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Novel Roles of Specific Isoforms of Protein Kinase C in Activation of the c-*fos* Serum Response Element

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Protein kinase C (PKC) is a multigene family of enzymes consisting of at least 11 isoforms. It has been implicated in the induction of c-*fos* and other immediate response genes by various mitogens. The serum response element (SRE) in the c-*fos* promoter is necessary and sufficient for induction of transcription of c-*fos* by serum, growth factors, and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). It forms a complex with the ternary complex factor (TCF) and with a dimer of the serum response factor (SRF). TCF is the target of several signal transduction pathways and SRF is the target of the rhoA pathway. In this study we generated dominant-negative and constitutively active mutants of PKC-α, PKC-δ, PKC-ε, and PKC-ζ to determine the roles of individual isoforms of PKC in activation of the SRE. Transient-transfection assays with NIH 3T3 cells, using an SRE-driven luciferase reporter plasmid, indicated that PKC-α and PKC-ε, but not PKC-δ or PKC-ζ, mediate SRE activation. TPA-induced activation of the SRE was partially inhibited by dominant negative c-Raf, ERK1, or ERK2, and constitutively active mutants of PKC-α and PKC-ε activated the transactivation domain of Elk-1. TPA-induced activation of the SRE was also partially inhibited by a dominant-negative MEKK1. Furthermore, TPA treatment of serum-starved NIH 3T3 cells led to phosphorylation of SEK1, and constitutively active mutants of PKC-α and PKC-ε activated the transactivation domain of c-Jun, a major substrate of JNK. Constitutively active mutants of PKC-α and PKC-ε could also induce a mutant c-*fos* promoter which lacks the TCF binding site, and they also induce transactivation activity of the SRF. Furthermore, rhoA-mediated SRE activation was blocked by dominant negative mutants of PKC-α or PKC-ε. Taken together, these findings indicate that PKC-α and PKC-ε can enhance the activities of at least three signaling pathways that converge on the SRE: c-Raf–MEK1–ERK–TCF, MEKK1-SEK1-JNK-TCF, and rhoA-SRF. Thus, specific isoforms of PKC may play a role in integrating networks of signal transduction pathways that control gene expression.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases which can be activated upon external stimulation of cells by various ligands including growth factors, hormones, and neurotransmitters (7, 51). Specific isoforms of PKC can be activated by calcium, various phospholipids, diacylglycerol (DAG) generated from phospholipase C (PLC) or PLD, and fatty acids generated from PLA_2, depending on the PKC isoforms (7, 17, 32, 36). Molecular cloning has identified 11 distinct isoforms of PKC in mammalian cells. Based on their structure, these isoforms are divided into three groups: (i) classical PKCs (α, βI, βII, γ), which can be activated by DAG or calcium; (ii) novel PKCs (δ, ε, η, θ, μ), which can be activated by DAG but not by calcium; and (iii) atypical PKCs (ζ, ι), which are not responsive to either DAG or calcium. Each of these isoforms contain an amino-terminal regulatory domain and a carboxy-terminal catalytic kinase domain. A number of studies have shown that the activation of cellular PKC by the potent phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol-13-ester (TPA) induces the expression of several immediate response genes including c-*fos*. The specific signaling pathways involved in this process are not known with certainty. In addition, since most cell types contain multiple isoforms of PKC and since there are no isoform-specific inhibitors of PKC, it is not certain which isoforms of PKC mediate these responses.

With respect to the above issues, the induced expression of the immediate-early response gene c-*fos* is of particular interest since this induction is usually rapid, within 30 min, and transient in various cell types following exposure to TPA, various growth factors, neurotrophins, or neurotransmitters. Furthermore, the signal transduction pathways leading to activation of the c-*fos* promoter have been studied in great detail and have served as an excellent model for studying the biochemical mechanisms by which extracellular signals generated at the plasma membrane activate gene transcription (30, 31) (also see Fig. 6). The serum response element (SRE) in the c-*fos* promoter is necessary and sufficient for rapid induction of the c-*fos* gene by serum, growth factors and TPA (29, 61). Two transcription factors, serum response factor (SRF) and ternary complex factor (TCF), bind to the SRE and mediate transcriptional activation. SRF is a ubiquitously expressed transcription factor that binds as a dimer to the CArG box of the c-*fos* SRE. It is a 67-kDa protein with a central core that contains the DNA binding and dimerization domains. SRF also has a C-terminal transcriptional activation domain and an N-terminal domain that can be phosphorylated by casein kinase II (CKII) and Ca^{2+}/calmodulin-dependent kinase (CaMK) (40, 41, 47). SRF forms a ternary complex with TCF on the SRE. In this ternary complex, TCF interacts with both the dimerization domain of SRF and a purine-rich sequence (CAGGAT) at the 5′ end of the SRE. TCF interacts with the c-*fos* SRE only if the SRE is already occupied by SRF. TCF is encoded by a family

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of ets-related genes which includes the genes encoding Elk-1, SRF accessory protein 1 (SAP-1), and SAP-2. The TCFs have three conserved regions, referred to as the A, B, and C boxes. The A box is the N-terminal ets-related DNA binding domain. The B box is the SRF binding domain. The A and B boxes are necessary and sufficient for ternary-complex formation with SRF on the SRE. The C box is the C-terminal transcriptional activation domain and contains a cluster of serine residues. Phosphorylation of TCF causes increased DNA binding, ternary-complex formation, and transcriptional activation (43, 60). TCF is phosphorylated by at least three major mitogen-activated protein (MAP) kinases, including ERK1/2, JNK, and p38. Serum, growth factors, and TPA induce the phosphorylation of Elk-1/SAP-1 through the Raf-MEK-ERK pathway (19, 26), whereas interleukin-1, tumor necrosis factor alpha, osmotic stress, H_2O_2, UV radiation, or anisomycin induce phosphorylation of TCF through the MEKK-SEK1-JNK (12, 20, 59, 66) or TAK1-MKK3-p38 pathways (27, 55, 67, 70). Mutants with mutations in the SRE that cannot bind TCF are not responsive to these MAP kinase pathways but remain responsive to serum induction through a TCF-independent pathway that requires SRF (28). In the absence of TCF, SRF can also mediate transcriptional activation by the serum mitogen lysophosphatidic acid (LPA) and also by intracellular activation of heterotrimeric G proteins by aluminum fluoride ion (AlF_4^-) (23, 50, 54). Functional rhoA is required for serum-, LPA-, and AlF_4^--induced transcriptional activation of SRE by SRF, and two other small GTPase rho family members, rac1 and cdc42Hs, also potentiate SRF activity (24, 50).

The precise roles of individual isoforms of PKC in the above-described signaling pathways that lead to activation of the SRE have not been elucidated. This was the goal of the present study. Our strategy was to introduce an SRE-luciferase reporter into NIH 3T3 mouse fibroblasts and to coexpress wild-type or various mutant forms of specific isoforms of PKC and/or dominant negative forms of components of these pathways. We present evidence that in this cell system PKC-α and PKC-ε are the major isoforms of PKC that play a role in activation of the SRE and that these PKC isoforms activate not only TCF through both the ERK and JNK pathways but also SRF through the rhoA pathway.

MATERIALS AND METHODS

Plasmid construction. The luciferase reporter plasmids pSRE-luc and pFSS-luc were described previously (28). pGAL4RE-luciferase and pGAL4DB–c-Jun were provided by A. G. Minden (46). pMA424 was obtained from M. Ptashne. pGAL4DB-Elk1 was purchased from Stratagene. pFos-wt-luc contains the −355 to −297 region of the mouse c-*fos* promoter fused to the −53 to −45 region of the human c-*fos* promoter, and pFos-pm18-luc and pFos-pm12-luc are derivatives of pFos-wt-luc with a point mutation that abolishes TCF binding or SRF binding, respectively (63). pCGN-SMS was also described previously (28).

pHANE is a mammalian expression vector that contains a cytomegalovirus promoter, Kozak translational initiation sequence, ATG start codon, N-terminal HA epitope tag, *Eco*RI cloning site, and stop codon. It was generated by ligating annealed synthetic oligonucleotides (upper strand, 5′-GATCCTCGAGGCCAC CATGGCTTATCCTTACGACGTGCCTGACTACGCCGAATTCTAAGGA TCC-3′; lower strand, 5′-AATTGGATCCTTAGAATTCGGCGTAGTCAGGC ACGTCGTAAGGATAAGCCATGGTGGCCTCGAG-3′) into pcDNA3 (Invitrogen) after digestion with *Bam*HI and *Eco*RI. pHANE was used to generate PKC mutants with an N-terminal HA tag. pHACE is a mammalian expression vector that contains a cytomegalovirus promoter, Kozak translational initiation sequence, ATG start codon, *Eco*RI cloning site, C-terminal HA epitope tag, and stop codon. It was generated by ligating annealed synthetic oligonucleotides (upper strand, 5′-GATCCTCGAGGCCACCATGGAATTCTATCCTTACGA CGTGCCTGACTACGCCTAAGGATCC-3′; lower strand, 5′-AATTGGATCC TTAGGCGTAGTCAGGCACGTCGTAAGGATAGAATTCCATGGTGGCC TCGAG-3′) into pcDNA3 after digestion with *Bam*HI and *Eco*RI. pHACE was used to generate PKC mutants with a C-terminal HA tag.

pHACE-PKC-WT expression plasmids were generated by ligating full-length open reading frames of different PKC isoforms into pHACE digested with *Eco*RI. pHACE-PKC-KR expression plasmids were generated by ligating full-length open reading frames of PKC isoforms with a K→R point mutation at the ATP binding site into pHACE digested with *Eco*RI. pHANE-PKC-CAT expression plasmids were generated by ligating cDNA fragments encoding only the catalytic domains of PKC isoforms into pHANE digested with *Eco*RI. pHANE-PKC-CAT-KR expression plasmids were generated by ligating cDNA fragments encoding only the catalytic domains of PKC isoforms with a K→R point mutation at the ATP binding site into pHANE digested with *Eco*RI. All the cDNA fragments of PKC mutants were generated by PCR and were analyzed to confirm their sequences with an automated DNA sequencer (ABI).

