MOLECULAR AND CELLULAR BIOLOGY, Feb. 1997, p. 732–741 Vol. 17, No. 2
0270-7306/97/$04.00+0

Role of Diacylglycerol-Regulated Protein Kinase C Isotypes in Growth Factor Activation of the Raf-1 Protein Kinase

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Received 9 September 1996/Returned for modification 28 October 1996/Accepted 12 November 1996

The Raf protein kinases function downstream of Ras guanine nucleotide-binding proteins to transduce intracellular signals from growth factor receptors. Interaction with Ras recruits Raf to the plasma membrane, but the subsequent mechanism of Raf activation has not been established. Previous studies implicated hydrolysis of phosphatidylcholine (PC) in Raf activation; therefore, we investigated the role of the ε isotype of protein kinase C (PKC), which is stimulated by PC-derived diacylglycerol, as a Raf activator. A dominant negative mutant of PKCε inhibited both proliferation of NIH 3T3 cells and activation of Raf in COS cells. Conversely, overexpression of active PKCε stimulated Raf kinase activity in COS cells and overcame the inhibitory effects of dominant negative Ras in NIH 3T3 cells. PKCε also stimulated Raf kinase in baculovirus-infected *Spodoptera frugiperda* Sf9 cells and was able to directly activate Raf in vitro. Consistent with its previously reported activity as a Raf activator in vitro, PKCα functioned similarly to PKCε in both NIH 3T3 and COS cell assays. In addition, constitutively active mutants of both PKCα and PKCε overcame the inhibitory effects of dominant negative mutants of the other PKC isotype, indicating that these diacylglycerol-regulated PKCs function as redundant activators of Raf-1 in vivo.

The Raf protein-serine/threonine kinases play central roles in the response of cells to extracellular growth factors. Activation of Raf downstream of protein-tyrosine kinase receptors is mediated by the Ras GTP-binding proteins, which are required for stimulation of Raf kinase activity (44, 50). Raf then phosphorylates and activates mitogen-activated protein kinase kinase (MEK) (8, 21, 28), initiating a protein kinase cascade that culminates in proliferation or differentiation of a variety of cell types. The molecular mechanism of Raf activation, however, remains unclear. Although Raf binds directly to the effector domain of activated GTP-bound Ras proteins, this interaction does not appear to stimulate Raf kinase activity (34, 43, 45–47, 51). Rather, it appears that the role of the Ras-Raf interaction is to recruit Raf to the plasma membrane, where it can be activated by membrane lipids or other protein kinases, the nature of which remain to be determined (30, 42). Phosphorylation of Raf on both tyrosine and serine/threonine residues is required for enzymatic activity (9), and it has been shown that the Src protein-tyrosine kinase can activate Raf by phosphorylation on Tyr 340 and 341 (13, 32). However, mutant Raf proteins in which Tyr 340 and 341 have been changed to Asp can still be activated following membrane association, indicating that an additional mechanism(s) of Raf activation exists (32).

Hydrolysis of phosphatidylcholine (PC) has previously been shown to induce Raf activation in NIH 3T3 cells (5, 10). The hydrolysis of PC is stimulated by a variety of growth factors, providing a second source of diacylglycerol in addition to that derived by phosphatidylinositide hydrolysis (12). However, neither synthetic diacylglycerols nor diacylglycerol produced by hydrolysis of PC isolated from NIH 3T3 cell membranes stimulates Raf kinase activity in vitro (4a, 15), suggesting the possibility that PC-derived diacylglycerol activates another protein kinase which in turn activates Raf. Because diacylglycerol activates several members of the protein kinase C (PKC) family, we have investigated the possible participation of PKC isotypes in Raf activation.

Members of the PKC family differ in both catalytic and regulatory properties (38). The most abundant PKC in NIH 3T3 cells is the classical PKC isotype PKCα (33), which has been reported to phosphorylate and activate Raf in vitro (19, 26, 40). However, it appears unlikely that PKCα is required for Raf activation in vivo because PKCα is efficiently downregulated by treatment of NIH 3T3 cells with phorbol esters, whereas this does not inhibit either Raf activation in response to growth factor stimulation (1, 7, 35) or the mitogenic effects of PC hydrolysis (29). NIH 3T3 cells also express the nonclassical PKC isotypes PKCε, -δ, and -ζ (11, 33). PKCζ is resistant to downregulation by phorbol esters (48), but it is unlikely to directly couple Raf to PC hydrolysis because it is not activated by diacylglycerol (37) and does not appear to phosphorylate Raf in vitro (40). On the other hand, PKCε has been shown to be activated by PC-derived diacylglycerol (18) and has been implicated in signaling cell proliferation because its overexpression (in contrast to that of PKCδ) can induce cell transformation (4, 33). PKCε thus appears to be a plausible candidate for involvement in mitogenic signaling, and so we have investigated its potential role in Raf activation.

MATERIALS AND METHODS

Plasmid constructs. Full-length cDNA encoding murine PKCε (a generous gift of Bernard Weinstein) was inserted into the pSVK3 vector (Pharmacia). pSVPKCε1-401 was constructed by cloning the PKCε cDNA fragment encoding amino acids 1 to 401 in pSVK3. pSVPKCε423-734 was constructed by using PCR to amplify the PKCε cDNA fragment encoding amino acids 423 to 734 with a 5′ primer containing ATG and a Kozak consensus sequence, followed by subclon-

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ing into pSVK3. Full-length cDNA encoding bovine PKCα was similarly subcloned into pSVK3, and deletion mutants (PKCα1-385 and PKCα302-672) were constructed by PCR amplification with appropriate primers.

Oncogene constructs and PKCζ cDNAs have been previously described (6, 11, 14, 27, 41). All constructs were subcloned into pSVK3 for expression in COS cells.

Transfection of NIH 3T3 cells. NIH 3T3 cells were transfected with plasmid DNAs in the presence of 20 μg of carrier calf thymus DNA (Sigma Chemical Co.) as described previously (14). Cells were subcultured into medium containing G418 (400 μg/ml) 3 days after transfection, and G418-resistant colonies were stained and photographed 14 to 16 days after G418 selection.

Transient expression assays in COS cells. COS-7 cells (3×10^5) were transfected with 10 μg of plasmid DNA in 2 ml of Dulbecco modified Eagle medium–10% NuSerum containing 400 μg of DEAE-dextran per ml and 100 μM chloroquine. One day after transfection, cells were incubated in 0.5% fetal calf serum for 24 h to induce quiescence. The activation of Raf-1 in response to growth factor stimulation was then assayed 48 h after transfection.

PKC immunoblot analysis. Cells were lysed in 20 mM Tris-HCl (pH 7.5)–0.5 mM EDTA–0.5% Triton X-100–25 μg of aprotinin per ml–1 mM phenylmethylsulfonyl fluoride (PMSF). Protein samples (30 μg) were electrophoresed in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and transferred to nitrocellulose filters. The filters were then blocked in 4% skim milk and probed with monoclonal anti-PKCα or -PKCε antibody (Transduction Laboratories). Antibodies were detected by using an Amersham ECL (enhanced chemiluminescence) kit.

