Diaz-Meco MT, Municio MM, Frutos S, Sanchez P, Lozano J, Sanz L and Moscat JThe product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms...

ARTICLE *in* CELL · OCTOBER 1996

Impact Factor: 32.24 · DOI: 10.1016/S0092-8674(00)80152-X · Source: PubMed

Cell, Vol. 86, 777–786, September 6, 1996, Copyright ©1996 by Cell Press

The Product of *par-4*, a Gene Induced during Apoptosis, Interacts Selectively with the Atypical Isoforms of Protein Kinase C

María T. Díaz-Meco, María M. Municio, Sonia Frutos, Pilar Sanchez, José Lozano, Laura Sanz, and Jorge Moscat
Centro de Biología Molecular
Consejo Superior de Investigaciones Científicas
Universidad Autónoma de Madrid
Canto Blanco
28049 Madrid
Spain

Summary

The atypical PKCs are involved in a number of important cellular functions, including cell proliferation. We report here that the product of the *par-4* gene specifically interacts with the regulatory domains of ζPKC and λ/ιPKC, which dramatically inhibits their enzymatic activity. This is particularly challenging, because expression of *par-4* has been shown to correlate with growth inhibition and apoptosis. Results are shown here demonstrating that the expression of *par-4* in NIH-3T3 cells induces morphological changes typical of apoptosis, which are abrogated by cotransfection of either wild-type ζPKC or λ/ιPKC, but not by their respective kinase-inactive mutants. These findings support a role for the atypical PKC subspecies in the control of cell growth and survival.

Introduction

The atypical subgroup of the protein kinase C (PKC) family of isozymes is composed of two members: λ/ιPKC and ζPKC (Akimoto et al., 1994; Ohno et al., 1989; Selbie et al., 1993). ιPKC is the human homolog of the mouse λ, and both show a high degree of homology in the catalytic as well as the regulatory domain with ζPKC from different species (Nishizuka, 1992). Contrary to other PKC isoforms, the atypicals cannot be regulated by Ca^{2+}, phorbol esters, or diacylglycerol (Akimoto et al., 1994; Ohno et al., 1989; Selbie et al., 1993; Ways et al., 1992), but are regulated by other lipid cofactors such as phosphatidylinositol (3,4,5)trisphosphate and ceramide (Lozano et al., 1994; Nakanishi and Exton, 1992; Nakanishi et al., 1993), which are generated following cell activation by inflammatory cytokines and growth factors (Hannun, 1994; Kolesnick and Golde, 1994; Exton 1994; Liscovitch and Cantley, 1994). Consistent with this, the atypical PKCs λ/ι and ζ are involved in a number of important cellular functions. Thus, microinjection of an inhibitor peptide with the sequence of the pseudosubstrate of the atypical PKC isotypes, but not of αPKC or εPKC, dramatically inhibited maturation (Dominguez et al., 1992) and NF-κB activation (Dominguez et al., 1993) in Xenopus oocytes, as well as the reinitiation of DNA synthesis in quiescent mouse fibroblasts (Berra et al., 1993). Also, down-regulation of ζPKC with antisense oligonucleotides or transfection of a kinase-inactive dominant-negative mutant of λ/ιPKC or ζPKC severely impairs cell proliferation (Berra et al., 1993), mitogen-activated protein kinase (MAPK) activation (Berra et al., 1995), and κB-dependent promoter activity (Diaz-Meco et al., 1993, 1996; Dominguez et al., 1993; Folgueira et al., 1996).

The major divergences in the sequence between the atypical PKC subfamily and the classical or novel PKCs are located in the regulatory domain. The identification of selective modulators for each PKC isotype will be of great help in the understanding of the function and regulation of the different PKC isoforms. In this regard, we have recently identified a novel protein, named λ-interacting protein (LIP), that is a selective activator of λ/ιPKC (Diaz-Meco et al., 1996). LIP binds to the zinc finger of λ/ιPKC, but does not bind to αPKC, εPKC, or even the highly related ζPKC (Diaz-Meco et al., 1996). Interestingly, overexpression of LIP stimulates κB-dependent promoter activity (Diaz-Meco et al., 1996), emphasizing the functional importance of this PKC regulator. Although the products of phosphatidylinositol (PI) 3-kinase activate ζPKC and λ/ιPKC (Nakanishi et al., 1993; Akimoto et al., 1996), they also stimulate other kinases such as εPKC (Moriya et al., 1996), ηPKC, (Toker et al., 1994), PRK1 (Palmer et al., 1995), and c-Akt (Franke et al., 1995). Ceramide activates both ζPKC and λ/ιPKC (Lozano et al., 1994; Müller et al., 1995), but it does also stimulate the ceramide-activated protein kinase (Yao et al., 1995; Zhang and Kolesnick, 1995) among other possible targets. This strongly indicates that the different lipid mediators are not selective for a given PKC. The fact that LIP specifically binds and activates the λ/ιPKC isoform suggests that the specificity of the regulation of the different PKC isotypes will most probably be mediated by protein regulators. The molecular characterization of those proteins would allow the dissection of specific pathways controlled by the different PKC isotypes. In this study, we have used the regulatory domain of ζPKC as the "bait" to identify potentially novel regulators of its kinase activity by the two-hybrid system. We report here that the product of the *par-4* gene specifically interacts with the zinc finger domain of ζPKC and λ/ιPKC and that it dramatically inhibits their enzymatic activity. Expression of *par-4* has been shown to correlate with growth inhibition and apoptosis (Sells et al., 1994). Results are shown here that strongly support a role for the atypical PKCs in the control of cell proliferation and survival.

Results

Yeast Two-Hybrid Assays

The regulatory domain of ζPKC (amino acids 1–250) was fused with the DNA-binding domain of the yeast LexA protein (pBTM116ζPKC^{REG}) to serve as the bait to screen a human placenta Matchmaker cDNA library. We selected colonies that grew on yeast dropout media lacking leucine, tryptophan, and histidine but containing 20 mM 3-amino-1,2,4-triazole and that were blue within 20 min when assayed by X-Gal colony filter assay. We

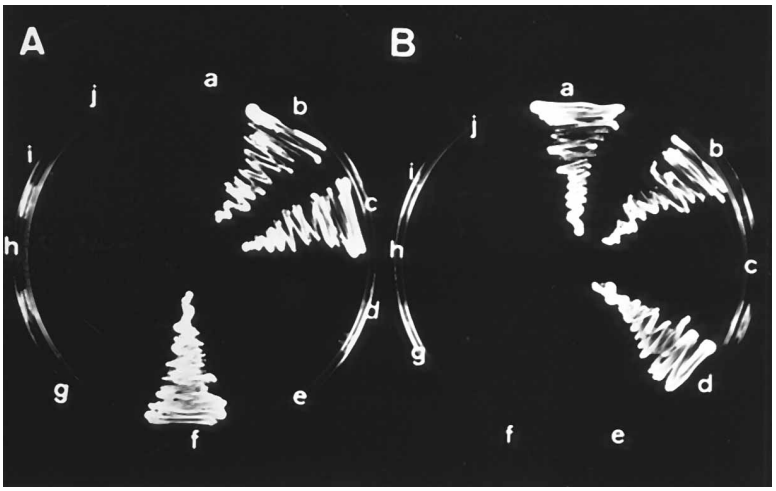

Figure 1. Specificity of the Interaction of Par-4 with ζPKC^{REG}

The Saccharomyces cerevisiae SFY526 reporter host strain was transformed with the following plasmids.

(A) pGAD10-Par-4 and pYTH9 (a); pYTH9ζPKC^{REG} (b); pYTH9λ/ιPKC^{REG} (c); pYTH9ζPKC^{CAT} (d); pYTH9ζPKC^{126} (e); pYTH9ζPKC^{ZF} (f); pYTH9ζPKC$^{REG-C167S}$ (g); pYTH9λ/ιPKC^{126} (h); pYTH9εPKC^{REG} (i); pYTH9αPKC^{REG} (j).

(B) pGAD10-Par-4 and pYTH9ζPKC^{REG} (a); pYTH9ζPKC^{ZF} (b); pYTH9ζPKC^{126} (c); pYTH9-Par-4 (d); pGBT9Raf^{REG} (e); pGBT9Raf^{CAT} (f); pGBT9Mos (g) or pGAD10-Par-4Δ and pYTH9ζPKC^{REG} (h); pYTH9ζPKC^{ZF} (i); pYTH9-Par-4 (j).

