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Induction of the Nuclear Proto-Oncogene c-fos by the Phorbol Ester TPA and c-H-Ras

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TPA is known to cooperate with an activated Ras oncogene in the transformation of rodent fibroblasts, but the biochemical mechanisms responsible for this effect have not been established. In the present study we used c-fos promoter-luciferase constructs as reporters, in transient transfection assays, in NIH3T3 cells to assess the mechanism of this cooperation. We found a marked synergistic interaction between TPA and a transfected v-Ha-ras oncogene in the activation of c-fos promoter and SRE. SRE has binding sites for TCF and SRF. A dominant-negative Ras (ras-N17) inhibited the TPA-Ras synergy by blocking the PKC-MAPK-TCF pathway. Dominant-negative RhoA and Rac1 (but not Cdc42Hs) inhibited the TPA-Ras synergy by blocking the Ras-Rho-SRF signaling pathway. Constitutively active PKCα and PKCε showed synergy with v-Ras. These results suggest that the activation of two distinct pathways such as Ras-Raf-ERK-TCF pathway and Rho-SRF pathway are responsible for the induction of c-fos by TPA and Ras in mitogenic signaling pathways.

INTRODUCTION

Ras proteins are involved in diverse cellular functions, including cell survival, proliferation, development, and immunity (Singh et al., 2005). The mammalian Ras family, composed of H-Ras, K-Ras and N-Ras, has been implicated in carcinogenesis. Mutation in the ras oncogene causes tumorigenesis and is found in about 30% of human cancers (Campbell and Der, 2004). Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) cooperates with Ras in tumor promotion in v-Ha-ras-transgenic mice (Ohara et al., 2003). Cooperation of Ras with TPA in 7,12-dimethylbenz[α]anthracene (DMBA)- or ($\pm$)-(E)-4-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1)-initiated tumorigenesis was also reported by several investigators (Malliri et al., 2002; Park et al., 2004; Satomi et al., 2003). The induction of Ras oncoprotein activates c-fos expression (Herman and Simonson, 1995). c-fos is an immediate-early response gene involved in cellular differentiation and proliferation and is overexpressed in many types of tumors. The c-fos gene encodes nuclear protein c-Fos that associates with c-Jun protein and forms a heterodimeric transcription factor AP1 (activator protein-1). c-Fos has DNA binding activity (Hashiramoto et al., 2006) and also can be induced by a number of proinflammatory cytokines, including interleukin-1, or by the stimulation of growth factors (Rivera et al., 1993; Schiller et al., 2006).

Expression of c-fos is modulated by various signal transduction pathways including Ras/Raf/ERK pathway (Hill and Treisman, 1995). GTP-bound Ras recruits Raf to the plasma membrane which in turn phosphorylates MEK, leading to ERK activation. The activated ERK translocates to the nucleus and regulates the activity of many transcription factors. Ternary complex factor (TCF) is one of the most studied transcription factors in this pathway. The ternary complex factor Elk-1 is involved in c-fos transcription (Li et al., 2001). The serum response element (SRE) in the c-fos promoter is required for induction of the c-fos gene. TCF and serum response factor (SRF) bind to SRE and activate the transcription. SRF can also be activated by the Rho family of small GTPases. The Rho family of small GTPases such as RhoA, Rac, and Cdc42 are members of the Ras superfamily and play essential roles in the regulation of diverse cellular functions. These GTPases cycle between an inactive, GDP-bound form and an active, GTP-bound form, thus functioning as molecular switches to downstream signal transduction processes (Bishop and Hall, 2000).