Expression vectors encoding human PKC-α (2) or mouse PKC-ε (9) have been described previously. cDNA for mouse PKC-δ was a gift from J. F. Mushinski (48). cDNA for rat PKC-ζ was a gift from Y. Ono (53). pMCL-MEK1-dN3/S218E/S222D was a gift from N. G. Ahn (42). pcDNA3-Raf-K375M was constructed by subcloning the *Bam*HI fragment of c-Raf-1 cDNA with a K375→M point mutation (provided by D. Morrison [15]) into pcDNA3. pCEP4-MEKK1-D1369A was a gift from M. Cobb (69). pCMV-ERK1-K71R and pCMV-ERK2-K52R were gifts from P. Shaw (35). pEF-TAK1-K63W was a gift from K. Matsumoto (70). pCMV-rhoA-Q63L and pCMV-rhoA-T19N were gifts from

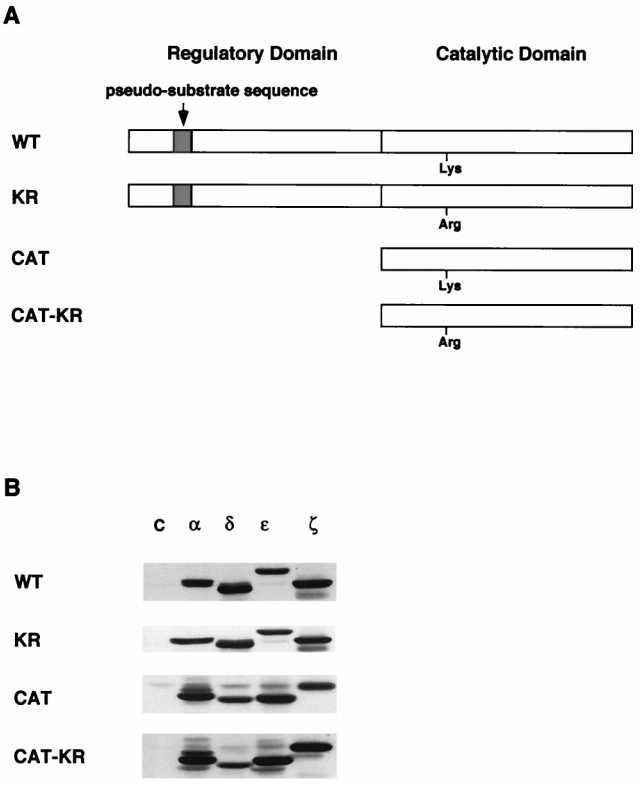

FIG. 1. Construction of dominant negative and constitutively active mutants of PKC-α, PKC-δ, PKC-ε, and PKC-ζ. (A) Structures of PKC mutants. PKC-WT constructs contain a full-length PKC open reading frame. Also shown are the pseudosubstrate sequence in the N-terminal regulatory domain and the essential lysine residue in the ATP binding region of the catalytic domain. PKC-KR constructs encode a full-length PKC with a point mutation that abolishes the ATP binding ability. PKC-CAT constructs encode a truncated protein in which the catalytic domain (CAT) of PKC is preserved and the regulatory N-terminal domain (REG) is deleted. PKC-CAT-KR constructs encode a catalytic domain of PKC with a point mutation that abolishes the ATP binding ability. PKC-WT and PKC-KR constructs were subcloned into the mammalian expression vector pHACE. PKC-CAT and PKC-CAT-KR constructs were subcloned into the mammalian expression vector pHANE. The PKC-δ–CAT construct was subcloned into the pHACE vector. (B) Western blot analysis of transiently expressed PKC wild-type and mutant constructs. An empty control vector (pcDNA3) or expression vectors containing PKC wild-type or mutant sequences (5 μg) were transiently transfected into COS-7 cells, and cell lysates were subjected to Western blot analysis with an anti-HA antibody. The apparent molecular masses of the corresponding proteins, based on the prestained molecular weight markers, were as follows: PKC-α–WT/KR, 82 kDa; PKC-δ–WT/KR, 76 kDa; PKC-ε–WT/KR, 90 kDa; PKC-ζ–WT/KR, 78 kDa; PKC-α–CAT/CAT-KR, 50 kDa; PKC-δ–CAT/CAT-KR, 47 kDa; PKC-ε–CAT/CAT-KR, 49 kDa; PKC-ζ–CAT/CAT-KR, 55 kDa. These values are consistent with the predicted sizes of these proteins.

J. S. Gutkind (13). pGEX-MARCKS was constructed by subcloning the cDNA fragment encoding amino acids 96 to 184 of murine MARCKS (provided by A. Aderem [57]) into the SmaI site of pGEX-3X (Pharmacia).

Transfection and reporter assays. NIH 3T3 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% calf serum. Triplicate samples of 10^5 cells in 35-mm plates were transfected with Lipofectin (Gibco BRL) with 1 μg of the reporter plasmid, 0.05 to 5 μg of various expression vectors, and 1 μg of pCMV–β-gal. pcDNA3 plasmid DNA was added to the transfections as needed to achieve the same total amount of plasmid DNA per transfection. At 6 h after transfection, the cells were fed with fresh medium (DMEM with 10% calf serum) and incubated overnight. The cells were then serum starved for 24 h in DMEM containing 0.5% calf serum. For TPA or LPA experiments, the cells were then treated with TPA (100 ng/ml; Sigma) or LPA (1 μg/ml; Sigma) for 3 h. Dimethyl sulfoxide (DMSO) (0.1%) was used as a solvent control. Cell extracts were then prepared, and luciferase assays were done with the luciferase assay system (Promega). Luciferase activities were normalized with respect to parallel β-galactosidase (β-gal) activities, to correct for differences in transfection efficiency. β-gal assays were performed with the β-galactosidase enzyme assay system (Promega).

Western blot analysis. NIH 3T3 cells were grown in DMEM containing 10% calf serum, and COS-7 cells were grown in DMEM containing 10% fetal bovine serum. With both cell types, 2×10^5 cells in 60-mm plates were transfected by Lipofectin (Gibco BRL) with 5 μg of the indicated expression vectors or the control vector pcDNA3. At 6 h after transfection, the cells were fed with DMEM containing 10% fetal bovine serum and incubated overnight. The cells were then trypsinized, transferred to 10-cm plates, and grown for 24 h before protein extraction. Cellular proteins were extracted by cell lysis in RIPA buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol) that contained protease inhibitors (10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate). Total-cell extracts (50-μg samples) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were then transferred to an Immobilon-P (Millipore) membrane at 60 V for 3 h at 4°C. The membranes were subsequently blocked with 5% dry milk in TBS-T (20 mM Tris HCl [pH 7.6], 137 mM NaCl, 0.05% Tween 20) and then probed with the indicated antibody. The immunoblots were visualized with the enhanced chemiluminescence Western blotting system (Amersham). The anti-HA antibody (Berkeley Antibody), anti-phospho-SEK1 antibody (New England Biolabs), and anti-SEK1 antibody (Santa Cruz) were used at a 1:1,000 dilution.

PKC assay. COS-7 cells were transfected with the indicated expression vectors or the control vector pcDNA3, as described above, and cellular proteins were extracted by cell lysis in PKC extraction buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol) that contained protease inhibitors (10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate). HA-tagged PKC proteins were immunoprecipitated from 300 μg of cell extracts with 3 μg of the anti-HA antibody and 30 μl of protein G-Sepharose, after a 3-h incubation at 4°C. The immunoprecipitates were washed twice with PKC extraction buffer and then twice with PKC reaction buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate) and resuspended in 20 μl of PKC reaction buffer. The kinase assay was initiated by adding 40 μl of PKC reaction buffer containing 10 μg of glutathione *S*-transferase (GST)-myristoylated alanine-rich C kinase substrate (MARCKS) and 5 μCi of [γ-^{32}P]ATP. The reactions were performed at 30°C for 30 min. The reactions were terminated by adding SDS sample buffer, and the mixtures were boiled for 5 min. The reaction products were analyzed by SDS-PAGE and autoradiography. Recombinant GST-MARCKS proteins were expressed in *Escherichia coli* BL21(DE3)/LysS and purified to homogeneity with glutathione S-Sepharose beads (Pharmacia).

