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EGF or PDGF receptors activate atypical PKCλ through phosphatidylinositol 3-kinase

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Overexpression of a TPA-insensitive PKC member, an atypical protein kinase C (aPKCλ), results in an enhancement of the transcriptional activation of TPA response element (TRE) in cells stimulated with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF). EGF or PDGF also caused a transient increase in the *in vivo* phosphorylation level and a change in the intracellular localization of aPKCλ from the nucleus to the cytosol, indicating the activation of aPKCλ in response to this growth factor stimulation. These immediate signal-dependent changes in aPKCλ were observed for a PDGF receptor add-back mutant (Y40/51) that possesses only two of the five major autophosphorylation sites and binds PI3-kinase, and were inhibited by wortmannin, an inhibitor of PI3-kinase. Furthermore, an N-terminal fragment of the catalytic subunit of PI3-kinase, p110α, inhibited aPKCλ-dependent activation of TRE in Y40/51 cells stimulated with PDGF. Overexpression of p110α resulted in an enhancement of TRE expression in response to PDGF and the regulatory domain of aPKCλ inhibited this TRE activation in Y40/51 cells. These results provide the first *in vivo* evidence supporting the presence of a novel signalling pathway from receptor tyrosine kinases to aPKCλ through PI3-kinase.

Keywords: EGF receptor/PDGF receptor/PI3-kinase/PKCλ

Introduction

The generation of a variety of lipid metabolites upon stimulation of a variety of cell surface receptors is one of the major biochemical events thought to elicit the pleiotropic effects of receptors (Cantley *et al.*, 1991; Nishizuka, 1992; Berridge, 1993; Kapeller and Cantley, 1994; Divecha and Irvine, 1995). The lipid metabolites intensively studied so far include diacylglycerols (DGs) (Nishizuka, 1992; Berridge, 1993; Divecha and Irvine, 1995) and phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3) (Cantley *et al.*, 1991), which are generated through receptor-stimulated activation of specific enzymes including phospholipase C (PLC) (Nishizuka, 1992) and phosphatidylinositol 3-kinase (PI3-kinase) (Kapeller and Cantley, 1994), respectively. PI3-kinase is a heterodimeric enzyme composed of a p85 regulatory subunit and a p110 catalytic subunit (Otsu *et al.*, 1991; Hiles *et al.*, 1992). PI3-kinase phosphorylates the D3 position of phosphatidylinositol, phosphatidylinositol-4-phosphate, or phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) and produces 3-phosphorylated inositol lipids such as PI-3,4,5-P_3 (Whitman *et al.*, 1987, 1988). Experiments on epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors as well as other receptors whose signal transduction involves tyrosine kinase activities have revealed that receptor activation causes the recruitment and activation of sets of proteins (Cantley *et al.*, 1991). These include PLCγ (Kumjian *et al.*, 1989; Morrison *et al.*, 1990) and PI3-kinase (Bjorge *et al.*, 1990; Kazlauskas and Cooper, 1990; Escobedo *et al.*, 1991a,b). In addition to its association with tyrosine kinase-type receptors such as PDGF and NGF, PI3-kinase associates with, and is stimulated by, insulin receptor substrate IRS1 (Backer *et al.*, 1992) and src-like kinases (Kypta *et al.*, 1990). In addition, receptors coupled to trimeric G-proteins have been shown to activate PI3-kinase (Stephens *et al.*, 1993a,b, 1994), suggesting the involvement of PI3-kinase in signalling pathways through a wide variety of cell surface receptors. However, the downstream effector molecules of PI3-kinase remain obscure.

Protein kinase C (PKC) was originally discovered as a serine/threonine kinase activated by DGs or tumour-promoting phorbol esters. Since the generation of DGs has been shown to be associated with the activation of a variety of receptors coupled to G-proteins as well as receptor tyrosine kinases and receptors coupled to cytosolic tyrosine kinases, PKC has been implicated in these signalling pathways (Nishizuka, 1988, 1992). Recent experiments have established that PKC comprises a protein family of at least 11 members that can be divided into three distinct classes: classical PKC (cPKC) comprising PKCα, βI, βII and γ; novel PKC (nPKC) comprising PKC δ, ε, η, θ and μ/PKD; and atypical PKC (aPKC), comprising aPKCζ and aPKCι/λ. cPKC and nPKC members are activated by DG and phorbol esters such as TPA *in vitro* and *in vivo* and are likely receptors for these agents in cells (Akita *et al.*, 1990; Ohno *et al.*, 1991, 1994; Nishizuka, 1992; Hata *et al.*, 1993; Johannes *et al.*, 1995; Lint *et al.*, 1995). aPKC members, however, are activated by neither DG nor phorbol esters and their mode of activation remains to be determined (Ono *et al.*, 1989b; Nakanishi and Exton, 1992; Selbie *et al.*, 1993; Akimoto *et al.*, 1994). Recent reports using a pseudo substrate peptide of aPKCζ or kinase-negative dominant negative form of aPKCζ raised the possibility that aPKCζ is

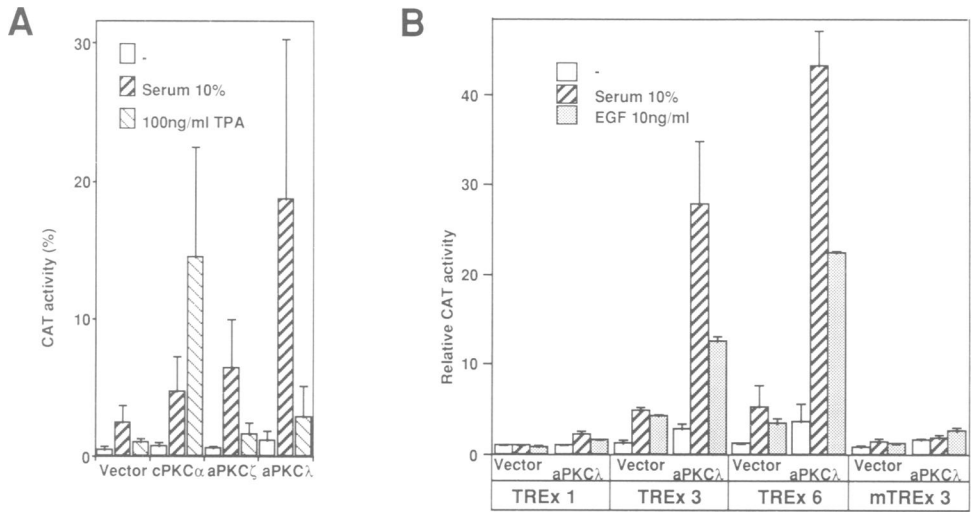

Fig. 1. Overexpression of aPKC enhances the serum- or EGF-stimulated activation of TRE. (**A**) PKC expression plasmids (12 μg/10 cm dish), cPKCα (SRDα), aPKCζ (SRDζ), aPKCλ (MLNP45) or an empty vector (SRD) were transfected with TRE ×6 CAT reporter (3 μg/10 cm dish). Values shown indicate the means (± SD) of the CAT conversion of three independent transfections. (**B**) CAT plasmids containing various numbers of TRE or mutant TRE sequences were used as reporters to monitor aPKCλ-dependent TRE activation. Values shown indicate the means (± SD) of relative CAT activities of three independent transfections. The value for unstimulated vector transfected control was taken as 1.

involved in *Xenopus* oocyte maturation and fibroblast proliferation (Dominguez *et al.*, 1992, 1993; Berra *et al.*, 1993; Diaz-Meco *et al.*, 1994). Furthermore, *in vitro*, Nakanishi has shown that enzymologically synthesized PI-3,4,5-P_3 modulates aPKCζ activity (Nakanishi *et al.*, 1993), raising the possibility that aPKCζ can be activated by PI-3,4,5-P_3. Although the *in vitro* results suggest a possible link between PI3-kinase and aPKC, there has been no *in vivo* experimental system to examine this possibility.

