal peptide of mouse PKC-θ (Santa Cruz Biotechnology) [21]. To assay for PKC activity, the immunoprecipitates were washed five times with 1 ml of 20 mM Tris-HCl, pH 7.6, containing 0.14 M NaCl, 2.5 mM EDTA and 2.5 mM EGTA. The pellet was suspended in 50 μl washing buffer and aliquots were diluted in 100 μl of a mixture containing 5 mM dithiothreitol, 15 mM $MgCl_2$, 10 μM ATP, 10 μCi [γ^{32}P]ATP, 1 μM okadaic acid in the absence or presence of a chromosome preparation obtained from 10^6 MEL cells as specified in Section 2.4. The presence of other additions in the assay mixture is specified elsewhere. After 30 min at 37°C, the reaction was stopped with electrophoresis sample buffer, followed by SDS-PAGE on a 8% polyacrylamide gel and autoradiography. The amount of radioactivity incorporated in a protein band was determined by the area of the densitometric peak [21].

2.3. Quantitative analysis of PKC-θ by Western blot

MEL cells were lysed in the sample buffer for electrophoresis and submitted to SDS-PAGE on a 8% polyacrylamide gel. Western blotting and the quantitative evaluation of PKC-θ present on each electrophoretic lane were carried out as specified previously [21].

2.4. Isolation of chromosomes from MEL cells

To obtain purified chromosomes, N23 MEL cells (2×10^7 cells) were synchronized at the metaphase as reported above, then collected by centrifugation and rinsed twice with PBS. Chromosomes were then purified as described [30] and stored at −70°C.

2.5. Characterization of the PKC-θ activating factor

To establish the molecular identity of the activating factor co-immunoprecipitated with PKC-θ, 3×10^7 MEL cells were synchronized at the metaphase and PKC-θ was immunoprecipitated as specified above. Immunoprecipitates were then divided into identical aliquots. The first one was incubated with trypsin (ratio 200:1, w:w) for 2 h at 37°C and the reaction was stopped by heating the mixture at 75°C for 3 min. A second aliquot was extracted with one volume of ether for 10 min at 20°C, then, the aqueous and ether phases were dried by lyophilization, diluted in the original volume and heated for 3 min at 75°C. A third aliquot was maintained for 2 h at 37°C followed by heating as above.

3. Results

In order to evaluate changes in the amount of PKC-θ expressed by MEL cells at different stages of the cell cycle, we measured the level of the kinase in cells collected from cultures containing 90% of cells in the interphase or 80% cells synchronized at the metaphase. As shown in Fig. 1A, Western blot analysis reveals that the amount of the 76 kDa PKC-θ immunoreactive band is very similar in both conditions and no degradation fragments, which produce 40–50 kDa immunoreactive bands containing the catalytic domain of this kinase [31], are detectable. These data indicate that subcellular redistribution of PKC-θ occurring during the MEL cell cycle is not accompanied by a proteolytic processing or a down-regulation of the kinase.

Due to the fact that at present, a rapid purification procedure is not available to obtain a high yield of native PKC-θ, we selected the single step immunoprecipitation method to separate PKC-θ from the other PKC isoenzymes present in MEL cells. PKC-θ immunoprecipitates, obtained using an anti-peptide antibody raised against the caboxy-terminal peptide of the kinase, were analyzed by a Western blot and resulted free of any other PKC isoenzyme previously reported to be expressed by MEL cells (data not shown) [21]. Furthermore, since the protein or peptide substrates of PKC-θ are poorly characterized, we used a chromosomal preparation to evaluate the presence of an intracellular substrate for PKC-θ localized in a cell structure on which the kinase is recruited during mitosis. As shown in Fig. 2, addition of PKC-θ immunoprecipitated from MEL cells to a chromosome prepara-

