Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 266-272

Overexpression of Mammalian Protein Kinase C-ζ Does Not Affect the Growth Characteristics of NIH 3T3 Cells

Piero Crespo, Harald Mischak[1] and J. Silvio Gutkind*

Molecular Signaling Unit, Laboratory of Cellular Development and Oncology, National Institute of Dental Research, NIH, Bethesda, Maryland 20892

[1]Institute of Clinical Molecular Biology, GSF, Marchioninisstr. 25,81377 Munich, Germany

Received June 22, 1995

The structure of protein kinase C ζ (PKCζ) is unusual with respect to other PKCs, as it lacks the C2 domain and possesses only one zinc finger region. Consequently, PKCζ can not be activated by diacylglycerol or phorbol esters and is not downregulated by prolonged treatment by phorbol esters nor blocked by commonly utilized PKC inhibitors. In this study, we have explored the idea that PKCζ might participate in proliferative pathways. Our findings show that marked overexpression of mammalian PKCζ does not alter the growth characteristics of NIH 3T3 cells, nor induces cellular transformation. Furthermore, mammalian PKCζ does not potentiate the transforming ability of oncogenes such as *ras, sis* and the muscarinic receptor m1. In this context, PKCζ or its dominant negative mutant do not affect MAP kinase activation by oncogenes or growth factors. Taken together, our findings demonstrate that mammalian PKCζ does not directly participate in signaling pathways involved in oncogenic transformation.

Protein kinase Cs (PKC) are a protein-family with a growing number of isozymes grouped in three major classes: Conventional (cPKCs) isoforms such as α, βI, βII and γ, novel PKCs (nPKCs), including isoforms δ, ε, η, and θ, and atypical PKCs (aPKCs), represented by the ζ and λ isozymes of PKC (for reviews, see 1,2). cPKCs are activated by diacylglycerol and tumor-promoting phorbol esters in a Ca^{2+}-dependent manner. In contrast, activation of nPKCs is Ca^{2+}-independent (1,2). The fact that each PKC isozyme possesses unique structural characteristics has suggested that individual isozymes may play distinct biological roles. As such, overexpression of PKC α and β in fibroblasts has been associated with deregulated cell growth (3), and PKCε can behave as a dominant acting oncogene (4).Conversely, overexpression of PKCδ leads to growth arrest (5).

*Corresponding author: National Institutes of Health,Building 30, Room 212
Bethesda, MD 20892. FAX:(301) 402-0823.

0006-291X/95 $12.00

PKCζ represents an atypical member of the PKC family. It lacks the C2 domain making its kinase activity Ca^{2+}-independent, and possesses only one zinc finger region in its regulatory domain (6). Consequently, PKCζ does not bind and can not be activated by diacylglycerol or phorbol esters (7). In addition, prolonged treatment with phorbol esters does not down regulate the PKCζ isoform (6,8), and most PKC inhibitors are ineffective on this PKC isozyme (9). The protein structure of PKCζ closely resembles that of $p72^{c\text{-}raf}$ (6) which is known to be critical for mitogenic signaling (10), thus raising the possibility that PKCζ might also participate in cell proliferation. Indeed, PKCζ has been shown to be required for maturation of *Xenopus* oocytes in response to insulin and *ras* (11). In the present study, we have explored a role for mammalian PKCζ in mitogenic or transforming pathways.

Materials and methods

Tissue culture products used were Dulbecco's modified Eagle medium (DMEM)(GIBCO), the neomycin analog G418 (GIBCO), and calf serum (Advanced Biotechnologies Inc.). Oligonucleotides were synthesized by Paragon Biotech, and PCR kit was from Perkin Elmer Cetus.

Expression vectors. Mouse-PKCζ cDNA was subcloned in pcDNAI-Amp vector (pcDNA) (Invitrogene), or pZipNeoSV(X) (pZN) expression vector. The kinase deficient mutant of PKCζ was obtained by mutating a critical lysine residue in position 281 to tryptophane (K281W) (12) using PCR methodology. The sequence of mutagenic oligonucleotides will be made available upon request.

Growth properties of NIH 3T3 cells. Plasmid DNA transfection of NIH 3T3 fibroblasts was performed by the calcium phosphate precipitation technique. Transfected cells were selected for their ability to grow in the presence of Geneticin (G418) (GIBCO) (0.750 to 1.5 mg/ml). Transformed foci were scored after 2-3 weeks as previously described (13). Colony formation in semisolid media, doubling time and [^{3}H]thymidine incorporation were determined as previously described (13).

