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Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKCδ

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Modulation of gene expression by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is thought to be mediated by protein kinase C (PKC), a major cellular receptor for TPA. We confirm this by showing that the overexpression of PKCδ enhances the TPA induction of the TRE-tk-CAT reporter gene in NIH3T3 cells. To investigate the mutual relationship between PKCδ- and Ras-dependent signal transduction pathways to a TRE binding transcription factor, AP1/Jun, we constructed constitutively active and dominant negative mutants of PKCδ. Activated Ras induced reporter gene expression in collaboration with overexpressed c-Jun or JunD, and this induction was insensitive to the dominant negative PKCδ. On the other hand, reporter gene expression induced by the constitutively active PKCδ was severely inhibited by dominant negative Ras, as well as by the dominant negative PKCδ. Thus, Ras activation must be indispensable for PKCδ to activate AP1/Jun. In the absence of overexpressed c-Jun or JunD, activated Ras was, however, clearly less effective than constitutively active PKCδ which showed full activation of reporter gene expression by itself. This suggests the presence of an additional, Ras-independent, signaling pathway downstream of PKCδ to activate AP1/Jun. In spite of the remarkable ability of constitutively active PKCδ to activate TRE-tk-CAT expression, this mutant suppressed cell growth.

Key words: c-*jun*/phorbol ester/protein kinase/transcription

Introduction

Protein kinase C (PKC) has been identified as a major cellular receptor for 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter in mouse skin (Castagna *et al.*, 1982; Blumberg, 1988). TPA is also known as a modulator of gene expression through *cis*-acting DNA elements such as TPA response element (TRE) or the κB site (Angel *et al.*, 1987; Baeuerle and Baltimore, 1988). Therefore, PKC is believed to modulate the activity of transcription factors that bind to these *cis* elements.

AP1/Jun is a major TRE binding transcription factor whose activity is thought to be controlled by several different mechanisms including: (i) heterodimer formation with other leucine zipper-containing proteins such as c-Fos (e.g. Chiu *et al.*, 1988; Schütte *et al.*, 1989; Hai and Curran, 1991); (ii) reduction–oxidation of cysteine residues located in the DNA binding domain (Abate *et al.*, 1990; Xanthoudakis *et al.*, 1992); (iii) interaction of a specific inhibitor with a negative regulatory region (δ region) (Baichwal and Tjian, 1990), (iv) phosphorylation of serine/threonine residues located in the activation domain or just upstream of the DNA binding domain. Among them, phosphorylation may be most tightly regulated by PKC because TPA induces site-specific phosphorylation (Pulverer *et al.*, 1991; Adler *et al.*, 1992) or dephosphorylation (Boyle *et al.*, 1991) of Jun. However, direct phosphorylation of Jun by PKC is not likely to occur *in vivo* because of differences in subcellular localization (Baker *et al.*, 1992). Among several protein kinases which phosphorylate Jun *in vitro*, mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) are regarded as Jun activators *in vivo* (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991). These serine/threonine kinases also phosphorylate and activate pp90^{rsk}, which could contribute to the modulation of AP1/Jun activity through Fos (Chen *et al.*, 1992). According to the recent understanding of the signal transduction pathway from receptor tyrosine kinase to MAPKs/ERKs, Ras plays key roles downstream of the receptor (e.g. Thomas *et al.*, 1992; Egan *et al.*, 1993; Lange-Carter *et al.*, 1993; Zhang *et al.*, 1993). In fact, it has been reported that Ras activates AP1/Jun *in vivo* by inducing the phosphorylation of serine residues that are phosphorylated by MAPKs/ERKs *in vitro* (Binetruy *et al.*, 1991). Since TPA also induces the phosphorylation of these residues, PKC may activate AP1/Jun through the activation of MAPKs/ERKs (Pulverer *et al.*, 1991; Adler *et al.*, 1992). However, the mechanism by which PKC activates these protein kinases is not clear.

Originally, PKC was reported as calcium-dependent protein kinases, which are now referred to as PKCα (type III), PKCβI,II (type II) and PKCγ (type I) (Nishizuka, 1988). In the course of the cDNA cloning of PKC, several other isozymes were found that show significant structural homology to the originally reported PKC, but lack the putative Ca^{2+} binding domain. These PKC homologues do not require Ca^{2+} for their kinase activity and are called novel PKC (nPKC), while Ca^{2+}-dependent isozymes were called conventional PKC (cPKC). Unlike the clear difference in Ca^{2+} dependency, phorbol esters such as TPA or diacylglycerols, natural PKC activators that can be replaced by phorbol esters, bind to both cPKC and nPKC, and induce their enzymatic activity (Ohno *et al.*, 1988; Ono *et al.*, 1988). The third group of PKC, called atypical PKC (aPKC), lack the phorbol ester/diacylglycerol binding domain and are thought to be activated by particular inositol phospholipids