Moreover, c-fos gene expression can be activated by the protein kinase C (PKC)-associated pathways (Soh and Weinstein, 2003; Soh et al., 1999). PKC represents a family of 10 protein serine/threonine kinases which is conserved in eukaryotes from yeast to human (Kazi et al., 2008; Mellor and Parker, 1998). PKCα and PKCε can activate SRE through at least three signaling pathways; Raf-MEK1-ERK-TCF, MEKK1-SEK1-JNK-TCF, and RhoA-SRF (Soh et al., 1999). TPA-induced phosphorylation of ERK is regulated by PKC (Choi et al., 2007). PKC can activate Raf by direct phosphorylation at Ser499 (Kolch et al., 1993). The synergy between Ras and the protein kinase A (PKA) pathway in c-fos induction was also reported (Seternes et al., 1998). In our present study we used transient transfection assays with c-fos promoter luciferase constructs as reporters to study the biochemical mechanism of the TPA-Ras synergy. We found a marked synergistic activation of c-fos promoter by TPA and activated Ras oncogene. This synergy was mediated by SRE in the c-fos promoter. We also studied the role of PKC isoforms in those pathways. We showed that PKC isoforms were necessary effectors downstream of the

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Rho family of small GTPases and that Raf was an important downstream target of PKC isoforms.

MATERIALS AND METHODS

Plasmids

Luciferase reporter plasmids, pSRE-luc and pfos-WT-luc, pHANE-PKCα-CAT, pHANE-PKCδ-CAT, pHANE-PKCε-CAT, pHANE-PKCζ-CAT and pCMV-rhoA-N19 were described previously (Soh et al., 1999). pM2N-ras-N17 (Lee et al., 2003a) and pcDNA3-rac1-N17 (Choi et al., 2006) were also described previously. pCEV29-cdc42Hs-N17, pcDNA3-RAF-CAAX and pcDNA3-v-Raf were kindly provided by Dr. J. S. Gutkind, Dr. D. Bar-Sagi and Dr. U. R. Rapp respectively. pcDNA3-v-ras was constructed by subcloning *Bam*HI fragment of v-ras cDNA (provided by Dr. G. Cooper) into pcDNA3. pcDNA3-FLAG-raf-WT and pcDNA3-FLAG-raf-d2 were also constructed by subcloning the BamHI fragment of c-raf-1 (provided by Dr. Morrison) and 53-132 deletion mutant of c-raf-1 (provided by Dr. R. Jove) into pcDNA3 vector respectively.

Cell culture

NIH3T3 and COS7 cells were cultured in Dulbecco's minimal essential medium (DMEM) containing 10% calf serum or fetal bovine serum (FBS), respectively, and antibiotics at 37°C in a humidified incubator with 5% carbon dioxide.

Cell transfection

NIH3T3 cells were grown in DMEM containing 10% calf serum. Triplicate of 1×10^5 cells in 35 mm plates were transfected by lipofectin (Gibco BRL) with 2 μg of reporter plasmid, 2 or 5 μg of expression vectors, and 1 μg of pCMV-β-gal. pcDNA3 plasmid DNA was added to the transfections as needed to achieve the same amount of plasmids per transfection. COS-7 cells were grown in DMEM containing 10% FBS. 2×10^5 cells in 60 mm plates were transfected by lipofectin (Gibco BRL) with 5 μg of expression vectors or control vector (pcDNA3).

Immunoprecipitation

Six hours after transfection, cells were fed with DMEM with 10% (FBS) and left overnight, then 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% NP-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol) containing protease inhibitors (10 μg/ml aprotinin, 10 μg /ml leupeptin, 0.1 mM PMSF) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate). FLAG-tagged proteins were immunoprecipitated from 300 μg of cell extracts by using 3 μg of anti-FLAG antibody and 30 μl of protein G-Sepharose, after a 3-h incubation at 4°C and analyzed by SDS-PAGE and Enhanced Chemiluminescence Western Blotting System (Amersham).

Luciferase reporter assay

Six hours after transfection, cells were fed with new media (DMEM with 10% calf serum), left overnight, then serum-starved for 24 h in DMEM with 0.5% calf serum. For TPA experiments, cells were treated with TPA for 3 h with a final concentration of 100 ng/ml. DMSO was used as a control. Luciferase Assays were performed with serum-starved or TPA-treated cells using the Luciferase Assay System (Promega). Luciferase activities were normalized by β-gal activities. β-gal assays were performed using the β-Galactosidase Enzyme Assay System (Promega).