Individual Leu$^+$ Trp$^+$ transformants were streaked to synthetic medium plates lacking tryptophan, leucine, and histidine. The plates were incubated at 30°C for 3 days. Essentially identical results were obtained in another two experiments.

obtained 5 positive colonies that stained intensely blue from 2.5 × 10^6 screened. The expression of His-3 and LacZ in these colonies was shown to depend on the LexA fusion protein by retransforming the recovered plasmids into yeasts containing the bait construct (Figure 1; Table 1). The sequences of the 5 clones revealed that they were identical and corresponded to a partial cDNA coding for the last 272 amino acids (positions 60–332) of the previously cloned *par-4*, which has been identified as a gene induced during apoptosis (Sells et al., 1994). Owing to the role of ζPKC in mitogenic signaling (see Introduction), this finding could be of great relevance. Therefore, the specificity of the interaction between ζPKC^{REG} and the *par-4* gene product was next tested with an unrelated molecule, pGBT9Lamin, which fails to transactivate the reporter constructs (Table 1). The catalytic domain of ζPKC did not interact with Par-4, whereas the full-length ζPKC, as well as the regulatory domain of λ/ιPKC, did show interaction (Figure 1; Table 1). Interestingly, Par-4 does not interact with the regulatory domains of αPKC or εPKC (Figure 1; Table 1), indicating that it is highly specific for the atypical PKC isotypes. On the other hand, Raf-1 is a serine/threonine kinase with an overall structure similar to that of the atypical PKCs (Ohno et al., 1989). Both types of kinases are critical components downstream of Ras in mitogenic cascades (Berra et al., 1993; Howe et al., 1992). Therefore, it was of great interest to determine whether Raf-1 would interact with Par-4. Neither full-length Raf-1 nor the catalytic domain or the regulatory region of Raf-1 interacted with Par-4 (Figure 1; Table 1). The product of c-*mos*, which is another serine/threonine kinase critically involved (like Raf-1 and the atypical PKCs) in mitogenic signal transduction in Xenopus oocytes and mammalian cells (Sagata et al., 1988), did not interact with Par-4 (Figure 1; Table 1). Therefore, these data collectively indicate that Par-4 specifically binds to the regulatory domain of ζPKC and λ/ιPKC, but not to other structurally and functionally related kinases. To map the region in the regulatory domain of ζPKC where Par-4 binds, we fused cDNA fragments corresponding to amino acids 1–126 and 127–194 (corresponding to the zinc finger region) of ζPKC or λ/ιPKC with the DNA-binding domain of the yeast GAL4 protein and determined their ability to interact with Par-4 in the two-hybrid system. Of note, only the zinc finger and the whole regulatory domain of ζPKC and λ/ιPKC displayed Par-4 binding activity (Figure 1; Table 1). Interestingly, disruption of the cysteine finger by mutation of Cys-167 to serine in the ζPKC regulatory domain completely abolished the binding of Par-4 (Figure 1; Table 1). The deduced amino acid sequence of *par-4* predicts a coiled-coil leucine zipper at the C-terminal part of the protein (Sells et al., 1994). Interestingly, this is located in a region that shows a significant degree of homology with the death domain of proteins that are critically involved in the induction of apoptosis (Chinnaiyan et al., 1995; Cleveland and Ihle, 1995; Hsu et al., 1995; Stanger

Table 1. β-Galactosidase Activity Induced by Interaction between the Regulatory Domain of ζPKC and Par-4

	GAL4 Activation Domain Fusion (Blue)	
GAL4 Binding Domain Fusion	pGAD10	pGAD10-Par-4
pYTH9	−	−
pYTH9λ/ιPKC^{REG}	−	+
pYTH9ζPKC$^{REG-C167S}$	−	−
pYTH9ζPKC^{CAT}	−	−
pYTH9ζPKC^{REG}	−	+
pYTH9ζPKC	−	+
pYTH9ζPKC^{126}	−	−
pYTH9λ/ιPKC^{126}	−	−
pYTH9ζPKC^{ZF}	−	+
pYTH9λ/ιPKC^{ZF}	−	+
pYTH9αPKC^{REG}	−	−
pYTH9εPKC^{REG}	−	−
pGBT9Raf^{CAT}	−	−
pGBT9Raf	−	−
pGBT9Mos	−	−
pGBT9Lamin	−	−

β-Galactosidase activity was determined by a filter assay for the yeast strains containing the indicated plasmids, as described in Experimental Procedures. Plus represents a positive indication of β-galactosidase activity (blue color) using filter assays. Essentially identical results were obtained in three other independent experiments.

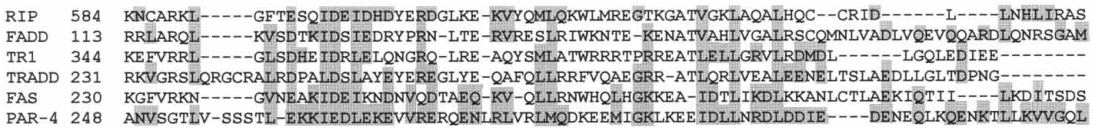

Figure 2. Alignment of Different Death Domains

Amino acids 248–330 of Par-4 are compared with the death domains of Fas, TNFR1 (TR1), TNFR1-associated death domain protein (TRADD), Fas-associating protein with death domain (FADD), and receptor-interacting protein (RIP). Shaded letters indicate amino acids that are conserved in Par-4, as described in Cleveland and Ihle (1995).

et al., 1995). Thus, the alignment of the Par-4 C-terminal region with the death domains of Fas, RIP, FADD, TRADD, and the tumor necrosis factor receptor 1 (TNFR1) using the CLUSTAL program of the PCGene package gives a pairwise similarity score for Par-4 with Fas and TRADD of 15 and 13, respectively. Although lower than that displayed by TRADD with TNFR1 (19), this score is identical to that given by Fas with FADD, TNFR1, or TRADD (15). Also of note, the pairwise similarity score for Par-4 with Fas and TRADD is higher than that for TRADD with RIP or FADD (12). Alignment of Par-4 residues 248–330 relative to residues 230–307 of Fas results in a 43% homology and 29% identity (Figure 2). This is comparable with that resulting from the alignment of, for example, TRADD with TNFR1 (23% identity; Cleveland and Ihle, 1995). However, the residue corresponding to Val-238 of Fas, which when mutated to asparagine abolishes cell death induction, is not conserved in Par-4 (Figure 2). It should be stressed that this residue is not conserved in other cell death proteins such as Reaper or RIP (Cleveland and Ihle, 1995). In the next series of experiments, this region of Par-4 was deleted and the ability of this mutant to interact with the regulatory domain of ζPKC was tested in the two-hybrid system. According to the results of this experiment, Par-4 homodimerizes (Table 2), but deletion of the C-terminal region completely abrogates the homodimerization, as well as the interaction with the regulatory domain of ζPKC (Table 2) or λ/ιPKC (data not shown). This represents evidence that the zinc finger domain of a PKC interacts with a leucine-zipper motif.

Binding of Par-4 to ζPKC In Vitro

To confirm the interaction observed in yeast, Par-4 and Par-4Δ, expressed as maltose-binding protein (MBP) fusion proteins and immobilized on amylose beads, were incubated with radiolabeled in vitro translated ζPKC. As expected, ζPKC interacted with MBP–Par-4 but not with MBP–Par-4Δ or MBP alone (Figure 3A). To investigate further the interaction between Par-4 and ζPKC, we incubated MBP–Par-4, MBP–Par-4Δ, or MBP with the regulatory domain of ζPKC or its zinc finger mutant ζPKC$^{REG\text{-}C167S}$, which were expressed as glutathione S-transferase (GST) fusion proteins (Figure 3B). Two different concentrations (100 or 400 nM) of the MBP fusion proteins were incubated with 800 nM GST fusion proteins for 1 hr at 4°C, after which MBP, MBP–Par-4, and MBP–Par-4Δ were immobilized on amylose resin. Following extensive washing with binding buffer plus 0.1% Triton X-100, bound MBP fusion proteins and any associated proteins were boiled in sample buffer, fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotted with anti-GST antibody to detect binding of the different GST fusion proteins to Par-4. Of note, GST–ζPKC^{REG} but not GST–ζPKC$^{REG\text{-}C167S}$ or GST bound to MBP–Par-4 (Figure 3B). Also, no binding was detected of the GST fusion proteins to MBP–Par-4Δ or MBP alone (Figure 3B). Similar results were obtained in experiments using GST–λ/ιPKC^{REG} (data not shown). Staining of a parallel gel confirms that all the reactions contained equal molar amounts of GST and MBP fusion proteins (data not shown).