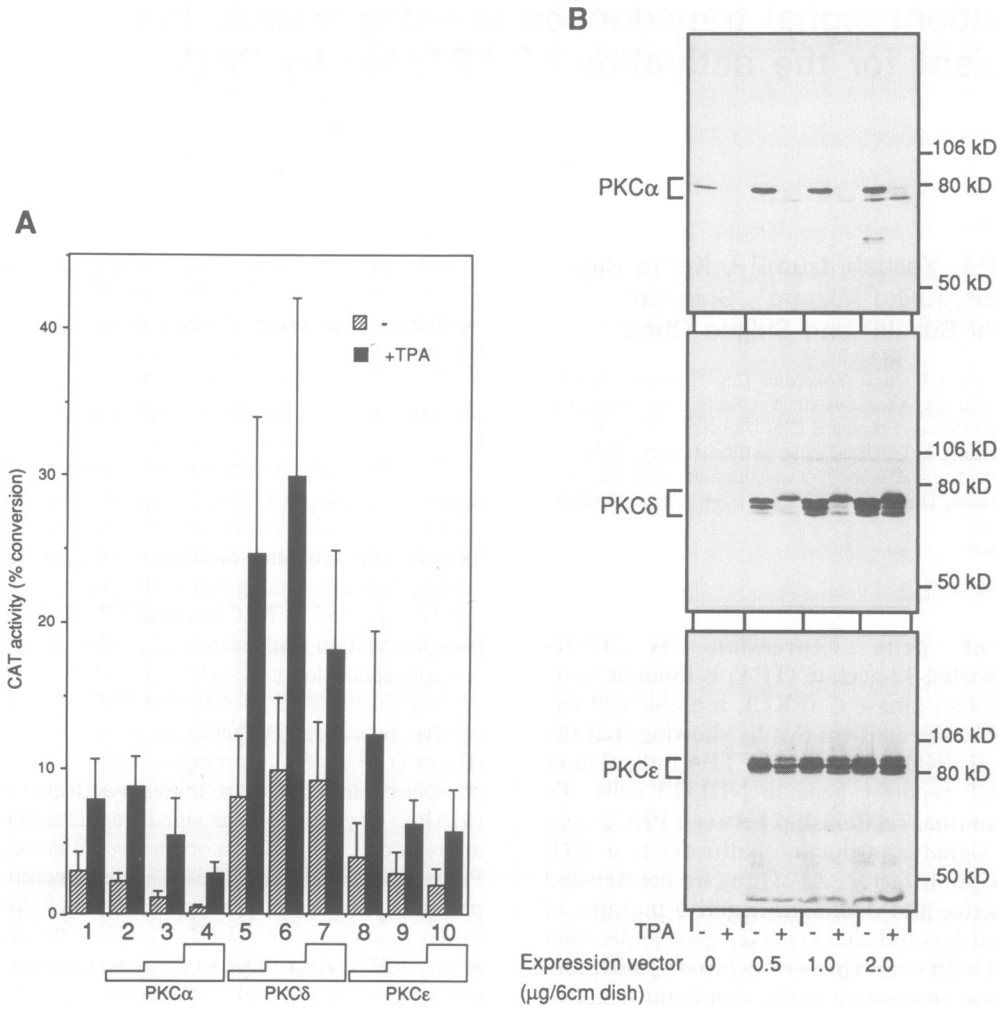

Fig. 1. PKCδ enhances TRE-tk-CAT expression. **(A)** NIH3T3 cells seeded in a 6 cm plate were co-transfected with 3.0 μg of TRE-tk-CAT reporter plasmid together with vector plasmid SRD (column 1) or expression vectors carrying cDNA for PKCα (columns 2–4), PKCδ (columns 5–7) or PKCε (columns 8–10). The amount of expression vectors used for the transfection of cells was 0.5 μg (columns 2, 5, 8), 1.0 μg (columns 3, 6, 9) or 2.0 μg (columns 4, 7, 10) and the total amount of DNA used for transfection was adjusted to 6 μg with SRD vector plasmid. After transfection, cells were cultured in 0.5% FCS/DMEM for 48 h and then exposed for 10 h to culture medium containing 100 ng/ml of TPA (solid columns) or vehicle alone (hatched columns). Mean values with standard deviations of CAT activity obtained from 3–5 independent experiments are shown. **(B)** The overexpression of each PKC isozyme was confirmed by Western blot analysis using specific antibodies. Cells were transfected as for CAT assay with different quantities for each PKC isozyme and harvested for SDS–PAGE after 48 h of serum starvation and 10 h of TPA or vehicle treatment as indicated at the bottom. Each panel shows endogenous (two lanes on the left) or overexpressed PKCα, PKCδ or PKCε as indicated.

(Nakanishi *et al.*, 1993). In addition to such differences in the activation mechanism, cPKC and nPKC also show differences in substrate specificity, at least *in vitro*. Furthermore, tissue distribution and levels of expression are different among the isozymes (Nishizuka, 1988). Taken together, these differences strongly suggest that each PKC isozyme plays distinct roles in living cells.

In this report, we show that PKCδ, an nPKC, is a potent activator of AP1/Jun in NIH3T3 cells and that Ras is indispensable but not sufficient for activation.

Results

PKCδ activates TRE-tk-CAT expression in mouse fibroblasts

The PKC isozyme that activates AP1/Jun in NIH3T3 cells was determined by co-transfection experiments using an expression vector of PKC isozymes and a TRE-tk-CAT reporter gene containing the *Escherichia coli* CAT gene downstream of the TPA response element and minimum promoter derived from the herpes simplex virus thymidine kinase gene. Three subspecies of PKC, PKCα, PKCδ and PKCε, detected in NIH3T3 cells by Western blot analysis (Mischak *et al.*, 1993; Figure 1B), were chosen to test the effect of overexpression. The reporter CAT gene expression was enhanced ~3-fold by TPA treatment of cells transfected with insertless expression vector together with the reporter gene; this expression might be mediated by endogenous PKC (Figure 1A, column 1). When an optimum quantity of PKCδ expression vector was used together with the reporter gene for co-transfection, CAT expression was enhanced ~3.5-fold on average without TPA treatment (Figure 1A, column 6). This might be due to a partial activation of exogenous PKCδ, which took place before cells were completely starved. Activation of the overexpressed PKCδ by TPA resulted in a further increase (~3-fold) in CAT