Statistical analysis

Statistical significance of the differences between groups was calculated by one-way analysis of variance (ANOVA), followed by Newman-Keuls post-test using GraphPad Prism 5.0 (GraphPad Software, Inc., USA).

RESULTS

Induction of c-fos by the phorbol ester TPA and activated Ras oncogene

It has long been known that TPA or oncogenic Ras can independently induce the transcriptional activation of c-fos proto-oncogene (Busam et al., 1993; Herman and Simonson, 1995). To determine whether TPA and Ras show synergy in the transcriptional activation of c-fos gene, we used transient transfection assays with c-fos promoter (pfos-WT-luc) and SRE (pSRE-luc) reporter plasmids in NIH3T3 mouse fibroblast cells. The c-fos promoter was activated by either treatment of TPA or transient transfection of pcDNA3-v-Ras (Fig. 1A). pcDNA3-v-ras encodes a constitutively active mutant of Ras (G12R/A59T mutation) (Feig and Cooper, 1988). We found a marked synergistic activation of c-fos promoter activity (5-fold with respect to individual activation, $p < 0.001$) when v-ras transfected cells were treated with TPA (Fig. 1A). Since SRE has been reported to be the major target of many growth factors, oncogenes, and TPA, we tested whether TPA and v-Ras could synergize in SRE activation as well. We observed a strong synergistic activation of SRE activity (9-fold inductions with respect to the individual, $p < 0.001$) by TPA and v-Ras (Fig. 1B). This synergy with TPA was insignificant ($p > 0.05$) with v-Raf, Raf-CAAX (Fig. 1C) or v-Abl oncogenes (data not shown).

Role of Ras in TPA-mediated SRE activation

Several reports on the role of Ras in PKC signaling pathways were contradictory and also cell type specific (Carnero et al., 1994; Hirai et al., 1994; Liu et al., 2002). In our previous study, we showed that Ras is involved in PKCε-induced ERK1/2 and MEK1/2 phosphorylation (Lee et al., 2003b). Though novel PKC isoforms can be activated by TPA (Kazi and Soh, 2007), we examined the role of Ras in the TPA-mediated SRE activation pathway using dominant-negative N17-Ras (S17N mutation). Dominant-negative N17-Ras (pM2N-ras-N17) was able to block TPA-induced SRE activation in a dose-dependent manner (Figs. 2A and 2B). TPA-induced Gal4-ElkC activity was also blocked by N17-Ras (Stewart and Guan, 2000). These results suggest that functional Ras is necessary for TPA to transduce the signal to SRE. One possible point of cooperation between TPA and Ras is the activation of Raf by PKC. It is possible to hypothesize that PKC activated by TPA could activate Raf only when Raf is brought to the vicinity of cytoplasmic membranes by the Ras-Raf interaction. To test this hypothesis, we generated expression vectors for wild-type Raf and mutant Raf lacking Ras-interaction domain. TPA is known to activate Raf (Liu et al., 2002) and cause mobility shift of Raf proteins due to its phosphorylation on Ser499 residue (Kolch et al., 1993). We generated expression vectors for wild-type c-Raf-1 (pcDNA3-FLAG-raf-WT) and Raf mutant devoid of Ras-binding activity (pcDNA3-FLAG-raf-d2). When COS-7 cells were treated with TPA after transient transfection of either wild-type or mutant Raf, we observed mobility shifts of mutant Raf as well as of wild-type Raf (Fig. 2C). It was also reported that PKC-mediated Raf activation was not blocked by N17-Ras (Marais et al., 1998). These data suggest that Ras-Raf interaction is dispensable for TPA-mediated Raf phosphorylation and that TPA may cooperate with Ras in other ways.