Immunoblotting. Lysates from NIH 3T3 cells were subjected to western blot analyses as decribed (13), using anti-PKCζ serum raised in rabbits against the final 10 carboxyl-terminal residues (GFEYINPLLLSAEESV).

Kinase assays. MAP kinase assays were performed in COS-7 cells upon transfection with an epitope tagged ERK2 cDNA (HA-MAPK), as previously described (14). PKCζ-autophosphorylation assays were determined in lysates from control and transfected NIH 3T3 cells upon immunoprecipitation with anti-PKCζ serum, utilizing a protocol identical to that of MAPK.

Results

NIH 3T3 cells were transfected with expression plasmids carrying mouse PKCζ cDNA, cloned in a sense (ζwt) or antisense (ζwt-AS) orientation. As shown in Table 1, each plasmid produced a similar number of G418-resistant colonies. We next examined the expression of PKCζ in lysates from mass populations of transfected, G418-selected cells. As shown in Fig. 1, the anti-

TABLE 1. Focus-forming activity of wild type PKCζ

DNA construct	Colonies per μg of transfected DNA	Foci per μg of transfected DNA
ZN-ζwt	364 ± 16	<1
ZN-ζwt-AS	352 ± 28	<1
ZN-c-H*ras*	868 ± 62	37 ± 6

Wild type, sense and anti-sense PKCζ cDNAs were cloned in the expression vector, pZipNeoSV(X) (ZN), and 0.05-2 μg of plasmid DNA was transfected into NIH 3T3 cells using the normal c-H*ras* potooncogene cloned in the same expression vector. Cultures were scored for both colony-forming activity, in the presence of G418 (GIBCO), or focus-formation 2-3 weeks after transfection. Data shown represent mean values ± S.E. of triplicate plates from three independent experiments.

PKCζ serum detected a weak 72 kD band in total cellular lysates or immunoprecipitates from vector-transfected NIH 3T3 cells, but a very intense band of 72 kDa was observed in ζwt transfectants and in lysates from rat brain, a reach source of PKCζ (8). Similarly, PKCζ kinase activity was also dramatically increased in ζwt transfectants, as judged by its autophosphorylating activity (Fig. 1).

In four independent transfections ζwt failed to induce focus-formation (Table 1), although a weakly transforming gene such as the human c-H*ras* proto-oncogene effectively induced cell transformation (Table 1). Thus, mammalian-PKCζ lacks demonstrable focus-forming activity. We next explored whether overexpression of PKCζ might alter the growth characteristics of NIH 3T3

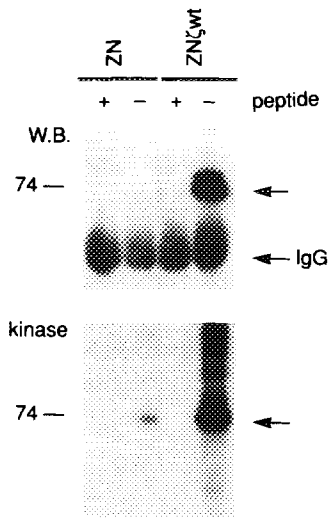

FIGURE 1. Expression of PKCζ in NIH 3T3 cells. G418 selected NIH 3T3 cells transfected with vector control (ZN), or with expression plasmids for PKCζ wild type were analyzed for expression of PKCζ either by western blotting with the anti-PKCζ serum (W.B.), or by PKCζ autophosphorylating activity (kinase). As a control, immunoprecipitating anti-PKCζ serum was blocked by preincubation with the homologous peptide. Immunoreacting or phosphorylated bands corresponding to PKCζ are indicated with arrows. Reacting bands corresponding to the IgG from the immunoprecipitating serum are indicated.