PKC kinase assays. Cells were lysed in 0.5 ml of 20 mM Tris-HCl (pH 7.5)–0.5 mM EDTA–0.5 mM EGTA–0.5% Triton X-100–25 μg each of aprotinin and leupeptin per ml. The cell extracts were incubated with polyclonal anti-PKCα (Gibco) or anti-PKCε (Santa Cruz Biotechnology) antibody for 1 h followed by incubation with protein A-Sepharose beads for 1 h. Immunoprecipitates were washed twice with lysis buffer, twice with 0.1 M Tris-HCl (pH 7.5) containing 0.5 M LiCl, and twice again with lysis buffer. PKCα immunoprecipitates were then incubated for 5 min at 30°C in a 40-μl reaction volume containing 50 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.0375% β-mercaptoethanol, 0.75 mM $CaCl_2$, 12.5 mM $MgCl_2$, 0.25% Triton X-100, 100 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 50 μg of phosphatidylserine, 125 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 50 μM synthetic peptide containing myelin basic protein (MBP) amino acids 4 to 14. PKCε immunoprecipitates were incubated for 30 min at 30°C in 40-μl reaction mixtures containing 50 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.75 mM EGTA, 0.0375% β-mercaptoethanol, 2.5 mM $MgCl_2$, 0.25% Triton X-100, 5 μg of bovine serum albumin, 100 ng of TPA, 50 μg of phosphatidylserine, 125 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 50 μM synthetic peptide containing MBP amino acids 4 to 14. The reactions were stopped by spotting 25 μl of reaction mixtures onto phosphocellulose filters. The filters were washed twice with 1% phosphoric acid and twice with H_2O. The incorporation of ^{32}P into MBP peptide was measured by scintillation counter.

Raf-1 protein kinase activity. Raf-1 was immunoprecipitated from extracts of NIH 3T3 or COS-7 cells by using monoclonal anti-Raf-1 antibody (Transduction Laboratories). Raf kinase activity was assayed by phosphorylation of MEK as described previously (5) except that 0.1 μg of MEK purchased from Santa Cruz Biotechnology Co. was used. Raf protein kinase was also assayed by using a coupled assay of ERK activation (21), with minor modifications. Briefly, Raf-1 immunoprecipitates were incubated with 0.5 μg of recombinant ERK2 (Santa Cruz) in reaction mixtures containing 200 μM ATP with or without MEK as indicated. Reaction mixtures were then electrophoresed in SDS–10% polyacrylamide gels containing 0.5 mg of MBP, and ERK activity was determined by an in-gel kinase assay (17).

Activation of Raf-1 in Sf9 cells. *Spodoptera frugiperda* Sf9 (2×10^6) cells were infected with recombinant baculoviruses at a multiplicity of infection of 10 (15, 25). Fifty hours after infection, cells were stimulated and lysed in 50 mM Tris-HCl (pH 7.2)–150 mM NaCl–0.1% SDS–0.5% sodium deoxycholate–1% Triton X-100–10 mM sodium pyrophosphate–25 mM sodium glycerophosphate–10% glycerol–0.1% mercaptoethanol–1 mM sodium vanadate–25 mM NaF–0.01% leupeptin–0.01% aprotinin–2 μM pepstatin. Raf-1 was immunoprecipitated and assayed for autophosphorylation activity and for phosphorylation of kinase-dead mutant K97M MEK (130 μg/ml) (31) in kinase buffer (25 mM HEPES [pH 7.5], 25 mM β-glycerophosphate, 1.5 mM EGTA, 5% glycerol, 1 mM dithiothreitol, 10 mM $MgCl_2$, 100 μM ATP, 10 μCi of [γ-^{32}P]ATP). Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Raf-1 autophosphorylation and MEK phosphorylation were quantified by PhosphorImager scanning. Immunostaining of Raf proteins was done with anti-RafSP63 antiserum.

Purification of PKCs. PKCs were purified from 5×10^8 Sf9 cells infected with recombinant viruses. Cells were lysed in 10 ml of extraction buffer (20 mM Tris-HCl [pH 7.5], 5 mM EDTA, 43 mM β-mercaptoethanol, 1% Triton X-100, 40 μg of leupeptin per ml, 0.3 mM PMSF, 10 mM benzamidine) and centrifuged at 15,000 × *g* for 20 min at 4°C. Lysates were loaded onto a 6-ml Fractogel DEAE (Merck Darmstadt) column equilibrated with buffer A (20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 43 mM β-mercaptoethanol, 0.1% Triton X-100, 0.3 mM PMSF, 10 mM benzamidine, 40 μg of leupeptin per ml). Proteins were eluted with 60 ml of a linear salt gradient to 1 M NaCl in buffer A. PKCε activity was found to elute at 30 to 50 mM NaCl, and PKCα activity was found to elute at 250 mM NaCl. PKC-containing fractions were pooled, concentrated with Centriplus 100 (Amicon) to a final volume of 1 ml, and loaded onto a Superdex 200 Pepgrade gel filtration column (120-ml bed volume; Pharmacia) run at a linear flow of 30 cm/h with buffer A containing 100 mM NaCl. Eluted fractions containing PKC activity were pooled and dialyzed against buffer A containing 50% glycerol. The content of PKC was about 10% of the total protein as estimated by analysis of silver-stained polyacrylamide gels.

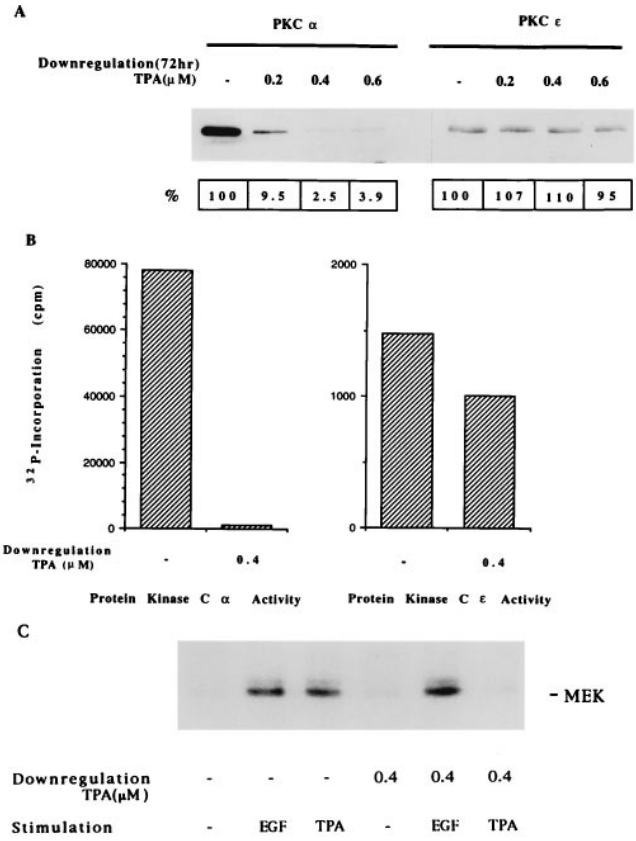

FIG. 1. Sensitivity of PKCα and PKCε to phorbol ester downregulation. NIH 3T3 cells were incubated with the indicated concentrations of TPA for 72 h and then assayed for PKCα and PKCε by either immunoblotting (A) or determination of protein kinase activities of immunoprecipitates (B). In panel C, control cells and cells that had been treated for 72 h with TPA were stimulated with TPA or EGF as indicated. Raf-1 activities were analyzed by immunokinase assays using MEK as the substrate. The data shown are representative of five independent experiments.

Enzymatic assays for PKC for monitoring the purification procedure were performed with MBP as the substrate. The PKCs were purified to a specific activity of 2,700 U of PKCε and 3,400 U of PKCα (1 U = 1 pmol of phosphate transferred to MBP under substrate saturation conditions) per mg.