During the course of our experiments examining the involvement of aPKCλ in the transcriptional activation of TPA response element (TRE) in rat 3Y1 fibroblasts, we found that mitogens of 3Y1 cells, such as EGF but not TPA, activate aPKCλ. aPKCλ can not be activated by TPA or membrane-permeable DG, which activates both cPKC and nPKC, suggesting that aPKCλ is activated through a signalling pathway distinct from those involving PLC. Thus, we examined the role of PI3-kinase in the signalling pathway from receptor tyrosine kinases to aPKCλ activation. Our results clearly indicate that aPKCλ is activated through PI3-kinase.

Results

Overexpression of aPKCλ causes enhanced transcriptional activation of TRE in response to serum or EGF but not TPA

Previous studies have shown that the overexpression of cPKCα, nPKCδ or nPKCε in rat 3Y1 fibroblasts enhances the TPA-induced activation of TRE-CAT reporter expression (Hata *et al.*, 1993; Ohno *et al.*, 1994). To determine whether aPKCλ is involved in this process, we overexpressed aPKCλ and monitored TRE-CAT expression. As shown in Figure 1A, overexpression of aPKCζ or aPKCλ augmented the serum-stimulated expression of TRE-CAT to a much greater extent than the TPA-stimulated response. In contrast, overexpression of cPKCα enhanced TRE-CAT expression in response to TPA, but did not greatly enhance the serum-stimulated response. The application of synthetic membrane-permeable DG to 3Y1 cells resulted in cPKCα, nPKCδ or nPKCε-dependent activation of TRE-luciferase (Ohno *et al.*, 1994) but not aPKC-dependent TRE activation (data not shown). Among the growth factors tested, EGF and TGFα were the most potent stimulators of TRE-CAT reporter in 3Y1 cells (Figure 1B and data not shown). The enhancement of TRE-CAT expression by overexpression of aPKCλ depends on the copy number of TRE in the reporter plasmid and is not observed for the mutant form of TRE, which does not bind AP1 (Figure 1B) (Angel *et al.*, 1987; Hata *et al.*, 1993). Thus, the TRE-CAT expression observed most likely reflects the transcriptional activation of TRE.

The results suggest that aPKCλ is involved in signalling from the EGF receptor to the nucleus, and demonstrate that the signalling events leading to aPKCλ activation can be monitored in intact cells. It appears that aPKCλ is activated in intact cells by signalling pathways that do not involve the PLCγ/DG pathway.

Overexpression of a PI3-kinase catalytic subunit, p110α, enhances aPKCλ-dependent TRE activation, while overexpression of the PI3-kinase p85α subunit causes suppression

Previous reports demonstrated that the amount of PIP_3 increased upon EGF stimulation of a rat fibroblast cell line that overexpresses human EGF receptor (Bjorge *et al.*, 1990) or in rat PC12 cells (Carter and Downes, 1992; Ohmichi *et al.*, 1992). To examine whether the level of cellular PIP_3 increases upon EGF stimulation in rat 3Y1 cells, the synthesis of cellular PIP_3 in response to EGF stimulation was analysed. EGF caused an increase in PIP_3 within 1 min (with a 2.3-fold net increase in the content in total cellular lipids) followed by a decrease within 10 min, indicating that PI3-kinase is transiently activated in response to EGF in 3Y1 cells (data not shown).

We next tried to examine the involvement of PI3-kinase in aPKC-dependent TRE activation. Overexpression of

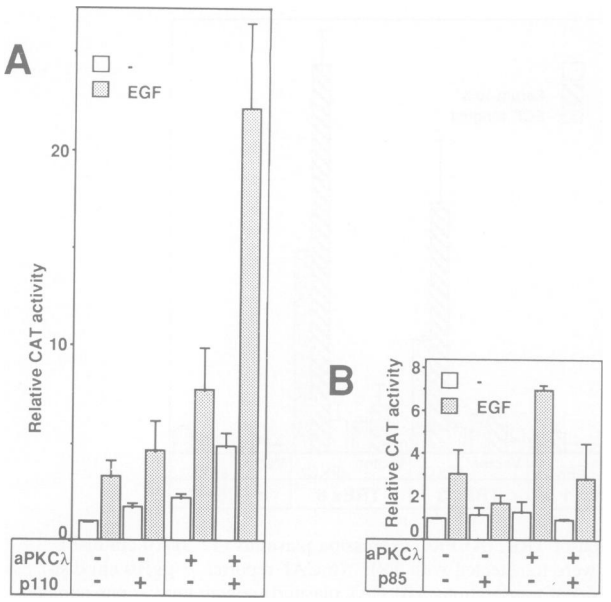

Fig. 2. Stimulation or inhibition of aPKCλ-dependent TRE-activation by co-transfected PI3-kinase subunits. Values are presented as for Figure 1B. (**A**) The expression plasmid encoding p110α (0 or 4 μg) was transfected with or without aPKCλ (6 μg). The reporter plasmid used was TRE ×6 CAT (3 μg). After serum starvation, cells were stimulated with EGF (10 ng/ml). (**B**) The reporter plasmid TRE ×6 CAT (3 μg) was co-transfected with the expression plasmid encoding p85α (0 or 1 μg) in the absence (–) or presence (+) of aPKCλ (6 μg).

the catalytic subunit of PI3-kinase, p110α, has been shown to cause an increased PI3-kinase activity in Sf9 cells (Hiles *et al.*, 1992), and the purified catalytic subunit has been shown to be fully active *in vitro* (Shibasaki *et al.*, 1991; Woscholski *et al.*, 1994). Thus, we overexpressed one of the PI3-kinase catalytic subunits, p110α, and monitored aPKCλ-dependent TRE activation. In these experiments a submaximal amount of aPKCλ was used so that any stimulatory effects of the co-expression of p110α could be more readily detected. As shown in Figure 2A, overexpression of p110α or aPKCλ alone had a slight effect on the basal level of activity and augmented EGF-stimulated TRE activation. However, co-expression of both p110α and aPKCλ resulted in a great enhancement in TRE activation in response to EGF. Similar results were also obtained when cells were stimulated with serum (data not shown). The enhancement of EGF-dependent TRE activation by co-expression of aPKCλ and p110α does not seem to be additive, but co-operative, suggesting some relationship between the two molecules in mediating EGF-stimulated TRE activation.