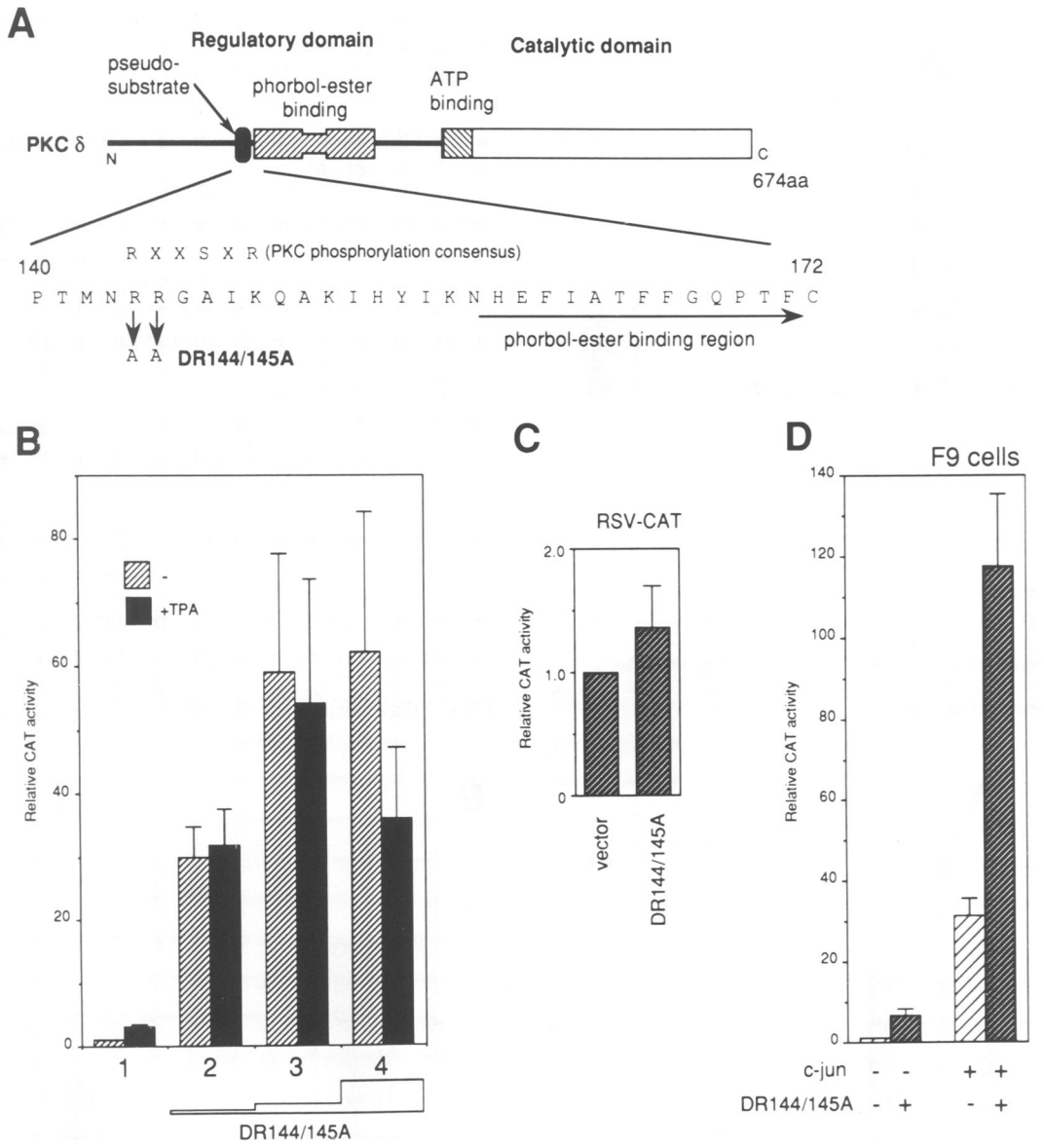

Fig. 2. Constitutively active mutant of PKCδ, DR144/145A. (**A**) The primary structure of PKCδ around the pseudosubstrate region and the positions of two arginines replaced by two alanines in DR144/145A are shown together with a schematic drawing of the PKCδ protein structure. (**B**) NIH3T3 cells were transfected with TRE-tk-CAT alone (column 1) or co-transfected together with 0.05 μg (column 2), 0.15 μg (column 3) or 0.5 μg (column 4) of DR144/145A expression vector and serum starvation and TPA treatment were performed as described for Figure 1A. Shown is the relative CAT activity over the activity observed for cells transfected with reporter plasmid alone without TPA. (**C**) RSV-CAT was used instead of TRE-tk-CAT as the reporter gene. NIH3T3 cells were co-transfected with SRD vector or with 0.1 μg of DR144/145A expression vector together with 3 μg of RSV-CAT. Cells were starved in 0.5% FCS/DMEM for 45 h and harvested for CAT assay. (**D**) Mouse embryonal carcinoma F9 cells seeded in a 6 cm plate were co-transfected with 2.0 μg of TRE-tk-CAT together with 0.2 μg of c-Jun expression vector or 0.2 μg of DR144/145A expression vector as indicated. Shown is the relative CAT activity over the activity observed for reporter transfection in the absence of expression vector.

activity. In contrast to PKCδ, which showed a significant effect on reporter gene expression, PKCε showed only a restricted effect (Figure 1A, columns 8−10), and we could not detect any positive effect of PKCα on this reporter gene. Furthermore, expression was actually suppressed when increased amounts of PKCα expression vector were used (Figure 1A, columns 2−4).

The overexpression of each isozyme was shown by Western blot analysis using isozyme-specific antibodies (Figure 1B). In this cell line, the endogenous level of PKCα is relatively high compared with PKCδ and PKCε, as reported by others using a different set of antibodies (Mischak *et al.*, 1993). The amount of each isozyme increased significantly when the corresponding expression vector was transfected, unless only a restricted fraction (<10%) of cells incorporated DNA and overexpressed PKC isozyme. When cells were treated with TPA, all of these endogenous and overexpressed enzymes underwent downregulation. In addition, PKCδ showed a TPA-induced mobility shift, which was most likely caused by hyperphosphorylation (Borner *et al.*, 1992). PKCε, including the endogenous enzyme, was much more resistant to downregulation than the others. Since the downregulation of PKC is believed to follow its activation, it is conceivable that TPA is not an effective activator of PKCε in NIH3T3 cells. However, further studies are required to prove this.

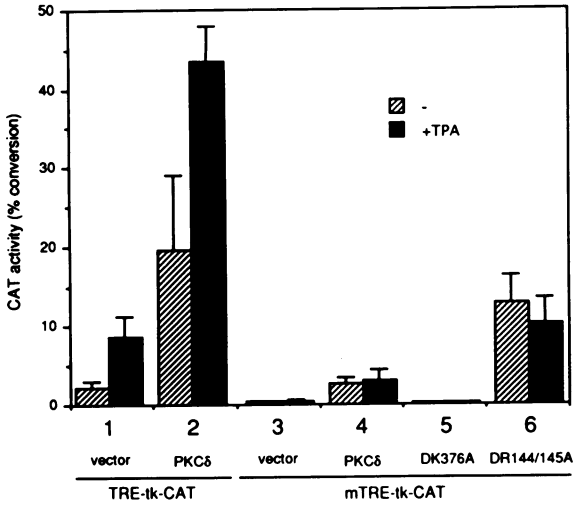

Fig. 3. TRE dependency of PKCδ-mediated activation. Transcriptional activation by PKCδ was monitored using TRE-tk-CAT reporter plasmid (columns 1, 2) or mTRE-tk-CAT reporter plasmid containing mutated TRE (columns 3–6). NIH3T3 cells were transfected with 3.0 μg of reporter plasmid together with vector plasmid SRD or expression vectors for PKCδ (1.0 μg), DR144/145A (0.15 μg) or dominant negative PKCδ, DK376A (1.0 μg), as indicated. Other conditions were the same as described for Figure 1A.

In any case, these observations confirm the conclusion that PKCδ is the most effective isozyme to mediate signal transduction from TPA to AP1/Jun.

A constitutively active mutant of PKCδ is a potent AP-1/Jun activator

To confirm the ability of PKCδ to activate TRE-tk-CAT expression, we designed a constitutively active PKCδ mutant (DR144/145A). This mutant harbors two amino acid substitutions in its pseudosubstrate region (Figure 2A), which is thought to act as an intramolecular suppressor of kinase activity in the absence of activators such as TPA (House and Kemp, 1987; Pears *et al.*, 1990). The expression of the reporter TRE-tk-CAT gene was strongly enhanced by DR144/145A and this enhancement no longer depended on TPA (Figure 2B). The expression of DR144/145A in NIH3T3 cells could not be observed clearly by Western blot analysis, most likely because of instability of the mutant protein. However, expression could be seen in individual cells by immunofluorescent microscopy (data not shown). To test the promoter specificity of this enhancement, the effect of DR144/145A on RSV-CAT reporter gene containing Rous sarcoma virus (RSV) long terminal repeat (LTR) with CAT gene was compared with that on TRE-tk-CAT. The basal level of RSV-CAT expression in serum-