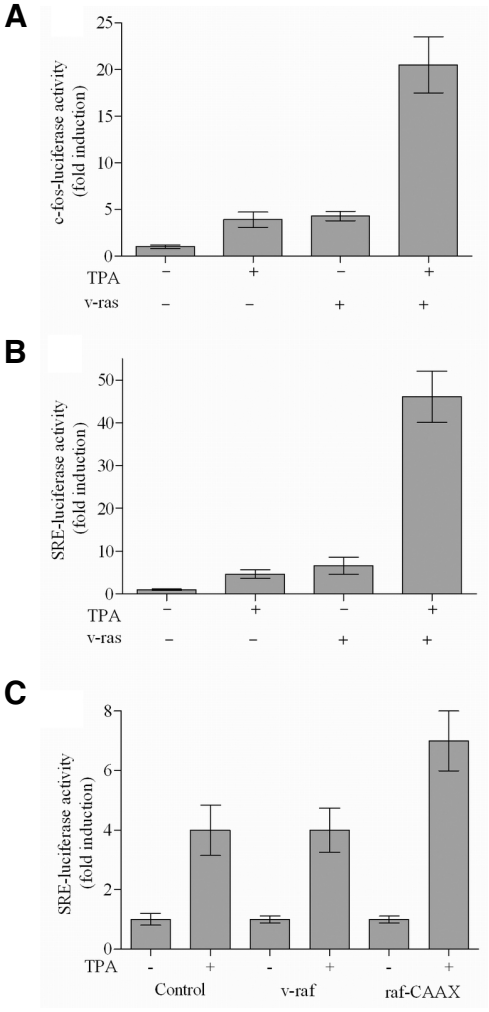

Fig. 1. Activation of SRE by TPA and activated Ras. (A) NIH3T3 cells were cotransfected with pfos-WT-luc reporter plasmid and pcDNA3-v-ras plasmid or empty control vector (pcDNA3). The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h and assayed for luciferase activities. (B) NIH3T3 cells were cotransfected with pSRE-luc reporter plasmid and pcDNA-v-ras plasmid or empty control vector (pcDNA3). The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h and assayed for luciferase activities. (C) NIH3T3 cells were cotransfected with pSRE-luc reporter plasmid and pcDNA3-v-Raf, pcDNA3-RAF-CAAX or empty control vector (pcDNA3). The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h and assayed for luciferase activities.