Table 2. β-Galactosidase Activity Induced by Interaction between the Wild-Type and Mutant Par-4 and the Regulatory Domain of ζPKC

GAL4 Binding Domain Fusion	GAL4 Activation Domain Fusion (Blue)		
	pGAD10	pGAD10-Par-4	pGAD10-Par-4Δ
pYTH9	–	–	–
pYTH9ζPKC^{REG}	–	+	–
pYTH9ζPKC^{ZF}	–	+	–
pYTH9-Par-4	–	+	–

β-Galactosidase activity was determined by a filter assay for the yeast strains containing the indicated plasmids, as described in Experimental Procedures. Plus represents a positive indication of β-galactosidase activity (blue color) using filter assays. Essentially identical results were obtained in three other independent experiments.

Inhibition of ζPKC by Par-4

Because Par-4 binds specifically to the zinc finger of ζPKC and λ/ιPKC and that region has been shown to mediate the binding of regulators (Diaz-Meco et al., 1996; Nishizuka, 1992; Quest and Bell, 1994), we sought to determine whether the interaction of Par-4 with ζPKC can modulate its enzymatic activity in vitro. Therefore, serum-starved COS cells were transfected with influenza hemagglutinin (HA) protein epitope–tagged ζPKC or λ/ιPKC, and the expressed protein was immunopurified with the anti-HA monoclonal antibody 12CA5. The addition of phosphatidylserine (a classical activator of all PKCs) to the immunoprecipitates significantly activated ζPKC (Figure 3C). Interestingly, the addition of 1 μg of MBP–Par-4, but not of MBP alone, produced a dramatic reduction of ζPKC (Figure 3C) and λ/ιPKC (data not shown) activities. MBP–Par-4 produced no effect on the activity of a highly purified preparation of classical PKCs (data not shown). Therefore, Par-4 not only binds specifically to the zinc finger of ζPKC and λ/ιPKC, but it is also a potent inhibitor of their kinase activity. As a control, it was shown that the Par-4Δ mutant expressed as an MBP fusion protein (MBP–Par-4Δ), which we showed

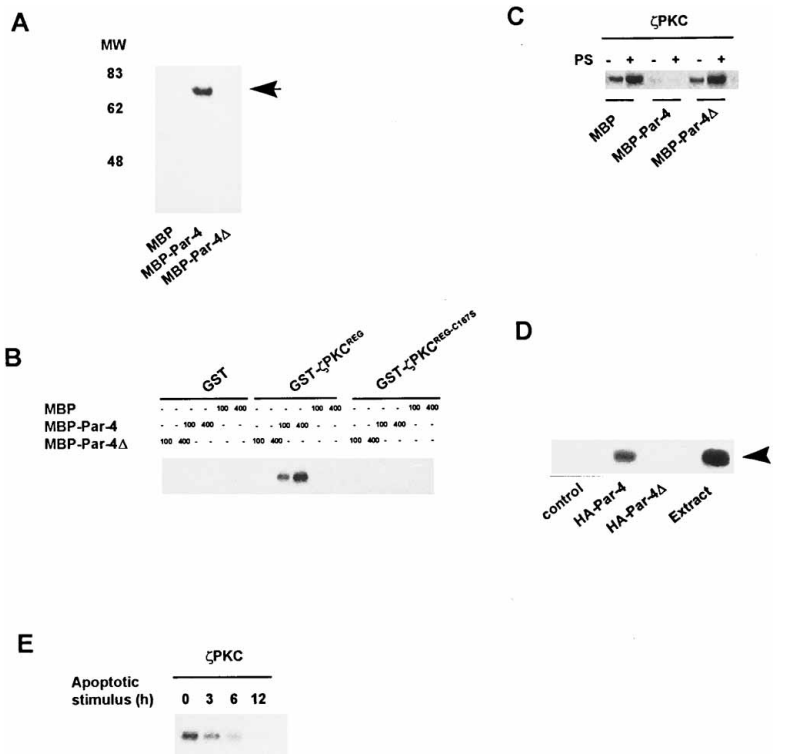

Figure 3. Interaction of Par-4 with ζPKC In Vitro and In Vivo

(A) Purified MBP, MBP–Par-4, or MBP–Par-4Δ (400 nM) was incubated with in vitro translated ^{35}S-labeled ζPKC at 4°C for 1 hr. The recombinant proteins recovered onto amylose beads were fractionated by SDS–PAGE, followed by autoradiography in an Instant-Imager. Essentially identical results were obtained in another two experiments.

(B) Purified GST or GST fusion proteins encoding the regulatory domain of ζPKC (GST–ζPKC^{REG}) or its zinc finger mutant (GST–ζPKC$^{REG-C167S}$) at a concentration of 800 nM were incubated with either 100 or 400 nM MBP fusion proteins for 1 hr at 4°C, after which the MBP fusion proteins were immobilized on amylose resin. MBP fusion proteins and any associated proteins were boiled in sample buffer, fractionated by SDS–PAGE, and immunoblotted with anti-GST antibody to detect binding of the different GST fusion proteins. Essentially identical results were obtained in another two experiments.

(C) COS cells were transfected with either control plasmid or an expression vector for HA-tagged ζPKC, after which they were made quiescent by serum starvation, and the expressed proteins were immunoprecipitated with an anti-HA antibody. Afterward, the effect of 1 μg of MBP, MBP–Par-4, or MBP–Par-4Δ on the unstimulated or phosphatidylserine-activated (50 μg/ml) enzymatic activity of HA-ζPKC was determined with hnRNPA1 as substrate. Essentially identical results were obtained in another two experiments.

(D) Subconfluent cultures of COS cells in 100 mm plates were transfected with 20 μg of either pCDNA3 (control), pCDNA3-HA-Par-4 (HA-Par-4), or pCDNA3-HA-Par-4Δ (HA-Par-4Δ). Plasmid DNA was removed 4 hr later, and cells were incubated in DMEM containing 10% FCS for 36 hr. Cell extracts (50 μg of protein) were immunoprecipitated with anti-HA antibody. The extract lane contains 20 μg of protein cell extract. Immunoprecipitates were resolved by SDS–PAGE and immunoblotted with an anti-ζPKC antibody. Essentially identical results were obtained in another two experiments.

(E) NIH-3T3 cells were induced to apoptosis by UV irradiation. Afterward, they were extracted at different times and immunoprecipitated with anti-ζPKC antibody, and the enzymatic activity was determined using hnRNPA1 as substrate. Essentially identical results were obtained in another two experiments.

above does not interact with ζPKC, was unable to inhibit ζPKC activity (Figure 3C).

Binding of Par-4 to ζPKC In Vivo

To determine whether Par-4 interacts in vivo with ζPKC, we transfected COS cells with either control plasmid, HA-tagged *par-4*, or HA-tagged *par-4*Δ expression vectors. Cell lysates were immunoprecipitated with an anti-HA monoclonal antibody 40 hr posttransfection. Immunoprecipitates were resolved by SDS–PAGE and analyzed by immunoblotting with an antibody against ζPKC. An immunoreactive band corresponding to ζPKC was clearly detected in immunoprecipitates only from cells transfected with the HA-*par-4* vector and not with the empty plasmid or with HA-*par-4*Δ (Figure 3D). Immunoblot analysis with anti-αPKC antibody revealed the lack of association of native αPKC with Par-4 (data not shown). To determine whether native Par-4 interacts with native ζPKC in vivo, we used the MBP–Par-4 fusion protein to immunize rabbits and generate an antibody against Par-4. Next, NIH-3T3 cells were induced to undergo apoptosis by ultraviolet (UV) irradiation (Brancolini et al., 1995), which was confirmed by measuring DNA fragmentation of parallel cultures (data not shown). Cell extracts were immunoprecipitated 4 hr after UV treatment with the anti-Par-4 antibody, which was either untreated or preabsorbed with MBP or MBP–Par-4. Immunoblot analysis of the immunoprecipitates with anti-Par-4 and anti-ζPKC antibody shows that UV irradiation induces the expression of Par-4, which coimmunoprecipitates with ζPKC (Figure 4). When the anti-Par-4 antibody was preadsorbed with MBP–Par-4 but not with MBP, the Par-4 protein was not immunoprecipitated, and ζPKC was absent (Figure 4). UV irradiation induces Par-4 at the gene transcriptional level (data not shown). Collectively, these results indicate that Par-4 binds to ζPKC in vitro and in vivo and that this association leads to a dramatic inhibition of the kinase activity.