Several reports support the notion that the activation of PI3-kinase involves the binding of the p85 subunit to tyrosine-phosphorylated proteins or peptides (Skolnik *et al.*, 1991; Ohmichi *et al.*, 1992). In fact, overexpression of p85α or microinjection of its N-terminal SH2 domain, has been reported to inhibit insulin-stimulated c-*fos* promoter activation (Yamauchi *et al.*, 1993; Jhun *et al.*, 1994). This suggests that the overexpression of p85 might uncouple the signal-dependent receptor/PI3-kinase interaction and consequently result in an inhibition of EGF-dependent PI3-kinase activation. Thus, we overexpressed the p85α regulatory subunit of PI3-kinase and monitored EGF-stimulated TRE activation in the presence and absence of exogenously expressed aPKCλ. As shown in Figure 2B, expression of p85α resulted in an inhibition of TRE activation in the presence or absence of aPKCλ. This suggests that both endogenous pathways leading to the activation of the TRE-CAT reporter as well as aPKCλ-dependent pathways involve the activation of PI3-kinase in 3Y1 cells.

aPKCλ-dependent TRE activation depends on the tyrosine residues of the βPDGF receptor required for PI3-kinase activation

Signal transduction pathways for βPDGFR have been studied in detail (Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989; Kumjian *et al.*, 1989; Wahl *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Kypta *et al.*, 1990; Morrison *et al.*, 1990; Li *et al.*, 1992; Lowenstein *et al.*, 1992). A panel of receptor mutants (the add-back series) has been constructed which enables the investigation of some of the receptor signalling pathways (Valius and Kazlauskas, 1993). When expressed in HepG2 cells, a βPDGF receptor mutant lacking five tyrosine phosphorylation sites (F5; Y740F, Y751F, Y771F, Y1009F, Y1021F) did not mediate βPDGF-dependent PIP_3 production or PI turnover. Restoration of Tyr740 and Tyr751 (Y40/51) or Tyr1021 (Y1021) enabled the receptor selectively to activate PI3-kinase or PLCγ, respectively. To examine the signalling pathway leading to aPKCλ activation, we used HepG2 cells that overexpress these βPDGFR add-back mutants (Figure 3B). We transfected aPKCλ with TRE-luciferase reporter gene and monitored luciferase expression after stimulation with PDGF. Western blots of the cell extracts prepared in parallel with the luciferase assay confirmed the expression of aPKCλ (Figure 3C). Multiple forms of aPKCλ were also seen when aPKCλ was overexpressed in COS1 cells (Akimoto *et al.*, 1994). The expression of aPKCλ in Y40/51 cells is lower than those in the other cell lines. This difference reflects that in transfection efficiency among the HepG2 cell lines (data not shown). However, the transfection efficiency of the Y40/51 cells remain constant in a series of co-transfection experiments (Figure 4A and B). As shown in Figure 3A, PDGF stimulation increased the luciferase activity in cells expressing wild-type βPDGFR, but this enhancement was not observed for the mutant form of TRE. The results suggest that aPKCλ is involved in signalling from the PDGF receptor to the nucleus. Also, PDGF stimulation enhanced aPKCλ-dependent TRE expression in HepG2 cells overexpressing the add-back mutant (Y40/51) that activates PI3-kinase but not PLCγ, whereas it did not enhance TRE expression in HepG2 cells overexpressing Y1021 or F5 receptors (Figure 3A). Although the overexpression of aPKCλ in F5 cells resulted in a slight increase in TRE activation, this was not dependent on PDGF stimulation. The results indicate that aPKCλ-dependent TRE activation requires the tyrosine residues of βPDGFR which are required for the association of PI3-kinase and imply that PI3-kinase is involved in the aPKCλ-dependent pathway. The results also suggest that the aPKCλ-dependent pathway does not involve the PLCγ/DG pathway.

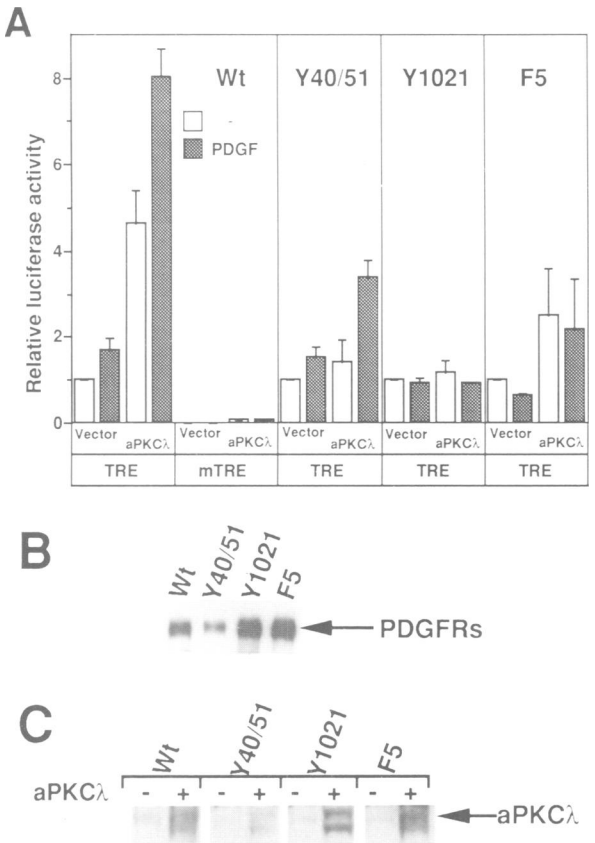

Fig. 3. aPKC-dependent TRE activation in HepG2 cells expressing βPDGFR add-back mutants. (**A**) HepG2 cells expressing βPDGFR mutants were transfected with expression vectors encoding aPKCλ (9 μg), and TRE ×3- or mTRE ×3-luciferase (3 μg). After serum starvation, cells were stimulated with PDGF (30 ng/ml) and the luciferase activity was evaluated. Values shown were normalized using those of unstimulated vector transfected cells as 1 and represent the means (± SD) of three independent transfections. (**B** and **C**) Western analysis of total cell extracts prepared in parallel with the luciferase assay shown in (A). Antibodies used are anti-PDGFR (B) or anti-PKCι (C). Ten micrograms of protein were analysed.

A dominant negative mutant of PI3-kinase suppresses aPKCλ-dependent TRE activation and a dominant negative mutant of aPKCλ suppresses p110α-dependent TRE activation

In order to examine directly the involvement of PI3-kinase in aPKCλ-dependent TRE activation in Y40/51 cells, we next examined the effects of the overexpression of PI3-kinase subunits and obtained results consistent with those obtained for EGF-stimulated 3Y1 cells shown in Figure 2 (Figure 4A). The co-expression of p110α with aPKCλ resulted in a co-operative enhancement in TRE expression. This further supports the co-operation of aPKCλ with PI3-kinase upon PDGF stimulation. We next examined the effect of some PI3-kinase mutants on aPKCλ-dependent TRE activation. A kinase knockout point mutant of p110α (Knp110), which does not possess PI3-kinase activity (Dhand *et al.*, 1994; data not shown), showed no co-operative effects. More importantly, the regulatory subunit of PI3-kinase, p85α, inhibited the aPKCλ-dependent activation of TRE to some extent. In addition, an N-terminal fragment of p110α (EcoS) also showed a dominant negative effect and completely suppressed aPKCλ-dependent TRE activation (Figure 4A). The N-terminal part of p110α has been shown to be involved in the interaction with its regulatory subunit during signal-dependent activation (Dhand *et al.*, 1994; Hu and Schlessinger, 1994; J.Takayanagi *et al.*, manuscript in preparation) and the direct interaction of EcoS with p85 was demonstrated in COS cells (data not shown). These results further support the notion that the aPKCλ-dependent TRE activation observed in Y40/51 cells depends on endogenous PI3-kinase.