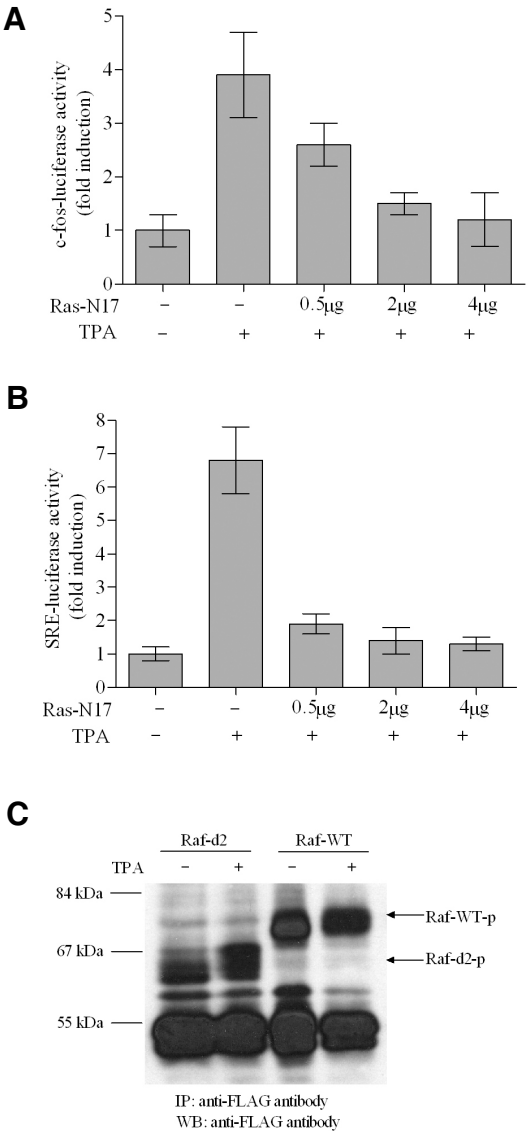

Fig. 2. Role of Ras in TPA-mediated SRE activation. (A) NIH3T3 cells were cotransfected with pfos-WT-luc reporter plasmid and different amounts of pM2N-ras-N17 plasmid or empty control vector. The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h and assayed for luciferase activities. (B) NIH3T3 cells were cotransfected with pSRE-luc reporter plasmid and different amounts of pM2N-ras-N17 plasmid or empty control vector. The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h and assayed for luciferase activities. (C) COS-7 cells were treated with TPA after transient transfection of either pcDNA3-FLAG-raf-WT or pcDNA3-FLAG-raf-d2. The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h. Cellular proteins were extracted by cell lysis. FLAG-tagged proteins were immunoprecipitated from 300 μg of cell extracts by using 3 μg of anti-FLAG antibody and then analyzed by SDS-PAGE and Enhanced Chemiluminescence Western Blotting System.

Role of the Rho family of small GTPases in TPA-Ras-mediated SRE activation

SRE can be activated by several signal transduction pathways including activation of TCF (Elk-1/Sap-1) by ERKs or JNKs (Soh et al., 1999). Another mechanism of SRE activation is the activation of SRF by protein kinase N or Rho-kinase, which are the downstream effectors of the Rho family of small GTPases, such as Cdc42, Rac1 and RhoA (Bishop and Hall, 2000; Sahai et al., 1998). Since Ras was reported to be also upstream of the Rho family of small GTPases in some signaling pathways (Kawano et al., 2000; Stice et al., 2002), we examined whether the Rho family of small GTPases play a role in the TPA-Ras synergy in c-fos induction. Dominant-negative mutants of RhoA (pCMV-rhoA-N19), Rac1 (pcDNA3-rac1-N17) and Cdc42Hs