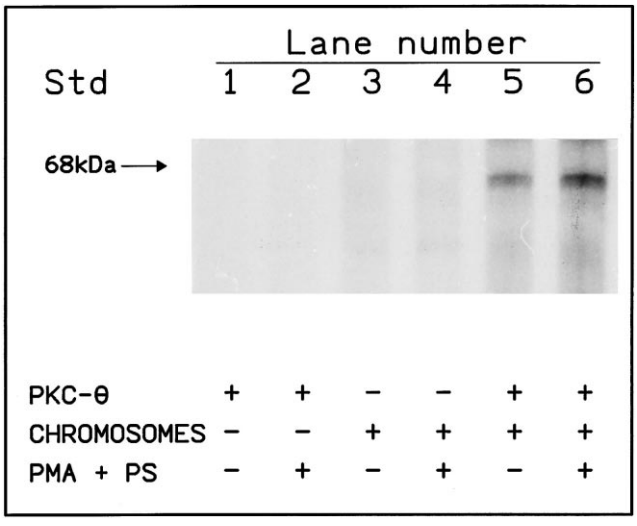

Fig. 2. Identification of PKC-θ substrates on isolated chromosomes. Chromosomes purified from 10^6 MEL cells at the metaphase were phosphorylated, as specified in Section 2, in the absence or presence of PKC-θ immunoprecipitated from 3×10^5 unsynchronized cells. After 30 min at 37°C, the reactions were stopped by addition of electrophoresis buffer and submitted to SDS-PAGE followed by autoradiography.

tion in the presence of [γ^{32}P]ATP results in the incorporation of ^{32}P in a protein band having a molecular mass of approximately 66 kDa. It has been reported that phorbol-12-myristate-13-acetate (PMA) and phosphatidylserine (PS) stimulate PKC-θ activity [6]. Addition of these lipid cofactors to the assay mixture enhances the incorporation of ^{32}P into the 66 kDa chromosomal protein 4-fold (cf. lanes 5 and 6). Chromosomes or PKC-θ alone do not show any appreciable phosphorylated band in these conditions.

These data indicate that the 66 kDa chromosomal protein is a true PKC-θ substrate, requiring lipid cofactors for maximal phosphorylation and that it can be used to assay PKC-θ activity alternatively to the commercial peptide substrates.

We evaluated the possibility that PKC-θ undergoes changes of catalytic properties following its translocation on the spindle pole during mitosis. The kinase was isolated by immunoprecipitation from MEL cells in the interphase or synchronized at the metaphase and the two PKC-θ preparations were then incubated with chromosomes in the absence or presence of lipid cofactors. As shown in Table 1, both PKC-θ samples exhibit a similar phosphorylating activity when assayed in the presence of PMA and PS. On the contrary, the enzyme isolated from cells in the interphase expresses 15% of its maximal activity in the absence of lipids, whereas the enzyme obtained from metaphasic cells expresses more than 60% of its maximal catalytic activity. This difference indicates that the kinase is more active during the metaphase, i.e. in the form associated with centrosomes and kinetochores.

To establish whether the PKC-θ activating agent, present in immunoprecipitates from metaphasic cells, was due to the presence of a protein or a lipid molecule, the immunoprecipitates were at first heated for 3 min at 75°C to obtain a complete inactivation of PKC-θ (data not shown) followed by incubation with trypsin or, alternatively, extraction with ether. Each immunoprecipitate was finally assayed for the presence of the PKC-θ activating factor by addition of chromosomes, as source of a protein substrate, and PKC-θ obtained from interphasic or metaphasic cells. As shown in Table 2, PKC-θ from metaphasic cells phosphorylates the 66 kDa chromosomal substrate with a comparable high efficiency both in the absence or in the presence of the heated immunoprecipitate. On the contrary, PKC-θ from cells in the interphase shows a 5-fold increase in the 66 kDa chromosomal protein phosphorylation when the heated immunoprecipitate is added to the assay mixture. Treatment of the heated immunoprecipitate with trypsin results in the disappearance of the PKC-θ activating effect, whereas, following extraction with ether, the activating factor is recovered in the aqueous phase. These data indicate that, in mitotic MEL cells, PKC-θ associates and co-immunoprecipitates together with a protein factor acting as a stimulating cofactor for the kinase activity. PKC-θ immunoprecipitates from cells in the interphase, on the contrary, do not contain any detectable PKC-θ activating factor (data not shown).