RESULTS

Generation of dominant negative and constitutively active mutants of specific isoforms of PKC. Activation of the c-*fos* SRE by the PKC agonist TPA has been previously described by several investigators (21, 56, 61, 66). However, as discussed in the introduction, the presence in mammalian cells of multiple PKC isoforms within a single cell type and the absence of isoform-specific inhibitors of PKC or isoform-specific PKC mutants has obscured the roles of individual isoforms in signal transduction pathways that lead to activation of this SRE. Since the NIH 3T3 mouse fibroblast cell line has been well characterized with respect to the signal transduction pathways that lead to activation of several immediate-early response genes, including c-*fos*, c-*jun*, and c-*myc*, it was used as our model cell line to examine the roles of individual PKC isoforms in the c-*fos* SRE signal transduction pathways. Initial Western blot analyses showed that the NIH 3T3 cells used in our studies express at least four isoforms of PKC, namely, PKC-α, PKC-δ, PKC-ε, and PKC-ζ (data not shown). Our strategy was to generate dominant negative and constitutively active mutants of PKC-α, PKC-δ, PKC-ε, and PKC-ζ (for details, see Materials and Methods). The respective cDNAs were inserted into the mammalian expression vector pHANE or pHACE. PKC-WT constructs contained the full-length open reading frames of PKC-α, PKC-δ, PKC-ε, or PKC-ζ. PKC-KR constructs contained full-length open reading frames with a K→R point mutation in the ATP binding site. PKC-CAT constructs contained only the respective catalytic domains, with the inhibitory N-terminal domains deleted. PKC-CAT-KR constructs contained the catalytic domains with a K→R point mutation in the ATP binding site. The structures of these PKC mutants are diagrammed in Fig. 1A, and the amino acid substituents in the mutants of individual PKC isoforms are summarized in Table 1. The expression vectors for PKC-WT, PKC-KR, PKC-CAT, or PKC-CAT-KR were transfected into COS-7 cells to verify that they expressed the predicted protein, by Western blot analyses. Figure 1B demonstrates that all of the constructs expressed the corresponding protein at comparable levels and that each of these proteins was of the expected size.

PKC-α and PKC-ε can activate the SRE. The roles of specific isoforms of PKC in activation of the c-*fos* SRE were studied by using transient-transfection reporter assays. NIH 3T3 mouse fibroblasts were transfected with the pSRE-luciferase reporter plasmid. The cells were serum starved for 24 h and then treated with either 0.1% DMSO (solvent control) or the specific PKC inhibitor Ro31-8220 (0.1 μM) or CGP41-251 (0.1 μM) for 3 h. They were then treated with either DMSO (0.1%) or TPA (100 ng/ml) for 3 h. Luciferase assays (Fig. 2A) showed that TPA markedly induced the expression of the pSRE-luciferase reporter, by about 10-fold, and that this induction was blocked by the PKC inhibitors. These experiments

TABLE 1. Coding sequences of the PKC mutants[a]

Mutant	Sequence in isoform:			
	PKC-α	PKC-δ	PKC-ε	PKC-ζ
WT	2–672	2–674	2–737	2–592
KR	2–672 (K368R)	2–674 (K376R)	2–737 (K437R)	2–592 (K281R)
CAT	326–672	334–674	395–737	239–592
CAT-KR	326–672 (K368R)	334–674 (K376R)	395–737 (K437R)	239–592 (K281R)

[a] Numbers represent the first and last amino acid residues encoded by the indicated PKC mutants. Point-mutated amino acid residues are indicated in parentheses.

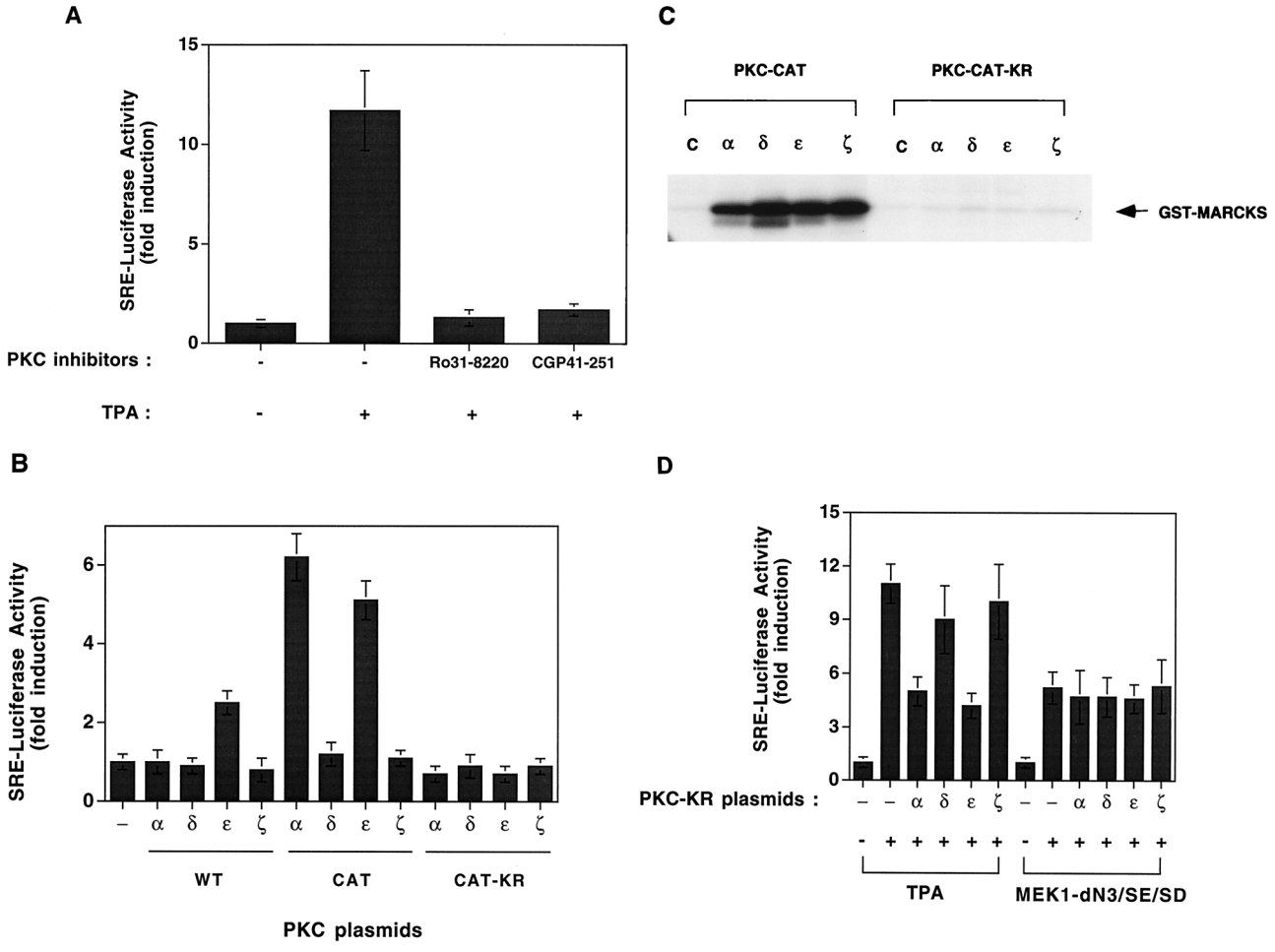

FIG. 2. PKC-α and PKC-ε activate the SRE. (A) NIH 3T3 cells were transfected with the pSRE-luciferase reporter plasmid (1 μg), which has a copy of the SRE followed by the c-*fos* TATA box and the firefly luciferase gene. The cells were then serum starved for 24 h and treated with either DMSO (0.1%) or the PKC inhibitor Ro31-8220 (0.1 μM) or CGP41-251 (0.1 μM) for 3 h. The cells were then treated with either DMSO (0.1%) or TPA (100 ng/ml) for 3 h. Cell extracts were prepared, and luciferase activities were measured and normalized with respect to β-galactosidase activities. (B) NIH 3T3 cells were transfected with the pSRE-luciferase reporter plasmid (1 μg) together with either the empty control vector (pcDNA3) or an expression vector (50 to 500 ng depending on the constructs) encoding various PKC sequences (PKC-WT, PKC-CAT, or PKC-CAT-KR), as indicated in the figure. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vector. The cells were then serum starved for 24 h and assayed for luciferase activities. (C) COS-7 cells were transfected with the indicated expression vectors or the control vector pcDNA3, and cellular proteins were extracted by cell lysis in PKC extraction buffer. HA-tagged PKC proteins were immunoprecipitated from 300 μg of cell extracts by using 3 μg of an anti-HA antibody and 30 μl of protein G-Sepharose, after a 3-h incubation at 4°C. Immune complex kinase reactions were performed at 30°C for 30 min in the presence of 10 μg of the GST-MARCKS substrate and 5 μCi of [γ-^{32}P]ATP. The reaction products were then analyzed by SDS-PAGE and autoradiography. The apparent molecular mass of the recombinant GST-MARCKS protein was about 50 kDa. For additional details, see Materials and Methods. (D) For TPA experiments, NIH 3T3 cells were transfected with the pSRE-luciferase reporter plasmid (1 μg) together with either the empty control vector (pcDNA3) or the indicated pHACE-PKC-KR vector (2 to 5 μg depending on the constructs). The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were serum starved for 24 h and then treated with either DMSO (0.1%) or TPA (100 ng/ml) for 3 h and assayed for luciferase activity. For MEK1-dN3/SE/SD experiments, NIH 3T3 cells were transfected with either the empty control vector (pcDNA3) or pMCL-MEK1-dN3/S218E/S222D (0.5 μg) together with the pSRE-luciferase plasmid (1 μg). The empty control vector (pcDNA3) or the indicated pHACE-PKC-KR vector (2-5 μg depending on the constructs) was also cotransfected with the reporter plasmid. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were then serum starved for 24 hours and assayed for luciferase activity. For all experiments, the data shown are representative of at least three independent experiments with each assay done in triplicate. The error bars indicate the standard deviations. Luciferase activities are expressed as fold induction after correction for β-galactosidase activities. For additional details, see Materials and Methods.

provide evidence that in this assay TPA can activate the SRE through the action of PKC.

