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Neuronal Differentiation of PC12 Cells Involves Changes in Protein Kinase C-θ Distribution and Molecular Properties

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In this study we demonstrate that the rat pheochromocytoma PC12 cell line expresses the novel protein kinase C isozyme designated PKC-θ. The isozyme is almost completely localized in the nuclear compartment of proliferating cells. Following stimulation with the nerve growth factor, PKC-θ is redistributed into the cytoplasm and the outgrowing neurite processes, mostly as a cytoskeletal associated kinase. This event is accompanied by an eightfold increase in the expression level and by the appearance of specific modifications of PKC-θ molecule. Conversely, the kinase is down-regulated once cells reach the terminally differentiated state displaying a neuron-like phenotype. These data suggest a functional role for the kinase in the regulation of cytoskeletal modeling along the multistage differentiation process of PC12 cells.

Key Words: **protein kinase C-θ; nerve growth factor; neuronal differentiation; PC12 cells.**

Multiple cell signaling events include steps mediated by members of the PKC enzyme family (1). The various PKC isozymes are characterized by different tissue distribution, intracellular localization, and substrate specificity (2). In addition, each PKC form undergoes complex regulation mechanisms, involving selective cofactor requirements, post translational reversible and irreversible modifications, and intracellular translocations, promoted by appropriate agonists and mediated by specific interaction with receptors for activated C kinases (3). Several PKC isozymes have been shown to play a key role in signal transduction networks activated by neurotrophic agents on neuronal progenitors such as immortalized raphe neurons (4), neuroblastoma cell lines (5, 6) and PC12 cells (7). The novel PKC-θ isozyme, highly expressed in the skeletal muscle and hematopoietic cells (8, 9), has been recently shown to be involved in the control of the expression level of an integrin regulatory protein, which modulates migration and proliferation of capillary endothelial cells (10), as well as in the activation mechanisms of IL-2 and FasL gene promoters (11). Moreover, in different cell lines, PKC-θ results recruited on the centrosomes and kinetochores of the spindle pole in a catalitically active form (12, 13), suggesting its direct participation to the mitotic phase of the cell cycle. In T cells, PKC-θ is clustered in a supramolecular activation complex (14), an event specifically induced by agonists stimulating cell proliferation.

Recently, PKC-θ has been also identified in cells of nerve origin such as the LAN-5 neuroblastoma cell line (12), microglial and astroglial cells (15), but very little is known about the physiological role of PKC-θ in nerve cells.

Here we provide evidence that in the pluripotent PC12 cell line, PKC-θ is involved in the long term program of neuronal differentiation. In agreement with observations indicating that PKC-θ becomes relocalized to specific cell sites during processes of angiogenesis (16), mitosis (11), and T cell receptor activation (14), our present results show that this kinase associates to the reorganizing cytoskeleton network during differentiation of PC12 cells towards the neuron-like non proliferating phenotype.

MATERIALS AND METHODS

Cell culture and induction of differentiation. PC12 cells were cultured in RPMI 1640 containing 15% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biospa, Milan, Italy) and incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2. Where indicated cells were induced to differentiate by reducing the serum concentration to 1% and by addition of 100 ng/ml NGF 7S (Sigma) (17). Terminally differentiated cells were defined as those possessing one or more neurites having a length of at least 500 μm. All cell cultures were free of mycoplasma contamination as established by a routine assay with a Mycoplasma Detection Kit (Roche Diagnostics).