Expression of Par-4 Inhibits AP1-Dependent Promoter Activity

Since ζPKC and λ/ιPKC are critically involved in the transactivation of AP1-dependent promoters (Akimoto et al., 1996; Bjorkoy et al., 1995), it was of great interest to determine the effect of *par-4* transfection on that parameter. Results from Figure 5A demonstrate that the transfection of a *par-4* expression vector dramatically inhibits the activation by serum of an AP1 enhancer–

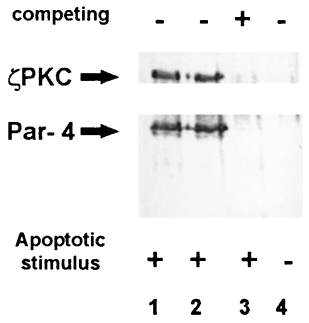

Figure 4. Interaction of Par-4 with ζPKC In Vivo

Cell protein from NIH-3T3 cells (100 μg), either induced to undergo apoptosis by UV irradiation (lanes 1–3) or untreated (lane 4), were immunoprecipitated with anti-Par-4 antibody that was either untreated (lanes 1 and 4) or preadsorbed with MBP (lane 2) or MBP–Par-4 (lane 3). Immunoprecipitates were fractionated by SDS–PAGE and immunoblotted with anti-Par-4 (lower) or anti-ζPKC (upper) antibodies. Essentially identical results were obtained in another two experiments.

directed luciferase reporter plasmid in a dose-dependent manner (Figure 5A). As a control, transfection of *par-4* produces little or no effect on a constitutive cytomegalovirus (CMV) promoter-based reporter vector (Figure 5A). Transfection of *par-4*Δ expression vector did not inhibit AP1 promoter activity (Figure 5B), consistent with its lack of effect on the atypical PKCs. Cotransfection of expression plasmid for λ/ιPKC, but not for αPKC, significantly suppressed the inhibition by Par-4 of the AP1 promoter activity (Figure 5C). All of the above indicates that the inhibition of the atypical PKCs by Par-4 is of physiological relevance.

Expression of Par-4 Induces Apoptosis in a ζPKC- and λ/ιPKC-Dependent Manner

Because *par-4* has been shown to be induced in cells undergoing apoptosis (Sells et al., 1994; Figure 4), a model can be proposed whereby the inducers of programmed cell death promote the expression of Par-4, which selectively interacts with and inhibits the atypical PKCs. This suggests that ζPKC and λ/ιPKC may be critically involved in the generation of survival signals. To test this hypothesis, we initially used a β-galactosidase transfection assay to determine cell viability (Hsu et al., 1995). NIH-3T3 cells transfected with an expression vector for a kinase-inactive dominant-negative mutant of ζPKC or λ/ιPKC (Berra et al., 1995; Diaz-Meco et al., 1996) gave a dramatic reduction in the number of β-galactosidase-positive (blue) cells, as compared with cells transfected with a control plasmid or an expression vector for wild-type ζPKC or λ/ιPKC (Table 3). Interestingly, transfection of *par-4* but not *par-4*Δ also provoked a dramatic reduction in the number of blue cells, indicating that inhibition of the atypical PKCs triggers cell death (Table 3). Cotransfection of wild-type ζPKC or λ/ιPKC (but not of their respective kinase-inactive mutants) with *par-4* increased the number of blue cells. This supports the notion that the mechanism by which *par-4* promotes cell death critically involves inhibition of the atypical PKCs. The results in Figure 6 show that expression of

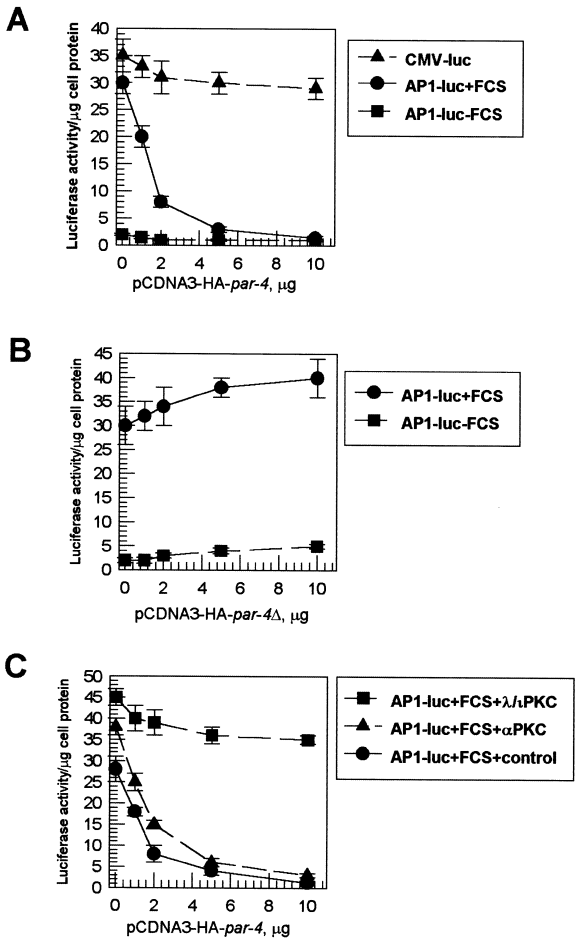

Figure 5. Transfection of a *par-4* Expression Vector Inhibits Serum-Induced AP1 Promoter Activity

(A) NIH-3T3 cells were transfected with different concentrations of plasmid pCDNA3-HA-par-4 along with 500 ng of the AP1 enhancer–directed luciferase reporter plasmid (AP1-luc) or a constitutive CMV-directed luciferase reporter (CMV-luc). After 4 hr, the DNA-containing medium was removed and cells were made quiescent by serum starvation for 24 hr. Following stimulation with serum (20% FCS for 4 hr), extracts were prepared and the luciferase activity was determined as described in Experimental Procedures. Results are the mean ± SD of three independent experiments with incubations in duplicate.

(B) As in (A), but transfecting pCDNA3-HA-par-4Δ. Results are the mean ± SD of three independent experiments with incubations in duplicate.

(C) As in (A), but cotransfected with 10 μg of expression vectors for λ/ιPKC or αPKC. The level of expression of both kinases was always comparable by immunoblot. Results are the mean ± SD of three independent experiments with incubations in duplicate.

Par-4 as well as of λ/ιPKC^{MUT} provokes DNA fragmentation consistent with the induction of apoptosis. Of note, the induction of apoptosis by λ/ιPKC^{MUT} does not induce Par-4 (data not shown), consistent with the notion that the atypical PKCs are critical downstream targets of Par-4. Transfection of Par-4Δ produces no effect on this parameter, and cotransfection of wild-type λ/ιPKC significantly suppresses DNA fragmentation induced by Par-4 (Figure 6). In addition, overexpression of Par-4 induces morphological changes that are typical of

Table 3. Cell Death Induced by Dominant-Negative ζPKC, Par-4 Expression, and UV Irradiation

	Number of Blue Cells per Well				
	Control	ζPKC	ζPKC^{MUT}	λ/ιPKC	λ/ιPKC^{MUT}
Control	3050 ± 280	4120 ± 310	325 ± 75	3800 ± 400	420 ± 50
Par-4	205 ± 50	2500 ± 320	110 ± 20	2900 ± 220	80 ± 19
Par-4Δ	3270 ± 230	4200 ± 300	ND	3900 ± 330	ND
UV Irradiation	450 ± 130	3800 ± 250	ND	3700 ± 300	ND