We then used the various PKC mutant constructs to examine their ability to mediate SRE activation in the absence of TPA. NIH 3T3 cells were transfected with the control plasmid, PKC-WT, PKC-CAT, or PKC-CAT-KR construct together with the pSRE-luciferase reporter, serum starved, and assayed for luciferase activity. Among the four PKC-WT constructs, only PKC-ε–WT was able to activate the SRE reporter (by about threefold) in the absence of TPA (Fig. 2B). However, when we transfected the PKC-CAT constructs, which are constitutively active since they lack the inhibitory regulatory domains, the PKC-α–CAT and PKC-ε–CAT mutants caused a six- and five-fold activation, respectively, of the SRE reporter (Fig. 2B). The results shown in Fig. 2B were obtained with optimal amounts of each plasmid. The amount of each plasmid that showed maximal activation was determined by concentration studies with 50 to 500 ng of plasmid DNA per transfection (data not shown). None of the PKC-CAT-KR mutants could activate the SRE reporter, indicating that the kinase activities of the PKC-α–CAT and PKC-ε constructs are required for SRE activation. To be certain that all of the PKC-CAT mu-

tants retained their functional integrity, we performed in vitro kinase assays to examine the kinase activities. For use as a PKC-specific in vitro substrate, we generated a bacterial expression vector which encodes a GST-MARCKS fusion protein. MARCKS is a filamentous actin-cross-linking protein that appears to function as an integrator of PKC and Ca^{2+}/calmodulin signals in the regulation of actin-membrane interactions and is phosphorylated by several PKC isoforms (1). Our GST-MARCKS construct contained the GST protein fused to the central domain (amino acids 96 to 184) of MARCKS, which contains three PKC phosphorylation sites (S152, S156, and S163). COS-7 cells were transiently transfected with either the control vector pcDNA3, PKC-CAT, or PKC-CAT-KR constructs, and the expressed PKC mutant proteins were then immunoprecipitated from the cell extracts with anti-HA antibodies. In vitro kinase assays of these immunoprecipitates, using GST-MARCKS as the substrate (Fig. 2C), demonstrated that all of the PKC-CAT constructs but none of the PKC-CAT-KR constructs had kinase activities toward this substrate, even in the absence of the usual PKC cofactors. The GST-MARCKS protein proved to be a quite specific in vitro substrate for PKCs, because other kinases including c-Raf, ERK2, JNK1, RSK1, and RSK2 could not phosphorylate GST-MARCKS in similar immune complex kinase assays (data not shown).

To further confirm the roles of these isoforms of PKC in the SRE pathway, we used kinase-inactive PKC-KR constructs as dominant negative mutants in SRE reporter assays in which endogenous PKCs were activated by treating the cells with TPA. Figure 2D shows that SRE activation by TPA was partially inhibited by the PKC-α–KR or PKC-ε–KR mutant but not by the PKC-δ–KR or PKC-ζ–KR mutant. The amount of each plasmid that gave maximal inhibition was determined by titrating the amount of plasmid in the range of 2 to 5 μg (data not shown). The inhibition by these two PKC-KR mutants appears to be due to specific inhibition of the endogenous isoforms of PKC-α and PKC-ε rather than nonspecific toxicity, because none of the PKC-KR mutants inhibited activated MEK1 (MEK1-dN3/SE/SD)-induced SRE activation (Fig. 2D). MEK1-dN3/SE/SD is a constitutively active mutant of MEK1 that can activate ERK1 and ERK2 (42).

Taken together, these experiments provide evidence that PKC-α and PKC-ε constitute the major endogenous PKC isoforms in NIH 3T3 cells involved in signal transduction pathways that lead to activation of the c-*fos* SRE. The results obtained with the wild-type constructs in Fig. 1A suggest that the activity of PKC-α is more tightly inhibited by its N-terminal regulatory domain than is the case with PKC-ε.

PKC-α and PKC-ε can activate the SRE through the c-Raf–MEK1-ERK-TCF pathway. Previous studies provide indirect evidence that PKC plays a role in activation of the c-Raf–MEK1-ERK pathway by enhancing the phosphorylation and activation of c-Raf (10, 11, 34, 45, 58). Therefore, we investigated the role of the c-Raf–MEK1-ERK pathway in PKC-mediated SRE activation by using dominant negative mutants of c-Raf, ERK1, and ERK2. NIH 3T3 cells were transfected with the SRE-luciferase reporter plasmid together with a control plasmid (pcDNA3) or expression vectors encoding kinase-inactive c-Raf, ERK1, or ERK2. The cells were then serum starved and treated with TPA for 3 h. Luciferase assays (Fig. 3A) showed that SRE activation by TPA was partially inhibited (about 50%) by the dominant negative c-Raf (K375M), ERK1 (K71R), or ERK2 (K52R) plasmid, indicating that the c-Raf–ERK pathway is necessary for optimum TPA-mediated SRE activation. Even when we added much larger amounts of these dominant negative constructs, we also obtained only partial inhibition, suggesting that ERK-independent signaling pathways also play a role in TPA- and PKC-mediated SRE activation. In contrast, these dominant negative constructs of c-Raf, ERK1, or ERK2 blocked activation of the transactivation domain of Elk-1 by TPA, almost completely, in transient-transfection reporter assays, and transfection of wild-type ERK1 or ERK2 reversed the inhibitory effects of the dominant negative mutants, which indicates the specificity of these dominant negative mutants (data not shown).

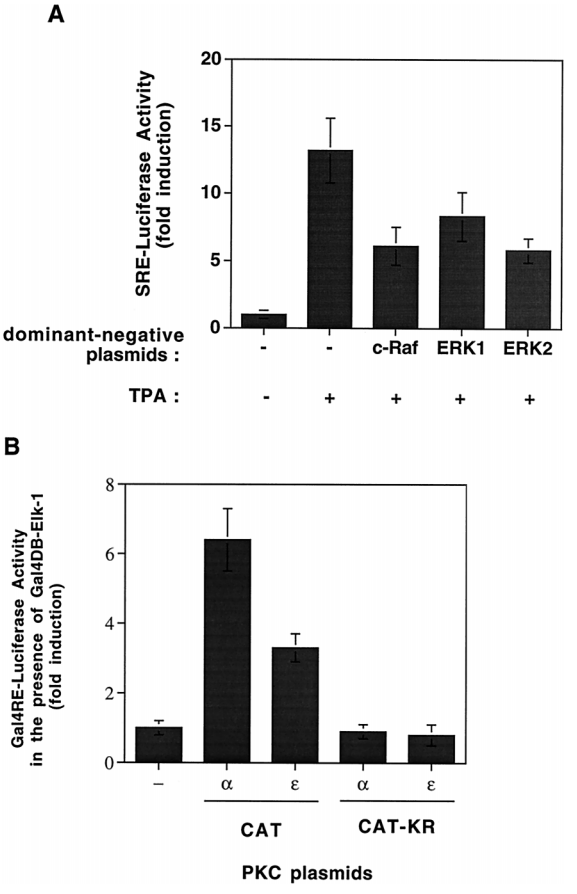

FIG. 3. PKC-α and PKC-ε activate the SRE through the c-Raf–MEK-ERK pathway. (A) NIH 3T3 cells were transfected with the pSRE-luciferase reporter plasmid (1 μg) together with either the empty control vector (pcDNA3) or one of the mammalian expression vectors (2 to 5 μg depending on the constructs) expressing dominant negative kinases (pcDNA3–c-Raf-K375M, pCMV-ERK1-K71R, or pCMV-ERK2-K52R), as indicated. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. Cells were serum starved for 24 h and then treated with either DMSO (0.1%) or TPA (100 ng/ml) and assayed for luciferase activity. (B) NIH 3T3 cells were transfected with the pGAL4RE-luciferase reporter plasmid (1 μg) and pGAL4DB–Elk-1 (50 ng). The empty control vector (pcDNA3), pHANE-PKC-α–CAT, pHANE-PKC-ε–CAT, pHANE-PKC-α–CAT-KR, or pHANE-PKC-ε–CAT-KR (50 to 500 ng) was also cotransfected with the reporter plasmid, as indicated. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were serum starved for 24 h and then assayed for luciferase activity. The data in this figure are representative of at least three independent experiments with all assays done in triplicate. The error bars indicate the standard deviations. The luciferase activities are presented as fold induction after correction for β-galactosidase activities.