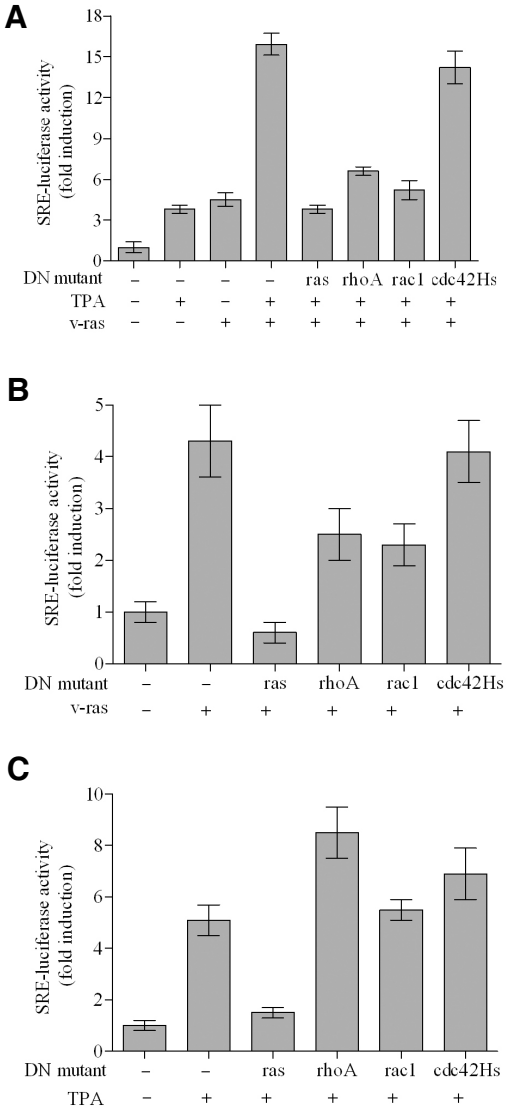

Fig. 3. Role of the Rho family of small GTPases in TPA-Ras-mediated SRE activation. (A) NIH3T3 cells were cotransfected with pSRE-luc reporter plasmid and pM2N-ras-N17, pCMV-rhoA-N19, pcDNA3-rac1-N17, pCEV29-cdc42Hs-N17 or empty control vector together with pcDNA3-v-ras or pcDNA3. The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h and assayed for luciferase activities. (B) NIH3T3 cells were cotransfected with pSRE-luc reporter plasmid and pM2N-ras-N17, pCMV-rhoA-N19, pcDNA3-rac1-N17, pCEV29-cdc42Hs-N17 or empty control vector together with pcDNA3-v-ras or pcDNA3. The cells were then serum starved for 24 h and assayed for luciferase activities. (C) NIH3T3 cells were cotransfected with pSRE-luc reporter plasmid and pM2N-ras-N17, pCMV-rhoA-N19, pcDNA3-rac1-N17, pCEV29-cdc42Hs-N17 or empty control vector. The cells were then serum starved for 24 h and treated with either 0.1% DMSO or 100 ng/ml TPA for 3 h and assayed for luciferase activities.

(pCEV29-cdc42Hs-N17) were used to determine whether the Rho family of small GTPases were important in TPA and Ras signaling pathways. Synergistic activation of SRE by TPA and v-Ras was dramatically inhibited by dominant-negative RhoA and Rac1 but not by dominant-negative Cdc42Hs (Fig. 3A). These data suggest that signals from RhoA and Rac1 in the SRE pathway might be necessary for TPA to cooperate with Ras. Inhibition of the TPA-Ras synergy by dominant-negative RhoA and Rac1 may be due to inhibition of the Ras pathway rather than of the TPA pathway, because dominant-negative RhoA and Rac1 could partially block SRE activation by v-Ras (Fig. 3B), whereas none of the dominant-negative Rho family of small GTPases could block SRE activation by TPA (Fig. 3C). Moreover, dominant-negative RhoA cooperated with TPA in SRE activation (Fig. 3C), although the cooperation between TPA and dominant-negative RhoA was not significant ($p > 0.05$). However, it is not clear how dominant-negative RhoA cooperate with TPA in mitogenic signaling pathways. Partial inhibition of v-Ras-mediated SRE activation by dominant-negative RhoA or Rac1 suggests that RhoA and Rac1 are on one branch of signal transduction pathways stemming from Ras in SRE activation pathways.

Activation of SRE by activated PKC and ras

It is well known that classical and novel PKC isoforms are the major receptors for TPA. The other TPA receptors include zinc finger domain containing proteins such as protein kinase D (PKD) (Valverde et al., 1994), RasGRP (Ebinu et al., 1998), chimaerin (Hall et al., 1990), Unc-13 (Brose et al., 1995) and DAG kinase (van Blitterswijk and Houssa, 2000). This diversity of TPA receptors led us to investigate whether the TPA-Ras synergy in c-fos induction is mediated by PKC or not. NIH3T3 cells were transfected with the constitutively active mutants of PKC isoforms, and pc-DNA3-v-ras plasmid, together with the pSRE-luciferase reporter plasmid. pHANE-PKCα-CAT, pHANE-PKCδ-CAT, pHANE-PKCε-CAT and pHANE-PKCζ-CAT plasmids encode catalytic domain mutants of PKCα, PKCδ, PKCε and PKCζ respectively. Within the four PKC mutants, PKCα and PKCε were able to synergize with v-Ras in SRE activation (Fig. 4). These results, which are consistent with previous studies indicating that catalytic domain of PKCδ and PKCζ did not activate SRE (Soh et al., 1999), and suggest that the TPA-Ras synergy is mediated by some but not all but specific PKC isoforms. The apparent inability of PKCδ to synergize with v-Ras in SRE activation is of interest because PKCδ shares considerable sequence homolog with PKCε, and is also activated by TPA. The inability of PKCζ to synergize with v-Ras in SRE activation is consistent with the fact that this isoform is not activated by TPA (Kazi and Soh, 2007).

DISCUSSION

We attempted to elucidate the c-fos transcription pathway which was stimulated by Ras and TPA. Overexpression of v-ras or TPA-treatment led to elevated activation of SRE in NIH3T3 cells. This activation was strongly potentiated in the presence of both TPA and v-ras, suggesting that TPA may cooperate with Ras in c-fos expression. The dominant negative mutant of v-ras (Ras-N17) blocked TPA-induced SRE activation in a dose dependent manner which indicates that functional Ras is required to transduce TPA-mediated signal to the SRE. To address the question how TPA cooperates with Ras in SRE activation we examined the possibility that Raf may mediate this synergy.