Activation of Raf-1 by PKC in vitro. Raf-1 was immunoprecipitated from Sf9 cells and incubated with purified PKCε or PKCα in 30 μl of reaction mixture containing 50 mM Tris (pH 7.4), 0.5 mM EDTA, 0.75 mM EGTA, 12.5 mM $MgCl_2$, 0.0375% β-mercaptoethanol, 0.25% Triton X-100, 3.75 μg of bovine serum albumin per ml, 1 mM $CaCl_2$, 100 μg of phosphatidylserine per ml, 200 ng of TPA per ml, and 125 μM ATP at 30°C for 4 min. Immunoprecipitates were washed once with lysis buffer and once with Raf kinase buffer before being used for assays of MEK phosphorylation.

Phosphorylation of kinase-dead MEK was assayed by the addition of K97M MEK and incubation for 15 min at 30°C in the presence of 5 μCi of [γ-^{32}P]ATP. MEK phosphorylation was then analyzed by SDS-PAGE, and blots were immunostained with anti-Raf antiserum. For assays of MEK activation, MEK was added with or without ERK in Raf kinase buffer with 10 mM $MgCl_2$, 100 μM ATP, and 5 μCi of [γ-^{32}P]ATP. After incubation for 15 min at 30°C, phosphorylation of MEK and ERK was determined by SDS-PAGE and quantified by PhosphorImager scanning.

RESULTS

Resistance of PKCε to phorbol ester downregulation. PKCε has been reported to be relatively resistant to phorbol ester

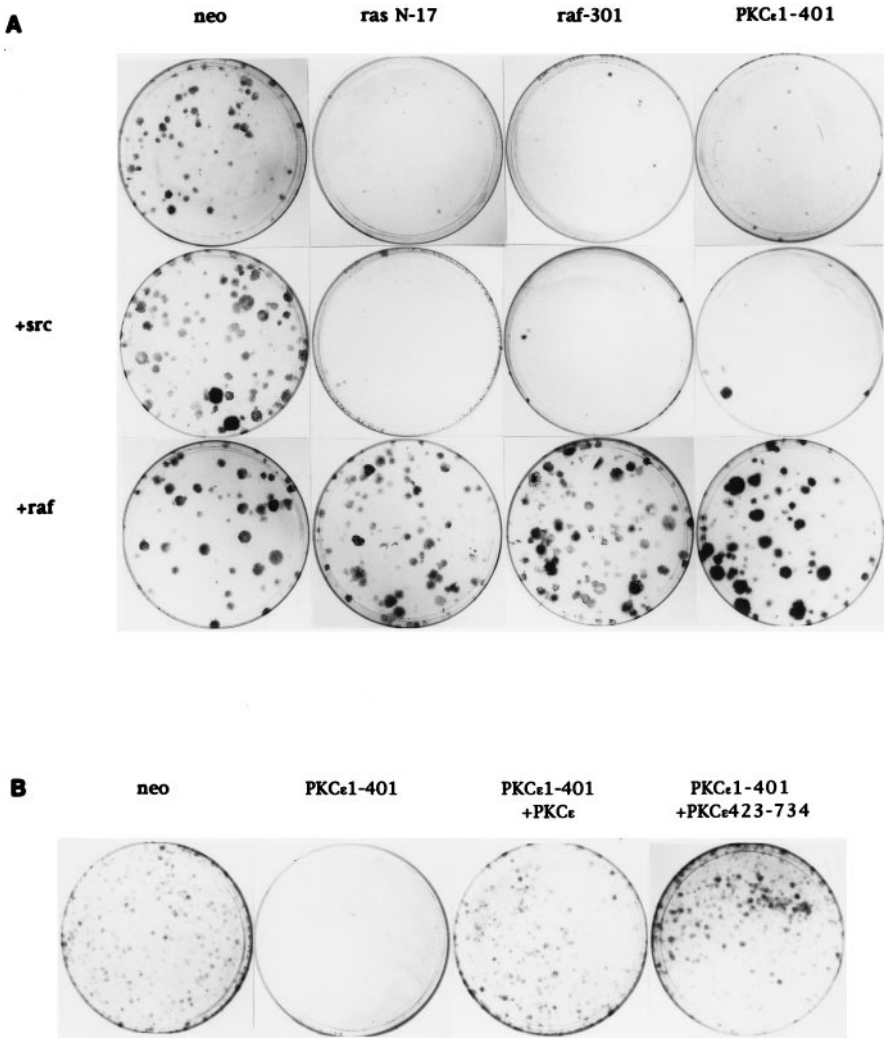

FIG. 2. Effect of a dominant negative PKCε mutant on proliferation of NIH 3T3 cells. NIH 3T3 cells were transfected with the indicated plasmid DNAs and subcultured into medium containing G418. G418-resistant colonies were stained and photographed 16 days after transfection. (A) Cultures designated neo were transfected with 0.1 μg of pZIPneoSV(X), and those designated rasN-17 were transfected with 0.1 μg of pZIPrasN-17. Cultures designated raf-301 and PKCε1-401 were cotransfected with 3 μg of pLTRraf-301 and pSVPKCε1-401, respectively, plus 0.1 μg of pZIPneoSV(X). Cultures designated +raf or +src were transfected with the plasmids described above plus 5 μg of plasmids expressing either oncogenic *raf* or oncogenic *src*. (B) Cultures designated neo were transfected with 0.1 μg of pZIPneoSV(X). Other cultures were cotransfected with pZIPneoSV(X) plus 3 μg of PKCε1-401 either alone or together with 5 μg of full-length PKCε or PKCε423-734 in pSVK3.

downregulation in some cell types, including NIH 3T3 cells (20). To initially assess the potential in vivo role of PKCε in Raf activation, we therefore compared the sensitivities of PKCε and PKCα to downregulation with the phorbol ester TPA and determined the effects of parallel TPA treatments on Raf-1 activation.

Immunoblot analysis of NIH 3T3 cell lysates indicated that PKCα was approximately 90% downregulated following 72 h of treatment with 200 nM TPA and completely downregulated (>95%) following treatment with 400 to 600 nM TPA (Fig. 1A). In contrast, PKCε was substantially more resistant to the effects of TPA treatment, with no significant downregulation observed after 72 h of treatment with TPA concentrations of up to 600 nM. PKCα, but not PKCε, was similarly downregulated after shorter times (24 and 48 h) of TPA treatment (data not shown). Similar results were obtained in protein kinase assays of PKCα and PKCε immunoprecipitates from cells that were treated for 72 h with 400 nM TPA (Fig. 1B). Such TPA downregulation reduced PKCα protein kinase activity >95% but resulted in only about a 30% reduction in the activity of PKCε.

