Atypical protein kinase C λ binds and regulates p70 S6 kinase

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p70 S6 kinase (p70 S6K) has been implicated in the regulation of cell cycle progression. However, the mechanism of its activation is not fully understood. In the present work, evidence is provided that an atypical protein kinase C (PKC) isotype, PKC λ , is indispensable, but not sufficient, for the activation of p70 S6K. Both the regulatory and kinase domains of PKC λ associate directly with p70 S6K. Overexpression of the kinase domain without kinase activity or the regulatory domain of PKC λ results

in the suppression of the serum-induced activation of p70 S6K. In addition, two types of dominant-negative mutants of PKC λ , as well as a kinase-deficient mutant of p70 S6K, suppress serum-induced DNA synthesis and E2F activation. The overexpression of the active form of PKC λ , however, fails to activate p70 S6K. These results suggest that PKC λ is a mediator in the regulation of p70 S6K activity and plays an important role in cell cycle progression.

INTRODUCTION

Atypical protein kinase C (PKC) comprise two members, PKC ζ and PKC ι/λ [1–4]. PKC ι is the human homologue of mouse PKC λ , and both show a high degree of sequence similarity with PKC ζ , including the pseudosubstrate sequence [1–3]. A series of studies using a pseudosubstrate peptide or kinase-deficient dominant-negative forms of PKC ζ and PKC λ suggest that atypical PKC plays important roles in *Xenopus* oocyte maturation [5], fibroblast proliferation [6], v-ras- or v-raf-induced transformation [7–9] and apoptosis [10,11]. Similar experiments also suggest that PKC ζ and PKC λ are involved in the activation of the PMA-response element TRE [9,12–14] and nuclear factor κ B [15,16], mitogen-activated protein kinase activation [8,17,18] and the differentiation of PC12 and leukaemic cells [19,20]. Unlike other PKC isotypes, atypical PKCs are not regulated by Ca²⁺, phorbol esters or diacylglycerols *in vitro* or *in vivo* [1–3,12,21]. Some studies have shown that ceramide regulates the activity of PKC ζ *in vitro* and *in vivo* [22,23]. Recent studies have also revealed that PKC ζ is regulated *in vitro* by PtdIns(3,4,5)P₃, a product of phosphatidylinositol 3-kinase (PI3-kinase) [24], and that PKC λ , as well as PKC ζ , is activated *in vivo* through a pathway involving PI3-kinase [12,25,26]. However, the downstream effectors and the cellular function of atypical PKCs remain to be clarified.

The p70 S6 kinase (p70 S6K) has been implicated in the progression of the cell cycle from G1 to S phase [27,28]. p70 S6K is activated by mitogenic stimuli, including growth factors, cytokines and phorbol esters. In many cases, p70 S6K activity remains elevated throughout G1. Microinjection of neutralizing antibodies against p70 S6K into rat fibroblasts inhibits DNA synthesis [29,30]. Rapamycin, an immunosuppressant that suppresses p70 S6K activity, causes G1 arrest in T-lymphocytes and other haematopoietic lineages and causes a significant delay in the entry into S phase in fibroblasts [31–33]. p70 S6K is also

involved in mitogen-induced 5'TOP mRNA translation [27,34] and transactivation of the cAMP-response element (CRE) activator CREM τ [35]. However, the role of the p70 S6K activation pathway in the regulation of the cell cycle is not fully understood. p70 S6K is co-ordinately activated through Ser/Thr phosphorylations of the enzyme by 3-phosphoinositide protein-dependent kinase 1 (PDK1) and other unknown kinase(s) in a complicated manner [27,28,36]. Activation of p70 S6K requires the multiple phosphorylation of a set of five (Ser/Thr) proline residues clustered in a pseudosubstrate autoinhibitory domain in the p70 S6K C-terminal noncatalytic tail [27,28]. These phosphorylations in the p70 S6K C-terminal tail do not directly activate the p70 S6K, but enable the subsequent phosphorylation of p70 S6K Thr-252 in the activation loop of the catalytic domain and Thr-412 in a hydrophobic segment C-terminal to the catalytic domain [28,37]. Once phosphorylated, these two phosphothreonine residues act together in a strongly co-operative manner to promote the full expression of p70 S6K catalytic activity; modification of either of Thr-412 or Thr-252 yields less than 5% of the activity generated by concomitant phosphorylation at both sites [28]. The phosphorylation of Thr-252 and Thr-412 is dependent on PI3-kinase. Co-expression of p70 S6K with a constitutively active PI3-kinase results in p70 S6K activation concomitant with phosphorylation at both Thr-252 and Thr-412 [38,39]; moreover, wortmannin inhibits mitogen-induced p70 S6K activation concomitant with dephosphorylation at both sites [37,38,40]. PDK1 can phosphorylate p70 S6K Thr-252 directly *in vitro*, resulting in partial activation of p70 S6K [28,36] and is a possible candidate for the physiological p70 S6K Thr-252 kinase, in as much as PDK1 contains a pleckstrin homology domain, binds directly to PtdIns(3,4,5)P₃ and also participates in the activation of protein kinase B (PKB) by phosphorylating PKB Ser-308 (in the activation loop), in a PtdIns(3,4,5)P₃-dependent manner [41]. Among the unanswered questions concerning the mechanism of p70 S6K activation are the identity of

Abbreviations used: PKC, protein kinase C; p70 S6K, p70 S6 kinase; PI3-kinase, phosphatidylinositol 3-kinase; PDK1, 3-phosphoinositide protein-dependent kinase 1; E2F, E2 promoter binding factor; TOR, target of rapamycin; PKB, protein kinase B; RD, regulatory domain; KD, kinase domain; HA, haemagglutinin; GST, glutathione S-transferase; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; PDGF, platelet-derived growth factor; FCS, fetal-calf serum; MBP, myelin basic protein; BrdU, 5-bromo-2'-deoxyuridine.