As observed for EGF-stimulated 3Y1 cells, overexpression of p110α also resulted in a slight enhancement of TRE activation in PDGF-stimulated Y40/51 cells (Figure 4B). We next examined the effect of some aPKCλ mutants on the activation and found that a kinase knockout point mutant of aPKCλ (Kn-aPKCλ) and a N-terminal fragment encoding the regulatory domain of aPKCλ (RD) inhibit the p110α-dependent enhancement of TRE in PDGF-stimulated 40/51 cells (Figure 4B). The effect of RD was more drastic than that of Kn-aPKCλ, presumably because of the level of expression; RD is expressed more abundantly than Kn-aPKCλ (data not shown). RD contains the cysteine-rich motif of aPKCλ that is conserved in all the PKC family members. As shown in lower panels of Figure 4A, co-transfection of PI3-kinase subunits or its mutants do not affect the expression levels of aPKCλ. Similarly, co-transfection of aPKCλ mutants does not affect the expression levels of p110α, as shown in the lower panels of Figure 4B. These results suggest that p110α-dependent TRE activation in 40/51 cells requires aPKCλ and/or its HepG2 cells homologue. Similar effects as those for Knp110, EcoS and Kn-aPKCλ described above were also observed for 3Y1 cells stimulated with EGF (data not shown).

EGF-dependent phosphorylation of aPKCλ is enhanced by overexpression of p110α

The results presented above clearly indicate the link between PI3-kinase and aPKCλ in receptor-stimulated TRE activation. The simplest explanation is that products of PI3-kinase, such as PIP_3, directly activate aPKCλ as demonstrated for purified aPKCζ and for nPKCε *in vitro* (Nakanishi *et al.*, 1993; Toker *et al.*, 1994). If the activated state of aPKCλ could be recovered from stimulated cells, we might be able to monitor directly the activation of aPKCλ. Thus, we immunoprecipitated aPKCλ from unstimulated and stimulated cells and compared their kinase activities *in vitro*. However, we failed to obtain reproducible results. To our knowledge, there are few reports where the activated state of any PKC family members have successfully been recovered from stimulated cells. This implies that the activated state of aPKCλ, as well as other PKC family members, may not be so stable as to be maintained during cell disruption and isolation, in clear contrast to phosphorylation-dependent kinases such as MAP kinases.

Many of the PKC family members including aPKCλ and aPKCζ have been shown to autophosphorylate *in vitro* (Nakanishi and Exton, 1992; Akimoto *et al.*, 1994). Furthermore, *in vivo* metabolic labelling experiments have shown that the *in vivo* phosphorylation levels of cPKCα, nPKCδ and nPKCε increase with cell stimulation by TPA, an activator of these PKC members, and by various cell

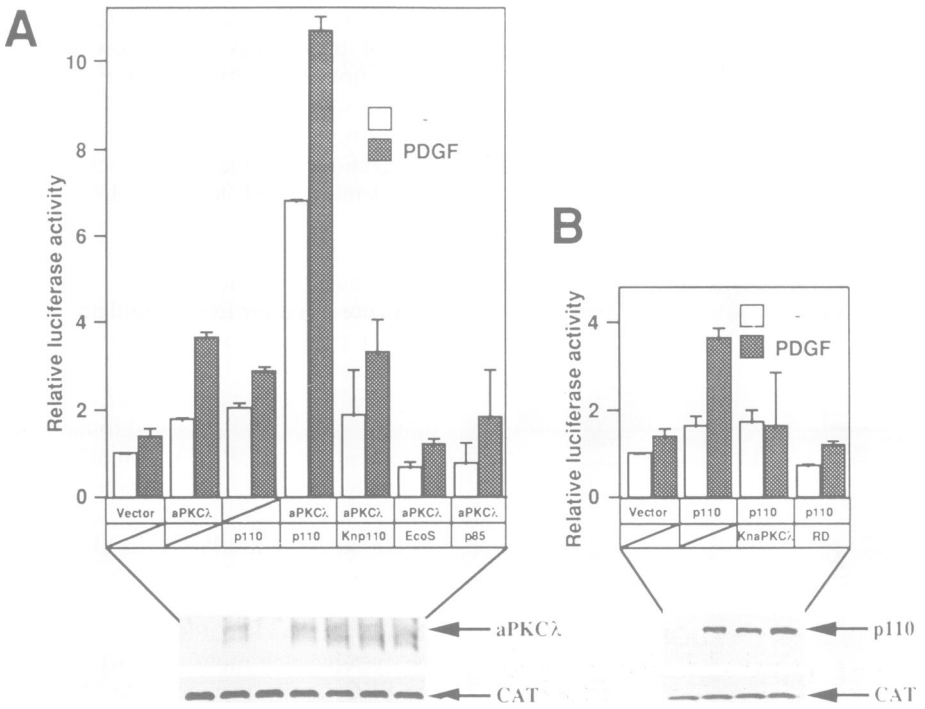

Fig. 4. A dominant negative form of p110α suppresses the aPKCλ-dependent TRE expression in HepG2 cells expressing Y40/51 βPDGFR mutant. (**A**) The expression plasmid encoding aPKCλ (0 or 9 μg) was transfected with or without p110α (6 μg), Knp110 (6 μg), EcoS (9 μg) or p85α (1 μg). (**B**) The expression plasmid encoding p110α or tag-p110α (0 or 9 μg) was transfected with or without Kn-aPKCλ (9 μg) or RD (6 μg). The values were normalized and represented as for Figure 3A. Lower panels show examples of the blots of the total cell extracts (10 μg) prepared in parallel with the luciferase assay. The blots were visualized using antibodies to aPKCι or to tag-p110α (B). In some of the repeated experiments, SV40-CAT expression plasmid (2 μg) was also co-transfected as an internal control of transfection efficiency. The lowest panels show examples of CAT expression.

stimuli which seem to activate these PKC members (Fry *et al.*, 1985; Mitchell *et al.*, 1989; Molina and Ashendel, 1991; Li *et al.*, 1994; Ohno *et al.*, 1994). It appears that the activation of PKC accompanies its autophosphorylation, although the relationship of PKC autophosphorylation to its activation remains unknown. As for aPKC members, the immunoprecipitated aPKCλ, as well as purified aPKCζ, has been shown to be autophosphorylated (Nakanishi and Exton, 1992; Akimoto *et al.*, 1994). Thus, we tried to measure the change in the *in vivo* phosphorylation level of aPKCλ upon stimulation with EGF. COS1 cells transfected with an expression plasmid encoding a tag-aPKCλ were metabolically labelled with ^{32}P-orthophosphate, left resting or stimulated with EGF, and the tag-aPKCλ was immunoprecipitated. The tag-aPKCλ was fully competent to enhance EGF-stimulated TRE activation in 3Y1 cells (data not shown). Figure 5A shows that tag-aPKCλ has a basal level of phosphorylation in serum-starved COS1 cells, indicating that aPKCλ is a phosphoprotein. Furthermore, EGF stimulation causes a transient increase in the level of phosphorylation. A maximal 1.3- to 2-fold increase was reproducibly observed peaking 5 min after stimulation. Although we do not exclude the possibility that this increase in the phosphorylation level of aPKCλ is caused by *trans*-phosphorylation by other unknown kinases, the change is EGF-dependent and possibly associated with the activation of aPKCλ in intact cells.