We then determined the effect of TPA downregulation on activation of the Raf-1 protein kinase, as assayed by MEK phosphorylation (Fig. 1C). In cells that had not been pretreated with TPA, Raf-1 kinase activity was stimulated by exposure to either TPA or epidermal growth factor (EGF). In contrast, TPA was no longer capable of activating Raf-1 in cells that had been pretreated with 400 nM TPA for 72 h. Activation of Raf-1 in response to stimulation by TPA therefore appeared to require PKCα, which had been downregulated by TPA pretreatment of these cells. In contrast, TPA pretreatment did not interfere with Raf-1 activation in response to stimulation by EGF, indicating that PKCα was not required for growth factor-mediated Raf-1 activation. However, since PKCε was not downregulated under these conditions, it remains a potential candidate for an in vivo Raf-1 activator. Consistent with the

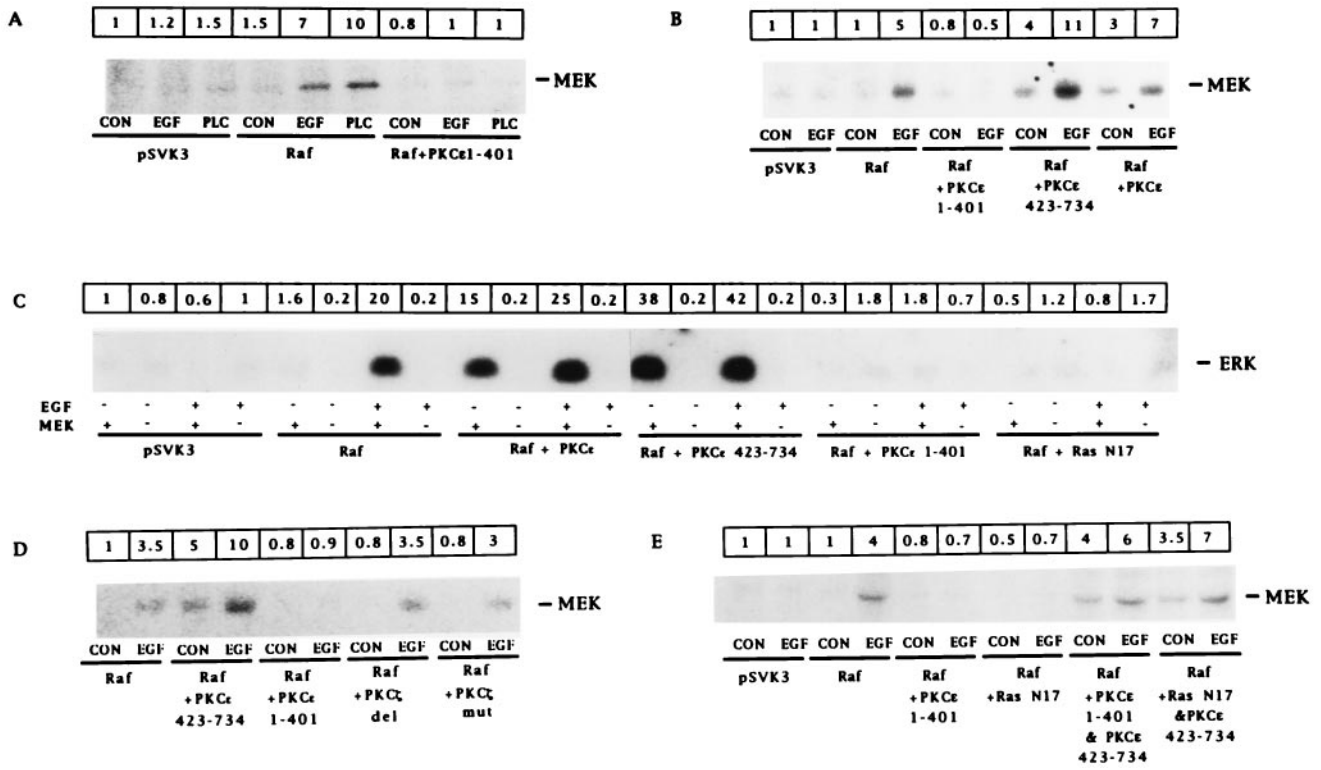

FIG. 3. Effect of PKCε on Raf-1 activation in COS cells. COS-7 cells were transfected with the indicated plasmid DNAs. One day after transfection, the cells were incubated in medium containing 0.5% fetal calf serum for 24 h to induce quiescence and then incubated with either EGF (10 ng/ml) or PC-PLC (0.5 U/ml) (5) for 5 min. Lanes: CON, unstimulated controls; pSVK3, cells transfected with 10 μg of pSVK3 vector alone; Raf, transfection with 5 μg of pSVKraf plus 5 μg of pSVK3; and Raf + PKC or Raf + Ras N17, transfection with 5 μg of pSVKraf plus 5 μg of the indicated PKC construct or Ras N17 in pSVK3. PKCζdel and PKCζmut are constitutively activated and dominant negative mutants of PKCζ, respectively. In panels A, B, D, and E, Raf-1 was immunoprecipitated and assayed for kinase activity by using MEK as the substrate. In panel C, Raf-1 immunoprecipitates were assayed for MEK activation by using a coupled assay of ERK activity. Data presented are representative of 2 to 10 independent determinations.

ability of PC-specific phospholipase C (PC-PLC), but not phorbol esters, to induce mitogenesis after downregulation of PKCα (29), treatment with PC-PLC also induced Raf-1 activation under these conditions (data not shown), suggesting that PC-derived diacylglycerol may be a more efficient in vivo activator of PKCε than TPA.

Effects of PKCε on proliferation and Raf-1 activation in mammalian cells. To further characterize the activity of PKCε, we constructed a dominant negative mutant (PKCε1-401) in which the C-terminal catalytic domain of PKCε was deleted. This mutant protein would be expected to interfere with normal PKCε activity, similar to analogous mutants of Raf (3, 11, 20, 27), in which N-terminal regulatory domains expressed in the absence of catalytic activity result in a dominant negative phenotype. Consistent with this prediction, expression of PKCε1-401 inhibited membrane translocation of endogenous PKCε in serum-stimulated COS cells (data not shown).

The role of PKCε in mammalian cell proliferation was then tested by transfection of NIH 3T3 cells with the PKCε1-401 mutant (Fig. 2). Cells were cotransfected with pZIPneoSV(X) and plasmids expressing dominant negative mutants of Ras, Raf-1, and PKCε, followed by selection of G418-resistant colonies. Cotransfection of PKCε1-401 inhibited proliferation of transfected NIH 3T3 cells and outgrowth of G418-resistant colonies, similar to the inhibitory effects of dominant negative mutants of Ras and Raf (Fig. 2A). Moreover, the inhibitory effect of PKCε1-401 was reversed by cotransfection with an active *raf-1* oncogene but not with *src*. As expected, the inhibitory effect of PKCε1-401 was also reversed by cotransfection with either full-length PKCε or a PKCε mutant containing only the C-terminal catalytic domain (PKCε423-734) (Fig. 2B). It thus appears that PKCε is required for NIH 3T3 cell proliferation. Furthermore, these results suggest that PKCε acts upstream of Raf-1 but downstream of protein-tyrosine kinases (e.g., Src), consistent with a role of PKCε in growth factor-stimulated Raf-1 activation.

The participation of PKCε in Raf-1 activation was further tested by transient expression assays in COS cells, in which plasmids containing simian virus 40 origins of replication are amplified and expressed at high levels (Fig. 3). Treatment with either EGF or PC-PLC stimulated Raf-1 kinase activity (assayed by phosphorylation of MEK) in cells transfected with a Raf expression plasmid. However, Raf-1 activation by either EGF or PC-PLC was abolished by cotransfection with the dominant inhibitory mutant PKCε1-401 (Fig. 3A). We then tested the effects of expression of full-length PKCε and of a PKCε mutant (PKCε423-734) in which the catalytic domain is intact but the regulatory domain has been deleted. Based on the effects of similar deletions in other PKCs (11, 23, 24, 36), the PKCε423-734 mutant would be expected to have constitutive catalytic activity. In contrast to the inhibitory effect of PKCε1-401, cotransfection with either full-length PKCε or PKCε423-734 stimulated Raf-1 in the absence of growth factor treatment and further increased Raf-1 activity in EGF-treated cells (Fig. 3B). To ensure that the phosphorylation of MEK detected in these assays resulted in a stimulation of its catalytic