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the PtdIns(3,4,5) P_3 -dependent kinase that phosphorylates p70 S6K Thr-412, and the role of the small GTPases, Cdc42 and Rac1 [42]. Moreover, PDK1 [and perhaps the p70 S6K (Thr-412) kinase as well] is likely to be bound to the membrane lipid, PtdIns(3,4,5) P_3 , during mitogen stimulation; the mechanism by which the p70 S6K is conveyed to the site of activation remains to be established. Finally, the elements through which mTOR (target of rapamycin) regulates p70 S6K activity are not yet known [43].

In the present study, we show that p70 S6K associates specifically *in vivo* with PKC λ , an atypical PKC isoform shown previously to be regulated by PI3-kinase. Moreover, although co-expression with active PKC λ is by itself insufficient to promote p70 S6K activation, co-expression of p70 S6K with catalytically inactive forms of PKC λ inhibits serum-stimulated p70 S6K activation. We also show that the catalytically inactive variants of PKC λ that inhibit p70 S6K activation *in vivo* also inhibit progression through G1 and E2 promoter binding factor (E2F)-mediated gene expression, as does a catalytically inactive p70 S6K.

EXPERIMENTAL

Plasmids

The mammalian expression vectors were constructed on SRD under the SR α promoter as described previously; w λ encodes mouse PKC λ cDNA (amino acid residues from M48 to the C-terminus, M48–V586); λ regulatory domain (RD) encodes the RD M48–G240 [12]; λ kinase domain (KD) encodes the KD M198–V586; λ KD–Kn encodes the KD (M198–V586) with a point mutation at the ATP binding site (K273E) [12]; λ 4RA encodes residues M48 to V586 and contains four point mutations at the pseudosubstrate region (R117,118,121,122A). The mammalian expression vectors for haemagglutinin (HA)-tagged p70 S6 kinase, HA–wS6K, HA– Δ CS6K and HA–S6K–Kn were constructed by inserting the *Eco*RI fragments of pMT2–p70, pMT2– Δ CT104, and pMT2–K123M to the *Eco*RI site of the SRD vector respectively. The construction of pMT2–p70, pMT2– Δ CT104 and pMT2–K123M were described previously [44]. Plasmid constructs for glutathione S-transferase (GST)-fusion proteins, GST– λ RD and GST– λ KD, were constructed by inserting the *Eco*RI–*Pst*I or the *Nsi*I–*Nhe*I fragment of MLNP45 into a pGEX vector (Pharmacia) respectively [1]. MBP– Δ CT104, was constructed by inserting the *Eco*RI fragment of pMT2– Δ CT104 into the *Eco*RI site of pMal–C2 vector (New England Biolabs). E2F–luciferase (E2P–luc) and the control reporter, mE2F–luciferase (mE2P–luc), which does not bind E2F, have been described previously [45]. Rous sarcoma virus (RSV)-chloramphenicol acetyltransferase (CAT) contains the RSV long terminal repeats upstream of the *CAT* gene. SRD–CAT contains the SR α promoter upstream of the *CAT* gene.

Cell lines and transfection

Human embryonic kidney (HEK) 293 (from Health Science Research Resources Bank), COS1 or HepG2 cells expressing β -platelet-derived growth factor (PDGF) receptor (a gift from Dr. Kazlauskas, Eye Research, Harvard Medical School, Boston, MA, U.S.A.) [46] were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-calf serum (FCS). Transfection of COS1 cells was by electroporation. The 293 cells and HepG2 cells were transfected by a standard calcium phosphate co-precipitation procedure. The total amount of transfected DNA [10 μ g/6-cm dish for immunoprecipitation, S6 kinase activity, 5-bromo-2'-deoxyuridine (BrdU) incorporation or

E2F activity measurements; 6 μ g/6-cm dish for immunofluorescence measurements; 7.5 μ g/10-cm dish for the binding assay] was adjusted to that of the corresponding empty vector. After transfection, COS1 cells were grown in 10% (v/v) FCS medium for 48 h. 293 cells were grown in 10% (v/v) FCS medium for 24 or 48 h and then starved in medium containing 0.5% (v/v) FCS for 20 h. HepG2 cells were starved in medium without FCS for 48 h. After starvation, 293 cells and HepG2 cells were stimulated by the addition of growth factors.

Antibodies and Western-blot analysis

Cell extracts or immunoprecipitates were separated on 8.5% polyacrylamide SDS gels, transferred and probed with the indicated antibodies using an enhanced chemiluminescence detection system (Amersham). The antibodies used were: anti-PKC λ [1], anti-PKC ι (Transduction Laboratories), anti-human p70 S6 kinase (Santa Cruz and Seikagaku Kogyo), anti-HA-epitope mouse monoclonal antibody (Boehringer Mannheim), anti-CAT (5 Prime-3 Prime Inc.), and anti-MBP (New England Biolabs).

Immunoprecipitation

Cells were harvested and suspended in 500 μ l of lysis buffer containing 10 mM Mops, 2 mM EDTA, 5 mM EGTA, 2 mM dithiothreitol, 50 mM β -glycerophosphate, 10 mM MgCl₂, 0.5% Triton X-100, 10 μ g/ml leupeptin, 2 mM PMSF, 2 mM vanadate and 20 mM NaF, pH 7.5. After 30 min incubation on ice and centrifugation at 14000 g, the supernatants were removed and aliquots containing the same amount of protein were incubated with the monoclonal anti-HA epitope antibody, 12CA5, and Protein G-Sepharose at 4 °C for 2 h. The immunocomplexes on Sepharose were washed three times with lysis buffer and once with 1M NaCl in lysis buffer.