NIH-3T3 cells were transfected with pCMV-βgal (2.5 μg) and 5 μg of either plasmid control pCDNA3 (control), pCDNA3-HA-Par-4 (Par-4), or pCDNA3-HA-Par-4Δ (Par-4Δ), together with 10 μg of pCDNA3 (control) pCDNA3-HA-ζPKC (ζPKC), pCDNA3-HA-ζPKC^{MUT} (ζPKC^{MUT}), pCDNA3-HA-λ/ιPKC(λ/ιPKC), or pCDNA3-HA-λ/ιPKC^{MUT} (λ/ιPKC^{MUT}). Cells were changed 24 hr posttransfection to 0.5% serum–containing medium for 12 hr, after which they were fixed and stained with X-Gal. In other experiments, cells were induced to undergo apoptosis by UV irradiation and 12 hr afterwards were fixed and stained as above. Results (number of blue cells per 35 mm dish) are the mean ± SD of three independent experiments with incubations in duplicate. ND, not determined.

apoptosis. This is illustrated in the experiment in Figure 7, where NIH-3T3 cells were transfected with the epitope-tagged HA-*par-4* expression vector, 24 hr posttransfection cells were reacted with the anti-HA antibody, and DNA was stained with Hoechst 33258. Par-4-expressing cells displayed a morphology consistent with apoptosis (Figure 7A, arrow) that includes apoptotic nuclei (Figure 7B, arrow). Nonfluorescent cells showed a completely normal morphology (Figures 7A and 7B). To determine whether overexpression of an atypical PKC overcomes the induction of apoptosis by Par-4, we cotransfected NIH-3T3 cells with the HA-*par-4* expression vector along with a 3-fold excess of a plasmid that expresses wild-type λ/ιPKC. Under these conditions, a clear suppression of the apoptotic phenotype was observed (Figures 7C and 7D). Transfection of wild-type λ/ιPKC alone produced no effects (Figures 7E and 7F), whereas the transfection of kinase-inactive λ/ιPKC induced apoptosis (Figures 7G and 7H). The results in Figure 3E are consistent with the proposed role of the atypical PKCs in apoptosis. Thus, the induction of apoptosis by UV irradiation leads to a dramatic reduction of the ζPKC enzymatic activity. Interestingly, transfection of wild-type λ/ιPKC suppresses the induction of apoptosis by UV irradiation (Table 3).

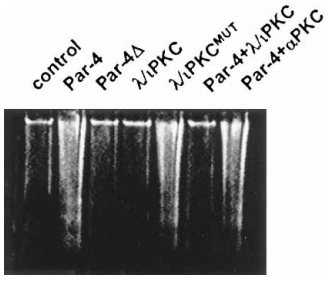

Figure 6. Induction of Apoptotic DNA Damage by Par-4 and λ/ιPKC^{MUT}

NIH-3T3 cells were transfected with 20 μg of either control plasmid or expression vectors for *par-4*, *par-4*Δ, λ/ιPKC, λ/ιPKC^{MUT}, *par-4* plus λ/ιPKC, or *par-4* plus αPKC. Transfection mixture was normalized to 40 μg of total plasmid DNA. Laddered electrophoretic patterns of oligonucleosomal DNA fragments were resolved 36 hr posttransfection by agarose gel electrophoresis. Essentially identical results were obtained in another two experiments.

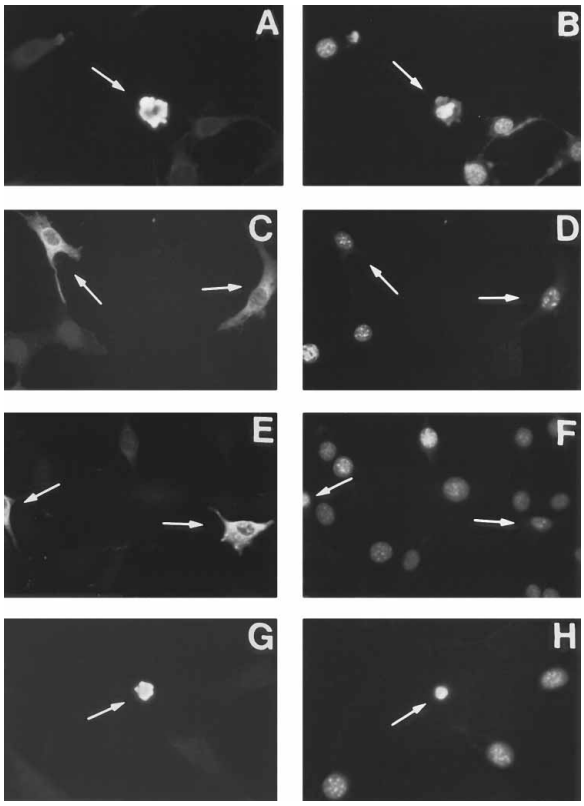

Figure 7. Morphological Changes Induced by Par-4 Expression

NIH-3T3 cells were transfected with 5 μg of pCDNA3-HA-Par-4 plus 15 μg of pRcCMV plasmids (A and B); with 5 μg of pCDNA3-HA-Par-4 plus 15 μg of pRcCMV-λ/ιPKC plasmids (C and D); with 5 μg of pCDNA3 plus 15 μg of pCDNA3-HA-λ/ιPKC (E and F); or with 5 μg of pCDNA3 plus 15 μg of pCDNA3-HA-λ/ιPKC^{MUT} (G and H). Cells were changed 24 hr posttransfection to 1% serum–containing medium for 12 hr, after which cells were immunostained with anti-HA antibody (A, C, E, and G) or Hoechst 3358 (B, D, F, and H). Essentially identical results were obtained in another three experiments.

Discussion

The different PKC isoforms are targets of important lipid second messengers. Thus, diacylglycerol is a major cofactor of the classical and novel isotypes (Nishizuka,

1992), whereas ceramide activates the atypicals (Lozano et al., 1994; Müller et al., 1995), which are also stimulated in vitro by phosphatidylinositol (3,4,5)trisphosphate (Nakanishi et al., 1993). Recently, Ohno and colleagues have demonstrated in vivo that the atypical PKC isoform λPKC, and possibly also ζPKC, are decisive steps downstream of PI 3-kinase in the mitogenic cascade (Akimoto et al., 1996). Interestingly, overexpression of ζPKC or λPKC results in an enhancement of AP1-dependent promoter activity, which is severely inhibited by expression of a dominant-negative mutant of λPKC (Akimoto et al., 1996). This is clearly consistent with the proposed role of the atypical PKCs in mitogenic signaling (Berra et al., 1993, 1995; Bjorkoy et al., 1995; Diaz-Meco et al., 1996). On the other hand, the activation of the atypical PKCs by ceramide appears to be of functional relevance. Thus, ceramide is a potent activator of NF-κB (Schütze et al., 1992; Yang et al., 1993), and it has been shown that expression of a dominant-negative mutant of the atypical PKCs severely impairs κB-dependent promoter activation by sphingomyelinase (Lozano et al., 1994). Taken together, these findings suggest that the stimulation of the atypical PKCs by different lipid second messengers has important functional consequences. However, although critical, these lipid mediators do not appear to be selective (see Introduction). We reasoned that the existence of substantial differences in the regulatory domains of the different PKC isotypes should permit the isolation of potentially selective activators of the different PKCs by the two-hybrid system in yeast. Following this experimental approach, we have recently succeeded in the identification of a novel protein, termed LIP, that specifically interacts with the regulatory domain of λ/ιPKC but not with many other structurally and functionally related kinases, including ζPKC (Diaz-Meco et al., 1996). What is really interesting about LIP is that it specifically activates λ/ιPKC but not ζPKC in vitro as well as in vivo and that its association with this PKC is dramatically activated following the mitogenic stimulation of quiescent cells. Therefore, selective regulation of the different PKCs could be mediated by protein–protein interactions.