Elk-1 is the major substrate of ERK1 and ERK2 in mammalian cells (19, 26). To test whether PKC-α or PKC-ε can activate Elk-1, NIH 3T3 cells were transfected with pGAL4RE-luc and pGAL4DB–Elk-1 plasmids, together with a control plasmid or PKC-CAT plasmids. The cells were serum

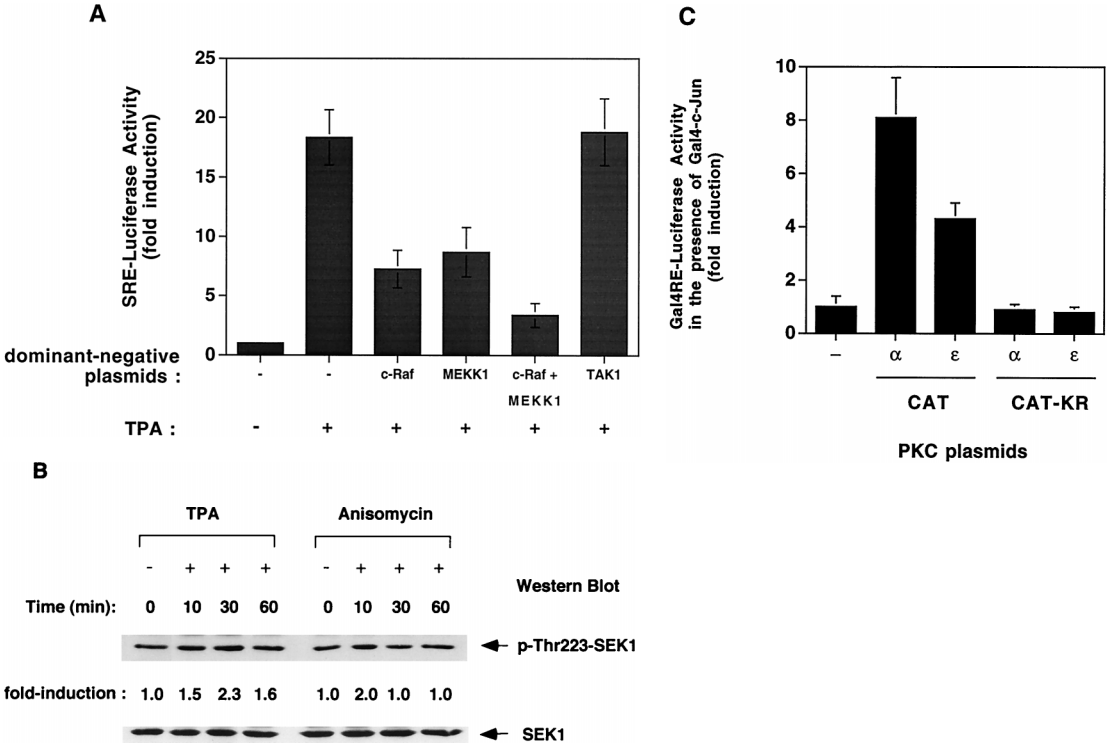

FIG. 4. PKC-α and PKC-ε also activate the SRE through the MEKK1-SEK1-JNK pathway. (A) NIH 3T3 cells were transfected with the pSRE-luciferase reporter plasmid (1 μg) together with either the empty control vector (pcDNA3) or pcDNA3–c-Raf-K375M, pCEP4-MEKK1-D1369A, pEF-TAK1-K63W (2 to 5 μg depending on the constructs), or both pcDNA3–c-Raf-K375M and pCEP4-MEKK1-D1369A (2 μg each), as indicated. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were serum starved for 24 h and then treated with either DMSO (0.1%) or TPA (100 ng/ml) for 3 h and assayed for luciferase activity. (B) NIH 3T3 cells were serum starved for 24 h and then treated with either TPA (100 ng/ml) or anisomycin (100 ng/ml) for 10, 30, or 60 min as indicated. Whole-cell extracts were prepared and subjected to Western blotting with an anti-phospho-Thr223-specific SEK1 antibody (top) or an anti-SEK1 antibody (bottom). (C) NIH 3T3 cells were transfected with the pGAL4RE-luciferase reporter plasmid (1 μg) and pGAL4DB–c-Jun. The empty control vector (pcDNA3), pHANE-PKC-α–CAT, pHANE-PKC-ε–CAT, pHANE-PKC-α–CAT-KR, or pHANE-PKC-ε–CAT-KR (50 to 500 ng) was cotransfected with the reporter plasmid, as indicated. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were serum starved for 24 h and then assayed for luciferase activity. Data shown here are representative of at least three independent experiments with all assays done in triplicate. The error bars indicate the standard deviations. The luciferase activities are presented as fold induction after correction for β-galactosidase activities.

starved for 24 h and then assayed for luciferase activity. pGAL4RE-luc is a luciferase reporter plasmid containing five copies of the GAL4 response element, and pGAL4DB–Elk-1 is an expression vector that encodes a fusion protein with a N-terminal GAL4 DNA binding domain and a C-terminal Elk-1 transactivation domain. Activation, by phosphorylation, of the Elk-1 transactivation domain of the GAL4DB–Elk-1 fusion protein, activates transcription of the GAL4RE-driven luciferase gene. Luciferase assays (Fig. 3B) showed that the transactivation domain of Elk-1 can be activated by either PKC-α–CAT (about sixfold) or PKC-ε–CAT (about threefold), but not by PKC-α–CAT-KR or PKC-ε–CAT-KR. Neither the PKC-δ–CAT nor PKC-ζ–CAT construct could activate this reporter (data not shown). pMA424, an expression vector containing only the GAL4 DNA binding domain, did not activate transcription of the GAL4RE-driven luciferase gene in the presence of either PKC-α–CAT or PKC-ε–CAT (data not shown), demonstrating the requirement in the above assay for the activated Elk-1 protein. These data provide evidence that PKC-α and PKC-ε, but not PKC-δ or PKC-ζ, can activate the c-Raf–MEK-ERK-TCF pathway.

PKC-α and PKC-ε can also activate the SRE through the MEKK1-SEK1-JNK-TCF pathway. At least three major MAP kinase pathways have been identified in mammalian cells; c-Raf–MEK-ERK, MEKK1-SEK1-JNK, and TAK1-MKK-p38 (30, 31, 70). All three pathways activate the SRE through phosphorylation of Elk-1 and SAP-1. Our finding that TPA-induced SRE activation was only partially inhibited by dominant negative c-Raf, ERK1, or ERK2 constructs (Fig. 3A) led us to investigate the roles of the JNK and p38 pathways in the PKC signaling pathway that leads to SRE activation. It is known that JNK and p38 can phosphorylate and activate Elk-1 and SAP-1 when cells are exposed to various environmental stimuli including osmotic stress and UV radiation (12, 20, 67). We first examined the effects of dominant negative MAPKKK (MAP kinase kinase kinase) plasmids on TPA-mediated SRE activation. Expression vectors for kinase-inactive c-Raf (K375M) (15), MEKK1 (D1369A) (69), or TAK1 (K63W) (70) were transfected into NIH 3T3 cells together with the SRE-luciferase reporter plasmid. The cells were serum starved and then treated with TPA for 3 h. Luciferase assays (Fig. 4A) showed that dominant negative MEKK1 and dominant negative c-Raf but not dominant negative TAK1 inhibited (by about 50%) TPA-induced SRE activation. Detectable amounts of proteins from the dominant negative c-Raf, MEKK1, and TAK1 constructs were expressed in NIH 3T3 cells (data not shown). Cotransfection of dominant negative c-Raf and MEKK1 further inhibited this activation (by about 80%). Transfection of larger amounts of the expression vectors for either dominant negative c-Raf or dominant negative MEKK1 did not cause further inhibition (data not shown). These findings suggest that optimum TPA-induced SRE activation re-

quires not only activation of c-Raf but also activation of MEKK1.

We then tested whether SEK1, the kinase downstream of MEKK1, could be activated by TPA. NIH 3T3 cells were serum starved for 24 h and then treated with TPA (100 ng/ml) for 10 to 60 min. Activation of SEK1 occurs through phosphorylation of two residues of this protein, Ser219 and Thr223, by MEKK. Western blot analysis with a phospho-Thr223-specific SEK1 antibody showed that treatment of the cells with TPA induced increased the phosphorylation of SEK1, within 10 min, without changing the total cellular level of the endogenous SEK1 protein (Fig. 4B). Anisomycin (100 ng/ml) was used as a positive control for activation of SEK1, and it also induced increased phosphorylation of SEK1. The fold induction was measured by densitometry and is indicated in Fig. 4B. Treatment of the cells with only the DMSO solvent did not induce phosphorylation of SEK1 (data not shown). We also used phospho-MKK3 and phospho-p38 specific antibodies to examine the effect of TPA on the TAK1-MKK3-p38 pathway, but we could not detect any changes in the levels of phosphorylation of these proteins (data not shown).

The role of JNK in PKC signaling was further studied in reporter assays by examining the transactivation of c-Jun, since c-Jun is the major substrate of JNK in mammalian cells. NIH 3T3 cells were transfected with pGAL4RE-luc and pGAL4DB–c-Jun constructs, together with a control plasmid or PKC-CAT plasmid. The cells were serum starved for 24 h and then assayed for luciferase activity. pGAL4DB–c-Jun is an expression vector that encodes a fusion protein with a C-terminal GAL4 DNA binding domain and a N-terminal c-Jun transactivation domain. Luciferase assays (Fig. 4C) showed that the transactivation domain of c-Jun could be activated by either PKC-α–CAT (about eightfold) or PKC-ε–CAT (about fourfold) but not by PKC-α–CAT-KR or PKC-ε–CAT-KR. Neither PKC-δ–CAT nor PKC-ζ–CAT constructs could activate this reporter (data not shown). These data provide evidence that PKC-α and PKC-ε, but not PKC-δ or PKC-ζ, can activate the MEKK1-SEK1-JNK-TCF pathway in NIH 3T3 cells.