S6 Kinase assay

The assay for p70 S6 kinase activity *in vivo* using 100 μ M S6 peptide (RRRLSSLRA) as a substrate was performed following the standard method [38,44]. Immune complexes were suspended in 50 μ l of kinase buffer (50 mM Tris/HCl, pH 7.5/10 mM MgCl₂/0.4 mM dithiothreitol/100 μ M unlabelled ATP/100 μ M S6 peptide containing 0.1 mg/ml of BSA, and 200 μ Ci/ml of [γ -³²P]ATP). The reaction was allowed to proceed for 15 min at 30 °C and was terminated by adding 20 μ l of 250 mM EDTA and boiling for 5 min. Following a brief centrifugation, the supernatant (30 μ l) was applied to p81 phosphocellulose paper. The papers were washed in 75 mM phosphoric acid and analysed by liquid scintillation counting.

PKC assay

The assay for PKC λ kinase activity *in vivo* using 25 μ M myelin basic protein (MBP) as a substrate was performed following the standard method [21]. Immune complexes were suspended in 50 μ l of kinase buffer (20 mM Tris/HCl, pH 7.5/5 mM MgCl₂/1 mM EGTA/25 μ M MBP/40 μ M unlabelled ATP containing 8 μ Ci/ml of [γ -³²P]ATP) with or without phosphatidylserine, and the reaction was allowed to proceed for 10 min at 30 °C. Reactions were terminated by adding sample buffer and boiling for 5 min. Following a brief centrifugation, the supernatant was subjected to SDS/PAGE and the radioactive bands were analysed by a radio image analyser (Fuji Film BAS2000).

Figure 1 Association of PKC λ with p70 S6K

(A) COS cells were co-transfected with expression plasmids of HA-wS6K (2.5 μ g/10-cm dish), wild-type PKC λ (w λ) (3 μ g/10-cm dish) or λ 4RA (3 μ g/10-cm dish) or with HA-wS6K + w λ or HA-wS6K + λ 4RA. Lysates were immunoprecipitated with anti-HA antibody, then subjected to immunoblotting with anti-PKC λ (top panel, anti- λ) or anti-p70 S6K (middle panel, anti-S6K). Whole-cell extracts of the same transfectants were immunoblotted with anti-PKC λ (bottom panel, anti- λ). Essentially identical results were obtained in three independent experiments. (B) 293 cells were co-transfected with expression plasmids of HA-wS6K (3.3 μ g/6-cm dish), w λ (3 μ g/6-cm dish) or both. Cells were treated and assayed as in (A). Essentially identical results were obtained in three independent experiments. (C) COS cells were co-transfected with expression plasmids of HA-wS6K (2.5 μ g/10-cm dish), w λ (3 μ g/10-cm dish), λ RD (5 μ g/10-cm dish) or λ KD (2 μ g/10-cm dish) or with HA-wS6K + λ RD, HA-wS6K + λ KD. Cells were treated and assayed as in (A). Essentially identical results were obtained in three independent experiments. (D) COS cells were co-transfected with expression plasmids of HA- Δ CS6K (2.5 μ g/10-cm dish), w λ (3 μ g/10-cm dish), λ RD (5 μ g/10-cm dish) or λ KD (2 μ g/10-cm dish) or with HA- Δ CS6K + λ RD, HA- Δ CS6K + λ KD. Cells were treated and assayed as in (A). Essentially identical results were obtained in three independent experiments. (E) Purified GST fusions of λ RD, λ KD, MBP or an MBP-fusion of Δ CS6K from *E. coli* were separated by SDS/PAGE and stained with Coomassie Blue (left panel). GST- λ RD or GST- λ KD at a concentration of 100 nM were incubated with 800 nM MBP or MBP- Δ CS6K for 30 min at 4 °C. The MBP fusion proteins were immobilized on amylose resin and the MBP fusion proteins and associated proteins were boiled in sample buffer, separated by SDS/PAGE and immunoblotted with anti-PKC λ (anti- λ) to detect the binding of GST fusion proteins (right panel). Essentially identical results were obtained in three independent experiments. (F) COS cells were transfected with w λ (3 μ g/10-cm dish), λ KD (2 μ g/10-cm dish) or λ 4RA (3 μ g/10-cm dish). Lysates were immunoprecipitated with anti-PKC λ , then subjected to immunocomplex kinase assay using MBP as a substrate. The amount of immunoprecipitated PKC λ proteins was normalized by means of immunoblotting (results not shown). Values shown indicate the means $\pm$ S.D. of relative atypical PKC (aPKC) activity of three independent transfections. Values shown were normalized by taking the value of empty plasmid-transfected cells without phosphatidylserine (PS) as 1. (G) The primary structures of w λ , λ 4RA, λ RD, λ KD and HA- Δ CS6K are shown together with schematic drawings of the PKC λ (w λ) and HA-p70 S6K (HA-wS6K) protein structures respectively. The positions of the four arginine residues replaced with alanine in λ 4RA and the anti-PKC λ (anti- λ) recognition sites are indicated.

In vitro binding assay

Purified GST fusion proteins encoding the RD (GST- λ RD) or KD (GST- λ KD) of PKC λ at a concentration of 100 nM were incubated with either 800 nM MBP or MBP- Δ CS6K for 1 h at 4 °C in lysis buffer containing 1 mg/ml BSA. The MBP fusion proteins were immobilized on amylose resin for 30 min at 4 °C, and then the complexes on resin were washed three times with lysis buffer and once with 1 M NaCl in lysis buffer. Bound MBP fusion proteins and associated proteins were subjected to immunoblotting using anti- λ or anti-MBP.

BrdU incorporation

The serum-stimulated incorporation of BrdU was assayed using a cell proliferation kit (Amersham). For 293 cells, the cells were plated on glass coverslips in 6-cm dishes, at 5×10^5 cells per dish, in growth medium overnight. After transfection, 293 cells were grown for 24 h in growth medium and starved for 20 h in Dulbecco's modified Eagle's medium containing 0.5% (v/v) FCS; 10% (v/v) FCS was then added and after 4, 7, 10, 13, 16, 19 or 22 h the cells were treated with BrdU for 3 h. Cells were fixed on to the coverslips with a solution of ethanol/acetic

acid/water (45:1:1; by vol.). After fixation, the cells were stained with anti-BrdU and anti-CAT antibodies by a standard procedure [47].