Western blot analysis. Cells were washed twice in phosphate-buffered saline (PBS). Proteins from total cell lysates or from cell

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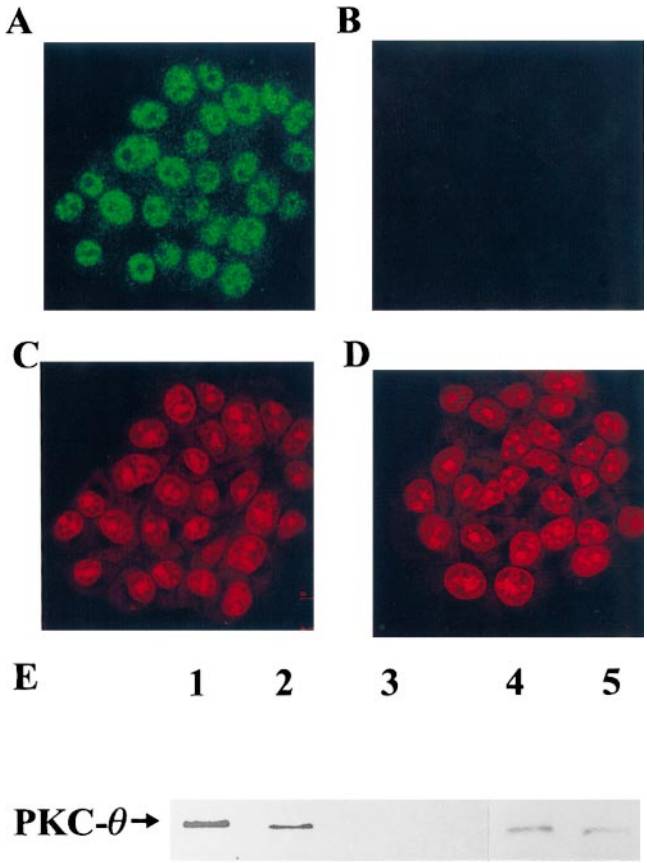

FIG. 1. Identification of PKC-θ in PC12 cells. Cells were processed for immunocytochemistry by using the anti-PKC-θ antibody directed to the C-terminal peptide in the absence (A and C) or presence (B and D) of the immunogen peptide. Stained nuclei of cells in A are shown in (C) and those of cells in B are shown in (D). (E) 5×10^4 PC12 cells (lanes 2, 3, 5), collected from non-confluent cell cultures, or Jurkat cells (8 μg of cell lysate, lanes 1, 4) were submitted to SDS–PAGE followed by Western blotting. The blots were probed with the same anti-PKC-θ antibody used in A, in the absence (lanes 1 and 2) or presence (lane 3) of the immunogen peptide. Alternatively, an antibody directed to the cystein rich region of the kinase was used (lanes 4 and 5).

fractions were separated by electrophoresis on 10% polyacrylamide gels in denaturing conditions (SDS–PAGE) (18). Protein were then transferred to nitrocellulose membranes (Bio Rad Laboratories) and Western-blotting was carried out as described previously (12). Blots were incubated with a policlonal anti-PKC-θ antibody raised against the C-terminal peptide of the mouse kinase (1:2000; Santa Cruz Biotechnologies) or, where indicated, with an anti-PKC-θ antibody raised against the cystein rich region of the kinase (1:250; Transduction Laboratories). Membranes were then incubated for 60 min with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Amersham Pharmacia) (19). The immunocomplexes were revealed by using an enhanced chemiluminescence detection system and Hyperfilm MP X-ray films (Amersham Pharmacia). Quantification of the immunoreactive bands was carried out with a dual-wavelength flying-spot scanner (Shimadzu Corporation).

PKC-θ immunocytochemistry. Cells were fixed and permeabilized by the Triton/paraformaldehyde method, as specified previously (20). Nonspecific interactions were blocked by a 30 min incubation in PBS containing 5% (v:v) fetal calf serum. Fixed cells were then incubated in PBS containing 0.5 μg/ml anti-PKC-θ antibody raised against the C-terminal peptide of the kinase for 16 h at 4°C, washed with PBS containing 2 mg/ml bovine serum albumin, followed by a 1 h incubation at 4°C in the same buffer containing 2.5 μg/ml FITC-conjugated goat anti-rabbit IgG (Amersham Pharmacia). Chromatin was stained by a 5 min incubation of fixed cells with 2 μg/ml propidium iodide (Sigma). The slides were washed again three times and then mounted in coverslips with FluoroGuard anti-fade reagent (Bio Rad Laboratories) before analysis. Images of the samples were collected as described (12), using a planapochromat $\times$60 oil-immersion objective with numerical aperture 1.4. The excitation/emission wavelengths were 488/522 nm for the FITC-conjugated antibody and 568/605 nm for stained chromatin.