TPA is known to activate serine/threonine protein kinase Raf through PKC (Kolch et al., 1993) and Raf is a downstream effector of Ras which transduces signal through Ras-Raf interaction (Campbell et al., 1998). However, the study with wild type and mutant Raf which lacks Ras interaction motif suggests that Ras-Raf interaction is not important for TPA-stimulated Raf

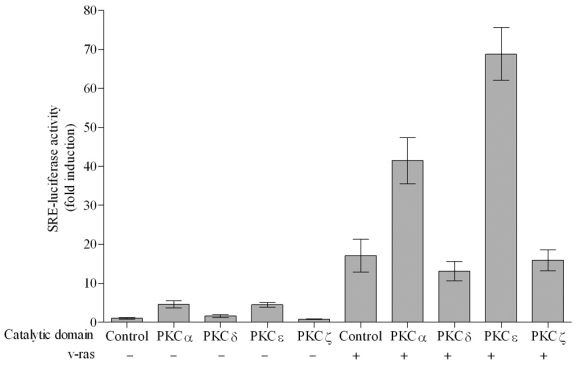

Fig. 4. Activation of SRE by activated PKC and Ras. NIH3T3 cells were cotransfected with pSRE-luc reporter plasmid and pHANE-PKCα-CAT, pHANE-PKCδ-CAT, pHANE-PKCε-CAT, pHANE-PKCζ-CAT or empty control vector, together with pcDNA3-v-ras or pcDNA3. The cells were then serum starved for 24 h and assayed for luciferase activities.

activation. Moreover, Raf was unable to cooperate with TPA in SRE activation. These findings suggest that TPA may synergize with Ras in other ways and other Ras effectors may play roles in this signaling pathway. One Ras effector PI3K activates PDK1 leading to the activation loop phosphorylation of PKC isoforms (Newton, 2003).

The other Ras effectors include Rho family small GTPases such as RhoA, Rac1 and Cdc42Hs (Kawano et al., 2000; Stice et al., 2002). Activation of these GTPases leads to SRE activation by SRF through protein kinase N or Rho-kinase (Bishop and Hall, 2000; Sahai et al., 1998). Dominant negative mutants of RhoA and Rac1 but not Cdc42Hs blocked the TPA and v-ras-mediated synergistic activation of SRE. Furthermore, dominant negative mutants of RhoA and Rac1 were able to partially block v-ras-mediated SRE activation. However, these mutants exhibited impaired ability to block TPA-induced SRE activation. These findings suggest that v-Ras may regulate SRE activation by activating Rho-SRF pathway. The inability to block the TPA-induced SRE activation indicates that TPA effectors might be the downstream of Rho GTPases. These observations were further supported by the evidence that regulatory domain of PKC isoforms partially blocked Rho family small GTPases-mediated SRE activation (Kazi and Soh, 2008) and that dominant negative mutants of PKCα and PKCε blocked RhoA-mediated SRE activation (Soh et al., 1999). PKC isoforms are the major TPA receptors, and classical and novel PKC isoforms contain a tandem repeat of zinc-finger domains in their regulatory domain for TPA binding.

Studies with the constitutively active mutants of PKC isoforms indicate that PKCα and PKCε cooperate with v-Ras in SRE activation. These findings suggest that PKCα and PKCε may play roles in tumorigenesis and are further supported by our recent studies that overexpression of the regulatory domain of PKCα and PKCε inhibited the anchorage-independent growth in MCF-7 cells (Soh et al., 2003). Thus this study may demonstrate that the activation of both Ras-Raf-ERK-TCF pathway and Rho-SRF pathways by TPA and v-Ras leads to the synergistic activation of SRE. These results further exemplify the importance of interaction among the signal transduction pathways in biological responses to external stimuli.

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