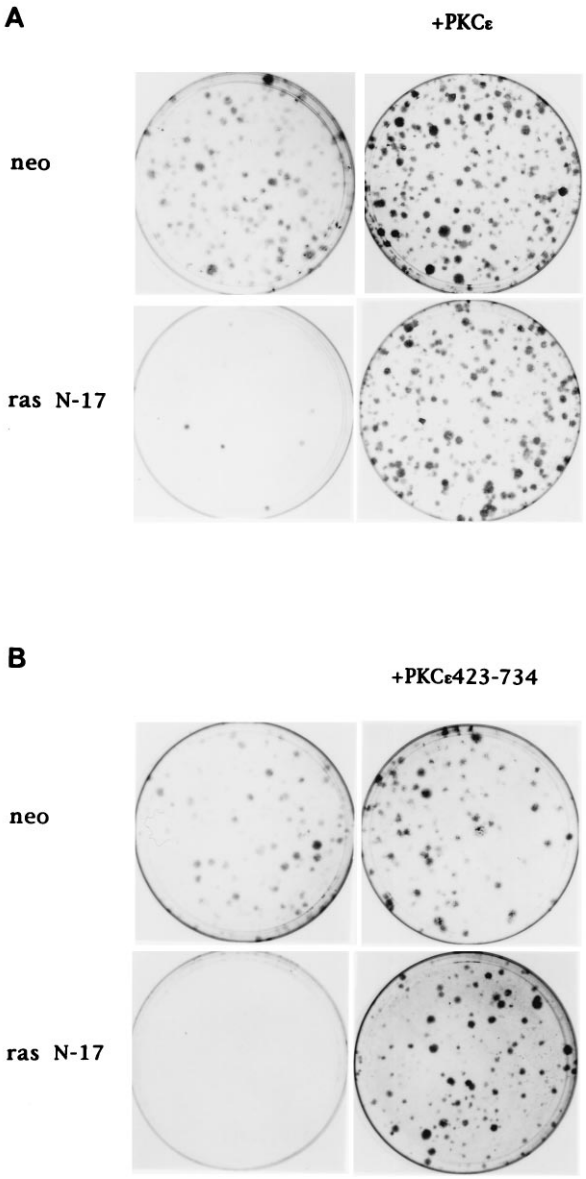

FIG. 4. Effect of PKCε on proliferation of NIH 3T3 cells downstream of Ras. Transfection of NIH 3T3 cells and selection of G418-resistant colonies were performed as described for Fig. 2. Cultures designated neo and ras N-17 were transfected with 0.1 μg of pZIPneoSV(X) and pZIPrasN17, respectively. Cells were cotransfected with 5 μg of the indicated PKC constructs in pSVK3.

activity, we performed similar experiments using a coupled assay in which Raf-1 immunoprecipitates were incubated with both MEK and ERK in the presence of unlabeled ATP and ERK activity was then determined by an in-gel protein kinase assay (Fig. 3C). These experiments confirmed that Raf-1 activation was stimulated by PKCε or PKCε423-734 but inhibited by PKCε1-401 or the dominant negative Ras N17 mutant.

The specificity of these effects for PKCε was indicated by the fact that Raf-1 activity in either control or EGF-treated cells was unaffected by cotransfection with inhibitory or activated mutants of PKCζ (Fig. 3D), consistent with the inability of PKCζ to phosphorylate Raf-1 in vitro (40). In addition, PKCε423-734 was able to overcome the inhibitory effects of either PKCε1-401 or Ras N17 (Fig. 3E). Thus, a dominant negative mutant of PKCε inhibited Raf-1 activation, while overexpression of wild-type or constitutively active PKCε was sufficient to stimulate the Raf kinase.

The role of PKCε in the Ras/Raf signaling pathway was further analyzed in terms of its effect on proliferation of NIH 3T3 cells. Cotransfection with either full-length or constitutively activated PKCε was sufficient to overcome the inhibitory effect of the dominant negative Ras N17 mutant on cell proliferation (Fig. 4). To confirm that transfection with PKCε allowed cotransfected cells to proliferate in the presence of Ras N17, several transformants obtained following cotransfection with plasmids expressing either activated Raf-1 or PKCε were analyzed for Ras N17 expression (Fig. 5). Such cotransfected cells expressed high levels of Ras N17, as well as either Raf-1 or PKCε, demonstrating that PKCε (like Raf-1) (14) is sufficient to overcome a block to cell proliferation resulting from inhibition of Ras signaling. These results are consistent with the COS cell experiments and indicate that PKCε can activate Raf-1 downstream of Ras.

Effects of PKCα on cell proliferation and Raf-1 activation. Since PKCα has been demonstrated to phosphorylate and activate Raf-1 in vitro, it was of interest to compare the effects of dominant negative and constitutively activated mutants of PKCα with those of PKCε in mammalian cells. Cotransfection of NIH 3T3 cells with a dominant negative mutant of PKCα (PKCα1-385) inhibited the outgrowth of G418-resistant colonies, similar to the effect of dominant negative PKCε (Fig. 6A). Also as for dominant negative PKCε, the inhibitory effect of PKCα1-385 was overcome by a *raf-1* oncogene but not by *src*. In addition, cotransfection of full-length or constitutively activated PKCα bypassed the inhibitory effect of Ras N17 (data not shown). Importantly, the inhibitory effect of dominant negative PKCα was overcome by constitutively active mutants of either PKCα (PKCα302-672) or PKCε, and constitutively active PKCα was reciprocally able to overcome the growth-inhibitory effect of dominant negative PKCε (Fig. 6A). These results suggest that dominant negative mutants of PKCα and PKCε, both of which bind diacylglycerol, can inhibit one another. In addition, it appears that active catalytic domains of

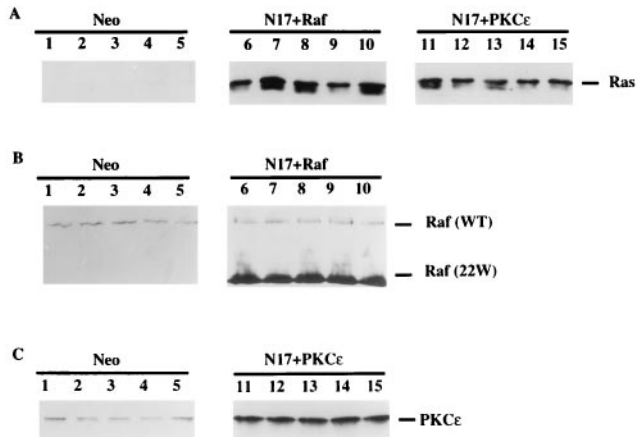

FIG. 5. Expression of Ras N17 by NIH 3T3 cells cotransfected with PKCε or Raf-1. Colonies of NIH 3T3 cells transfected with pZIPneoSV(X) (Neo) alone (lanes 1 to 5) or cotransfected with Ras N17 and oncogenic Raf 22W (from Fig. 2; lanes 6 to 10) or with Ras N17 and PKCε (from Fig. 4; lanes 11 to 15) were picked and cultured for analysis of Ras, Raf, and PKCε expression. For detection of Ras N17 (A), 100 μg of cell extract was immunoprecipitated with anti-Ras antibody YA6-172 (16), which is specific to human Ras (from which Ras N17 was derived). Immunoprecipitates were electrophoresed in SDS-polyacrylamide gels and immunoblotted with anti-Ras antibody Ras10. Raf (B) and PKCε (C) were detected by immunoblotting of cell lysates with anti-Raf SP63 (Santa Cruz) and anti-PKCε antibodies, respectively.