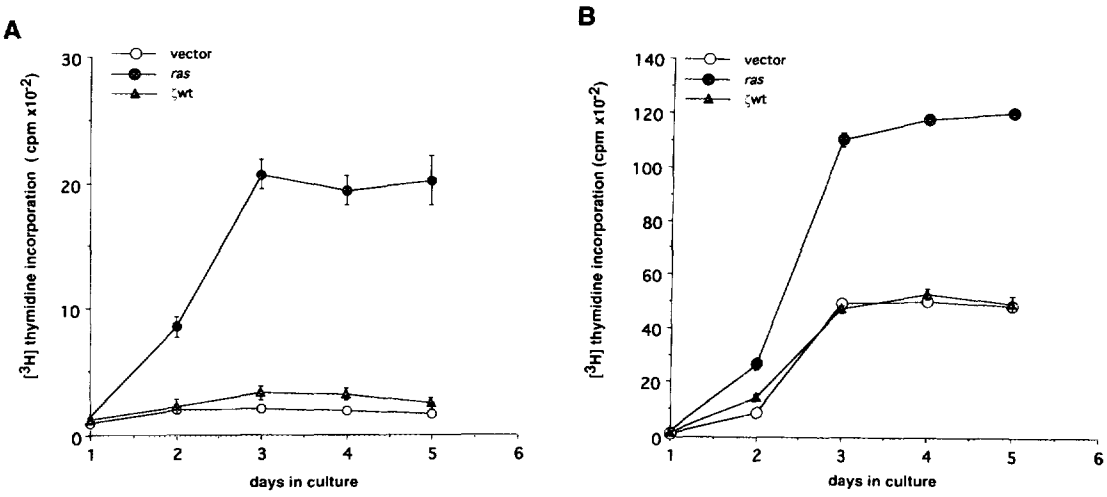

FIGURE 2. Growth curves of transfected NIH 3T3 cells. Mass cultures of G418-selected NIH 3T3 cells transfected with vector control (vector), or with expression plasmids for ζwt and the human ras oncogene (*ras*) were cultured for the indicated time in DMEM supplemented with 0.1% (A) or 5% (B) of calf serum, in the presence of [^{3}H]thymidine. Incorporation of label to cellular DNA was determined as described in Materials and Methods. Data represent mean ± SEM of triplicate samples of a representative assay. Similar results were obtained in three independent experiments.

cells, using mass populations of G418 selected cells. Cells overexpressing PKCζ were not able to grow in low serum containing media (Fig. 2), and they did not exhibit any difference in DNA-synthesis when compared to parental NIH 3T3 cells, nor they display any difference in either doubling time or saturation cell density (Table 2). In addition, PKCζwt overexpressing cells were not able to yield colonies capable of anchorage-independent growth in soft agar nor they induced tumor formation in nude mice (data not shown). In contrast, parallel cultures of NIH 3T3 cells transfected with the human *ras* oncogene were capable of growing in serum-poor medium (Fig. 2), exhibited increased growth rate and saturation density (Table 2), grew efficiently in semi solid

TABLE 2. Growth characteristics of NIH 3T3 cells transfected with wild type or mutated PKCζ

Cell line	Doubling time (h)	Saturation density (x10^4)
ZN	22.4 ± 1	101 ± 13
ZN-ζwt	21.2 ± 3	103 ± 19
ras	12.3 ± 2	291 ± 53

Mass cultures of NIH 3T3 cells transfected with the indicated expression vectors were analyzed for exponential cell growth and saturation density as described in "Methods". Data represent mean ± SEM from three independent experiments.

TABLE 3. Effect of wild type or mutant PKCζ on oncogene-induced focus-formation in NIH 3T3 cells

DNA	ZN	ZN-ζwt
vector	<1	<1
ras	318 ± 58	259± 47
m1+agonist	364 ± 82	300 ± 67
v-*sis*	379 ± 61	319 ± 21
v-*mos*	186 ± 26	187 ± 35

100 ng of plasmid DNA carrying the human *ras* oncogene, m1 , v-*sis*, or v-*mos* was transfected into NIH 3T3cells in combination with 1 μg of pZN , or ZN-wt PKCζ . m1-transfected cells were maintained in the presence of the muscarinic cholinergic agonist, carbachol (10^{-4} M). Foci of transformation were scored 2-3 weeks after transfection.

medium and were highly tumorigenic in nude mice (data not shown, see 13). Because mammalian PKCζ does not appear to harbor transforming potential we set out to explore whether PKCζ affects focus-formation by known oncogenes. As shown in Table 3, cotransfection with PKCζwt had little or no effect on the focus-forming activity of *v- ras*, v-*sis* , v-*mos* or the agonist-activated m1 G protein-coupled receptor (Table 3). Taken together, these data demonstrate that overexpression of PKCζ does not alter the growth properties of NIH 3T3 cells, nor it affects the transforming abilities of known oncogenes.