Cytoskeleton extraction. PC12 cells (4×10^6 cells) were washed three times with PBS and cytoskeletons were prepared as described (21). The Triton soluble and insoluble (cytoskeleton) cell fractions were immediately suspended in the electrophoresis sample buffer and heated at 95°C for 1 min.

RESULTS

PKC-θ Expression in PC12 Cells

An immunocytochemical analysis, carried out on PC12 cells by using an anti-PKC-θ antibody directed to the C-terminal peptide of the kinase, revealed that these cells express the PKC-θ isozyme localized in the nuclear compartment (Figs. 1, cfr. A and C). The specificity of this staining was demonstrated by disappearance of the signal by pretreatment of the antibody with the immunogen peptide (Fig. 1, B and D). A Western

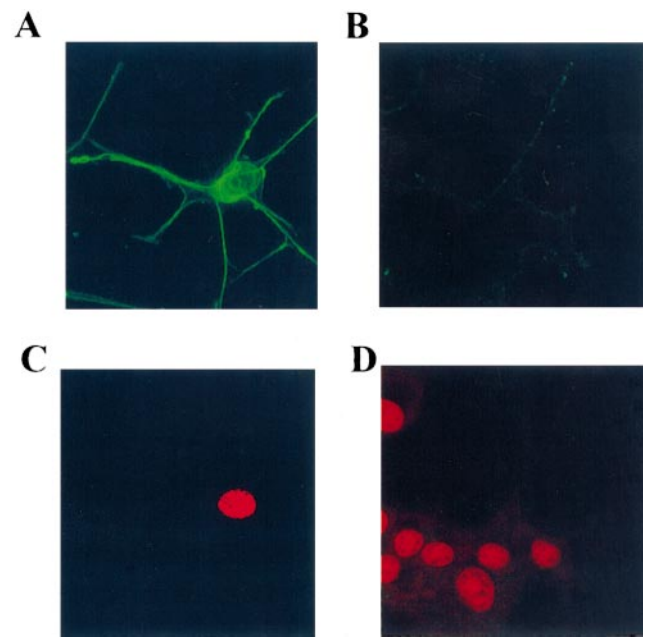

FIG. 2. Immunocytochemistry of PKC-θ in differentiating and neuronal differentiated PC12 cells. Cells were cultured in the presence of the inducer for 72 h (A and C) or 240 h (B and D) and immunocytochemistry analysis was carried out as in the legend to Fig. 1. Stained nucleus of cell in A is shown in (C) and those of cells in B are shown in (D). Representative images of three independent experiments are shown.

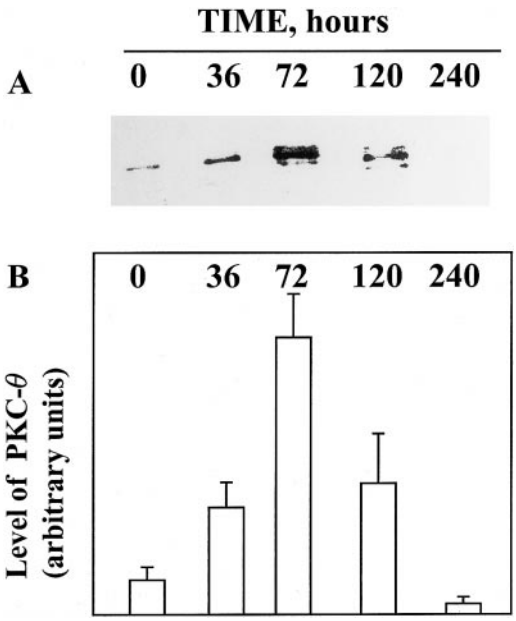

FIG. 3. Levels of PKC-θ in neuronal differentiation of PC12 cells. (A) Cells were collected at increasing times of exposure to the inducer and submitted to Western blotting as in the legend to Fig. 1, by using the anti-PKC-θ antibody directed to the C-terminal peptide. (B) Quantification of the immunoreactive signal shown in A was carried out by a densitometric analysis and shown as means ± S.D. from three independent experiments.