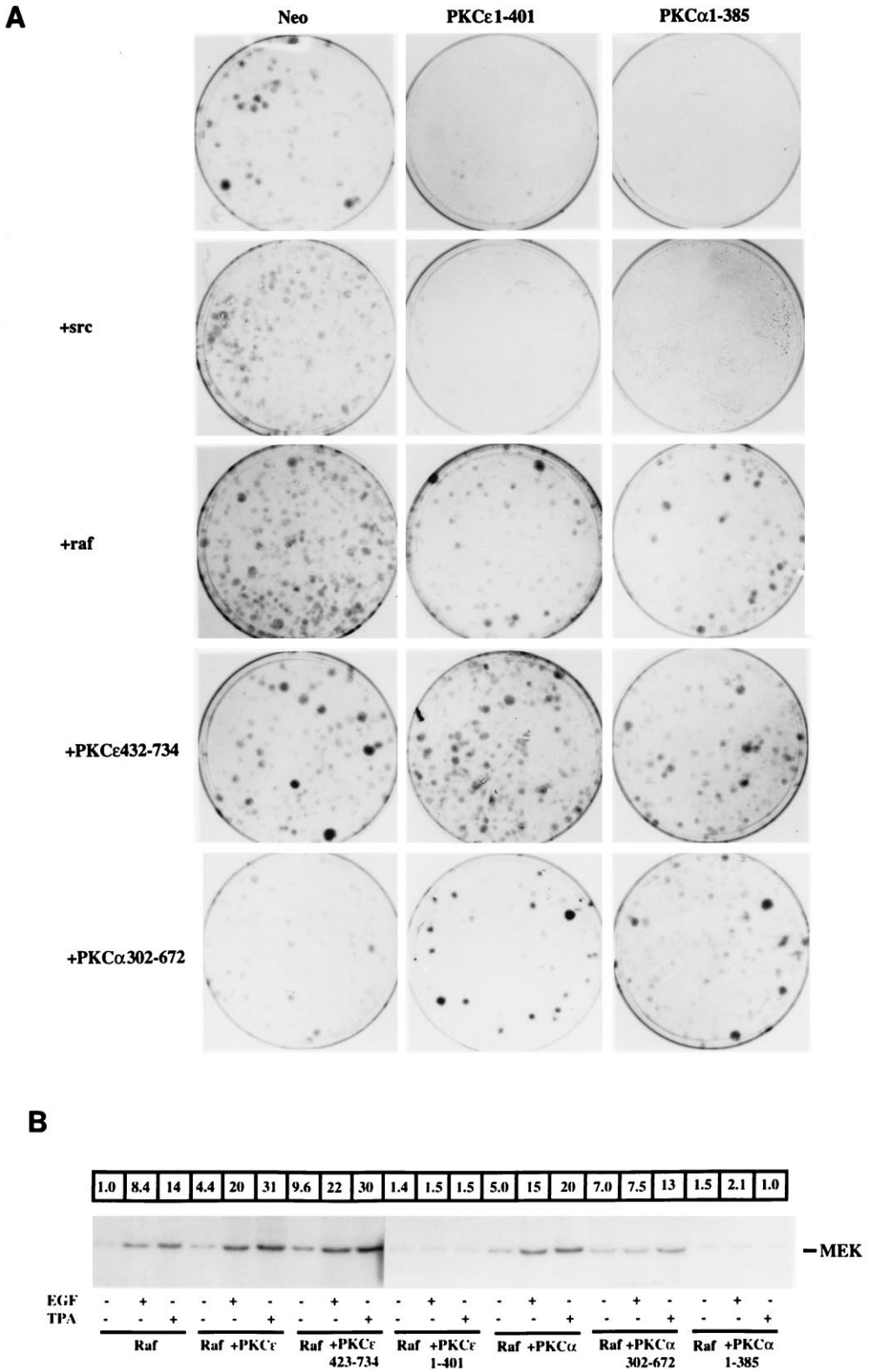

FIG. 6. Effect of PKCα on cell proliferation and Raf-1 activation. (A) NIH 3T3 cells were transfected with the indicated plasmid DNAs, and formation of G418-resistant colonies was assayed as described for Fig. 2. PKCα1-385 and PKCα302-672 are dominant negative and activated mutants, respectively. (B) COS cells were transfected and treated with 10 ng of EGF or 50 nM TPA as described for Fig. 3. Raf-1 kinase activity was assayed by using MEK as the substrate.