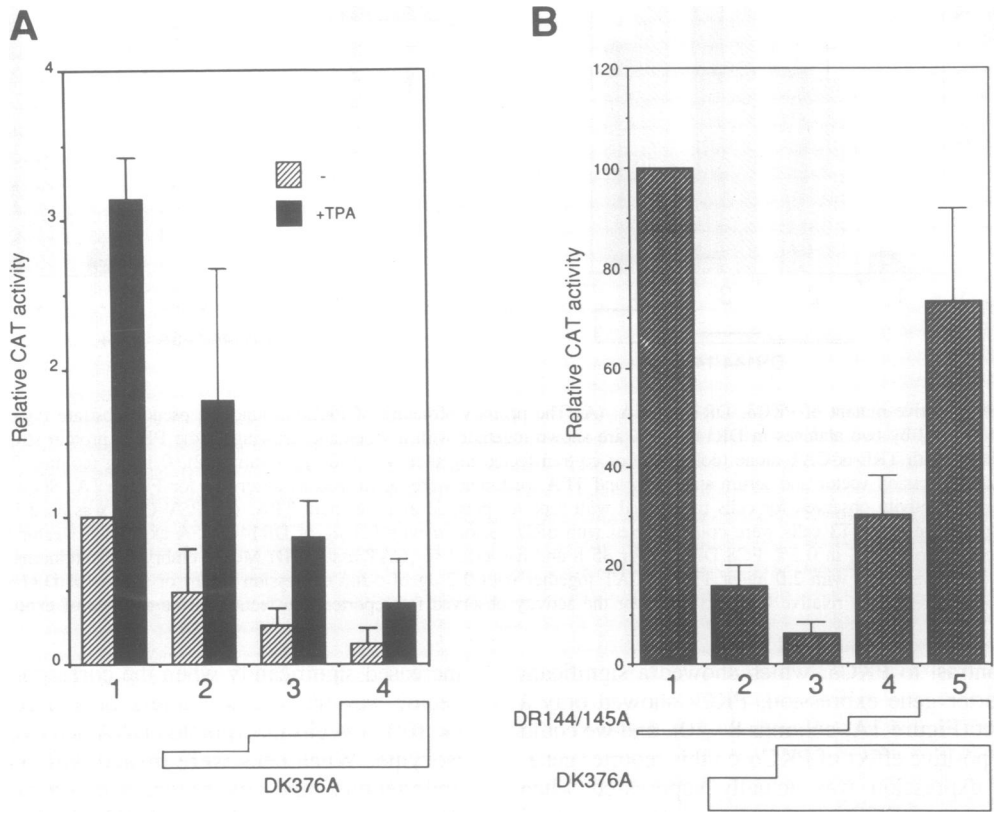

Fig. 4. Effect of dominant negative mutants. (**A**) The effect of DK376A on the TPA-induced level (solid column) or non-induced level (hatched column) of TRE-tk-CAT expression was tested by co-transfection of NIH3T3 cells with DK376A expression vector together with reporter gene. The DNA used for the transfection was 3 μg of TRE-tk-CAT and insertless vector alone (column 1), or 0.5 μg (column 2), 1.0 μg (column 3) or 2.0 μg (column 4) of DK376A expression vector. (**B**) The effect of DK376A on the TRE-tk-CAT expression activated by DR144/145A is shown. The CAT activity shown is the percentage of the activated level. Expression vectors used for the co-transfection with reporter gene were 0.1 μg (columns 1–3), 0.5 μg (column 4) or 1.0 μg (column 5) of DR144/145A expression vector and 1.0 μg (column 2) or 2.0 μg (columns 3–5) of DK376A expression vector. Cells were starved in 0.5% FCS/DMEM for 45 h and harvested for CAT assay.