blot analysis was also carried out to define the molecular properties of the protein identified by this antibody. As shown in Fig. 1E, in PC12 cells a single immunoreactive band was detected having an electrophoretic mobility corresponding to that present in Jurkat cells, known to be very rich in PKC-θ, and used as control (Fig. 1E, cfr. lane 1 and 2). The immunoreactivity disappeared if Western blot was carried out in the presence of the immunogen peptide (Fig. 1E, lane 3). Furthermore, also a different anti-PKC-θ antibody, directed to the cystein-rich region of the kinase, identified a single protein band with identical migration in PC12 and Jurkat cells (Fig. 1E, cfr. lane 4 and 5). It can be concluded that PKC-θ is expressed in PC12 cells and is almost completely confined in the nuclear cell compartment.

Distribution and Level of PKC-θ in Differentiating PC12 Cells

To elucidate if PKC-θ undergoes changes in intracellular localization during the acquirement of the nonproliferating neuronal phenotype, we induced PC12 cells with NGF and analyzed the subcellular localization of the kinase by immunocytochemistry and confocal microscope examination. As shown in Fig. 2A, after 72 h of exposure to the inducer, PKC-θ was localized outside the nucleus and in the elongating neurite processes. However, the change of PKC-θ intracellular localization is an early event in the program of neuronal differentiation of PC12 cells promoted by NGF, being already detectable after 24 h from induction, and persisted at least for 1 week (data not shown). On the contrary, in terminal neuron-like differentiated cells, obtained after 10–14 days of exposure to the inducer, PKC-θ resulted down-regulated (Fig. 2B), indicating that PKC-θ activity is not essential for the survival functions of ungrowing differentiated neurons.

Additional experiments were performed to determine the molecular mechanisms promoting the change in the distribution of PKC-θ observed in PC12 cells induced with NGF. Western blot analysis, carried out at different times of exposure to the inducer, revealed that the intracellular amount of PKC-θ increased, reaching a maximum after 72 h (Fig. 3A). At this time, the level of PKC-θ was found to be eightfold higher as compared to that measured in uninduced cells. Conversely, a prolonged exposure to the inducer resulted in a decreased intracellular amount of the kinase which, after 240 h, when cells displayed neurites with lengths in the range of 500–1000 μm, was reduced at undetectable levels.

Localization of PKC-θ in Differentiating PC12 Cells

In order to identify the cytoplasmic localization of PKC-θ during neuronal differentiation, PC12 cells

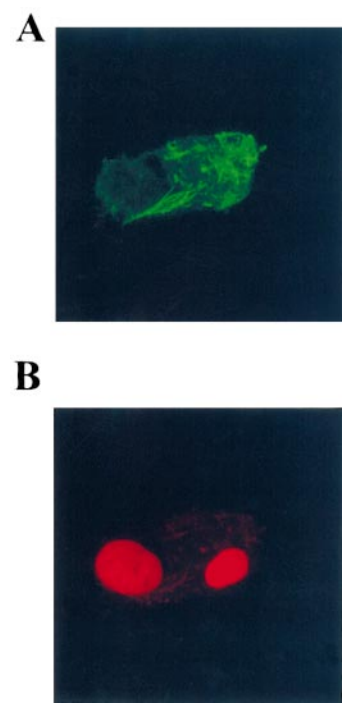

FIG. 4. Subcellular localization of PKC-θ in differentiating PC12 cells. Following 72 h of induction with NGF, cells were incubated for 5 min with 5 μM cytochalasin B and immunocytochemistry analysis was carried out as in the legend to Fig. 1A.