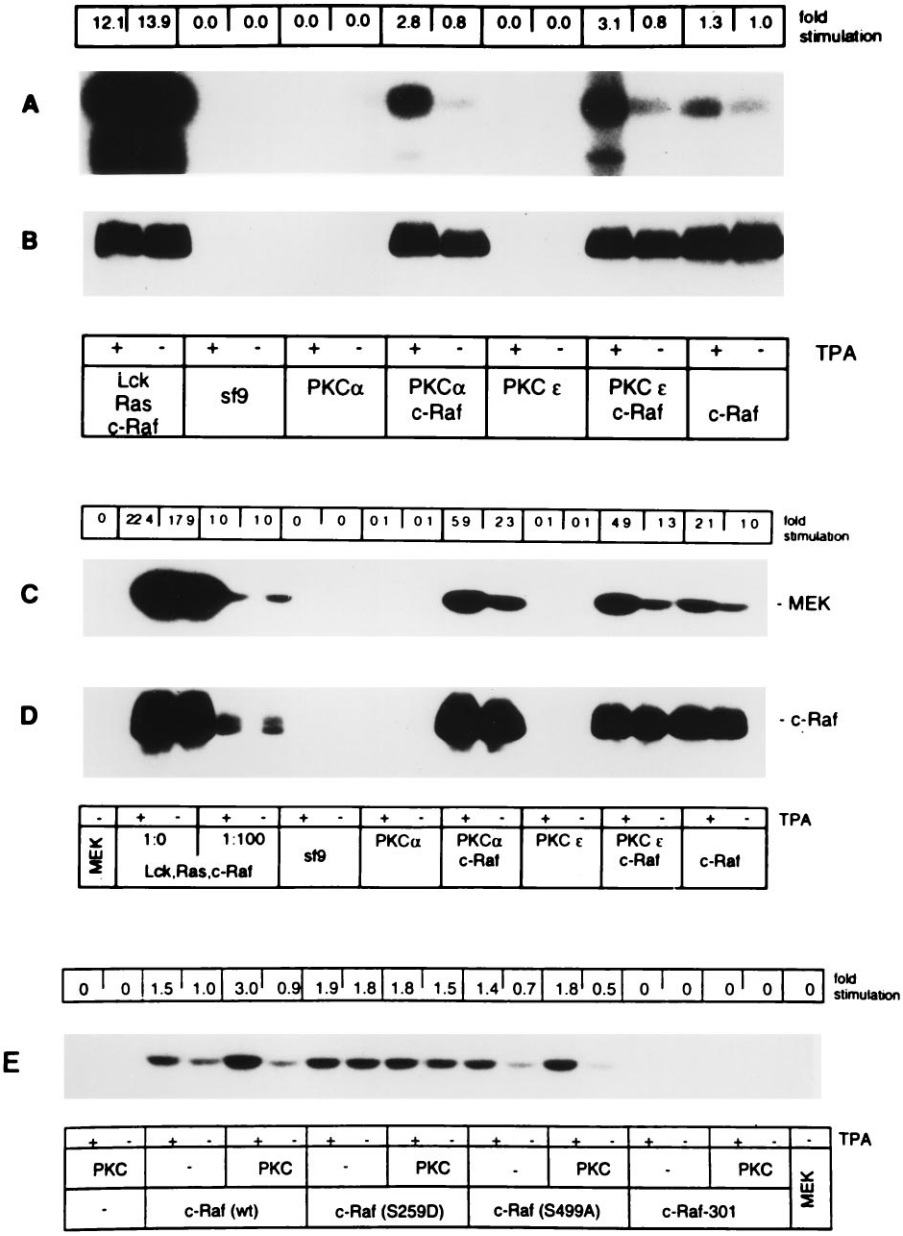

FIG. 7. Activation of Raf-1 by PKCε in Sf9 cells. Sf9 cells were infected with recombinant viruses at a multiplicity of infection of 10. After 50 h, the cells were stimulated by incubation with 200 ng of TPA per ml for 15 min. Raf-1 immunoprecipitates were assayed for autophosphorylation activity (A) and for phosphorylation of kinase-dead mutant K97M MEK (C). Blots were immunostained for Raf proteins with anti-Raf SP63 antiserum by using an ECL kit (B and D). In panel E, PKCε was expressed in Sf9 cells alone or in combination with wild-type Raf-1 or the indicated Raf-1 mutants. Raf proteins were immunoprecipitated and assayed for MEK phosphorylation.

either PKCα or PKCε are sufficient to allow NIH 3T3 cell proliferation.

PKCα also functioned similarly to PKCε when tested for Raf-1 activation in transient assays in COS cells (Fig. 6B). In particular, dominant negative PKCα inhibited Raf-1 activation in response to either EGF or TPA, whereas full-length PKCα or PKCα302-672 stimulated Raf-1 activity in the absence of EGF or TPA and further increased Raf-1 activity after mitogenic stimulation. These results are consistent with the similar effects of PKCα and PKCε on proliferation of NIH 3T3 cells and indicate that both of these PKC isotypes can function as Raf activators in mammalian cells.

Activation of Raf-1 by PKCε in insect cells. The ability of PKCε to activate Raf-1 was then tested in insect cells infected with recombinant baculoviruses (Fig. 7). Raf-1 was expressed in Sf9 cells, either alone or in combination with PKCα or PKCε. Cells were then stimulated by incubation with TPA, and Raf-1 immunoprecipitates were assayed for kinase activity, either by autophosphorylation (Fig. 7A) or by phosphorylation of kinase-inactive MEK K97M (31) (Fig. 7C). Coinfection with either PKCα or PKCε resulted in an approximately threefold stimulation of Raf-1 kinase activity, which was dependent on TPA treatment. Raf-1 activation by PKCε was thus comparable to activation by PKCα, which has previously been reported

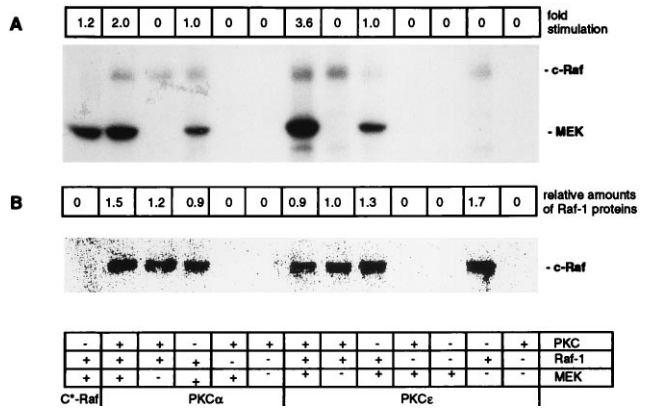

FIG. 8. Activation of Raf-1 by PKCε in vitro. Raf-1 was immunoprecipitated from singly infected Sf9 cells. Uninfected Sf9 cells were used as a control. C*-Raf indicates Raf-1 activated by coinfection of Sf9 cells with Ras and Lck recombinant viruses. Raf-1 immunoprecipitates were incubated with 1.34 U of purified PKCε or 1.68 U of purified PKCα in kinase buffer for 4 min at 30°C. The immunoprecipitates were then washed and incubated with K97M MEK for 15 min at 30°C in the presence of 5 μCi of [γ-^{32}P]ATP. MEK phosphorylation was analyzed by SDS-PAGE (A), and blots were immunostained with anti-Raf SP63 antiserum by using a ^{35}S-labeled secondary antibody (B). Data were quantified by PhosphorImager scanning. The amount of C*-Raf used in kinase assays was 1/100 the amount of Raf-1.

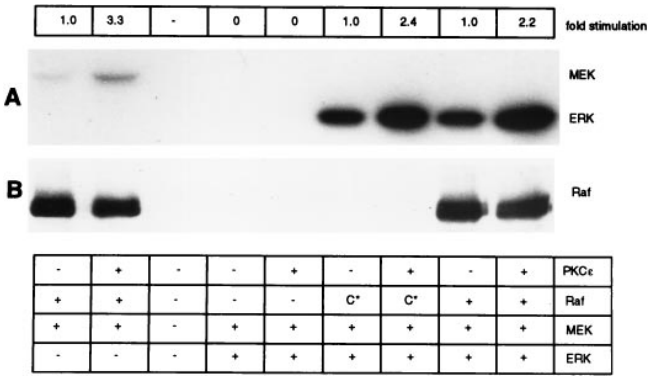

FIG. 9. Raf-1 activated by PKCε activates MEK in vitro. Raf-1 was immunoprecipitated from singly infected Sf9 cells and incubated with purified PKCε as for Fig. 8. Raf-1 immunoprecipitates were then washed before addition of MEK with (lanes 4 to 9) or without (lanes 1 to 3) ERK in Raf kinase buffer containing [γ-^{32}P]ATP. After incubation for 15 min at 30°C, MEK activation was analyzed by SDS-PAGE and data were quantified (A) with a BAS 2000 Bio Imaging Analyzer (Fuji). Immunoprecipitated Raf proteins were detected on Western blots by Raf-specific antisera and ^{35}S-labeled secondary antibodies. Quantification with the BAS 2000 Bio Imaging Analyzer revealed that the variation between different samples was <20% (B). C* indicates Raf-1 activated by coinfection of Sf9 cells with Ras and Lck recombinant viruses. The amount of C*-Raf used in kinase assays was 1/100 the amount of Raf-1.

to phosphorylate and activate Raf-1 in this system (26, 40). However, the activation of Raf-1 by both PKCα and PKCε was less than that attained by Ras plus the Lck protein-tyrosine kinase in Sf9 cells (49). Analysis of Raf-1 phosphorylation sites indicated that Ser 259 and Ser 499 are required for optimal activation by PKCε, with amino acid substitution at position 259 having a more pronounced effect than substitution at position 499 (Fig. 7E).

In vitro activation of Raf-1 by PKCε. We next sought to determine whether purified PKCε was capable of activating Raf-1 by direct phosphorylation in vitro. PKCα and PKCε were purified from Sf9 cells infected with recombinant viruses as described in Materials and Methods. Purified recombinant PKCα and PKCε were then incubated with immunoprecipitated Raf-1 in the presence of phosphatidylserine, TPA, and unlabeled ATP (Fig. 8). Reactions were stopped by addition of lysis buffer, immunoprecipitates were washed, and Raf-1 kinase activities were then determined by using kinase-inactive MEK as the substrate. Raf-1 kinase activity was increased approximately twofold by incubation with PKCα and three- to fourfold by PKCε, indicating that phosphorylation by both of these PKC isotypes stimulates Raf-1 kinase activity in vitro.