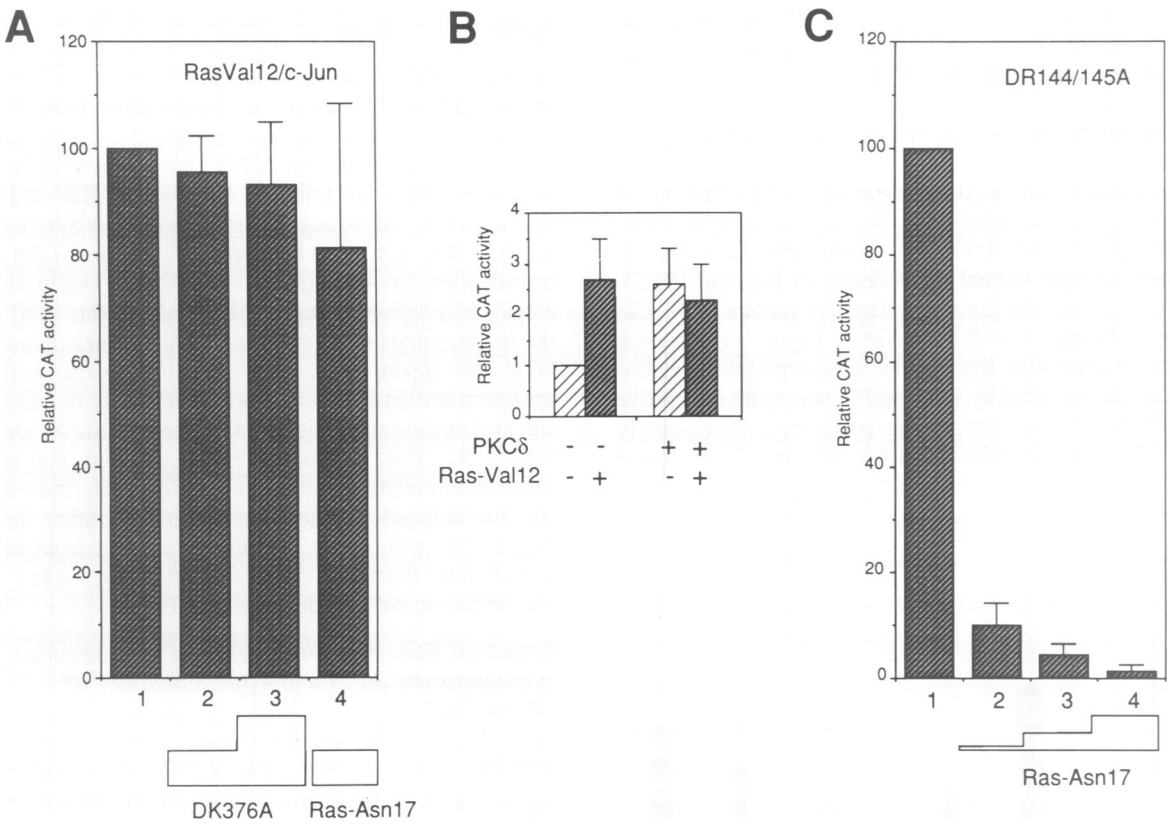

Fig. 5. Mutual dependency of PKCδ and Ras for the activation of TRE-tk-CAT expression in NIH3T3 cells. (**A**) The effect of dominant negative PKCδ, DK376A and dominant negative Ras, Ras-Asn17, on TRE-tk-CAT expression enhanced by activated Ras, Ras-Val12 and c-Jun is shown. NIH3T3 cells were co-transfected with 3.0 μg of TRE-tk-CAT, 1.0 μg of Ras-Val12 expression vector and 0.5 μg of c-Jun expression vector together with insertless vector alone (column 1), 1.0 μg (column 2) or 2.0 μg (column 3) of DK376A expression vector or 1.0 μg of Ras-Asn17 expression vector (column 4). Cells were starved in 0.5% FCS/DMEM for 45 h and harvested for CAT assay. (**B**) Collaboration of activated Ras and wild-type PKCδ to induce TRE-tk-CAT expression was tested by a co-transfection experiment using 3 μg of the reporter gene together with 1.0 μg of PKCδ- or Ras-Val12 expression vectors as indicated. (**C**) Inhibition of DR144/145A-induced TRE-tk-CAT expression by dominant negative Ras is shown. Cells were co-transfected with 3 μg of TRE-tk-CAT and 0.1 μg of DR144/145A expression vector together with insertless vector alone (column 1), or 0.1 μg (column 2), 0.5 μg (column 3) or 1.0 μg (column 4) of Ras-Asn17 expression vector.

starved cells was comparable to that of TRE-tk-CAT expression, but the effect of DR144/145A on RSV-CAT was much less (Figure 2C). When mTRE-tk-CAT gene containing mutated TRE was used as a reporter, the basal level of CAT expression was nearly 10 times lower than that observed with TRE-tk-CAT and the induction by TPA was not observed (Figure 3, column 3). The overexpression of PKCδ or DR144/145A resulted in some enhancement of the mTRE-tk-CAT expression in a TPA-independent manner, but the enhanced level of CAT expression was $<10\%$ of that observed with the reporter gene including intact TRE (Figures 2B and 3). Therefore, the major target of PKCδ on TRE-tk-CAT must be TRE. However, we cannot rule out the possibility that a certain transcription factor binding to the thymidine kinase promoter region is a minor target of PKCδ and collaborates with AP1/Jun for the enhancement of TRE-tk-CAT expression. We further tested the Jun/AP1 dependency of enhancement by employing mouse embryo carcinoma F9 cells which express very low levels of AP1/PEA1 (Kryszke *et al.*, 1987). The expression of TRE-tk-CAT gene was only weakly induced by DR144/145A in F9 cells; however, ectopic expression of c-Jun enabled DR144/145A reporter expression to be activated to a level several times higher than that attained by DR144/145A or c-Jun alone (Figure 2D). Taken together, these results strongly suggest that PKCδ in its active form activates gene expression through transcription factor AP1/Jun.

Dominant negative mutant of PKCδ inhibits PKCδ-dependent AP1/Jun activation

As an approach to understand the signal transduction pathway from PKCδ to AP1/Jun, a dominant negative mutant of PKCδ (DK376A) was constructed. This mutant harbors an amino acid substitution at Lys376 in the catalytic domain, which is crucial for ATP binding and is conserved among several protein kinases (Zoller *et al.*, 1981). As shown in Figure 4A, DK376A showed a dominant negative effect against TRE-tk-CAT expression in a dose-dependent manner and TPA could not neutralize this effect. Furthermore, the enhancement of TRE-tk-CAT expression by constitutively active PKCδ, DR144/145A, was also diminished by DK376A (Figure 4B, columns 1–3). Therefore, this dominant negative mutant most likely titrates PKCδ substrate or binding protein required for the signal transduction to TRE. Considerable inhibition of CAT expression ($\sim 50\%$) by DK376A was observed even with mTRE-tk-CAT (Figure 3, column 5) or RSV-CAT reporter (data not shown). This inhibition could be due to some non-specific effect of DK376A. However, the majority of the dominant negative effect observed with TRE-tk-CAT reporter must

be specific because the inhibition of CAT expression was almost completely overcome by the overexpression of active PKCδ (Figure 4B, columns 3–5).

Ras is required for the transcriptional activation by PKCδ

As reported previously (Binetruy *et al.*, 1991) and shown in Figure 6A, activated Ras, Ras-Val12, is a strong activator of TRE in collaboration with overexpressed c-Jun or JunD. To understand the mutual dependency of Ras and PKCδ, we tested whether the dominant negative mutant of PKCδ, DK376A, blocks the Ras-activated signal transduction pathway. In contrast to the reporter gene expression induced by PKCδ, that induced by Ras and Jun was quite insensitive to DK376A and was inhibited just partly by a high quantity of DK376A expression vector that is sufficient for the more severe inhibition of PKC-induced reporter gene expression (Figure 5A, columns 1–3). Then, we tested whether Ras activates PKCδ. The activation of reporter gene expression by wild-type PKCδ was further enhanced by TPA, as shown in Figure 1A; however, activated Ras did not show the TPA-like effect on PKCδ (Figure 5B). These results imply at least two possibilities as follows. One is that Ras and PKCδ activate AP1/Jun through independent pathways; the other is that Ras acts downstream of PKCδ in the signal transduction to AP1/Jun. To test these possibilities, we employed a dominant negative Ras mutant, Ras-Asn17. This ras mutant binds GDP with an affinity higher than that for GTP and appears to compete with endogenous Ras for upstream activators while lacking the ability to transduce a signal downstream (Feig and Cooper, 1988). As shown in Figure 5C, this mutant severely inhibited the reporter expression enhanced by active PKCδ, while the effect on Ras/Jun-induced reporter expression was rather moderate (Figure 5A, column 4). This suggests the presence of signal transduction from PKCδ to Ras, which plays a key role in the activation of AP1/Jun through PKCδ.

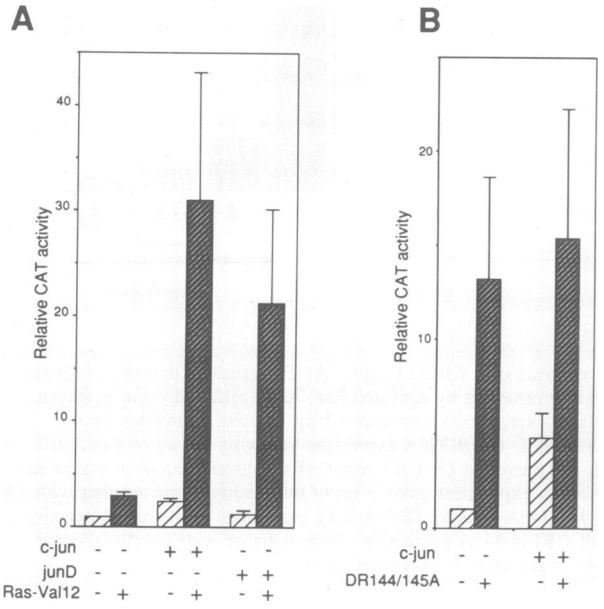

Fig. 6. Activated Ras, but not activated PKCδ, collaborates with Jun to induce TRE. (**A**) NIH3T3 cells were co-transfected with 3 μg of TRE-tk-CAT reporter gene together with c-Jun (0.5 μg), JunD (0.5 μg) or Ras-Val12 (1.0 μg) expression vectors as indicated. Cells were starved in 0.5% FCS/DMEM for 45 h after transfection and harvested for CAT assay. Shown is the relative CAT activity over the basal level of reporter gene expression. (**B**) The amount of expression vector DNA used for transfection was 0.5 μg for the c-Jun expression vector and 0.05 μg for the DR144/145A expression vector. Other conditions were the same as described in (A).