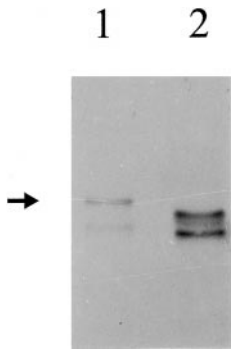

FIG. 5. Electrophoretic properties of PKC-θ in differentiating PC12 cells. Aliquots of Triton soluble (lane 1) and Triton insoluble (cytoskeleton; lane 2) cell fractions, obtained from PC12 cells induced for 72 h with NGF, were submitted to Western blotting by using the anti-PKC-θ antibody directed to the C-terminal peptide. The arrow indicates the migration of the single PKC-θ form present in control uninduced cells.

were grown for 72 h in the presence of NGF, followed by a 5 min exposure to the actin filament disassembling agent cytochalasin B. As shown in Fig. 4, in this condition PKC-θ protein resulted partially diffused and the axon-like structures completely disorganized. This observation is consistent with the existence of a tight association between the kinase and the actin cytoskeleton filaments undergoing a profound reorganization during the neurite outgrowth process.

The time course analysis reported in Fig. 3A revealed that PKC-θ, represented as a single immunoreactive protein band in uninduced cells, showed a different migration pattern in cells recovered after 72 or 120 h of treatment with NGF, suggesting the existence of multiple forms of the kinase. This finding indicates that PKC-θ may undergo specific molecular modifications during cell differentiation and association to the actin cytoskeleton network. Next, we considered the possibility that distinct forms of the enzyme could be differently distributed between the cytoskeletal and soluble fraction in 72 h NGF stimulated cells. In the cell soluble fraction PKC-θ was recovered as two immunoreactive bands (Fig. 5, lane 1). Conversely, in the cytoskeletal fraction (Fig. 5, lane 2), PKC-θ was present in a higher amount and showed a different electrophoretic migration pattern. In fact, the two most represented forms were present only in a cytoskeletal-bound state and displayed a faster migration as compared to the form specifically localized in the soluble fraction. These findings strongly suggest that association to the actin cytoskeleton network of differentiating PC12 cells requires post-transcriptional or post-transductional modified PKC-θ.

DISCUSSION

A number of recent reports indicate that the expression of PKC-θ isozyme is not restricted to a few cell types, such as skeletal muscle, endothelial or hematopoietic cells, but is also detectable in several other cells and tissues (4, 15, 22). In this study we demonstrate that two different anti-PKC-θ antibodies, directed to domains localized in the catalytic or regulatory regions of the kinase, identify this PKC isozyme in PC12 cells. Specifically, PKC-θ is localized in the nuclear compartment of proliferating control cells, as previously shown for an unrelated cell line (12). It has been demonstrated that PKC-θ plays several specific roles in T-cell activation (14) and is involved in the regulation of the cell cycle (16). Its catalytic activity has been shown to be necessary for the maintenance of a normal actin cytoskeleton network (16) and a specific substrate for PKC-θ has been also identified among the proteins acting as linkers between the plasma membrane and the actin cytoskeleton (23). Because PC12 cells induced with NGF undergo a large reorganization of the cytoskeletal network which accompanies the outgrowth of axon-like structures, we have investigated the involvement of PKC-θ in this process. A crucial function for PKC-θ in neuronal differentiation of PC12 cells is indicated by the following evidences: (i) the expression of PKC-θ largely increases in the early steps of neuronal differentiation; (ii) specifically modified forms of PKC-θ are accumulated, through a tight association, on the actin cytoskeleton during the elongation of the axon-like cell processes; (iii) at the completion of the terminal differentiation program a down-regulation of PKC-θ occurs. Altogether, these results indicate that PKC-θ could be involved in two alternative functions in the same cell, depending on its localization inside or outside the nucleus. The effect of cytochalasin B demonstrates that PKC-θ outside the nucleus is associated to the actin cytoskeleton filaments. However, PKC-θ recovered from these cell structures shows molecular properties different from the nuclear kinase form. This finding suggests the existence of a biochemical mechanism which adapts PKC-θ to its new intracellular localization. At present it can be only tentatively hypothesized that a post-translational modification of PKC-θ, such as phosphorylation which has been reported previously in other cells (11), or an alternative splicing, due to the large increase in the synthesis of PKC-θ in differentiating PC12 cells, could produce the observed structural changes. Although no precise information is yet available concerning the type of modifications responsible for the changes of apparent molecular mass of the kinase, we exclude that an uncontrolled proteolytic degradation occurs during the preparation of cell samples, as identical results were obtained when cells were lysed directly in boiling buffer or in the presence of different cocktails of proteinase inhibitors (data bot shown).