PKC-α and PKC-ε can also activate the SRE through the rhoA-SRF pathway. The small GTPase protein rhoA is required for serum-, LPA-, and AlF_4^--induced SRF activation (23, 24, 50). To test the hypothesis that SRF activation may also contribute to activation of the SRE by specific PKC isoforms, we examined the ability of TPA or the constitutively active mutants of PKC to activate the SRE in the absence of TCF binding. pFos-wt-luc is a reporter plasmid containing positions −355 to −297 of the truncated mouse c-*fos* promoter with a wild-type SRE sequence. pFos-pm18-luc is a derivative of pFos-wt-luc plasmid and has a mutation that abolishes TCF binding, but SRF binding ability is still retained (21). pFos-pm12-luc is also a derivative of pFos-wt-luc plasmid and has a mutation that abolishes SRF binding (22) and therefore also TCF binding (63). NIH 3T3 cells were transfected with either pFos-wt-luc, pFos-pm18-luc, or pFos-pm12-luc and starved for 24 h. The cells were then treated with either DMSO (0.1%), TPA (100 ng/ml), or LPA (1 μg/ml) for 3 h and assayed for luciferase activity. LPA was previously shown to activate the SRE by activating rhoA in a TCF-independent manner (23, 24, 50). The truncated wild-type c-*fos* promoter was strongly activated by either TPA (about 14-fold) or LPA (about 10-fold), whereas the pm12 mutant c-*fos* promoter which lacks the SRF binding site was not activated by either TPA or LPA (Fig. 5A). These data indicate that activation of the truncated wild-type c-*fos* promoter by either TPA or LPA was due specifically to activation of the SRE in this promoter. The pm18 mutant c-*fos* promoter, which lacks a TCF binding site, was activated not only by LPA (by about eightfold) but also by TPA (by about fivefold) (Fig. 5A). We then performed similar experiments with constitutively active mutants of PKC (PKC-α–CAT or PKC-ε–CAT) instead of TPA and constitutively active rhoA (rhoA-Q63L) instead of LPA. The pCMV-rhoA-Q63L construct encodes a constitutively active rhoA protein that maintains the GTP-bound state (13). NIH 3T3 cells were transfected with either pFos-wt-luc, pFos-pm18-luc, or pFos-pm12-luc, together with either a control plasmid, the PKC-CAT plasmids, or pCMV-rhoA-Q63L. The cells were then starved for 24 h and assayed for luciferase activity. The truncated wild-type c-*fos* promoter was activated strongly by rhoA-Q63L (about 11-fold) and less strongly by either PKC-α–CAT (about 4-fold) or PKC-ε–CAT (about 5-fold) (Fig. 5B). In view of the stronger induction of this c-*fos* promoter by TPA than by LPA (Fig. 5A), the weaker induction of the c-*fos* promoter by the individual PKC isoforms, compared to that obtained with rhoA, suggests that both endogenous PKC-α and PKC-ε are responsible, in combination, for the TPA effect. The pm18 mutant c-*fos* promoter, which lacks the TCF binding site, was activated strongly by rhoA-QL (about 10-fold) but only modestly by either PKC-α–CAT (about 3-fold) or PKC-ε–CAT (about 4-fold) (Fig. 5B). The pm12 mutant c-*fos* promoter, which lacks the SRF binding site, was not activated by either PKC-α–CAT, PKC-ε–CAT, or rhoA-Q63L. PKC-δ–CAT and PKC-ζ–CAT constructs did not activate any of these c-*fos* promoter constructs (data not shown). Taken together, these data demonstrate that PKC-α and PKC-ε can activate the SRF independently of activation of TCF.

We then examined the ability of the constitutively active mutants of PKC to directly activate SRF in transient-transfection assays. NIH 3T3 cells were transfected with the pFSS-luc and pCGN-SMS constructs together with either a control plasmid, the PKC-CAT plasmids, or pCMV-rhoA-Q63L plasmid. The cells were serum starved for 24 h and then assayed for luciferase activity. pFSS-luc is a reporter plasmid containing a mutated version of the SRE sequence (termed FSS) designed to bind poorly to SRF but strongly to MCM1, the yeast homolog of SRF (28, 68). pCGN-SMS is an expression vector containing a mutant SRF (termed SMS) that has DNA binding specificity for MCM1 (28). Thus, the pFSS-luc reporter plasmid is responsive to a transfected SRF but is not affected by the endogenous SRF protein, even when it is present at high levels. Luciferase assays (Fig. 5C) showed that either PKC-α–CAT (about 3-fold) or PKC-ε–CAT (about 6-fold), as well as rhoA-Q63L (about 11-fold), could activate the transfected SRF but that PKC-α–CAT-KR and PKC-ε–CAT-KR were inactive. The PKC-α–CAT or PKC-ε–CAT construct did not activate this reporter in the absence of the transfected SRF and also did not cause induction of SMS expression (data not shown). The expression of SMS had a minimal effect on a reporter lacking the FSS sequence (28). PKC-δ–CAT and PKC-ζ–CAT constructs did not activate this reporter in the presence or absence of the transfected SRF (data not shown). These data, together with the previous data (Fig. 5A and B), demonstrate that PKC-α and PKC-ε can activate SRF.

To test the hypothesis that specific isoforms of PKC might also act upstream of rhoA, we examined whether an expression vector containing a dominant negative rhoA (pCMV-rhoA-T19N) inhibited PKC-induced SRE activation. NIH 3T3 cells were transfected with the pSRE-luciferase reporter together with 0, 1, or 5 μg of pCMV-rhoA-T19N. The cells were then serum starved for 24 h, treated with TPA (100 ng/ml) for 3 h, and assayed for luciferase activity. TPA-induced SRE activation was not inhibited by the dominant negative rhoA (Fig.