Activated Ras, but not activated PKCδ, requires overexpressed Jun for the full activation of TRE-tk-CAT

Even though Ras was indispensable for the induction of AP1/Jun, activated Ras, Ras-Val12, alone induced the reporter gene expression only 3-fold on average and this value rarely exceeded 10-fold in several independent experiments using a different quantity of expression vector for transfection (see Figure 5B or 6A). On the other hand, the effect of constitutively active PKCδ, DR144/145A, on reporter gene expression was more significant, showing up to an 80-fold induction (see Figure 2B). Overexpression of c-Jun or JunD augmented the induction of the reporter gene expression by Ras-Val12, to the level attained by DR144/145A (Figure 6A), while DR144/145A showed full activity by itself and no collaboration with overexpressed c-Jun was observed (Figure 6B). These results suggest the presence of a Ras-independent signal transduction pathway downstream of PKCδ, which is required for the efficient activation of AP1/Jun and can be replaced by the overexpression of c-Jun or JunD.

Constitutively active PKCδ suppresses cell growth

The significant ability of the active mutant of PKCδ to induce TRE-tk-CAT gene expression allows us to test if this mutant

Table I. Effect of PKC and its mutant on colony formation

Cells		Number of G418-resistant colonies in one (10 cm) plate					
		SRD[a]	PKCα	PKCδ	PKCε	DK376A	DR144/145A
NIH3T3	ex. 1	205	161	155	160	302	11
	ex. 2	281	261	203	117	178	33
29-1		180	ND	139	ND	140	47
29-2		186	ND	182	ND	186	76
29-3		157	ND	93	ND	129	12

[a]Expression vector without cDNA insert.

NIH3T3 or Ras-transformed cells (29-1, 29-2, 29-3) were seeded at a concentration of 2×10^5 cells/10 cm plate and co-transfected 24 h later with 1 μg of pSV_2-Neo together with 10 μg of expression vectors as indicated. After selection in G418-containing medium, the number of colonies was counted. For NIH3T3 cells, the results from two independent transfections (ex. 1, ex. 2) are shown. For Ras-transformed cells, typical results from three independent transfections are shown.

is oncogenic. Contrary to our expectation, no foci were formed with DR144/145A-transfected NIH3T3 cells under conditions where Ras-Val12-transfected cells form several foci (data not shown). Similarly, no growth in soft agar was observed with DR144/145A-transfected cells. When NIH3T3 cells were co-transfected with a neomycin-resistant gene, pSV_2-Neo, and DR144/145A expression vector, the number of neomycin-resistant colonies was severely reduced compared with the number of colonies formed by cells transfected with pSV_2-Neo alone (Table I). Wild-type PKC or DK376A, however, showed no significant effect on the colony formation. Then, we tested the growth-suppression activity of DR144/145A on Ras-transformed cell lines. Three Ras-transformed cell lines (29-1, 29-2, 29-3) were established from independent foci formed by NIH3T3 cells transfected with Ras-Val12 expression vector. All overexpressed transfected Ras and showed transformed phenotypes in several criteria, including cell shape, increased growth rate and saturation density and anchorage-independent cell growth. As shown in Table I, colony formation with these cells was also inhibited by DR144/145A, while wild-type PKCδ or DK376A did not show a significant effect.

Discussion

TRE has been identified as a common *cis*-acting DNA element found in the promoter-enhancer region of TPA-inducible genes (Angel *et al.*, 1987). Since the major cellular receptor for TPA is PKC (Blumberg, 1988), it is believed that this serine/threonine kinase activates gene expression through TRE. We show here that PKCδ is a potent AP1/Jun activator in mouse cells and that Ras plays an essential role in signal transduction from PKCδ to AP1/Jun.

We employed three different approaches to confirm the ability of PKCδ to activate AP1/Jun. First, selected PKC isozymes were overexpressed in mouse fibroblasts and the effect on the induction of TRE-tk-CAT expression by TPA was tested. Among seven TPA-sensitive PKC isozymes now identified, PKCα, PKCδ and PKCε are most common in fibroblastic cells such as Rat 6 cells or mouse NIH3T3 cells (Borner *et al.*, 1992; Mischak *et al.*, 1993; see also Figure 1B). We chose these three isozymes to overexpress in NIH3T3 cells and found that the overexpression of PKCδ enhances the response of TRE to TPA. This indicates that PKCδ mediates signal transduction from TPA to TRE. Second, a constitutively active PKCδ mutant (DR144/145A) was designed to show that the activation of PKCδ alone can cause the activation of gene expression. Even though a large part of TPA incorporation into living cells depends on PKC (Blumberg, 1988), a minor receptor for TPA could play critical roles in TRE activation. The TPA-independent activation of TRE by DR144/145A negates this possibility and demonstrates that the activation of PKCδ is sufficient for TRE activation. Finally, a dominant negative mutant of PKCδ (DK376A) was constructed to test the requirement of PKCδ for the activation of TRE. When a conserved lysine residue in the ATP binding site (Lys376) was replaced by an alanine residue, PKCδ became not only an inactive molecule, but also a suppressor of gene expression. TPA did not neutralize this dominant negative effect and the constitutively active mutant, DR144/145A, was also inhibited. Therefore, the negative effect of DK376A most likely reflects the titration of the PKCδ substrate or binding protein required for signal transduction to TRE, rather than the titration of a cellular activator, such as diacylglycerol. Since PKCδ lacking the catalytic domain also showed a dominant negative effect on gene expression (S.-i.Hirai, unpublished data), the regulatory domain may interact not only with phospholipid or diacylglycerol, but also with those proteins.

In comparison with PKCδ, PKCε was less effective and PKCα showed strong inhibition of TRE-tk-CAT expression. These differences are likely to depend on the substrate specificity of each isozyme and the inhibition of AP1/Jun by PKCα can be explained in a similar way to the inhibition by DK376A. Since PKCα contains a well-conserved cysteine-rich region in its regulatory domain (Nishizuka, 1988), this molecule may titrate PKCδ substrate or binding protein. However, we cannot conclude that PKCα is a suppressor of TRE. This isozyme may activate TRE combined with other promoters and may be active in different cell lines containing different sets of substrates for PKC isozymes (Pears *et al.*, 1990; Hata *et al.*, 1993).