In the study reported here, we identify Par-4 as a novel and selective inhibitor of both λ/ιPKC and ζPKC. Although a number of PKC-interacting proteins have recently been identified (Klauck et al., 1996; Konishi et al., 1994; Mochly-Rosen et al., 1991; Ron et al., 1994; Staudinger et al., 1995), only LIP (Diaz-Meco et al., 1996) and Par-4 have been shown to interact with the zinc finger domain of the atypical PKCs and have effects on their kinase activities. Interestingly, the triggering of the apoptotic pathway promotes the induction of Par-4 (Sells et al., 1994), which according to the data presented here binds to and inhibits ζPKC as well as λ/ιPKC. This suggests that the atypical PKCs are subjected to a finely tuned balance of positive and negative signals that modulate their enzymatic activities through the binding of different protein regulators to the zinc finger region. Although we and others (Sells et al., 1994) have found that apoptotic stimuli induce the production of Par-4, we have found at least one example where Par-4 is expressed constitutively. That is the case of the HeLa cell line. In those cells, Par-4 is not associated with ζPKC unless cells are treated with an apoptotic stimulus, such as UV light, that triggers the association of the preexisting Par-4 with ζPKC. This correlates with a dramatic reduction of its enzymatic activity (M. M. M., J. M., and M. T. D.-M., unpublished data). Therefore, it seems that cells that are able to live in the presence of Par-4 have mechanisms that prevent its interaction with ζPKC. Under these conditions, the apoptotic stimulus may lead to the inactivation of that mechanism. Alternatively, a signal could be required for the Par-4–ζPKC interaction to occur. Therefore, UV irradiation, besides inducing the expression of Par-4 in NIH-3T3 cells, may trigger the mechanism that allows the interaction of Par-4 with ζPKC. Par-4 binding to ζPKC in transfection experiments may be due to the fact that overexpression of the protein lowers the requirement of the second signal necessary for this binding to occur in a more physiological environment. Ceramide also activates this association in HeLa cells (data not shown), suggesting the bifunctional role of this lipid second messenger. Thus, it transiently activates ζPKC at early times of cell activation, whereas at later times it promotes the association of ζPKC with Par-4. Because ceramide binds directly to ζPKC but only activates it at low concentrations (Müller et al., 1995), it is possible that high levels of ceramide produced at relatively later times induce a conformational change in ζPKC that makes it competent to interact with Par-4, leading to its enzymatic inactivation, which is required for apoptosis to proceed. Therefore, ceramide emerges as a bifunctional molecule that activates both NF-κB and MAPK, on the one hand, and apoptosis on the other. TRADD, a novel TNFR1-associated protein, is another interesting example of a bifunctional messenger (Hsu et al., 1996).

Expression of Par-4 inhibits AP1 promoter activation in a λ/ιPKC- and ζPKC-dependent manner. Preliminary unpublished results from this laboratory demonstrate that expression of Par-4 inhibits MAPK, which may account for the effects on AP1 promoter activity, and are consistent with the recently suggested role of the atypical PKCs in the activation of MAPK (Berra et al., 1995). Inhibition of MAPK has been proposed to be important for induction of cell death (Xia et al., 1995). On the other hand, PI 3-kinase plays a critical role in the generation of cell survival signals (Yao and Cooper, 1995). The fact that the atypical PKCs appear to be located downstream of that enzyme (Akimoto et al., 1996; Nakanishi et al., 1993) is potentially of great relevance. Since PI 3-kinase–derived second messengers activate several kinases (see above), a critical question is the relative role of the atypical PKCs compared with the other effectors in the control of cell survival. The fact that Par-4 induces apoptosis most probably through inhibition of ζPKC and λ/ιPKC suggests that the atypical PKCs may be at least one of the relevant transducers of PI 3-kinase–derived survival signals. It has recently been reported that the activation of apoptosis triggers the proteolytic stimulation of δPKC (Emoto et al., 1995), the growth-inhibitory properties of which have been documented (Watanabe et al., 1992). Collectively, these results indicate that different PKC isotypes are critical players in the control of

programmed cell death. The precise mechanisms underlying the interactions among 3′-phosphoinositides, Par-4, and the atypical PKCs are not clear at this moment and will be a matter for future studies.

An interesting aspect of the work presented here and that on LIP (Diaz-Meco et al., 1996) is the fact that both LIP and Par-4 bind to the same region in λ/ιPKC yet do not display an apparent homology at the amino acid level. It is possible that similar secondary or tertiary structures (or both) in LIP and Par-4 could account for their ability to interact with the zinc finger of λ/ιPKC. In this regard, the PKC-binding domains of LIP and Par-4 have coiled-coil structures that may be important for that interaction. Further work should clarify this point, as well as the mechanism whereby LIP and Par-4 connect λ/ιPKC and ζPKC to upstream signaling molecules and cytokine receptors. In any case, the data presented here further support the role of the atypical PKCs in cell growth and strongly suggest that the selective modulation of the different PKC isotypes will be mediated by protein–protein interactions. The role of the lipid mediators in this scenario could be to act as cofactors that allow or impede the binding of the atypical PKCs to their respective protein regulators.

Experimental Procedures

Two-Hybrid Screening and cDNA Isolation

For the yeast two-hybrid screening, pBTM116ζPKC^{REG} was cotransformed with the human placenta cDNA Matchmaker library in the pGAD10 vector (Clontech Laboratories) into the L40 yeast strain, and the transformants were plated to synthetic medium lacking histidine, leucine, and tryptophan and containing 20 mM 3-amino-1,2,4,triazole. The plates were incubated at 30°C for 5 days. His$^+$ colonies were assayed for β-galactosidase activity by a filter assay as described below. Oligonucleotides were synthesized by Isogen Biosciences (The Netherlands). pBTM116ζPKC^{REG}, containing the rat brain ζPKC regulatory domain, was constructed by polymerase chain reaction (PCR) with the oligonucleotides 5′-GGAGAATTCATG CCCAGCAGGACC-3′ and 5′-AGGAATTCTCACTGAGAGATTTTGAT CCCATC-3′ and subcloned into the EcoRI site of pBTM116 vector. DNA sequencing was done by the dideoxynucleotide chain termination method.

β-Galactosidase Filter Assays

Yeast strains were patched to synthetic medium lacking leucine and tryptophan, incubated for 3 days at 30°C, and then transferred to a nitrocellulose filter. The filter was placed in an aluminum foil atop a sea of liquid nitrogen for 20 s and then immersed in liquid nitrogen for 1–2 s. The filter was allowed to come to room temperature and then was placed on top of Whatman 1 paper that had been prewet in Z buffer containing 0.75 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The filters were incubated for 3 hr at 30°C. Blue coloration is indicative of β-galactosidase activity.

Plasmids

pYTH9λ/ιPKC, pYTH9λ/ιPKC^{REG}, pYTH9ζPKC$^{REG\text{-}C167S}$, pYTH9λ/ιPKC^{126}, pYTH9λ/ιPKC^{ZF}, pYTH9ζPKC, pYTH9ζPKC^{REG}, pYTH9ζPKC^{126}, pYTH9ζPKC^{ZF}, pYTH9αPKC^{REG}, pYTH9εPKC^{REG}, pGBT9-Raf^{REG}, pGBT9Raf, pGBT9Raf^{CAT}, pGBT9Mos, pGBT9Lamin, pCDNA3-HA-ζPKC, pCDNA3-HA-λ/ιPKC, pCDNA3-HA-ζPKC^{MUT}, pCDNA3-HA-λ/ιPKC^{MUT}, GST–ζPKC^{REG}, GST–λ/ιPKC^{REG}, and pRcCMVλ/ιPKC have previously been described (Berra et al., 1995; Diaz-Meco et al., 1996). The αPKC expression vector was obtained by insertion of the full-length cDNA of αPKC into the pMT-2 vector. GST–ζPKC$^{REG\text{-}C167S}$ was made by PCR using pYTH9ζPKC$^{REG\text{-}C167S}$ as a template and the following primers: 5′-ATTGGATCCAGCAGGACCG ACCCCAA-3′ and 5′-AGGAATTCTCACTGAGAGATTTTGATCCC ATC-3′. The PCR product was digested with BamHI and EcoRI and cloned into BamHI–EcoRI-digested pGEX-2TK. To generate pcDNA3-HA-Par-4, an EcoRV–XbaI fragment excised from pBluescript–Par-4 was subcloned into EcoRV–XbaI sites of pcDNA3-HA. pcDNA3-HA-Par-4Δ was obtained by PCR using pBluescript–Par-4 as a template and the following primers: 5′-ATTAACCCTCACTA AAG-3′ and 5′-GCTCTAGATCATTTCCAGTGTGCTACTTG-3′. The PCR product was digested with EcoRV and XbaI and subcloned into the same sites of pcDNA3-HA. pGEX-2TK constructs were transformed into E. coli JM101, and expression of GST fusion proteins and their purification on glutathione–Sepharose were carried out according to the procedures recommended by the manufacturer. Par-4 was inserted in the EcoRI site of pMALc2 (New England Biolabs). Par-4Δ was amplified by PCR using pGAD-Par-4 as a template and the following primers: 5′-TACCACTACAATGGATG-3′ and 5′-GCTCTAGATCATTTCCAGTGTGCTACTTG-3′. The PCR product was digested with EcoRI and XbaI and subcloned into pMALc2. MBP–Par-4 and MBP–Par-4Δ fusion proteins were expressed in E. coli and purified by binding to an amylose resin according to the procedures recommended by the manufacturer. Expression and purification of hnRNPA1 have been previously described (Diaz-Meco et al., 1996).