The ability of PKCε-activated Raf-1 to activate MEK was further demonstrated by using a coupled assay for ERK phosphorylation (Fig. 9). Purified recombinant PKCε was first incubated with immunoprecipitated Raf-1 in the presence of phosphatidylserine, TPA, and unlabeled ATP as described above. Immunoprecipitates were then washed, and Raf-1 kinase activities were determined by incubation with MEK and kinase-inactive ERK (39). Incubation of Raf-1 with purified PKCε resulted in approximately a twofold increase in ERK phosphorylation, indicating that phosphorylation by PKCε-activated Raf-1 stimulated MEK activity. Interestingly, activated Raf-1 prepared from Sf9 cells that were coinfected with Ras and Lck recombinant viruses (designated C*-Raf) was further stimulated by PKCε, suggesting that phosphorylation of Raf-1 by PKCε may act synergistically with phosphorylation by the Lck protein-tyrosine kinase.

Finally, we determined the effects of mutations of serines 499 and 259 on Raf-1 activation by purified PKCε (Fig. 10). Mutation of serine 499 to alanine (S499A) did not significantly affect the basal level of Raf-1 activity but prevented activation by PKCε. Mutation of serine 259 to aspartic acid (S259D) activated Raf-1 kinase approximately fourfold and prevented further activation by PKCε. These effects are similar to those observed for activation of Raf-1 by PKCε in Sf9 cells (Fig. 7) as well as to results of previous studies of Raf-1 activation by PKCα (26).

DISCUSSION

In this study, we have investigated the role of the nonclassical PKC isotype PKCε in activation of the Raf-1 protein kinase. The possibility that PKCε can serve to activate Raf-1 was suggested by previous studies showing that Raf-1 could be activated as a result of PC hydrolysis, which yields the second messenger diacylglycerol (5, 10). In addition, the classical PKC isotype PKCα has been shown to be capable of phosphorylating and activating Raf-1 in vitro (19, 26, 40). However, a number of experiments indicate that the activation of Raf-1

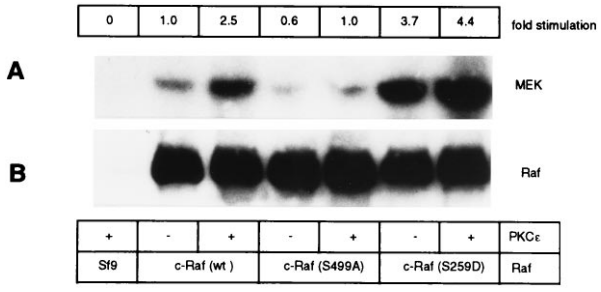

FIG. 10. Activation of Raf-1 mutants by PKCε in vitro. Wild-type (wt) or mutant (S499A and S259D) Raf-1 proteins were immunoprecipitated from singly infected Sf9 cells and incubated with purified PKCε as for Fig. 8. MEK phosphorylation (A) was analyzed after the samples were resolved by SDS-PAGE. Blots were immunostained with anti-Raf sera and the ECL detection system to compare amounts of precipitated Raf proteins (B).

downstream of growth factor receptors is not inhibited by downregulation of PKCα with phorbol esters (1, 7, 35). Consequently, PKCα does not appear to be generally required for Raf-1 activation in growth factor-stimulated cells.

Importantly, the mitogenic activity of PC hydrolysis is also resistant to phorbol ester downregulation of PKCα (29), leading us to pursue the potential involvement of PKCε, which is activated by PC-derived diacylglycerol (18), in cell proliferation and Raf-1 activation. Consistent with previous results (20), PKCε was resistant to treatment of NIH 3T3 cells with phorbol esters under conditions that resulted in essentially complete downregulation of PKCα. Such phorbol ester downregulation did not inhibit Raf-1 activation in response to EGF stimulation, consistent with the possible role of PKCε, but not PKCα, as a Raf activator under these conditions.

The biological role of PKCε was then investigated by transfection of both NIH 3T3 and COS cells with dominant negative and constitutively activated PKCε mutants. Dominant negative PKCε inhibited NIH 3T3 cell proliferation, similar to dominant negative mutants of Ras and Raf, and blocked Raf activation in response to mitogenic stimulation in COS cell transient expression assays. Conversely, activated PKCε overcame a block to proliferation of NIH 3T3 cells resulting from expression of dominant negative Ras N17 and was sufficient to activate Raf-1 in COS cells in the absence of growth factor stimulation. Thus, PKCε appeared to function as a Raf-1 activator in mammalian cells.

In both NIH 3T3 and COS cell assays, PKCα behaved similarly to PKCε, consistent with the previously reported activity of PKCα as a Raf-1 activator in vitro (26, 40). In contrast, neither dominant negative nor constitutively active mutants of PKCζ, which does not activate Raf-1 in vitro (40), affected Raf-1 activation in mammalian cells. It is noteworthy that constitutively activated mutants of both PKCα and PKCε overcame the inhibitory effects of dominant negative mutants of the other PKC isotype, suggesting that PKCα and PKCε can function as redundant activators of Raf-1 in vivo.

The combined results for NIH 3T3 and COS cells indicate that PKCε is a component of the mitogenic signaling pathway leading to Raf-1 activation in response to growth factor stimulation. To determine whether this resulted from direct phosphorylation of Raf-1 by PKCε, we first coinfected insect cells with recombinant viruses expressing Raf-1 and PKCε or PKCα. As previously reported for PKCα, expression of PKCε was sufficient to activate Raf-1 in this system. These experiments were then extended by demonstrating that purified PKCε phosphorylated and activated Raf-1 in vitro, again with an activity comparable to that of PKCα.

The effects of PKCε mutants, combined with these in vitro results, indicate that activation of Raf-1 by PKCε plays a critical role in mitogenic signaling. Previous studies have led to the model that interaction with Ras recruits Raf-1 to the plasma membrane, where it can be activated by phosphorylation. One mechanism of Raf-1 activation appears to be phosphorylation on tyrosine residues (13, 32). However, mutant Raf-1 proteins lacking tyrosine phosphorylation sites are still activated in growth factor-stimulated cells, and so alternative mechanisms of Raf-1 activation must also exist (32). The present results indicate that phosphorylation of Raf-1 on serine residues by PKCα or PKCε is at least one such activation mechanism. In this regard, it is noteworthy that the kinase activity of Raf-1 that had already been activated by tyrosine phosphorylation was further stimulated by PKCε. Phosphorylation on tyrosine and serine residues may thus have synergistic effects on Raf-1 kinase activity in growth factor-stimulated cells.

The biological significance of Raf-1 phosphorylation by PKCε, which is stimulated by diacylglycerol produced by PC hydrolysis, is further indicated by the observations that both PC-PLC (2, 22) and PKCε (4, 33) can act as oncogenes, with their overexpression being sufficient to induce cell transformation. It is also noteworthy that PC hydrolysis provides a sustained source of diacylglycerol in growth factor-stimulated cells, compatible with the persistent signaling activity required for long-term responses such as mitogenesis (12, 38). Activation of Raf-1 via PC hydrolysis and PKCε phosphorylation may therefore play an important role in maintaining sustained activity of the Raf/MEK/ERK signaling pathway following growth factor stimulation.

ACKNOWLEDGMENTS

We are grateful to I. B. Weinstein for supplying the PKCε cDNA, to M. G. Kazanietz and P. M. Blumberg for providing the recombinant PKCα and PKCε baculoviruses, and to M. Schuler for protein purification.

This research was supported by NIH grant RO1 CA18689, by DFG grants TR 348/1-1 and SFB 176B19, and by a NATO collaborative research grant.

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