We next examined the effect of p110α overexpression on EGF-dependent changes in aPKCλ. As shown in Figure 5B, overexpression of p110α caused an enhancement of tag-aPKCλ phosphorylation in both resting and EGF-stimulated cells. When normalized by the aPKCλ amount (Figure 5C), the phosphorylation of aPKCλ was enhanced 1.6-fold by co-expression of p110α. In cells overexpressing p110α, EGF caused a 1.7-fold increase in the phosphorylation of tag-PKCλ when normalized by the aPKCλ protein amount. While this is not a dramatic increase, it was highly reproducible. These data clearly indicate the signal-dependent immediate change in aPKCλ and support the notion that aPKCλ is activated through a PI3-kinase pathway.

PDGF-stimulated phosphorylation of aPKCλ depends on the tyrosine residues of βPDGF receptor required for PI3-kinase activation

To verify the possibility that the activation of PI3-kinase leads to the phosphorylation of aPKCλ, we used a series of βPDGFR mutants. For this purpose, the receptor mutant (Y40/51), which activates PI3-kinase, was transiently co-expressed with tag-aPKCλ in COS1 cells, and PDGF-dependent phosphorylation of tag-aPKCλ was tested. COS1 cells do not express detectable levels of βPDGFR (data not shown). PDGF-stimulation caused a 1.7-fold increase in the phosphorylation state of tag-aPKCλ when normalized for the amount of protein as measured by Western analysis (Figure 5D). This response seems to be dependent on the activation of PI3-kinase, since pre-treatment of cells with 100 nM wortmannin, an inhibitor of PI3-kinase, completely blocked PDGF-stimulated tag-aPKCλ phosphorylation (Figure 5D). These experiments

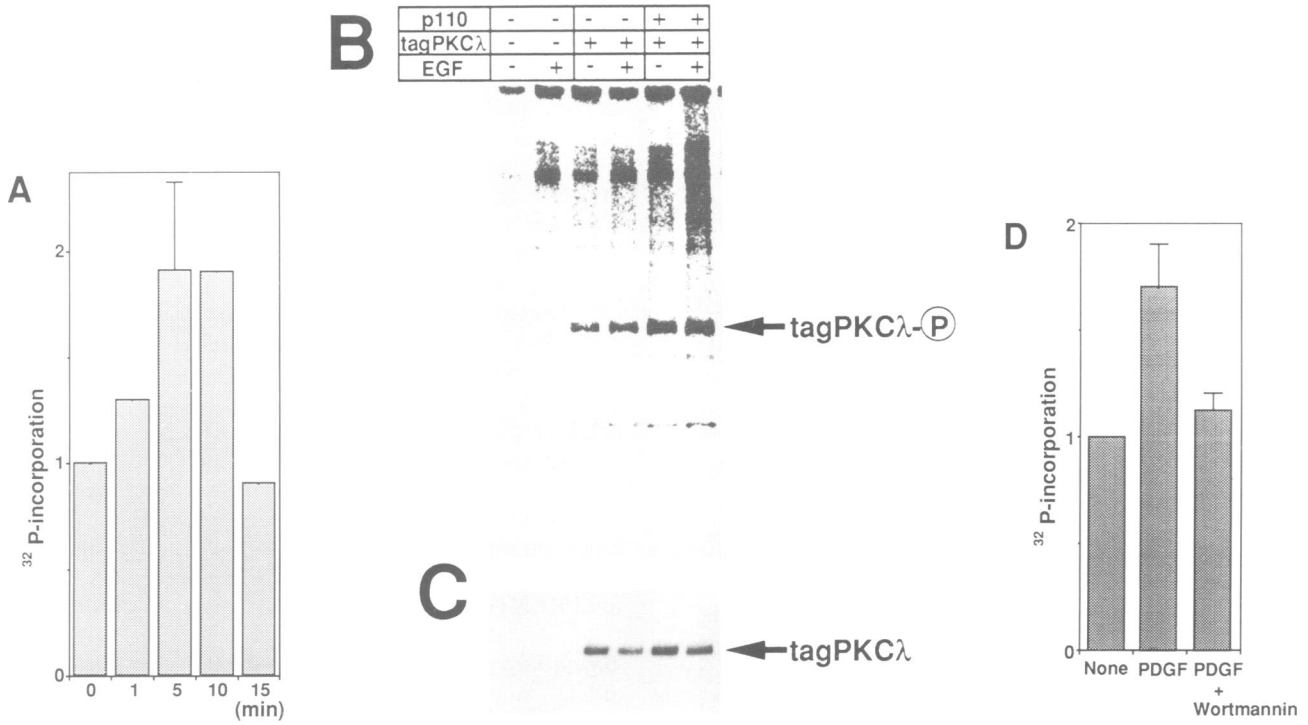

Fig. 5. Phosphorylation of aPKCλ increases upon EGF stimulation and p110α co-transfection. (**A**) Serum-starved COS1 cells expressing tag-aPKCλ were metabolically labelled with [^{32}P]phosphate and stimulated with EGF (30 ng/ml) for the indicated times. The cells were lysed and the tag-aPKCλ was immunoprecipitated and its phosphorylation level evaluated. Values shown were normalized with the amount of aPKCλ evaluated by the Western blotting. Values time 0 and 5 represent the means (± SD) of three independent transfections; others are the means of two. (**B**) Serum-starved COS1 cells expressing tag-aPKCλ and/or p110α were metabolically labelled and stimulated with EGF as in (A) for 5 min. The cells were lysed and the tag-aPKCλ was immunoprecipitated. The samples were subjected to SDS–PAGE, and the gels were visualized by a radioimage analyser. The arrow points to the phosphorylated tag-aPKCλ. (**C**) Western blot analysis of the same gel shown in (B) probed with anti-T7-tag antibody. (**D**) COS1 cells were co-transfected with expression vectors encoding Y40/51 βPDGFR and tagged aPKCλ. The cells were left resting (–) or stimulated for 5 min with 40 ng/ml PDGF (+) and the incorporation of into ^{32}P into tag-aPKCλ was evaluated. Where indicated, wortmannin (100 nM) was added 30 min before PDGF stimulation. Values indicate means (± SD) of three independent transfections.

indicate that PDGF stimulation causes an immediate change in the phosphorylation state of aPKCλ through the PI3-kinase pathway.