Binding Studies

Purified MBP, MBP–Par-4, or MBP–Par-4Δ (400 nM) were incubated with in vitro translated ^{35}S-labeled ζPKC at 4°C for 1 hr. The recombinant proteins were recovered on amylose beads, which were washed eight times with binding buffer and eluted with 10 mM maltose, and the eluates were fractionated by SDS–PAGE followed by autoradiography in an InstantImager.

Purified GST or GST fusion proteins encoding the regulatory domain of ζPKC (GST–ζPKC^{REG}) or the zinc finger mutant (GST–ζPKC$^{REG\text{-}C167S}$) at a concentration of 800 nM were incubated with either 100 or 400 nM MBP fusion proteins for 1 hr at 4°C according to a previously published procedure (Diaz-Meco et al., 1996), after which the MBP fusion proteins were immobilized on amylose resin. After extensive washing, bound MBP fusion proteins and any associated proteins were boiled in sample buffer, fractionated by SDS–PAGE, and immunoblotted with anti-GST antibody to detect binding of the different GST fusion proteins.

COS cells were transfected with either control plasmid or an expression vector for HA-tagged ζPKC or λ/ιPKC (pCDNA3-HA-ζPKC or pCDNA3-HA-λ/ιPKC), after which they were made quiescent by serum starvation, and the expressed proteins were immunoprecipitated with an anti-HA antibody as described previously (Diaz-Meco et al., 1996). Afterward, we determined the effect of 1 μg of MBP, MBP–Par-4, or MBP–Par-4Δ on the unstimulated or phosphatidylserine-activated (50 μg/ml) enzymatic activity of HA-ζPKC. The activity of ζPKC was measured essentially as described previously (Diaz-Meco et al., 1996) using hnRNPA1 as substrate. A similar experiment was performed with a highly purified preparation of classical PKC (Boehringer Mannheim).

Cellular Studies

Subconfluent cultures of COS cells in 100 mm plates were transfected by the calcium phosphate method (GIBCO BRL) with 20 μg of either pCDNA3 (control), pCDNA3-HA-Par-4 (HA-Par-4), or pCDNA3-HA-Par-4Δ (HA-Par-4Δ). Plasmid DNA was removed 4 hr later, and cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) for 36 hr. Cultures were then extracted with lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, and protease inhibitors), and extracts were immunoprecipitated with 2 μg of anti-HA antibody (12CA5; Boehringer Mannheim) per milligram of protein extract. Immunoprecipitates were resolved by SDS–PAGE and immunoblotted with anti-ζPKC or anti-αPKC antibodies (GIBCO BRL).

In another set of experiments, NIH-3T3 cells were transfected with 5 μg of pCDNA3-HA-Par-4 plus 15 μg of pRcCMV plasmids or with 5 μg of pCDNA3-HA-Par-4 plus 15 μg of pRcCMV-λ/ιPKC plasmids, or with 5 μg of pCDNA3 plus 15 μg of pCDNA3-HA-λ/ιPKC or with 5 μg of pCDNA3 plus 15 μg of pCDNA3-HA-λ/ιPKC^{MUT}. Cells were changed 24 hr posttransfection to 1% serum–containing medium for 12 hr, after which cells were immunostained with anti-HA antibody or Hoechst 3358.

Cell viability assays were performed in NIH-3T3 cells that were transfected with pCMV-βgal (2.5 μg) and 5 μg of either plasmid control pCDNA3, pCDNA3-HA-Par-4, or pCDNA3-HA-Par-4Δ, together with 10 μg of pCDNA3, pCDNA3-HA-ζPKC, pCDNA3-HA-ζPKC^{MUT}, pCDNA3-HA-λ/ιPKC, or pCDNA3-HA-λ/ιPKC^{MUT}. Cells were changed 24 hr posttransfection to 0.5% serum–containing medium for 12 hr, after which they were fixed and stained with X-Gal, and the number of blue cells per 35 mm dish was determined microscopically.

Subconfluent cultures of NIH-3T3 cells were transfected with different amounts of plasmid pCDNA3-HA-par-4 or pCDNA3-HA-par-4Δ, together with 500 ng of either 3AP1-Luc or CMV-Luc reporter plasmids either with or without expression vectors for wild-type λ/ιPKC or αPKC. After 4 hr, the DNA-containing medium was removed and cells were made quiescent by serum starvation (0.5%) for 24 hr. Afterward, cells were either untreated or stimulated with 20% FCS for 4 hr. Extracts were prepared and luciferase activity was determined as described previously (Diaz-Meco et al., 1996). Data are expressed in terms of relative luciferase activity units, calculated as (light emission from experimental sample − light emission of lysis buffer alone)/μg of cellular protein in the sample.

For UV treatment, culture medium was removed, dishes were washed once with PBS and UVC irradiated (180 J/m^2), and fresh medium was added to the cells.

To assess the apoptotic DNA damage, cells treated according to the experiments were collected, rinsed with Ca^{2+}- and Mg^{2+}-free PBS, lysed in lysis buffer (10 mM EDTA, 50 mM Tris [pH 8.0], 0.5% sodium lauryl sarcosine, 0.5 mg of proteinase K per milliliter) and then incubated at 50°C for 1 hr. RNase A (0.5 mg/ml) was added, and lyses were incubated for an additional hour. DNA was electrophoresed in a 1% agarose gel in 0.5× TBE running buffer for 1 hr at 90 V.

Acknowledgments

Correspondence should be addressed to J. M. This work was supported by grants SAF93-0018 from the Comision Interministerial de Ciencia y Tecnologia, PB93-180 from the Dirección General de Investigación Científica y Tecnica, 94/1910-E from the Fondo de Investigaciones Sanitarias, and AE00011/95 from Comunidad de Madrid (CAM). M. T. D.-M. has a postdoctoral contract from the Consejo Superior de Investigaciones Científicas (CSIC)–Fundación Ramon Areces. M. M. M. and P. S. are Fellows from Glaxo–CSIC and CAM, respectively. J. L. is a fellow from the Ministerio de Educación. We also want to thank Glaxo Spain for partially funding this work and the Fundación Ramón Areces for an Institutional Grant to the Centro de Biología Molecular. We are indebted to Esther Garcia and Carmen Ibañez for technical assistance.

Received March 25, 1996; revised July 18, 1996.

References

Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., Suzuki, K., and Ohno, S. (1994). A new member of the third class in the protein kinase C family, PKCλ, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells. J. Biol. Chem. *269*, 12677–12683.

Akimoto, K., Takahashi, R., Moriya, S., Nishioka, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S.-I., Mizuno, K., Hirai, S.-I., Kazlauskas, A., and Ohno, S. (1996). EGF or PDGF receptors activate atypical PKCλ through phosphatidylinositol 3-kinase. EMBO J. *15*, 788–798.

Berra, E., Diaz-Meco, M.T., Dominguez, I., Municio, M.M., Sanz, L., Lozano, J., Chapkin, R.S., and Moscat, J. (1993). Protein kinase C ζ isoform is critical for mitogenic signal transduction. Cell *74*, 555–563.

Berra, E., Diaz-Meco, M.T., Lozano, J., Frutos, S., Municio, M.M., Sanchez, P., Sanz, L., and Moscat, J. (1995). Evidence for a role of MEK and MAPK during signal transduction by protein kinase Cζ. EMBO J. *14*, 6157–6163.

Bjorkoy, G., Overvatn, A., Díaz-Meco, M.T., Moscat, J., and Johansen, T. (1995). Evidence for a bifurcation of the mitogenic signaling pathway activated by ras and phosphatidylcholine-hydrolyzing. J. Biol. Chem. *270*, 21299–21306.