Because it has been recently suggested that PKCζ is involved in a MAP kinase (MAPK) related pathway (15), we have explored a role for this PKC isozyme in communicating receptors or Ras to MAPK, utilizing transient expression in COS-7 cells as an experimental model. We also generated kinase defficient mutant of PKCζ (ζK281W), which would be expected to act as a dominant negative PKCζ (16). As shown in Fig. 3A, coexpression of v-*ras* or triggering cells with EGF potently stimulated the phosphorylating activity of an epitope tagged-MAPK (HA-MAPK). In contrast, marked overeexpression (Fig.3B) of either PKCζ construct did not induce activation of MAPK, nor they affected MAPK activation in response to EGF or v-*ras*. However, under identical experimental conditions the dominant-negative mutant of c-*raf*, Raf-301 (10) nearly abolished activation of MAPK by both *ras* and EGF (Fig.3A). Thus, PKCζ appears to participate in a route distinct from that of the MAPK signaling pathway.

Discussion

A role for protein kinase C in mediating cell proliferation by growth factors or oncogenes is still controversial. For instance, depletion of PKC does not prevent mitogenesis induced by

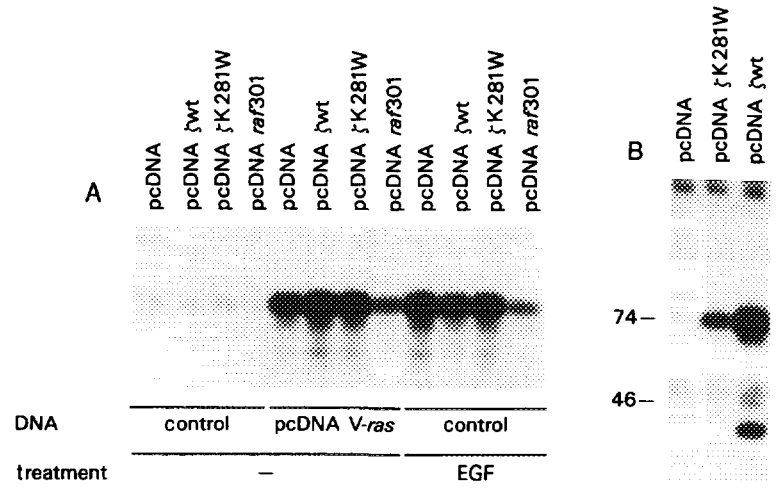

FIGURE 3. MAP kinase assays in COS-7 cells. In A, COS-7 cells cotransfected with an epitope-tagged ERK2 together with the indicated DNA constructs were left untreated or exposed to EGF (100 ng/ml, 5 min) and assayed for MAP kinase activity using myelin basic protein (MBP) as a substrate, which represents the major phosphorylated band in the autoradiograms. In B, COS-7 cells transfected with the indicated expression plasmids were analyzed for expression of PKCζ by western blotting with the anti-PKCζ serum. Similar results were obtained in three independent experiments.

agonist-activated tyrosine kinases (17) or G protein-coupled receptors (8). Thus, it is tempting to hypothesize that some non-conventional PKC which can not be activated nor downregulated by phorbol esters, such as PKCζ, might be involved in these mitogenic pathways. As an approach towards testing this hypothesis, we have explored whether PKCζ plays a role in proliferative signaling in NIH 3T3 cells. Our findings demonstrate that marked overexpression of mammalian PKCζ does not alter the growth characteristics of NIH 3T3 cells, nor does it induces cellular transformation. These data differ from those of a previously published report (16), in which cells overexpressing *Xenopus*' PKCζ were shown to display deregulated cell growth and to acquire certain characteristics of transformed cells. Perhaps this disparity can be explained by the different approaches taken to explore biological properties conferred by overexpression of PKCζ. Several hundred G418-selected colonies were pooled to give rise to the population of cells which was used throughout our study, and this mass culture overexpressed 10-20 folds more PKCζ than control cells. In contrast, others (16) focused their study on transformed cell clones which expressed only 2-5 folds more PKCζ than control cells. Thus, it appears that certain clonal derived NIH 3T3 cells might be prone to growth alterations when overexpressing PKCζ (16). However, this behavior is not apparent when studying large populations of cells. Nevertheless, as natural substrates for PKCζ are not yet known, we can not exclude the possibility that distinct activities of amphibian as compared to mammalian PKCζ might be reflected in distinctive biological activities.

Recent studies have shown that PKCζ can be activated by phosphatidylinositol-3,4,5-phosphate (PIP_3) (17), which has been implicated in cell growth and in other biological processes involving redistribution of cytoskeletal components, such as chemotaxis (18) and protein trafficking (19). Thus, the activation of PI(3) kinase, and thereby PKCζ, may represent a point of convergence for multiple signals inducing cytoskeletal changes. Whether PKCζ plays a role in this process warrants further investigation.

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