PDGF-induced translocation of aPKCλ depends on the tyrosine residues of βPDGF receptor required for PI3-kinase activation

Another criterion used frequently for monitoring the activation of PKC members in intact cells is the signal-dependent change in intracellular localization (Ha and Exton 1993; Tippmer *et al.*, 1994; Wooten *et al.*, 1994). We examined whether or not PDGF stimulation causes a change in the intracellular localization of aPKCλ. We overexpressed aPKCλ in HepG2 cells expressing wild-type or mutant βPDGFRs and examined the intracellular localization of aPKCλ by an immunofluorescence technique using anti-aPKCλ antibodies. Quite interestingly, aPKCλ immunostaining was found in both the cytoplasm and the nucleus in resting cells (>90% of cells expressing aPKCλ) (Figure 6). Confocal immunofluorescent microscopy revealed that the nuclear staining of aPKCλ locates inside the nuclei (data not shown). PDGF-stimulation of cells expressing the wild-type βPDGFR drastically changes the pattern of staining within 5 min; there is a decrease in the nuclear signal and an increase in cytosolic staining (60–70% of cells showed a drastic change) (Figure 6). This change could be detected within 1 min after PDGF stimulation (data not shown). Confocal immunofluorescent microscopy confirmed the difference and further revealed that the nuclear staining had not completely disappeared but had translocated to structures inside the nucleus (data not shown). Parallel experiments on HepG2 cells expressing the add-back mutant (Y40/51), which activates PI3-kinase, showed a similar pattern of change in aPKCλ localization (>60% of cells changed the pattern of immunostaining), whereas cells expressing Y1021 or F5 receptors, which do not activate PI3-kinase, did not. These results clearly indicate that the PDGF-induced translocation of aPKCλ requires the tyrosine residues of βPDGFR, which are required for the association of PI3-kinase. Furthermore, the βPDGF-induced translocation of aPKCλ observed in cells expressing wild-type or Y40/51 mutants was completely inhibited by 100 nM wortmannin (>95% of cells showed the pattern of the resting state) (Figure 6), further supporting the notion that PI3-kinase is involved in this event.

Discussion

PI3-kinase is an upstream mediator of aPKCλ activation

EGF treatment of serum-starved COS1 cells results in increased phosphorylation of aPKCλ (Figure 5). Further, EGF treatment of quiescent 3Y1 cells results in aPKC-dependent transcriptional activation of TRE (Figure 1). This implies that aPKCλ is activated following EGF

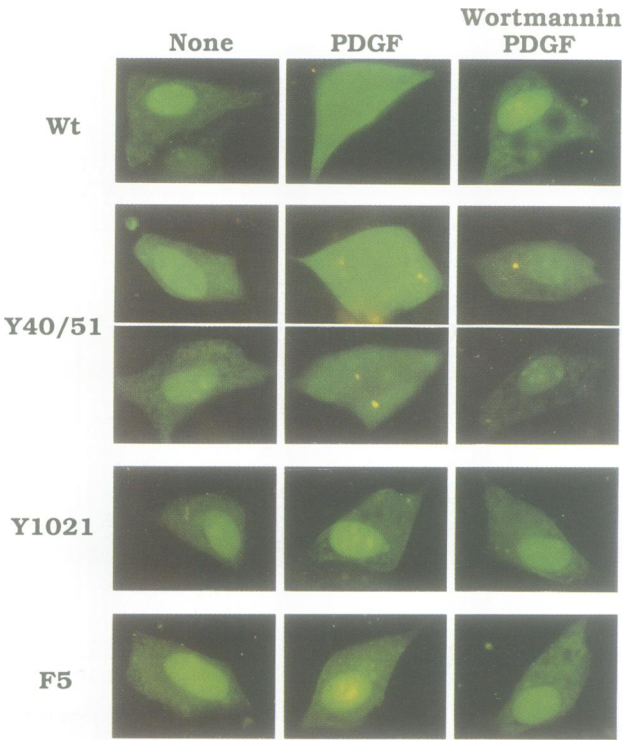

Fig. 6. PDGF-stimulated translocation of aPKCλ in HepG2 cells expressing βPDGFR add-back mutants. HepG2 cells transfected with aPKCλ were starved for 48 h, pre-treated with wortmannin (100 nM) for 30 min where indicated, and stimulated for 5 min with PDGF (40 ng/ml). The cells were then fixed and stained with anti-aPKCλ antibody as described in Materials and methods. Shown are cells expressing transfected aPKCλ.

receptor stimulation to cause transcriptional activation of TRE. EGF receptor stimulation also results in a transient increase in cellular PIP_3 (data not shown) in 3Y1 cells as reported previously in rat PC12 cells (Carter and Downes, 1992; Ohmichi *et al.*, 1992). The aPKCλ-dependent activation of TRE and phosphorylation of aPKCλ with EGF stimulation are enhanced by the overexpression of the PI3-kinase catalytic subunit (Figures 2 and 5). Further, aPKC-dependent TRE activation is inhibited by the overexpression of inhibitory p85α subunit (Figure 2). These results indicate that PI3-kinase and aPKCλ co-operate in a signalling pathway from EGFR to TRE. The association of p85 with a sequence including a tyrosine-phosphorylated YXXM motif has been demonstrated for PDGF receptors (Kazlauskas and Cooper, 1989, 1990; Kazlauskas *et al.*, 1990; Escobedo *et al.*, 1991a; Otsu *et al.*, 1991; Kashishian *et al.*, 1992). However, EGFR lacks a tyrosine-phosphorylated YXXM motif and binds p85 very weakly compared with PDGFR (Hu and Schlessinger, 1992). Recently the activation of PI3-kinase upon stimulation of EGF receptors was shown to be mediated through Erb B3, which contains a tyrosine-phosphorylated YXXM motif (Fedi *et al.*, 1994; Soltoff *et al.*, 1994). Thus, the EGF-stimulated activation of PI3-kinase and aPKCλ in 3Y1 cells as demonstrated in the present study might involve Erb B3.

Experiments using a series of PDGF add-back mutants (Valius and Kazlauskas, 1993) showed that the activation of aPKCλ, monitored in terms of aPKCλ-dependent TRE-activation, aPKCλ phosphorylation, and aPKCλ translocation, requires the receptor tyrosine residues (Y740 and Y751) responsible for binding to and activation of PI3-kinase (Figures 3–6). Although residue 751 has also been shown to bind Nck (Nishimura *et al.*, 1993), this PDGF-dependent activation of aPKCλ through the PDGFR mutant (Y40/51) is inhibited by 100 nM wortmannin, an inhibitor of PI3-kinase (Figures 5 and 6). Since long exposure to 100 nM wortmannin induced cell death accompanied with DNA fragmentation in HepG2 cells (data not shown), it could not be used for long-term experiments such as TRE activation. However, aPKCλ-dependent TRE activation was inhibited by a dominant negative mutant of PI3-kinase. Furthermore, p110-dependent TRE activation was completely inhibited by a dominant negative mutant of aPKCλ, aPKCλ RD.

These results provide *in vivo* evidence showing that aPKCλ is a signal-dependent kinase that is activated upon stimulation by growth factors such as EGF and PDGF. Furthermore, the results also show that the signalling pathway downstream of EGF or PDGF receptors leading to aPKCλ activation involves PI3-kinase but not PLCγ.