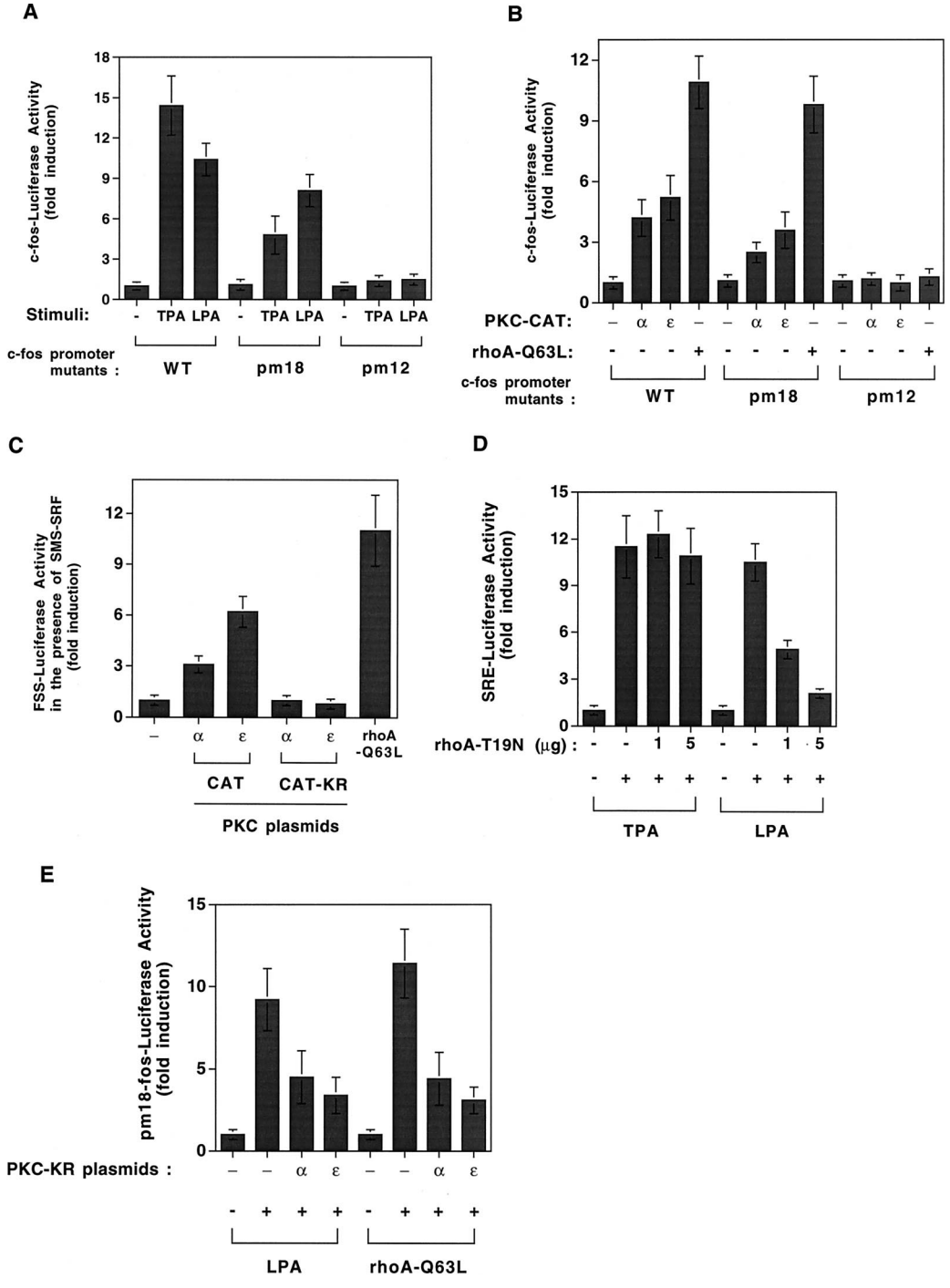

FIG. 5. PKC-α and PKC-ε also activate the SRE through the rhoA pathway. (A) NIH 3T3 cells were transfected with either pFos-wt-luc, pFos-pm18-luc, or pFos-pm12-luc (1 μg) as indicated and serum starved for 24 h. The cells were then treated with either DMSO (0.1%), TPA (100 ng/ml), or L-α-oleoyl-LPA (1 μg/ml) for 3 h and assayed for luciferase activity. (B) NIH 3T3 cells were transfected with either pFos-wt-luc, pFos-pm18-luc, or pFos-pm12-luc (1 μg) as indicated, together with either a control plasmid (pcDNA3), the PKC-CAT plasmids, or pCMV-rhoA-Q63L (50 to 500 ng) as indicated. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were then serum starved for 24 h and assayed for luciferase activity. (C) NIH 3T3 cells were transfected with pFSS-luc (1 μg) and pCGN-SMS (50 ng). The empty control vector (pcDNA3), pHANE-PKC-α–CAT, pHANE-PKC-ε–CAT, pHANE-PKC-α–CAT-KR, pHANE-PKC-ε–CAT-KR, or pCMV-rhoA-Q63L (50 to 500 ng) was cotransfected with the reporter plasmid, as indicated. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were serum starved for 24 h and assayed for luciferase activity. (D) NIH 3T3 cells were transfected with the pSRE-luciferase reporter plasmid (1 μg) together with either the empty control vector (pcDNA3) or pCMV-rhoA-T19N (1 or 5 μg), as indicated. The total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. The cells were serum starved for 24 h, treated with either DMSO (0.1%), TPA (100 ng/ml), or L-α-oleoyl-LPA (1 μg/ml) for 3 h, and assayed for luciferase activity. (E) For LPA experiments, NIH 3T3 cells were transfected with either a control plasmid (pcDNA3) or the PKC-KR constructs (2 to 5 μg depending on the constructs), together with the pFos-pm18-luc plasmid (1 μg). The cells were serum starved for 24 h, treated with either DMSO (0.1%) or LPA (1 μg/ml) for 3 h, and assayed for luciferase activity. For rhoA-Q63L experiments, NIH 3T3 cells were transfected with either the empty control vector (pcDNA3) or pCMV-rhoA-Q63L (0.5 μg) together with the pFos-pm18-luc plasmid (1 μg). The empty control vector (pcDNA3), pHACE-PKC-α-KR, or pHACE-ε-KR (2 to 5 μg depending on the constructs) was also cotransfected with the reporter plasmid, as indicated. Total amounts of transfected plasmid DNA were kept constant by addition of empty control vectors. Cells were then serum starved for 24 h and assayed for luciferase activity.

5D), suggesting that rhoA is not required for PKC-mediated SRE activation. In contrast, when the same experiment was performed with LPA (1 μg/ml) instead of TPA as the inducer, dominant negative rhoA was able to markedly inhibit LPA-mediated SRE activation (Fig. 5D).

We then tested the possibility that specific isoforms of PKC are downstream effectors of rhoA by using dominant negative mutants of the specific isoforms of PKC. NIH 3T3 cells were transfected with either a control plasmid or the PKC-KR constructs, together with the pFos-pm18-luc reporter. The cells were serum starved for 24 h, treated with either DMSO (0.1%) or LPA (1 μg/ml) for 3 h, and assayed for luciferase activity. As expected, LPA strongly activated the pm18 mutant c-*fos* promoter, which lacks the TCF binding site (Fig. 5E). Cotransfection with the dominant negative PKC-α–KR or PKC-ε–KR construct markedly inhibited the effect of LPA, by about 50 and 70%, respectively (Fig. 5E). Similar experiments with rhoA-Q63L instead of LPA showed that the dominant negative PKC-α–KR or PKC-ε–KR construct markedly inhibited the effect of rhoA-Q63L, by about 70 and 80%, respectively (Fig. 5E). Similar results were obtained with either pSRE-luciferase reporter or pCGN-SMS/pFSS-luciferase reporter assays (data not shown). These data provide evidence that both PKC-α and PKC-ε are required for optimal rhoA-mediated activation of the SRE pathway.

Taken together, our data suggest that the rhoA-mediated pathway of activation of the SRE requires functional PKC-α and PKC-ε for optimal activity. However, PKC-α or PKC-ε may not be sufficient for activation of the SRF by rhoA, because the level of activation of the SRE (Fig. 5A and B) or SRF (Fig. 5C) by PKC was significantly lower than that obtained with rhoA, suggesting the importance of other rhoA effector proteins such as PKC or PRK2.

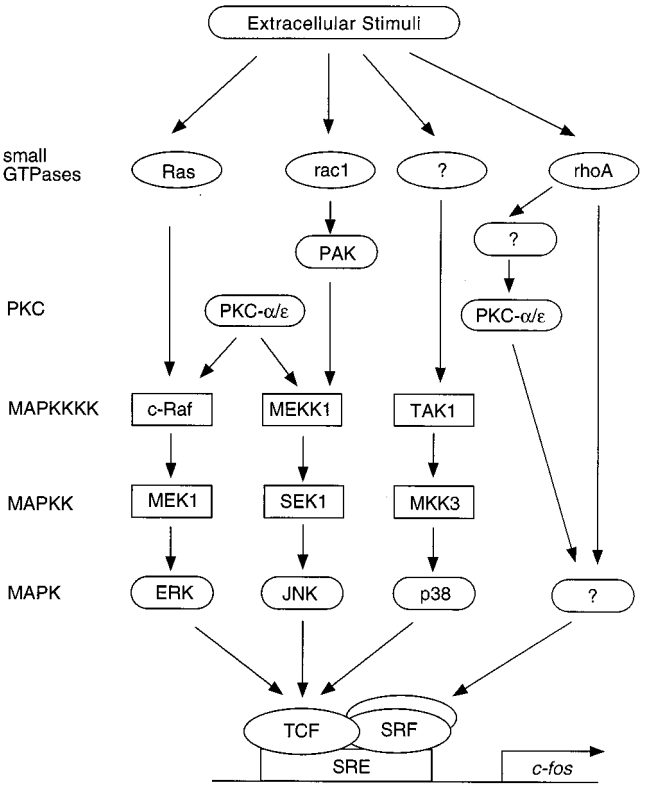

FIG. 6. Proposed signal transduction pathways involved in PKC-mediated SRE activation. Various external stimuli can lead to activation of the three indicated MAP kinase (MAPK) pathways (p38, ERK, and JNK) and also the rhoA pathway. These, in turn, lead to activation of the transcription factors TCF and SRF, which form a ternary complex with the SRE promoter element of the c-*fos* gene. The data presented in this paper provide evidence that PKC-α and PKC-ε play important roles in both the c-Raf-MEK1-ERK and MEKK1-SEK1-JNK pathways, leading to activation of the TCF transcription factor. PKC-α and PKC-ε also appear to act downstream of rhoA in the pathway leading to activation of the SRF transcription factor. The precise mechanism by which PKC-α and PKC-ε activate c-Raf and MEKK1 is not known, nor are the intermediate steps in the rhoA pathway.

DISCUSSION

As described in the introduction, remarkable progress has recently been made in elucidating the details of the signal transduction pathways and transcription factors that control the transcription of the immediate-early response gene c-*fos*. There is also indirect evidence that PKC plays a role in this process, but it was not known with certainty which isoforms of PKC are involved and at what level they interact with these complex pathways. Indeed, studies on the specific cellular effects of individual isoforms have, in general, been hampered by several factors, including the fact that individual cells often express several isoforms of PKC, the PKC activator TPA can activate all of the isoforms of PKC except PKC-ζ and PKC-ι, and isoform-specific inhibitors of PKC are not yet available. Our laboratory and other groups have previously addressed the roles of specific isoforms of PKC in growth control and oncogenic transformation by stably overexpressing individual isoforms of PKC in rodent fibroblasts. With Rat6 fibroblasts, we found that cells overexpressing PKC-ε displayed oncogenic transformation, apparently due to the activation of c-Raf (9, 10). Stable overexpression of PKC-βI in the same cell line resulted in partial transformation (25), while stable overexpression of PKC-α did not enhance growth (8). Results consistent with these findings were obtained by other investigators with NIH 3T3 fibroblasts. These and other results (8, 9, 25, 51) established the principle that individual isoforms of PKC can exert very different biological effects, even in the same cell type. However, this approach does not readily lend itself to an analysis of how individual isoforms of PKC directly affect the expression of a single gene like c-*fos*.