PKC-θ is no more expressed by differentiated neuron-like cells. The disappearance of the kinase was also reported for the completely unrelated line of mu-

rine erythroleukemia cells, following the acquirement of the terminal differentiated erythroid phenotype (12), indicating that, at least in these cell models, the kinase is not necessary for the survival of ungrowing cells.

The present results are consistent with a specific function of PKC-θ in the reorganization of the cytoskeletal network. Several proteins have been shown to interact directly with this kinase form and considered RACKs proteins (24). At present, among these proteins only the membrane-organizing-extension spike protein, moesin, has been demonstrated to interact specifically with PKC-θ and to be also phosphorylated in the actin-binding sequence (23). The identification of specific cytoskeletal targets for PKC-θ in PC12 cells is at present under investigation. In conclusion, our findings indicate that PKC-θ is involved in the mechanisms of cytoskeletal reorganization not only in short time processes, typical of cytokinesis in endothelial or hematopoietic cell lineage, but also in the long time process of rearrangement of filamentous actin and axonal sprouting triggered by neurotrophic agents in PC12 cells.

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REFERENCES

1. Mellor, H., and Parker, P. J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332,** 281–292.
2. Blobe, G. C., Stribling, S., Obeid, L. M., and Hannun, Y. A. (1996) Protein kinase C isoenzymes: Regulation and function. *Cancer Surveys* **27,** 213–248.
3. Mochly-Rosen, D., and Gordon, A. S. (1998) Anchoring proteins for protein kinase C: A means for isozyme selectivity. *FASEB J.* **12,** 35–42.
4. Rind, H. B., and Whittemore, S. R. (1999) Protein kinase C and cAMP-dependent protein kinase regulate the neuronal differentiation of immortalized raphe neurons. *J. Neurosci. Res.* **56,** 177–188.
5. Ponzoni, M., Lucarelli, E., Corrias, M. V., and Cornaglia-Ferraris, P. (1993) Protein kinase C isoenzymes in human neuroblasts. Involvement of PKC epsilon in cell differentiation. *FEBS Lett.* **322,** 120–124.
6. Buchner, K., Lindschau, C., and Hucho, F. (1997) Nuclear localization of protein kinase Cα and its association with nuclear components in Neuro-2a neuroblastoma cells. *FEBS Lett.* **406,** 61–65.
7. Borgatti, P., Mazzoni, M., Carini, C., Neri, L. M., Marchisio, M., Bertolaso, L., Previati, M., Zauli, G., and Capitani, S. (1996) Changes in nuclear protein kinase C activity and isotype composition in PC12 cell proliferation and differentiation. *Exp. Cell. Res.* **224,** 72–78.
8. Baier, G., Telford, D., Giampa, L., Coggeshall, K. M., Baier-Bitterlich, G., Isakov, N., and Altman, A. (1993) Molecular cloning and characterization of PKC theta, a novel member of the protein kinase C (PKC) gene family expressed predominantly in hematopoietic cells. *J. Biol. Chem.* **268,** 4997–5004.
9. Osada, S., Mizuno, K., Saido, T. C., Suzuki, K., Kuroki, T., and Ohno, S. (1992) A new member of the protein kinase C family, nPKC, predominantly expressed in skeletal muscle. *Mol. Cell Biol.* **2,** 3930–3948.