It was previously reported that Ras protein activates c-Jun by stimulating the phosphorylation of its activation domain or by removing a cell-specific inhibitor (Baichwal *et al.*, 1991; Binetruy *et al.*, 1991). Furthermore, the activation of Ras protein upon TPA treatment of T lymphocytes has been reported (Downward *et al.*, 1990). Therefore, we thought that PKCδ may stimulate AP1/Jun through the activation of Ras proteins. This possibility was tested by using dominant negative mutants of Ras and PKC. We showed that the dominant negative Ras mutant (Ras-Asn17) blocks the activation of TRE by PKCδ. Since the dominant negative PKCδ mutant (DK376A) showed no significant effect on TRE activation by activated Ras (Ras-Val12) and Jun, it is conceivable that Ras acts downstream of PKCδ in the signal transduction to AP1/Jun. Recently, it has been shown that the dominant negative Ras, Ras-Asn17, blocks several TPA-induced events, including the activation of MAPKs/ERKs (Wood *et al.*, 1992), the activation of TGFβ promoter (Wotton *et al.*, 1993) and the activation of TRE (Alexandropoulos *et al.*, 1993). Together with our results, these indicate that Ras is a dominant effector of PKC. Now, the question arises as to how PKCδ activates Ras protein. In T lymphocytes, a decrease in Ras-GAP (GTPase-activating protein) activity upon TPA treatment has been reported (Downward *et al.*, 1990). On the other hand, the overexpression of GAP in NIH3T3 cells blocks the induction of MAPKs/ERKs activity by TPA, without changing the ability of serum to activate MAPKs/ERKs (Nori *et al.*, 1992). These data indicate that PKC modulates Ras activity through GAP. However, whether Ras-GAP, GDS (guanine nucleotide dissociation stimulator) or Ras itself are directly phosphorylated and controlled by PKC remains to be clarified.

Recently, it was reported that Raf-1 is directly phosphorylated and activated by PKCα *in vivo* as well as *in vitro* (Sözeri *et al.*, 1992; Kolch *et al.*, 1993). Since Raf-1 is believed to activate MAPKs/ERKs, a candidate for a direct c-Jun activator, through MAPK kinase (MAPKK) or MEK (Lange-Carter *et al.*, 1993; Moodie *et al.*, 1993), the activation of TRE by PKCδ could depend on the phosphorylation of Raf-1 by PKCδ. However, this is not likely for the following reasons. First, PKCδ phosphorylates

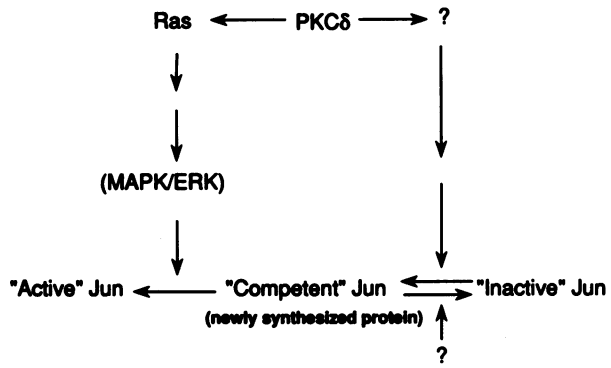

Fig. 7. Schematic drawing of the proposed mechanism of AP1/Jun activation by PKCδ.

Raf-1 protein poorly compared with PKCα *in vitro* (Sözeri *et al.*, 1992). Second, the dominant negative effect of Ras-Asn17 observed in the present work cannot be explained by this model, since this mutant Ras does not interact with Raf-1 even in the GTP binding form (Warne *et al.*, 1993). Finally, it has recently been reported that PKCα stimulates the auto kinase activity of Raf-1, but not the ability to phosphorylate MAPKK/MEK (Macdonald *et al.*, 1993). However, we cannot rule out the possibility that another activator of MAPKK/MEK, such as MEK kinase (MEKK) (Lange-Carter *et al.*, 1993), is a substrate for PKCδ and transmits a signal to AP1/Jun.

Ras-dependent signal transduction is indispensable for the activation of TRE by PKCδ, but by itself is not sufficient. Ras-Val12 enhances the expression of TRE-tk-CAT gene in NIH3T3 cells only moderately (Binetruy *et al.*, 1991) and the overexpression of c-Jun or JunD is required for the activation of reporter gene to the level attained by the overexpression of activated PKCδ (Figure 6A). As we have shown in the experiment using F9 cells (Figure 2D), PKCδ also requires Jun to activate TRE; however, its overexpression is not required in NIH3T3 cells. Therefore, it is conceivable that Ras cannot activate the AP1/Jun present in serum-starved NIH3T3 cells, while PKCδ can. This unique ability of PKCδ most likely depends on a Ras-independent signal transducer that makes endogenous Jun competent to accept activation by Ras (see Figure 7). Since such a Ras-independent pathway can be replaced by the overexpression of Jun, it may result in the removal of negative modification on Jun, such as by the interaction with an inhibitory protein or the phosphorylation of the C-terminal part of Jun, which inhibits DNA binding activity (Auwerx and Sassone-Corsi, 1991; Lin *et al.*, 1992). In addition, the enhancement of TRE-tk-CAT expression by activated PKCδ may partly depend on the induction of endogenous c-*jun* gene expression which is positively autoregulated by AP1/Jun (Angel *et al.*, 1988).

The ability of PKCδ to activate TRE or Ras implies the possibility that constitutively active PKCδ, DR144/145A, transforms NIH3T3 cells. The results, however, show that this activated PKCδ is a suppressor of cell growth. This is consistent with observations, recently reported by others, that show the phorbol ester-induced arrest of cell division in CHO or NIH3T3 cells overexpressing PKCδ (Watanabe *et al.*, 1992; Mischak *et al.*, 1993). On the other hand, it has been reported that the overexpression of PKCε transforms NIH3T3 cells or Rat 6 embryo fibroblasts (Cacace *et al.*, 1993; Mischak *et al.*, 1993). Since PKCε was not as effective as PKCδ at activating TRE (Figure 1A), the abilities to activate TRE and to transform cells seem not to be correlated, as suggested by studies on c-Jun mutants (Håvarstein *et al.*, 1992; Oliviero *et al.*, 1992; Métivier *et al.*, 1993). The transformation of cells by activated Ras cannot completely neutralize the inhibition of cell growth by activated PKCδ (Table I), showing that the inhibition of cell growth is mostly independent of Ras activity. Taken together, these results emphasize the presence of additional effector(s) of PKCδ other than Ras.