E2F activity

The measurement of E2F activity was carried out as described previously [45]. Briefly, after the transfected 293 cells were starved using the same conditions as those for BrdU incorporation, 10% (v/v) FCS was added to the dishes. After 21 h, the cells were harvested and used for luciferase assay. The same extracts were also used for immunoblotting with anti-CAT antibody.

Measurement of translocation

The translocation of PKC λ was examined by immunofluorescence as described previously [12].

RESULTS

Direct association of PKC λ and p70 S6K

In order to examine the possible functional link between PKC λ and p70 S6K, we first examined whether PKC λ associated with p70 S6K *in vivo*. Extracts of COS or 293 cells transfected with expression plasmids encoding HA-tagged p70 S6K (HA-wS6K) and PKC λ (w λ) were used as the starting material to immunoprecipitate HA-wS6K (Figure 1). The immunoprecipitates were then probed with antibodies against PKC λ . Figures 1(A) and 1(B) show that the S6K immunoprecipitate contains PKC λ , indicating the physical association of p70 S6K and PKC λ *in vivo*. The PKC λ mutant, λ 4RA, contains point mutations in the

pseudosubstrate region (Figure 1G). When λ 4RA was immunoprecipitated from COS cells and its kinase activity was evaluated using a peptide from MBP as a substrate, λ 4RA showed increased basal kinase activity in the absence of the phospholipid co-factor, phosphatidylserine, although it still showed a partial dependence on the phospholipid co-factor (Figure 1F). This indicates that λ 4RA is a partially activated mutant. The λ 4RA was also co-immunoprecipitated with HA-wS6K (Figure 1A). The difference in the intensity of the bands in Figure 1(A) between PKC λ and λ 4RA suggests that the association between PKC λ and p70 S6K might require the activated conformation of PKC λ . The reverse experiment, where PKC λ or λ 4RA was first immunoprecipitated and the immunoprecipitate was probed with anti-HA, gave similar results (not shown).

Next, an attempt was made to identify the domain of PKC λ required for association with p70 S6K. As shown in Figure 1(C) both the λ RD and λ KD of PKC λ (Figure 1G), as well as wild-type PKC λ , associated with p70 S6K. Similar experiments using a C-terminal deletion mutant of p70 S6K (HA- Δ CS6K) (Figure 1G) showed that the mutant associated with PKC λ or λ KD but not with λ RD (Figure 1D). Taken together, the N-terminal region of p70 S6K, including the KD, interacted with the KD of PKC λ , and the C-terminal region of p70 S6K was required for interaction with the RD of PKC λ . Thus there might be at least two independent interactions between PKC λ and p70 S6K. Reverse experiments, where the PKC mutants were first immunoprecipitated and the immunoprecipitates were then probed with anti-HA antibodies, confirmed this conclusion (results not shown).

To examine whether the association between PKC λ and p70 S6K was direct or indirect, Δ CS6K (MBP- Δ CS6K) immobilized on an amylose resin was incubated with GST- λ RD or GST- λ KD. Figure 1(E) shows that GST- λ KD but not GST- λ RD

Figure 2 Suppression of the serum-induced activation of p70 S6K in 293 cells by both RD and KD-Kn of PKC λ .

(A) 293 cells were transfected with an expression plasmid of HA-wS6K (3.3 μ g/6-cm dish). Cells were starved for 20 h (open bars), then stimulated with 10% (v/v) FCS for 20 min (shaded bars). Wortmannin (100 nM) (Wort.) or rapamycin (50 nM) (Rapa.) were added 15 min before serum stimulation where indicated. Lysates were immunoprecipitated with anti-HA antibody and then subjected to immunocomplex kinase assay using p70 S6 peptide as a substrate. The lower panels indicate the amount of HA-wS6K immunoprecipitated by anti-p70 S6K. (B) 293 cells were co-transfected with expression plasmids of HA-wS6K (3.3 μ g/6-cm dish) (wS6K), wS6K + λ RD (3 μ g/6-cm dish) or wS6K + λ KD-Kn (1.5 μ g/6-cm dish). Cells were treated and assayed as described in (A). (C) 293 cells were co-transfected with HA-wS6K (3.3 μ g/6-cm dish) (wS6K), wS6K + wild-type PKC λ (w λ) (3.3 μ g/6-cm dish), wS6K + λ KD (3.3 μ g/6-cm dish) or wS6K + λ 4RA (3.3 μ g/6-cm dish). Cells were treated and assayed as in (A). In all experiments, the values shown indicate the means $\pm$ S.D. of the relative p70 S6 kinase activity obtained in three independent transfections. Values shown were normalized by taking the value obtained for HA-wS6K-transfected cells without serum stimulation as 1.

Figure 3 Failure of rapamycin to inhibit the PDGF-stimulated translocation of PKC λ in HepG2 cells expressing β PDGF receptor

(A) HepG2 cells transfected with PKC λ were starved for 48 h, pre-treated with wortmannin (100 nM) (Wort.) or rapamycin (50 nM) (Rapa.) for 30 min where indicated, and stimulated for 20 min with PDGF-BB (40 ng/ml). The cells were then fixed and stained with anti-PKC λ . (B) Values (the percentage of translocated cells among PKC λ -overexpressing cells) shown indicate the means $\pm$ S.D. of three independent experiments. (C) Both wortmannin and rapamycin inhibited the activation of endogenous p70 S6K in HepG2 cells. A p70 S6K mobility shift was detected by immunoblotting using whole cell extracts (20 μ g) and anti-p70 S6K. The positions of p70 S6K and the p85 S6K isoform are indicated.

bound to MBP- Δ CS6K, indicating that the interaction between PKC λ and p70 S6K was direct.