Table 1
Requirement of lipid cofactors by PKC-θ isolated from MEL cells in the interphase or metaphase

Cell condition	Addition	PKC-θ activity (cpm/sample)
Interphase	None	150 ± 30
	PMA+PS	980 ± 80
Metaphase	None	650 ± 70
	PMA+PS	1 000 ± 100

Each PKC-θ assay mixture (100 μl) contained the additions specified in Section 2, PKC-θ immunoprecipitated from the indicated cell source, a chromosome preparation from 10^7 MEL cells and, where indicated, 160 nM PMA and 100 μg/ml PS. After 30 min at 37°C, the samples were stopped and submitted to SDS-PAGE as reported in Section 2. The ^{32}P band corresponding to the 66 kDa chromosomal protein was excised and counted in a scintillation counter. Data represent mean ± S.D. of three replicate determinations.

Table 2
Properties of a PKC-θ activating factor

Cell condition	Addition	PKC-θ activity (cpm/sample)
Interphase	None	240 ± 40
	h.i.p.	1 250 ± 90
	Trypsin-treated h.i.p.	300 ± 30
	Ether-extracted h.i.p.	
	Aqueous phase	1 110 ± 130
	Organic phase	260 ± 50
Metaphase	None	1 420 ± 120
	h.i.p.	1 560 ± 100

Each PKC-θ assay mixture (100 μl) contained the additions specified in Section 2, PKC-θ immunoprecipitated from the indicated cell source, a chromosome preparation from 10^7 MEL cells and, where indicated, a sample of heated PKC-θ immunoprecipitate (h.i.p.) untreated or previously treated with trypsin or extracted with ether (see Section 2). After 30 min at 37°C, the samples were stopped and submitted to SDS-PAGE as reported in Section 2. The ^{32}P band corresponding to the 66 kDa chromosomal protein was excised and counted in a scintillation counter. Data represent mean ± S.D. of three replicate determinations.

We also explored the possibility that this PKC-θ activating protein could operate on the auto-phosphorylation process of the kinase, a specific event accompanying PKC activation [32]. The heated PKC-θ immunoprecipitate from metaphasic cells was then added to PKC-θ isolated both from interphasic or metaphasic cells in the absence or presence of the classical lipid cofactors PMA and PS. As shown in Fig. 3, in the absence of lipids, the incorporation of ^{32}P into PKC-θ isolated from interphasic cells (76 kDa phosphorylated band, lane 1) is very poor. However, addition of PKC-θ-heated immunoprecipitate increases the extent of auto-phosphorylation of the kinase 5-fold. A similar enhancement of the auto-phosphorylating activity is obtained by addition of the lipid cofactors to the assay mixture. Conversely, auto-phosphorylation of PKC-θ obtained from metaphasic cells occurs at a high extent in the absence of any addition and is not further stimulated by lipid cofactors. The identity between the phosphorylated protein band showing a molecular mass of 76 kDa and the PKC-θ molecule was also confirmed by overlapping of the ^{32}P band and PKC-θ immunoreactive band detectable by a Western blot on the same electrophoretic run (data not shown).

4. Discussion

We recently established that, although many PKC isoenzymes are located in the nucleus of MEL cells, PKC-θ is the only isoform completely recovered on mitotic spindle structures [21]. A specific association of PKC-θ to recognition sites on centrosomes and kinetochores occurs in mitotic cells both of human and murine origin, suggesting that this PKC-θ targetting process is probably involved in cell cycle progression. This conclusion is also supported by results demonstrating that PKC-θ is absent from the nucleus of ungrowing cells