Therefore, in the present study, we adopted an alternative strategy. We first generated mammalian expression vectors that could be used to transiently overexpress either wild-type, constitutively active mutants, or dominant negative mutants of PKC-α, PKC-δ, PKC-ε, or PKC-ζ and used these constructs in transient-transfection assays to address the question of the specificity of PKC isoforms in SRE activation pathways, using a SRE-luciferase reporter construct in NIH 3T3 mouse fibroblasts. The SRE activation pathway plays a major role in controlling the expression of c-*fos* and provides a very good model system to study the role of PKC isoforms in signal transduction pathways, because at least four different signaling pathways converge on the SRE: (i) c-Raf–MEK1-ERK-TCF, (ii) MEKK1-SEK1-JNK-TCF, (iii) TAK1-MKK3-p38-TCF, and (iv) rhoA-SRF (Fig. 6). We found that constitutively active forms of PKC-α or PKC-ε can activate the SRE in the absence of TPA and that dominant negative forms of PKC-α or PKC-ε can inhibit TPA-induced SRE activation (Fig. 2A). Similar assays with mutant forms of PKC-δ and PKC-ζ gave negative results (Fig. 2A). These data suggest that PKC-α and PKC-ε are the major PKC isoforms that mediate SRE activation in NIH 3T3 cells. We also found that in the absence of TPA, the PKC-α–WT construct was totally inactive in SRE activation whereas the PKC-ε–WT construct did activate the SRE (Fig.

2B). These data suggest that in the absence of TPA (or other activators), the activity of PKC-α is completely inhibited by its regulatory domain whereas the activity of PKC-ε is only partially inhibited by its regulatory domain. These findings are consistent with the above-mentioned previous studies indicating that stable overexpression of wild-type PKC-ε (9) but not wild-type PKC-α (8) is oncogenic in Rat6 fibroblasts. The apparent inability of PKC-δ to activate the SRE in the present studies is of interest because PKC-δ has considerable sequence homology to PKC-ε and is also activated by TPA. Consistent with our finding are several reports describing an anti-proliferative role of PKC-δ in mammalian cells (49, 64). We also found that transfection of a PKC-δ–CAT construct into NIH 3T3 cells caused growth inhibition (unpublished data). The inability of PKC-ζ to activate the SRE is consistent with the fact that this isoform is not activated by either TPA or calcium (7, 51). Arai et al. also reported that angiotensin II activates PKC-α and PKC-ε and induces c-*fos* in CHO cells (5). Our results do not, however, rule out the possibility that PKC-δ or PKC-ζ plays a role in activating the SRE in response to other agonists.

We found that TPA-induced SRE activation was partially blocked by a dominant negative c-Raf or by dominant negative ERK1 or ERK2 and that the transactivation domain of Elk-1 could be activated by either PKC-α or PKC-ε (Fig. 3A). These findings directly implicate the c-Raf–MEK1-ERK-TCF pathway and PKC-α and PKC-ε in mediating the effects of TPA (Fig. 6). It seems most likely that these isoforms of PKC act by enhancing the activation of c-Raf, either directly or indirectly (34, 45, 58). The precise mechanism of activation of c-Raf is not known. There is evidence that c-Raf is recruited to the plasma membrane through direct interaction with an activated Ras protein and that certain protein kinases, such as PKC and/or src family tyrosine kinases, then activate c-Raf kinase activity by phosphorylating c-Raf (44). PKC-α was shown to activate c-Raf, presumably by direct phosphorylation of its Ser499 residue in NIH 3T3 cells (34). PKC-α, PKC-β and PKC-γ, but not PKC-δ, PKC-η, or PKC-ζ, were shown to activate c-Raf in a TPA-dependent manner in insect cells when these isoforms of PKC and c-Raf were coexpressed with recombinant baculoviruses (45). However, in these studies it was not clear that PKC directly phosphorylates and activates the c-Raf protein. We have previously reported that PKC-ε functions as an oncogene in Rat6 cells by enhancing the activation of c-Raf (10). However, the precise role of c-Raf phosphorylation by PKC in signal transduction pathways remains to be determined, because although a mutation of Ser499 impeded c-Raf activation by PKC-α, it did not abrogate the stimulation of c-Raf activity by a combination of Ras plus the src tyrosine kinase Lck (34).

We found that TPA-induced SRE activation could also be partially blocked by a dominant negative MEKK1 and that TPA induced the phosphorylation of the Thr223 residue of the SEK1 protein (Fig. 4A and B). We also found that the transactivation domain of c-Jun could be activated by constitutively active mutants of PKC-α or PKC-ε (Fig. 4C). These observations suggest that the JNK pathway is also one of the signaling pathways used by PKC to mediate SRE activation in NIH 3T3 fibroblasts (Fig. 6). Our results are consistent with the finding that in COS cells the kinase activity of a transfected JNK1 was moderately elevated by TPA treatment (16). However, it remains to be established whether PKC activates MEKK1 through direct phosphorylation or through a more indirect mechanism. The involvement of specific isoforms of PKC in multiple MAP kinase signaling pathways could provide cells with a mechanism for coordinating or integrating the activities of these pathways, thus ensuring the proper response to various extracellular stimuli, in a cell-type-specific manner. It is of interest that activation of certain membrane-associated tyrosine kinase receptors, for example the epidermal growth factor receptor, leads to at least two events: (i) direct activation of c-*ras* activity through the GRAB-SOS proteins and then c-*ras* enhancement of the activation of c-Raf, and (ii) direct activation of phospholipase C, which generates DAG, an activator of both PKC-α and PKC-ε. According to the above scheme, the later events would also enhance the activation of c-Raf. Thus, the convergence of these two sets of events would coordinate the activation of the c-Raf–MEK1-ERK-TCF pathway.

We found that constitutively active mutants of PKC-α and PKC-ε could also induce transactivation activity of the SRF transcription factor and that rhoA-mediated SRE activation was markedly inhibited by dominant negative mutants of PKC-α or PKC-ε (Fig. 5C and E). These results provide the first evidence that specific isoforms of PKC also function as downstream effectors in the rhoA-mediated SRE activation pathway. Several rhoA-binding proteins have recently been identified, including PKN/PRK1, PRK2, and rhophilin (3, 4, 62, 65). PKN and PRK2 have been shown to mediate LPA-induced formation of stress fibers and focal adhesions, two important cellular responses that involve rhoA (3, 4, 65). However, the precise roles of these rhoA-binding proteins in the SRE activation pathway are not known.

Nonaka et al. (52) reported that a mutation that abolished the pseudosubstrate site of *PKC1*, a yeast homolog of mammalian conventional PKCs, rescued a recessive temperature-sensitive growth phenotype in a yeast strain in which *RHO1*, a yeast homolog of mammalian rhoA, was replaced with mammalian rhoA. They also found that only the GTP-bound form of Rho1p interacted with the C1 domain (DAG binding domain) of PKC1p in yeast two-hybrid assays. However, we could not detect any interaction between mammalian rhoA and mammalian PKC-α or PKC-ε in yeast two-hybrid assays (data not shown). We cannot, however, rule out the possibility that these proteins interact in mammalian cells in the presence of other proteins or specific activation factors. It is of interest that PKC might be activated indirectly by PLD since in vitro rhoA can activate PLD (37–39). The hydrolysis of phosphatidylcholine by PLD produces phosphatidic acid (PA) and choline. PA can then be dephosphorylated by a PA phosphatase, generating DAG, which can activate several isoforms of PKC (17, 36). In addition, PKC can activate PLD in some cell lines (14). Therefore rhoA, PKC, and PLD could play complex roles in mediating the activation of the SRF and other transcription factors. We are currently searching for putative downstream effectors of PKC-α and PKC-ε in the rhoA-mediated SRF activation pathway.

Several studies have demonstrated that SRF can be phosphorylated by CKII (40, 41) or the CaMK (47). Enhanced levels of cytoplasmic Ca^{2+} trigger SRE-dependent transcription via a Ras-independent signaling pathway that appears to involve a CaMK (47). Therefore, in future studies it will be of interest to examine the possible roles of specific isoforms of PKC in regulating the activities of CKII or CaMK. Several SRF-associated transcription factors such as p65/NF-κB (18), ATF6 (71), and TFII-I (33) have recently been identified. It will also be of interest to investigate the possibility that specific isoforms of PKC activate SRF indirectly, by activating these SRF-associated transcription factors.

The present study implicates PKC-α and PKC-ε as critical protein kinases that can modulate at least three signal transduction pathways that converge on the SRE transcriptional

enhancer element (Fig. 6), presumably through a complex network of interactions. It seems likely that these, and other isoforms of PKC, also play a role in modulating additional pathways of signal transduction and the activities of additional transcriptional enhancer elements, but these details remain to be elucidated. Previous studies provide evidence that in addition to these effects on gene transcription, specific isoforms of PKC can also directly phosphorylate cytoskeletal and cytoskeleton-associated proteins, thus directly modulating cytoplasmic and membrane-associated cell functions (6, 7, 51). We should stress that our findings are confined to NIH 3T3 murine fibroblasts, and it seems likely that the roles of specific isoforms of PKC in signal transduction and the control of gene expression may differ between cell types. Nevertheless, it is apparent that specific isoforms of PKC can play multiple roles within networks of signal transduction pathways, presumably to provide integrated and specific responses to various external stimuli.

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