Both λ RD and λ KD without kinase activity suppressed the serum-induced activation of p70 S6K

The association between PKC λ and p70 S6K raises the possibility that PKC λ is involved in the activation of p70 S6K. To evaluate this possibility, the effect of the overexpression of PKC λ mutants on the serum-induced activation of p70 S6K was examined. The S6K that immunoprecipitated from 293 cells transfected with HA-wS6K showed S6-peptide kinase activity, which increased upon serum stimulation of the cells and was completely suppressed by pretreating the cells with wortmannin or rapamycin (Figure 2A), consistent with previous reports [37,44]. As shown in Figure 2(B), overexpression of the KD of PKC λ without kinase activity (λ KD-Kn) almost completely suppressed the serum-induced activation of p70 S6K. Further, overexpression of the RD of PKC λ partially suppressed the serum-induced activation of p70 S6K (HA-wS6K). These results

strongly support the idea that PKC λ or a related protein is involved in the serum-induced activation of p70 S6K. Another atypical PKC, PKC ζ , also associated with HA-wS6K in COS cells, and its KD without kinase activity (ζ KD-Kn) suppressed the serum-induced activation of HA-wS6K (results not shown). Thus PKC λ and/or PKC ζ is implicated in the regulation of p70 S6K.

We next examined whether the overexpression of activated PKC λ mutants enhanced p70 S6K activity. For this purpose, two kinds of activated mutants of PKC λ , λ 4RA and λ KD were used. Both mutants showed increased basal activity in an *in vitro* kinase assay (Figure 1G) and associated with p70 S6K in COS cells (Figures 1A, 1C and 1D). As shown in Figure 2(C), the overexpression of the PKC λ mutants λ KD and λ 4RA, as well as the wild-type PKC λ , had no apparent effect on the basal activity of HA-wS6K. This suggests that PKC λ activity is not sufficient for the activation of p70 S6K.

The dominant negative effects of PKC λ mutants on the serum-induced activation of p70 S6K and the failure of the activated mutants of PKC λ to enhance the basal activity of p70 S6K

Figure 4 Suppression of serum-stimulated DNA synthesis in 293 cells by both the RD and KD-Kn of PKC λ .

(A) 293 cells were transfected with SRD-CAT (3.3 μ g/6-cm dish). Cells were starved and stimulated with 10% (v/v) FCS for 4, 7, 10, 13, 16, 19, or 22 h. After serum stimulation, the cells were treated for 3 h with BrdU. The cells were then fixed and stained with anti-BrdU and anti-CAT antibodies. The numbers of BrdU-incorporating cells among 200 CAT protein-expressing cells were counted. Values shown indicate the means $\pm$ S.D. of relative BrdU incorporation in three independent transfections. The value in cells treated for 16–19 h was taken as 1. (B) 293 cells were co-transfected with expression plasmids encoding PKC λ mutants (6.6 μ g/6-cm dish), p70 S6K mutants (6.6 μ g/6-cm dish), or an empty vector, and SRD-CAT (3.3 μ g/6-cm dish) (—). After serum starvation, the cells were stimulated with 10% (v/v) FCS for 16 h and then treated with BrdU for 3 h. Wortmannin (100 nM) (Wort.) or rapamycin (50 nM) (Rapa.) was added 30 min before serum stimulation. Values shown indicate the means $\pm$ S.D. of relative BrdU incorporation in three independent transfections.

suggest that PKC λ is required but not sufficient for the activation of p70 S6K. This notion is consistent with the following observation that PKC λ and PKC ζ fail to phosphorylate recombinant p70 S6K *in vitro*. To evaluate the possibility that PKC λ directly phosphorylates p70 S6K, p70 S6K (MBP-wS6K) was expressed and purified in *Escherichia coli* cells and used as a substrate for immunoprecipitated λ KD, λ 4RA from COS cells, or PKC ζ purified from recombinant baculovirus-infected insect cells. However, the maximum incorporation of phosphates into MBP-wS6K was 0.054 mol/mol of MBP-wS6K (results not shown), suggesting that p70 S6K was not a good substrate for atypical PKCs. These results, on the other hand, also suggest the possible presence of a p70 S6K kinase that acts downstream of PKC λ and directly phosphorylates and activates p70 S6K.

PKC λ lies on the rapamycin-insensitive pathway

Both rapamycin and wortmannin completely suppressed the serum-induced activation of p70 S6K [37,44] and Figure 2(A). Since wortmannin and rapamycin inhibit PI3-kinase and mTOR [27,37,44] respectively, the above observation led us to hypothesize that the serum-induced activation of p70 S6K required at least two independent signalling events, one sensitive to wortmannin and the other sensitive to rapamycin. On the other hand, previous studies of the activation of PKC λ downstream of PDGF receptors have provided evidence that PKC λ is activated through a pathway involving PI3-kinase [12]. Taken together with our present demonstration that dominant negative PKC λ suppressed the serum-induced activation of p70 S6K, PKC λ lies on the wortmannin sensitive pathway leading to the activation of p70 S6K. However, a recent report that wortmannin inhibited not only PI3-kinase but also mTOR [48] raises the question of the effect of rapamycin on the activation of PKC λ . As reported previously, the activation of the PDGF receptor results in increased phosphorylation and translocation of PKC λ , and these changes in PKC λ require the PI3-kinase pathway and are

Figure 5 Inhibition of serum-stimulated E2F activity in 293 cells by both the RD and KD-Kn of PKC λ .