Signalling pathway involving aPKC

aPKC comprises a subgroup of the PKC family that is characterized by the possession of a conserved kinase domain and cysteine-rich domain (CRD) (Ono *et al.*, 1989b; Nishizuka, 1992; Selbie *et al.*, 1993; Akimoto *et al.*, 1994). cPKC and nPKC possess a tandem repeat of CRD and have been shown to bind DG and tumour-promoting phorbol esters such as phorbol dibutyrate (PDBu) in a phospholipid-dependent manner, while aPKC binds neither DG nor PDBu (Ono *et al.*, 1989a,b; Akita *et al.*, 1990; Akimoto *et al.*, 1994; Quest *et al.*, 1994). Our results showing that aPKCλ does not respond to TPA (although it does respond to EGF or PDGF in a PI3-kinase-dependent manner) are quite interesting in that some products of PI3-kinase, such as PIP_3, might be involved in the activation of aPKCλ as proposed for DG in the activation of cPKC and nPKC members. The structural similarity between DG and PIP_3 strongly supports this possibility. Nakanishi *et al.* (1993) reported that enzymatically synthesized PIP_3 stimulates aPKCζ kinase activity *in vitro*. On the other hand, Toker *et al.* (1994) have reported that PIP_3 has no significant effect on the kinase activity of aPKCζ *in vitro*. Although we do not have purified aPKCλ and are unable to examine the direct effect of PIP_3 on the kinase activity of aPKCλ, the close sequence similarity between aPKCζ and aPKCλ especially in CRD (69% amino acid identity in CRD), supports the possibility that, like aPKCζ, aPKCλ might be activated by PIP_3. Alternatively, the signal-dependent increase in the phosphorylation of aPKCλ might suggest the possibility that aPKCλ could be activated directly by PI3-kinase or indirectly through other kinases which were recently suggested to be activated through PI3-kinase which include Akt (Burgering and Coffer 1995; Franke, *et al.* 1995) or PAK (Manser, *et al.* 1994) (see below).

The intracellular localization of aPKCλ revealed in the present study is quite interesting in that stimulation with PDGF results in an immediate translocation of aPKCλ within the nucleus and from the nucleus to the cytosol. This suggests that the activation of aPKCλ occurs in the nucleus as well as in the cytosol, although more detailed

studies on the location of the activated aPKCλ are required. The presence of aPKCλ in the nucleus as demonstrated in our overexpression experiments suggests some additional role for aPKCλ in the nucleus.

Moscat's laboratory has made observations on the function of aPKCζ. Suppression of aPKCζ inhibits *Xenopus* oocyte maturation (Dominguez *et al.*, 1992, 1993). aPKCζ has also been suggested to be involved in NF-κB-CAT activity induced by *Bacillus cereus* PC-PLC or TNFα and in the proliferation of NIH 3T3 cells (Berra *et al.*, 1993; Diaz-Meco *et al.*, 1993). The same authors also reported that aPKCζ immunoprecipitated from TNFα-stimulated NIH 3T3 cells contains a putative IκBα-kinase activity (Diaz-Meco *et al.*, 1994), and that aPKCζ is involved in PDGF-dependent stromelysin promoter activation (Sanz *et al.*, 1994). It should be noted that both aPKCζ and aPKCλ are expressed in almost all cell lines examined (Ono *et al.*, 1989b; Selbie *et al.*, 1993; Akimoto *et al.*, 1994). Since the sequence of the putative pseudosubstrate region of aPKCζ is completely conserved in aPKCλ and the sequences of the kinase domains of aPKCζ and aPKCλ are 86% identical (Akimoto *et al.*, 1994), the observed effects of agents used in the above experiments targeted for aPKCζ might include the inhibition of aPKCλ. Our present demonstration that aPKCλ is involved in TRE activation raises the question as to the signalling pathway involved which should be clarified in future experiments.

Specificity and diversity of signalling pathways involving PKC family members

The generation of a variety of lipid metabolites upon activation of a variety of cell surface receptors is one of the major biochemical events thought to elicit the pleiotropic effects of receptors (Nishizuka, 1992; Liscovitch and Cantley, 1994; Divecha and Irvine, 1995). Although PKC family members have been implicated in the mediation of signals elicited by these lipid metabolites (Nishizuka, 1992; Liscovitch and Cantley, 1994; Divecha and Irvine, 1995), there are a number of ambiguities. The major uncertainty arises from the fact that an important basis for assuming PKC is a cellular receptor for these metabolites is data obtained *in vitro* that many of the metabolites activate PKC kinase activity. In other words, technical difficulties in evaluating the activation of a specific PKC member, as well as the involvement of individual lipid metabolites in intact cell systems, severely hamper the clarification of the involvement of PKC and these metabolites.

Two major lipid metabolites that have been studied extensively are DG and PIP_3. Since membrane-permeable DG activates cPKCα, nPKCδ and nPKCε but not aPKCζ (Ohno *et al.*, 1994), the downstream mediator of DG involves c/nPKC but not aPKC. The present study provides evidence that aPKCλ is activated through PI3-kinase, not through the PLCγ pathway. Thus, two major signalling pathways involving related lipid second messengers seem to activate two distinct, but evolutionarily related, sets of protein kinases, c/nPKC and aPKC.

Since the activation of PI3-kinase is observed upon stimulation of a variety of cell surface receptors, the present results suggest that aPKCλ might be activated downstream of a wide variety of receptors including G-protein coupled receptors. Another intriguing question about the significance of aPKCλ in mediating the PI3-kinase pathway involves whether aPKC is the only target for PI3-kinase. The structural similarity between aPKCλ and c/nPKC members, as well as other proteins sharing the CRD motif, such as vav, raf and n-chimerin, raises the possibility that these proteins, like aPKCλ, might interact in some way with the PI3-kinase pathway. Recent reports by Toker, showed that some members of the PKC family, such as nPKCε, are activated *in vitro* by PIP_3 (Toker *et al.*, 1994). In addition, our recent *in vivo* experiments on nPKCε, similar to those shown in the present study, provided evidence supporting the observation that nPKCε is activated through the PI3-kinase pathway as well as the PLCγ pathway (Moriya *et al.*, 1996). This raises the possibility that two separate signalling pathways involving different lipid metabolites activate distinct but overlapping sets of downstream signalling kinases, the PKC family members.