Brancolini, C., Benedetti, M., and Schneider, C. (1995). Microfilament reorganization during apoptosis: the role of Gas-2, a possible substrate for ICE-like proteases. EMBO J. *14*, 5179–5190.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505–512.

Cleveland, J.L., and Ihle, J.N. (1995). Contenders in FasL/TNF death signaling. Cell *81*, 479–482.

Diaz-Meco, M.T., Berra, E., Municio, M.M., Sanz, L., Lozano, J., Dominguez, I., Diaz-Golpe, V., Lain de Lera, M.T., Alcamí, J., Payá, C.V., Arenzana-Seisdedos, F., Virelizier, J.L., and Moscat, J. (1993). A dominant negative protein kinase C ζ subspecies blocks NF-κB activation. Mol. Cell. Biol. *13*, 4770–4775.

Diaz-Meco, M.T., Municio, M.M., Sanchez, P., Lozano, J., and Moscat, J. (1996). λ-Interacting protein, a novel protein that specifically interacts with the zinc finger domain of the atypical protein kinase C isotype λ/ι and stimulates its kinase activity *in vitro* and *in vivo*. Mol. Cell. Biol. *16*, 105–114.

Dominguez, I., Diaz-Meco, M.T., Municio, M.M., Berra, E., Garcia de Herreros, A., Cornet, M.E., Sanz, L., and Moscat, J. (1992). Evidence for a role of protein kinase C ζ subspecies in maturation of *Xenopus laevis* oocytes. Mol. Cell. Biol. *12*, 3776–3783.

Dominguez, I., Sanz, L., Arenzana-Seisdedos, F., Diaz-Meco, M.T., Virelizier, J.L., and Moscat, J. (1993). Inhibition of protein kinase ζ subspecies blocks the activation of a NF-κB-like activity in *Xenopus laevis* oocytes. Mol. Cell. Biol. *13*, 1290–1295.

Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W.W., Kamen, R., Weichselbaum, R., and Kufe, D. (1995). Proteolytic activation of protein kinase Cδ by an ICE-like protease in apoptotic cells. EMBO J. *14*, 6148–6156.

Exton, J.H. (1994). Phosphatidylcholine breakdown and signal transduction. Biochim. Biophys. Acta *1212*, 26–42.

Folgueira, L., McElhinny, J.A., Bren, G.D., MacMorran, W.S., Diaz-Meco, M.T., Moscat, J., and Payá, C.V. (1996). Protein kinase C-ζ mediates NF-κB activation in human immunodeficiency virus-infected monocytes. J. Virol. *70*, 223–231.

Franke, T.F., Yang, S.-H., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., and Tsichlis, P.N. (1995). The protein kinase encoded by the proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell *81*, 727–736.

Hannun, Y.A. (1994). The sphingomyelin cycle and the second messenger function of ceramide. J. Biol. Chem. *269*, 3125–3128.

Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C.J. (1992). Activation of MAP kinase pathway by the protein kinase Raf. Cell *71*, 335–342.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1–associated protein TRADD signals cell death and NF-κB activation. Cell *81*, 495–504.

Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D.V. (1996). TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell *84*, 299–308.

Klauck, T.M., Faux, M.C., Labudda, K., Langeberg, L.K., Jaken, S., and Scott, J.D. (1996). Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science *271*, 1589–1592.

Kolesnick, R., and Golde, D.W. (1994). The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell *77*, 325–328.

Konishi, H., Kuroda, S., and Kikkawa, U. (1994). The pleckstrin homology domain of RAC protein kinase associates with the regulatory domain of protein kinase Cζ. Biochem. Biophys. Res. Commun. *205*, 1770–1775.

Liscovitch, M., and Cantley, L.C. (1994). Lipid second messengers. Cell *77*, 329–334.

Lozano, J., Berra, E., Municio, M.M., Diaz-Meco, M.T., Dominguez, I., Sanz, L., and Moscat, J. (1994). Protein kinase C ζ isoform is

critical for κB-dependent promoter activation by sphingomyelinase. J. Biol. Chem. *269*, 19200–19202.

Mochly-Rosen, D., Khaner, H., and López, J. (1991). Identification of intracellular receptors for activated protein kinase C. Proc. Natl. Acad. Sci. USA *88*, 3997–4000.

Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S.-I., Mizuno, K., Takenawa, T., Fukui, Y., Watanabe, Y., Ozaki, S., and Ohno, S. (1996). Platelet-derived growth factor activates protein kinase C ϵ through redundant and independent signaling pathways involving phospholipase Cγ or phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. USA *93*, 151–155.

Müller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D., and Pfizenmaier, K. (1995). PKC ζ is a molecular switch in signal transduction of TNFα, bifunctionally regulated by ceramide and arachidonic acid. EMBO J. *14*, 1961–1969.

Nakanishi, H., and Exton, J.H. (1992). Purification and characterization of the ζ isoform of protein kinase C from bovine kidney. J. Biol. Chem. *267*, 16347–16354.

Nakanishi, H., Brewer, K.A., and Exton, J.H. (1993). Activation of the ζ isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *268*, 13–16.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science, *258*, 607–614.

Ohno, Y., Fuji, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1989). Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. Proc. Natl. Acad. Sci. USA *86*, 3099–3103.

Palmer, R., Dekker, L.V., Woscholski, R., Le Good, J.A., Gigg, R., and Parker, P. (1995). Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *270*, 22412–22416.

Quest, A.F.G., and Bell, R.M. (1994). The regulatory region of protein kinase Cγ. J. Biol. Chem. *269*, 20000–20012.

Ron, D., Chen, C.-H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994). Cloning of an intracellular receptor for protein kinase C: a homolog of the b subunit of G protein. Proc. Natl. Acad. Sci. USA *91*, 839–843.

Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J., and Vande Woude, F. (1988). Function of c-mos protooncogene product in meiotic maturation in *Xenopus* oocytes. Nature *335*, 519–525.

Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krönke, M. (1992). TNF activates NF-κB by phosphatidylcholine-specific phospholipase C–induced "acidic" sphingomyelin breakdown. Cell *71*, 765–776.

Selbie, L.A., Schmitz-Peiffer, C., Sheng, Y., and Biden, T.J. (1993). Molecular cloning and characterization of PKCι, and atypical isoform of protein kinase C derived from insulin-secreting cells. J. Biol. Chem. *268*, 24296–24302.

Sells, S.F., Wood, D.P., Joshi-Barve, S.S., Muthukumar, S., Jacob, R.J., Crist, S.A., Humphreys, S., and Rangnekar, V.M. (1994). Commonality of the gene programs induced by effectors of apoptosis in androgen-dependent and -independent prostate cell. Cell Growth Diff. *5*, 457–466.

Stanger, B.Z., Leder, P., Lee, T.-H., Kim, E., and Seed, B. (1995). RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell *81*, 513–523.

Staudinger, J., Zhou, J., Burgess, R., Elledge, S., and Olson, E.N. (1995). PICK1: a perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. J. Cell. Biol. *128*, 263–271.

Toker, A., Meyer, M., Reddy, K.K., Falck, J.R., Aneja, R., Aaneja, S., Parra, A., Burns, D.J., Ballas, L.M., and Cantley, L.C. (1994). Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. J. Biol. Chem. *269*, 32358–32367.

Watanabe, T., Ono, Y., Taniyama, Y., Hazama, K., Igarashi, K., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992). Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-δ subspecies. Proc. Natl. Acad. Sci. USA *89*, 10159–10163.

Ways, D.K., Cook, P.P., Webster, C., and Parker, P.J. (1992). Effect of phorbol esters on protein kinase C-ζ. J. Biol. Chem. *267*, 4799–4805.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., and Greenberg, M.E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science *270*, 1326–1331.

Yang, Z., Costanzo, M., Golde, D.W., and Kolesnick, R.N. (1993). Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor κB translocation in intact HL-60 cells. J. Biol. Chem. *268*, 20520–20523.

Yao, R., and Cooper, G.M. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science *267*, 2003–2006.

Yao, B., Zhang, Y., Delikat, S., Mathias, S., Basu, S., and Kolesnick, R. (1995). Phosphorylation of Raf by ceramide-activated protein kinase. Nature *378*, 307–310.

Zhang, Y., and Kolesnick, R. (1995). Signalling through the sphingomyelin pathway. Endocrinology *136*, 4157–4159.