Cells (293) were co-transfected with expression plasmids encoding λ RD (3.3 or 6.6 μ g/6-cm dish), λ KD-Kn (3.3 or 6.6 μ g/6-cm dish), w λ (3.3 or 6.6 μ g/6-cm dish), S6K-Kn (3.3 or 6.6 μ g/6-cm dish), wS6K (3.3 or 6.6 μ g/6-cm dish) or empty vector and E2F (3.3 μ g/6-cm dish) or mE2F-luciferase reporter (3.3 μ g/6-cm dish). The total amount of DNA (10 μ g/6-cm dish) was adjusted with empty vector. After serum starvation, the cells were stimulated with 10% (v/v) FCS for 21 h and the E2F activity was evaluated by luciferase assay. The results presented are relative E2F activity. Values shown represent the luciferase activities relative to those obtained for vector transfected cells and represent the means $\pm$ S.D. of three independent transfections. In some of the repeated experiments, the RSV-CAT expression plasmid was also co-transfected as an internal control of transfection efficiency. The lower panel shows an example of CAT expression by anti-CAT (20 μ g of protein).

suppressed by wortmannin or the dominant negative mutant of PI3-kinase [12]. Figure 3 shows that wortmannin, but not rapamycin, inhibits the translocation of PKC λ upon PDGF stimulation, whereas both wortmannin and rapamycin suppress the activation of endogenous p70 S6K. The results clearly indicated that the activation of PKC λ was insensitive to rapamycin and that the activation of PKC λ was not sufficient for the activation of p70 S6K.

Suppression of cell-cycle progression by dominant-negative mutants of PKC λ , as well as by the kinase-deficient mutant of p70 S6K

If PKC λ is required for the activation of p70 S6K, and if p70 S6K is required for cell cycle progression, then the dominant negative mutants of PKC λ should suppress cell cycle progression. To test this hypothesis, λ RD or λ KD-Kn was overexpressed in 293 cells, and the effect of this overexpression on serum-induced DNA synthesis was evaluated. Cells overexpressing the PKC λ mutants were identified by their expression of co-transfected CAT plasmid, and the percentage of cells incorporating BrdU among those expressing CAT was calculated. In 293 cells, the maximal incorporation of BrdU by serum stimulation occurred at 16–19 h (Figure 4A) and this was suppressed by pretreating the cells with wortmannin or rapamycin (Figure 4B). Overexpression of λ RD or λ KD-Kn, but not wild-type PKC λ or the KD of PKC λ , resulted in the suppression of BrdU incorporation (Figure 4B). Further, the kinase-deficient mutant of p70 S6K

(S6K-Kn), but not wild-type p70 S6K, also suppressed serum-induced BrdU incorporation, consistent with previous findings that micro-injection of antibodies against p70 S6K suppressed DNA synthesis [29,30]. These results support our hypothesis that PKC λ is involved in the activation of p70 S6K.

It is well known that E2F plays an important role in the progression of G1 phase of the cell cycle. In early G1 phase, E2F forms an inactive complex with the retinoblastoma-susceptibility gene product to suppress its transcriptional activity. In late G1 phase, retinoblastoma-susceptibility gene product is phosphorylated and loses its ability to bind to E2F. Consequently, E2F is released from the complex resulting in the induction of genes required for DNA synthesis [49]. To further confirm our hypothesis, the effect of the overexpression of dominant negative mutants of PKC λ on the serum-induced activation of E2F in 293 cells was examined. For this purpose, a reporter plasmid in which synthetic E2F-binding sequences are located upstream of the luciferase gene was used. This system permits the detection of the transcriptional activity of E2F isotypes regulated under the control of the retinoblastoma-susceptibility gene and retinoblastoma-susceptibility-gene-related proteins in intact cells [45]. Figure 5 shows that both λ RD and λ KD-Kn, as well as S6K-Kn, suppressed the expression of luciferase, whereas the expression of RSV-CAT, encoded by the co-transfected plasmid, was not affected, suggesting that PKC λ , as well as p70 S6K, is involved in the activation of E2F.

DISCUSSION

PKC λ as a crucial regulator of p70 S6K activity

In the present study, we provide evidence in strong support of PKC λ being a crucial mediator involved in the activation of p70 S6K. Both the KD and RD of PKC λ associate with p70 S6K *in vivo* (Figure 1). Further, both the λ KD-Kn and the λ RD show dominant negative effects on the activation of p70 S6K (Figure 2B). However, the constitutive active forms of PKC λ (λ KD and λ 4RA) fail to activate p70 S6K (Figure 2C). These results led us to propose that the activity of PKC λ is indispensable but not sufficient for the activation of p70 S6K. Rapamycin fails to inhibit the PDGF-induced translocation of PKC λ (Figure 3), whereas it completely suppresses the activation of p70 S6K [37,44] (Figures 2 and 3). How then does PKC λ fit into regulatory apparatus controlling the activity of p70 S6K? The multisite phosphorylation that underlies the insulin/mitogen activation of the p70 S6K is controlled by PI3-kinase-dependent and PI3-kinase-independent inputs, each indispensable to p70 S6K activation [37,44]. The PI3-kinase-regulated inputs are provided by (at least) two PtdIns(3,4,5) P_3 regulated protein kinases, PDK1, which phosphorylates p70 S6K Thr-252 in the activation loop, and an, as yet, unidentified p70 S6K (Thr-412) kinase [28,36]. The PI3-kinase-independent inputs include an array of proline-directed kinases, which phosphorylate Ser-394 and the cluster of (Ser/Thr) proline residues (Ser-434, Ser-441, Ser-447, Ser-452 and Thr-444) in the autoinhibitory pseudosubstrate domain of the p70 S6K C-terminal non-catalytic tail; these kinases include the mitogen-activated protein kinases, stress-activated protein kinases, p38 and Cdc2 [28]. In addition, rapamycin, through inhibition of the mTOR kinase, also controls the phosphorylation of multiple sites on p70 S6K [37,44], however the mechanism is not known. Rapamycin-induced p70 S6K dephosphorylation is not accompanied by an alteration in the activity of any of the (Ser/Thr) kinases enumerated above, which are known to act directly on p70 S6K [37].