Materials and methods

Expression vectors and reporter gene

Expression vectors for PKC isozymes were constructed with vector plasmid SRD and cDNA of rabbit PKCα, mouse PKCδ or rabbit PKCε, as described elsewhere (Ohno *et al.*, 1988; Mizuno *et al.*, 1991). Expression vectors for c-Jun or JunD were constructed with a vector plasmid containing RSV LTR and mouse c-Jun or JunD cDNA, as described elsewhere (Hirai *et al.*, 1989). Ha-Ras-Val12 and Ki-Ras-Asn17 were made by site-directed mutagenesis and expression vectors for these mutants were constructed with SRD vector (Fukumoto *et al.*, 1990). TRE-tk-CAT contains the *E.coli* chloramphenicol acetyl transferase (CAT) gene downstream of TRE derived from the human collagenase gene (−72, TGAGTCA) and minimum promoter derived from the herpes simplex virus thymidine kinase gene (−109 to +57) (Angel *et al.*, 1987). mTRE-tk-CAT containes mutated TRE (TTACTTA) which binds slightly to AP1/Jun (Hirai and Yaniv, 1989). RSV-CAT contains RSV LTR and CAT gene (Gorman *et al.*, 1982).

Construction of mutant PKCδ

The spontaneously active PKCδ mutant, DR144/145A, and dominant negative PKCδ mutant, DK376A, were constructed directly on the PKCδ expression vector by site-directed mutagenesis using a heteroduplex method described elsewhere (Hirai and Yaniv, 1989). Briefly, two different linearized plasmid DNAs were prepared by removing either a part of the ampicillin resistance gene or a cDNA insert. Equal quantities (molar) of DNA were mixed (total ~5 μg) and denatured in 40 μl of 0.2 M NaOH for 10 min at room temperature. To generate heteroduplexes, the mixture was neutralized with 180 μl of 100 mM Tris−HCl (pH 7.5), 30 mM HCl and incubated at 68°C for 2 h. Then, 1 μg of the phosphorylated oligonucleotides shown below was mixed with 50 μl of heteroduplex solution and hybridized at 42°C for 1 h. To this hybridization mix were added 1 μl each of 200 mM $MgCl_2$, 10 mM ATP, 5 mM dNTP mix and 200 mM dithiothreitol (DTT), and the heteroduplex was repaired with 5 U of Klenow enzyme and 1 U of T4 ligase at room temperature for 5 h. The repaired DNA was used to transfect bacteria and a mutant clone was selected by colony hybridization using the mutant probe oligonucleotide shown below. After checking the DNA sequence of the mutated region, the mutant expression vectors were used for the transfection experiment. Oligonucleotides used for mutagenesis and colony hybridization were as follows.

Mutant oligonucleotides:

DR144/145A:

AAGTTTCCAACCATGAACGCAGCTGGAGCCATTAAACAGGCC

DK376A:

GATAAGTACTTTGCAATCGCATGCCTGAAGAAGGACGTGGT

Probe oligonucleotides:

DR144/145A: CATGAACGCAGCTGGAG

DK376A: GCAATCGCATGCCTGAA

Transfection and CAT assay

NIH3T3 cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 7% calf serum (CS). Cells were seeded 24 h before transfection at a concentration of 2−3 × 10^5 cells/6 cm plate and fed with fresh medium at 4−6 h before transfection. Transfection by calcium phosphate co-precipitation was carried out for 8 h using 3 μg of reporter plasmid and 0.1−2.0 μg of appropriate expression vectors, as shown in the figure legends. The culture medium was then changed to DMEM supplemented with 0.5% fetal calf serum (FCS) to starve the cells. When required, TPA treatment was performed after 48 h of starvation by changing the medium to 0.5% FCS/DMEM containing TPA at a concentration of 100 ng/ml. DMEM/0.5% FCS containing 0.1% dimethyl sulfoxide (DMSO), the vehicle for TPA, was used for control treatment. Cells were

further cultured for 10 h and harvested for CAT assay. When TPA treatment was not required, cells were harvested after 45 h of starvation.

Mouse embryonal carcinoma F9 cells were routinely grown in DMEM supplemented with 10% FCS and seeded for transfection at a concentration of 5×10^5 cells/6 cm plate. Transfection was performed as described for NIH3T3 cells, except that cells were continuously fed with 10% FCS/DMEM until they were harvested for CAT assay 24 h after transfection.

CAT activity was measured by a standard procedure described elsewhere (Hirai *et al.*, 1989); the results of TLC were quantified with a Bio-image analyzer (Fuji BAS2000).

Western blotting

NIH3T3 cells were transfected, serum starved and TPA treated as described above and collected directly in SDS−PAGE sample buffer. Following SDS−PAGE, the separated proteins were electrophoretically transferred to a PVDF membrane. The membrane was soaked in PBS containing 5% skimmed milk for 1 h at room temperature and incubated with rabbit polyclonal antibodies against PKCα (Gibco 3222SA), PKCδ (Mizuno *et al.*, 1991) or PKCε (Gibco 3198SA) appropriately diluted with TBST [150 mM NaCl, 0.05% Tween 20, 20 mM Tris−HCl (pH 7.5)] containing 0.1% bovine serum albumin for 1 h at 37°C. These specific antibodies were raised against synthetic peptides whose sequences were derived from the joining region of the regulatory and catalytic domains of PKCα or the C-terminal part of PKCδ or PKCε. After washing thoroughly with TBST containing 500 mM NaCl, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (TAGO 6500) diluted 1000 times with TBST. After washing the membrane as described above, antigen−antibody complexes were visualized with an artificial substrate for alkaline phosphatase (Vector Lab. SK-5100).

Colony-forming assay

NIH3T3 cells or Ras-transformed cells (see below) were seeded at a concentration of 2×10^5 cells/10 cm plate and transfected with 1 μg of pSV_2-Neo and 10 μg of expression vector for PKC or Ras by calcium phosphate co-precipitation. After 2 days culture in 7% CS/DMEM, cells were selected with 7% CS/DMEM containing G418 (Gibco Geneticin) at a concentration of 300 μg/ml. The selection medium was changed once after 3 days and culturing was continued for 3 more days. Cells were then fixed with methanol and stained with Giemsa's solution (Sigma) for colony counting.

Ras-transformed cell lines, 29-1, 29-2 and 29-3, were established from foci formed with NIH3T3 cells transfected with Ha-Ras-Val12 expression vector. NIH3T3 cells were seeded at a concentration of 2×10^5 cells/10 cm plate and transfected with 10 μg of Ha-Ras-Val12 expression vector. Cells were cultured in 7% CS/DMEM for 14 days with the medium changed every 3 days. Then, focus-forming cells were picked up using a micro-pipette tip and clone purified. All the cell lines used can grow in low-serum medium and in soft agar (data not shown).

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