10. Tang, S., Gao, Y., and Ware, J. A. (1999) Enhancement of endothelial cell migration and *in vitro* tube formation by TAP20, a novel integrin-modulating, PKCθ-dependent protein. *J. Cell. Biol.* **147,** 1073–1083.
11. Villunger, A., Ghaffari-Tabrizi, N., Krumbock, N., Bauer, B., Schneider, T., Kasibhatla, S., Greil, R., Baier-Bitterlich, G., Uberall, F., Green, D. R., and Baier, G. (1999) Synergistic action of protein kinase C theta and calcineurin is sufficient for Fas ligand expression and induction of a crmA-sensitive apoptosis pathway in Jurkat T cells. *Eur. J. Immunol.* **29,** 3549–3561.
12. Passalacqua, M., Patrone, M., Sparatore, B., Melloni, E., and Pontremoli, S. (1999) Protein kinase C-θ is specifically localized on centrosomes and kinetochores in mitotic cells. *Biochem. J.* **337,** 113–118.
13. Passalacqua, M., Patrone, M., Sparatore, B., Pedrazzi, M., Melloni, E., and Pontremoli, S. (1999) Protein kinase C-θ is specifically activated in murine erythroleukemia cells during mitosis. *FEBS Lett.* **453,** 249–253.
14. Monks, C. R. F., Kupfer, H., Tamir, I., Barlow, A., and Kupfer, A. (1997) Selective modulation of protein kinase C-theta during T-cell activation. *Nature* **385,** 83–86.
15. Slepko, N., Patrizio, M., and Levi, G. (1999) Expression and translocation of protein kinase C isoforms in rat microglial and astroglial cultures. *J. Neurosci. Res.* **57,** 33–38.
16. Tang, S., Morgan, K. G., Parker, C., and Ware, J. A. (1997) Requirement for protein kinase C θ for cell cycle progression and formation of actin stress fibers and filopodia in vascular endothelial cells. *J. Biol. Chem.* **272,** 28704–28711.
17. Greene, L. A., and Tischler, A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73,** 2424–2428.
18. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
19. Palejwala, S., and Goldsmith, L. T. (1992) Ovarian expression of cellular Ki-ras p21 varies with physiological status. *Proc. Natl. Acad. Sci. USA* **89,** 4202–4206.
20. Coligan, S. E., Knisbeck, A. M., Margulies, D. H., Shevach, E. M., and Strober, W. (1991) Immunofluorescence and cell sorting. Current Protocols in Immunology, Unit 1.5, Wiley, New York.
21. Payrastre, B., van Bergen en Henegowen, P. M. P., Breton, M., den Hartigh, J. C., Plantavid, M., Verkleij, A. J., and Boonstra, J. (1991) Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: effect of epidermal growth factor. *J. Cell Biol.* **115,** 121–128.
22. Uberall, F., Giselbrecht, S., Hellbert, K., Fresser, F., Bauer, B., Gschwendt, M., Grunicke, H. H., and Baier, G. (1997) Conventional PKC-alpha, novel PKC-epsilon and PKC-theta, but not atypical PKC-lambda are MARCKS kinases in intact NIH 3T3 fibroblasts. *J. Biol. Chem.* **272,** 4072–4078.
23. Pietromonaco, S. F., Simons, P. C., Altman, A., and Elias, L. (1998) Protein kinase C-θ phosphorylation of moesin in the actin-binding sequence. *J. Biol. Chem.* **273,** 7594–7603.
24. Ron, D., Napolitano, E. W., Voronova, A., Vasquez, N. J., Roberts, D. N., Calio, B. L., Caothien, R. H., Pettiford, S. M., Wellik, S., Mandac, J. B., and Kauvar, L. M. (1999) Direct interaction in T-cells between theta PKC and the tyrosine kinase p59fyn. *J. Biol. Chem.* **274,** 19003–19010.