Integrating the results of the present study with these earlier observations, and with the evidence that PKC λ is itself regulated

by PI3-kinase [12], we infer that PKC λ participates in the PI3-kinase-dependent regulation of p70 S6K. Significant direct phosphorylation of p70 S6K *in vitro* by activated forms of PKC λ has not been observed. We cannot yet exclude the possibility that this result reflects inappropriate experimental conditions, e.g. the lack of some necessary cofactor or an unsuitable p70 S6K substrate. Nevertheless, several other plausible roles for PKC λ in p70 S6K activation are readily foreseen. Thus PKC λ may be the activator of a p70 S6K-kinase, e.g. the p70 S6K (Thr-412) kinase. Alternatively, but not exclusively, PKC λ , through its ability to associate stably with p70 S6K, may serve as a shuttle to convey p70 S6K to sites of PtdIns(3,4,5) P_3 synthesis, where p70 S6K is brought into contiguity with PDK1 and perhaps with the p70 S6K (Thr-412) kinase also. The present data are in fact consistent with a role for PKC λ in both functions. The observation that both a kinase-negative PKC λ catalytic domain (λ KD-Kn) deleted of its λ RD, which is perhaps the PtdIns(3,4,5) P_3 -binding fragment of PKC λ , and λ RD are each capable of inhibiting p70 S6K activation suggests that PKC λ acts in a complex, multifunctional manner in mediating p70 S6K activation. It is of considerable interest that PKC ζ , which is highly similar to PKC λ , also associates with p70 S6K and has been shown previously to associate with PKB (results not shown) [50]. The PtdIns(3,4,5) P_3 -dependent activation of PKB is highly similar to that of p70 S6K; both PKB and p70 S6K require a PtdIns(3,4,5) P_3 -dependent phosphorylation in the activation loop (p70 S6K, Thr-252; PKB, Ser-308), a modification catalysed by PDK1 for both enzymes [28,41]. In addition, both p70 S6K and PKB require the PtdIns(3,4,5) P_3 -dependent phosphorylation at a site (p70 S6K, Thr-412; PKB, Ser-473) C-terminal to the catalytic domain, where residues in a hydrophobic segment have an amino acid sequence which is very similar in p70 S6K and PKB [28]. The primary difference between PKB and p70 S6K in their PtdIns(3,4,5) P_3 -dependent activation is that PKB itself binds directly to PtdIns(3,4,5) P_3 , whereas p70 S6K does not [28,51]. These analogies suggest that the atypical PKC λ and PKC ζ may function as indispensable elements in the PtdIns(3,4,5) P_3 -dependent activation of PKB, as well as p70 S6K.

Function of the PKC λ /p70 S6K signalling pathway

We have revealed that the PKC λ -p70 S6K signalling pathway is involved in G1/S progression of the cell cycle. Two dominant inhibitory forms of PKC λ , as well as a kinase-deficient form of p70 S6K, suppress DNA synthesis and E2F activity (Figures 4 and 5). Similar results were obtained for PKC ζ (results not shown). Thus atypical PKC isotypes act similarly, at least in the experimental system used in the present study. These results are consistent with the results of previous studies, obtained using neutralizing antibodies and rapamycin, that p70 S6K is involved in G1/S progression of the cell cycle [29–33]. It has also been reported that neutralized antibodies against PI3-kinase or dominant-negative forms of PI3-kinase inhibit DNA synthesis [52,53]. The use of a dominant-negative form or inhibitory pseudosubstrate peptide of atypical PKC isotypes has been shown to inhibit DNA synthesis and cell proliferation in fibroblasts [6,8]. Taken together with the observation that PI3-kinase is an upstream mediator of both atypical PKC and p70 S6K, the present study strongly supports the notion that the PKC λ -p70 S6K pathway regulates the progression of the cell cycle.

However, the manner in which the PKC λ -p70 S6K signalling pathway regulates E2F activity remains to be clarified. Since p70 S6K is involved in the CRE or PMA-response-element transcriptional activation ([35] and M. Nakaya, K. Akimoto and S. Ohno, unpublished work) and specific protein synthesis [27,34],

which play important roles in cell proliferation, E2F activity may be indirectly regulated by these events. In addition, rapamycin has been shown to inhibit the down-regulation of p27 cdk inhibitor [54,55], retinoblastoma-susceptibility gene product phosphorylation [56], and cyclin D1-Cdk4 and cyclin E-Cdk2 activation [54,55], which are involved in the regulation of E2F activity. It remains to be clarified whether the effects of rapamycin are mediated through the atypical PKC-p70 S6K signalling pathway.