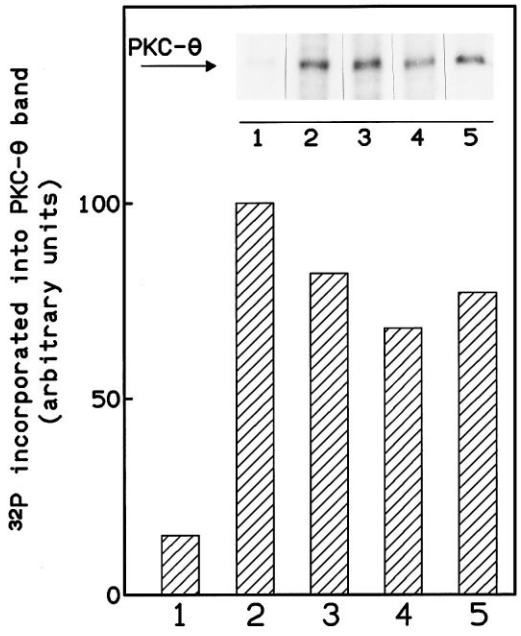

Fig. 3. Auto-phosphorylation of PKC-θ. PKC-θ was immunoprecipitated from interphasic (lanes 1, 2 and 3) or metaphasic (lanes 4 and 5) MEL cells. The immunoprecipitated material from 10^7 cells was auto-phosphorylated as specified in Section 2 in the absence of other additions (lanes 1 and 4), in the presence of PMA and PS (lane 2) or in the presence of heated PKC-θ immunoprecipitate from 10^7 metaphasic cells (lanes 3 and 5). The reaction mixtures were then submitted to SDS-PAGE. The inset shows the autoradiography of the PKC-θ band, a densitometric analysis of the phosphorylated PKC-θ bands is also shown.

and is also almost completely down-regulated in terminal differentiated erythroid cells [21]. We now provide experimental evidence indicating that in mitotic MEL cells, PKC-θ is present in an active form. In fact, a chromosomal protein with a molecular mass of 66 kDa is phosphorylated by PKC-θ isolated from mitotic cells with a four times higher efficiency, in the absence of lipid cofactor, as compared to the kinase obtained from interphasic cells. This activation of PKC-θ at the metaphase is also detectable as auto-phosphorylation of the kinase molecule. The loss of lipid requirement, shown by PKC-θ from mitotic cells, is not due to accumulation of free catalytic fragments produced by proteolytic events during mitosis, as indicated by the presence of similar levels of the native form of kinase in metaphasic and interphasic cells and also by the absence of low Mr PKC-θ immunoreactive forms in both conditions. A heat-stable protein factor, which co-immunoprecipitates with PKC-θ in MEL cells at the metaphase, has been found to be responsible for the catalytic activation of this kinase and substituting the classical lipid cofactors. The presence of intracellular complexes between PKC isoenzymes and PKC activating proteins has been yet identified and proposed as an alternative mechanism to localize active PKCs in a specific cell site. It has recently been demonstrated that PKC-α binds and is activated by syndecan-4 transmembrane heparan sulfate [33], whereas PKC-βII and PKC-ε bind on different domains and are activated by F-actin [34,35]. Another stimulating agent of PKC is the HMG1 protein, known as a DNA binding molecule [36]. This protein stimulates both PKC-α and PKC-β activity increasing their maximal catalytic activity and substituting their requirement for diacylglycerol [37]. A similar effect on cPKC isoenzymes has also been described for stratifin, a member of the 14.3.3 protein family [38]. It has been reported that some PKC isoenzymes operate as modulators of different cell functions following their interaction with specific proteins, such as phosphatidylinositol-4-kinase and phosphatidylinositol-4-phosphate-5-kinase, which bind PKC-μ at specific membrane sites [39] and the metalloprotease-disintegrin MDC9 involved in the regulated shedding through binding to PKC-δ [40].

Taken together, our results show that PKC-θ is recovered in an active form when it is associated to the mitotic apparatus and a heat stable protein factor is responsible for the catalytic activation in the absence of lipid cofactors. Experiments aimed to identify this PKC-θ stimulating protein are in progress.

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