Signalling pathway involving PI3-kinase

In addition to the involvement of tyrosine kinases in the activation of PI3-kinase, recent reports suggest the existence of new mechanisms regulating PI3-kinase activity in cells. These include ras, cdc42Hs and the βγ-subunit of trimeric G-proteins (Rodriguez-Viciana *et al.*, 1994; Stephens *et al.*, 1994; Zheng *et al.*, 1994). In contrast to the mode of activation of PI3-kinase, much less is known about the cellular function of PI3-kinase and its product, PIP_3. Experiments using PDGF receptor mutants indicated the involvement of Tyr740 and Tyr751 in the association of PI3-kinase subunits, cellular PIP_3 production, and the PDGF-dependent induction of DNA synthesis in HepG2 cells (Valius and Kazlauskas, 1993), supporting the notion that PI3-kinase is involved in inducing DNA synthesis. The use of wortmannin, an inhibitor of PI3-kinase, has produced observations suggesting the involvement of PI3-kinase in a variety of cell functions (Arcaro and Wymann, 1993; Yano *et al.*, 1993; Kimura *et al.*, 1994; Okada *et al.*, 1994a,b; Thelen *et al.*, 1994; Ui *et al.*, 1995), although the specificity of the reagent for PI3-kinase remains to be clarified. Microinjection of a dominant negative mutant of PI3-kinase, p110α, resulted in the inhibition of insulin/IGF-I-induced membrane ruffling (Kotani *et al.*, 1994). The use of the p85 subunit as a dominant negative mutant has also suggested that PI3-kinase is involved in the maturation of *Xenopus* oocytes, in c-*fos* transcription upon insulin stimulation, and in PDGF receptor trafficking (Muslin *et al.*, 1993; Yamauchi *et al.*, 1993; Joly *et al.*, 1994). Recent reports suggesting the involvement of the PI3-kinase pathway in pp70 S6-kinase activation (Cheatham *et al.*, 1994; Chung *et al.*, 1994; Ming *et al.*, 1994; Weng *et al.*, 1995) raises the possibility that aPKCλ might be involved in the activation of pp70 S6-kinase. During revision of the manuscript there appeared reports that a protein kinase, Akt/PKB, whose kinase domain shares significant sequence similarities to the PKC family, is activated downstream of the PDGF receptor through the PI3-kinase pathway (Burgering and Coffer, 1995; Franke *et al.*, 1995). Another report suggests that PDGF activates rac through PI3-kinase (Hawkins *et al.*, 1995). This raises a possibility that a protein kinase, PAK, which binds to and is activated by rac (Manser *et al.*, 1994), might also be involved in the signalling downstream of PI3-kinase.

Materials and methods

Plasmids

PKC expression plasmids encoding cPKCα (YK504), aPKCζ (M246) and aPKCλ (MLNP45) were described previously (Akimoto *et al.*, 1994; Ohno *et al.*, 1994). An expression vector encoding tag-aPKCλ was constructed by inserting the tag sequences in front of aPKCλ. The resulting plasmid, SRHisCλ, encodes a protein with N-terminally extended sequences that contain His ×6 and a 12 amino acid sequence from the T7 gene 10 leader sequence of the original pBlueBacHis. A kinase-negative point mutant of aPKCλ (Kn-aPKCλ), where K at position 273 was substituted with E, was constructed following the standard procedure. RD of aPKCλ encoding nucleotides 1–759 was constructed by *Eco*RI and *Pst*I digestion of MLNP45. Bovine cDNA clones encoding full-length PI3-kinase subunits, p85α (Otsu *et al.*, 1991) and p110α (Hiles *et al.*, 1992), were isolated and inserted into an expression plasmid, pMIKNeo (K.Maruyama, personal communication) and SRHis vector. EcoS plasmids encoding nucleotides 1–379 were constructed by *Eco*RI digestion of the p110α expression plasmids (J.Takayanagi *et al.* manuscript in preparation). CAT and luciferase-reporter plasmids were described previously (Hata *et al.*, 1993; Ohno *et al.*, 1994). Briefly, various number of synthetic human collagenase TRE motif (5′-AAGCATGAGTCAGACA-3′) or its mutant, mTRE (5′-AAGCTGGAGTCAGACA-3′), in tandem were inserted upstream of the human interferon β gene promoter (–55 to +19) followed by CAT or luciferase structural gene.

Cell culture, transfection and CAT or luciferase activity measurements

Rat embryonic fibroblast 3Y1-B clone 1-6 cells (provided by Dr Genki Kimura, Kyushu University) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) as described previously (Ohno *et al.*, 1994). Cells were rendered quiescent by incubation in DMEM containing 0.5% FCS for 48 h before stimulation. COS1 cells were maintained in the same medium and starved by incubation for 16 h in serum-free medium before stimulation. HepG2 cells expressing βPDGFR mutants were maintained and starved for 48 h before stimulation as described previously (Valius and Kazlauskas, 1993). Transfections were carried out as described previously (Hata *et al.*, 1993). The total amount of transfected DNA (15 μg/10 cm dish for CAT activity, 27 μg/10 cm dish for luciferase activity measurements and 18 μg/10 cm dish for immunofluorescent measurements) was adjusted to the corresponding empty vector. CAT and luciferase activities were evaluated 10 and 4 h after stimulation, respectively, as described previously (Ohno *et al.*, 1994) using 25 μg protein. The values for CAT or luciferase activity represent means of more than three independent transfections. The efficiency of transfection into cells were examined by use of SV40-luciferase or SV40-CAT which constitutively express luciferase or CAT protein, respectively. EGF was obtained from Takara Shuzo, TGFα from Life Technologies and PDGF-BB from UBI.

Antibodies and Western blot analysis

Cell extracts or immunoprecipitates were separated on a 4–20% polyacrylamide–SDS gel, transferred, and probed with the indicated antibodies using an ECL detection system (Amersham). Antibodies used were anti-aPKCι (Transduction Laboratories), anti-human βPDGFR(UBI), anti-CAT(5 prime-3 prime, Inc.) and T7-Tag mouse monoclonal antibody (Novagen).

Metabolic labelling and immunoprecipitation

Transfection of COS1 cells was done by electroporation as previously described (Akimoto *et al.*, 1994). At 48 h after transfection, cells were serum-deprived for 16 h, the medium was changed to phosphate-free DMEM and the cells were incubated for 1 h. The cells were then incubated in medium containing ^{32}P-labelled orthophosphate (125 μCi/ml) for 5 h before stimulation. After treatment with EGF (30 ng/ml), the cells (in 6 cm dishes) were harvested and suspended in 100 μl of buffer containing 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 10 μg/ml leupeptin, 2 mM PMSF, 2 mM pyrophosphate, 2 mM vanadate and 20 mM NaF. After 30 min incubation on ice, the lysates were centrifuged in a microfuge (6000 r.p.m. ×4 min) and the supernatants were recovered. The lysates were incubated with anti-aPKCλ antibodies (Akimoto *et al.*, 1994) preadsorbed with Protein A–Sepharose, and incubated for 2 h at 4°C. The immunocomplexes on Sepharose were washed in buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP40, 1 mM EDTA and 0.25% gelatin. The immunoprecipitates were separated by SDS–PAGE. After electrophoresis, the proteins were transferred to a membrane (Clearblot P, ATTO), probed with anti T7-tag antibody and the radioactivities were analysed by an radioimage analyser (Fuji Film BAS2000).

Immunofluorescent microscopy

HepG2 cells transfected with aPKCλ were starved for 48 h. The cells were pre-treated with vehicle (DMSO, final concentration 0.01%) or wortmannin (100 nM) (Wako Chemical) for 30 min (Kimura *et al.*, 1994), stimulated with recombinant human PDGF-BB (40 ng/ml, R&D system) for 5 min and fixed with 3% formaldehyde. The intracellular localization of aPKCλ was visualized by immunofluorescent microscopy using anti-aPKCλ antibody (Akimoto *et al.*, 1994) and fluorescein-conjugated anti-rabbit IgG as the first and second antibodies, respectively. Only cells expressing transfected aPKCλ were stained clearly.

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