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REFERENCES

- Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., Suzuki, K. and Ohno, S. (1994) *J. Biol. Chem.* **269**, 12677–12683
- Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3099–3103
- Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y. and Biden, T. J. (1993) *J. Biol. Chem.* **268**, 24296–24302
- Nishizuka, Y. (1992) *Science* **258**, 607–614
- Dominguez, I., Diaz-Meco, M. T., Municio, M. M., Berra, E., de Garcia, H. A., Cornet, M. E., Sanz, L. and Moscat, J. (1992) *Mol. Cell. Biol.* **12**, 3776–3783
- Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J., Chapkin, R. S. and Moscat, J. (1993) *Cell* **74**, 555–563
- Kieser, K., Seitz, T., Adler, H. S., Coffey, P., Kremmer, E., Crespo, P., Gutkind, J. S., Henderson, D. W., Mushinski, J. F., Kolsh, W. and Mischak, H. (1996) *Genes Dev.* **10**, 1455–1466
- Bjorkoy, G., Perard, M., Overvatn, A. and Johansen, T. (1997) *J. Biol. Chem.* **272**, 11557–11565
- Bjorkoy, G., Overvatn, A., Diaz-Meco, M. T., Moscat, J. and Johansen, T. (1995) *J. Biol. Chem.* **270**, 21299–21306
- Diaz-Meco, M. T., Municio, M. M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L. and Moscat, J. (1996) *Cell* **86**, 777–786
- Murray, N. R. and Fields, A. P. (1997) *J. Biol. Chem.* **272**, 27521–27524
- Akimoto, K., Takahashi, R., Moriya, S., Nishioka, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S., Mizuno, K., Hirai, S. et al. (1996) *EMBO J.* **15**, 788–798
- Huang, C., Ma, W., Bowden, G. T. and Dong, Z. (1996) *J. Biol. Chem.* **271**, 31262–31268
- Huang, C., Ma, W. and Dong, Z. (1997) *Oncogene* **14**, 1945–1954
- Dominguez, I., Sanz, L., Arenzana-Seisdedos, F., Diaz-Meco, M. T., Virelizier, J. L. and Moscat, J. (1993) *Mol. Cell. Biol.* **13**, 1290–1295
- Diaz-Meco, M. T., Berra, E., Municio, M. M., Sanz, L., Lozano, J., Dominguez, I., Diaz-Golpe, V., Lain de Lera, M. T., Alcamí, J., Paya, C. V. et al. (1993) *Mol. Cell. Biol.* **13**, 4770–4775
- Berra, E., Diaz-Meco, M. T., Lozano, J., Frutos, S., Municio, M. M., Sanchez, P., Sanz, L. and Moscat, J. (1995) *EMBO J.* **14**, 6157–6163
- Van Dijk, M. C. M., Hilkmann, H. and van Blitterswijk, W. J. (1997) *Biochem. J.* **325**, 303–307
- Ways, D. K., Posekany, K., deVent, J., Garriss, T., Chen, J., Hooker, J., Qin, W., Cook, P., Fletcher, D. and Parker, P. J. (1994) *Cell Growth Differentiation* **5**, 1195–1203
- Wooten, M. W., Zhou, G., Seibenhener, M. L. and Coleman, E. S. (1994) *Cell Growth Differentiation* **5**, 395–403
- Nakanishi, H. and Exton, J. H. (1992) *J. Biol. Chem.* **267**, 16347–16354
- Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L. and Moscat, J. (1994) *J. Biol. Chem.* **269**, 19200–19202
- Muller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D. and Pfizenmaier, K. (1995) *EMBO J.* **14**, 1961–1969
- Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993) *J. Biol. Chem.* **268**, 13–16
- Standaert, M. L., Galloway, L., Karnam, P., Bandyopadhyay, G., Moscat, J. and Farese, R. V. (1997) *J. Biol. Chem.* **272**, 30075–30082
- Toker, A. and Cantley, L. C. (1997) *Nature (London)* **387**, 673–676
- Pullen, N. and Thomas, G. (1997) *FEBS Lett.* **410**, 78–82
- Alessi, D., Kozlowski, M. T., Weng, Q.-P., Morrice, N. and Avruch, J. (1997) *Curr. Biol.* **8**, 69–81
- Lane, H. A., Fernandez, A., Lamb, N. J. C. and Thomas, G. (1993) *Nature (London)* **363**, 170–172
- Reinhardt, C., Fernandez, A., Lamb, N. J. C. and Thomas, G. (1994) *EMBO J.* **13**, 1557–1565
- Chung, J., Kuo, C. J., Crabtree, G. R. and Blenis, J. (1992) *Cell* **69**, 1227–1236
- Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J. and Crabtree, G. R. (1992) *Nature (London)* **358**, 70–73
- Price, D. J., Grove, J. R., Calvo, V., Avruch, J. and Bierer, B. E. (1992) *Science* **257**, 973–977
- Jefferies, H. B. J., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B. and Thomas, G. (1997) *EMBO J.* **16**, 3693–3704
- de Groot, R. P., Ballou, L. M. and Sassone-Corsi, P. (1994) *Cell* **79**, 81–91
- Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A. and Thomas, G. (1998) *Science* **279**, 707–710
- Dennis, P. B., Pullen, N., Kozma, S. C. and Thomas, G. (1996) *Mol. Cell. Biol.* **16**, 6242–6251
- Weng, Q.-P., Andrabi, K., Klippel, A., Kozlowski, M. T., Williams, L. T. and Avruch, J. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5744–5748
- Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M.-A. and Williams, L. T. (1996) *Mol. Cell. Biol.* **16**, 4117–4127
- Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A. and Blenis, J. (1994) *Nature (London)* **370**, 71–75
- Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D. et al. (1997) *Curr. Biol.* **7**, 776–789
- Chou, M. M. and Blenis, J. (1996) *Cell* **85**, 573–583
- Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B. and Schreiber, S. L. (1995) *Nature (London)* **377**, 441–446
- Weng, Q.-P., Andrabi, K., Kozlowski, M. T., Grove, J. R. and Avruch, J. (1995) *Mol. Cell. Biol.* **15**, 2333–2340
- Nakaigawa, N., Hirai, S., Mizuno, K., Shuin, T., Hosaka, M. and Ohno, S. (1996) *Biochem. Biophys. Res. Commun.* **222**, 95–100
- Valius, M. and Kazlauskas, A. (1993) *Cell* **73**, 321–334
- Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S.-I., Mizuno, K., Takenawa, T., Fukui, Y., Watanabe, Y., Ozaki, S. and Ohno, S. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 151–155
- Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, Jr., J. C. and Abraham, R. T. (1996) *EMBO J.* **15**, 5256–5267
- Helin, K. (1998) *Curr. Opin. Genet. Dev.* **8**, 28–35
- Konishi, H., Shinomura, T., Kuroda, S., Ono, Y. and Kikkawa, U. (1994) *Biochem. Biophys. Res. Commun.* **205**, 817–825
- James, S. R., Downes, C. P., Gigg, R., Grove, S. J. A., Holmes, A. B. and Alessi, D. R. (1996) *Biochem. J.* **315**, 709–713
- Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. L. and Olefsky, J. M. (1994) *Mol. Cell. Biol.* **14**, 7466–7475
- Roche, S., Koegel, M. and Courtneidge, S. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9185–9189
- Kato, J., Matsuoka, M., Polyak, K., Massague, J. and Sherr, C. J. (1994) *Cell* **79**, 487–496
- Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M.-H., Massague, J., Crabtree, G. and Roberts, J. M. (1994) *Nature (London)* **372**, 570–573
- Terada, N., Lucas, J. J., Szepesi, A., Franklin, R. A., Domenico, J. and Gelfand, E. W. (1993) *J. Cell. Physiol.* **154**, 7–15