

# Protein kinase C $\mu$ selectively activates the mitogen-activated protein kinase (MAPK) p42 pathway

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**Abstract** Here we show that human protein kinase C  $\mu$  (PKC $\mu$ ) activates the mitogen-activated protein kinase (MAPK). Transient expression of constitutive active PKC $\mu$  leads to an activation of Raf-1 kinase as demonstrated by *in vitro* phosphorylation of MAPK. PKC $\mu$  enhances transcriptional activity of a basal thymidine kinase promoter containing serum response elements (SREs) as shown by luciferase reporter gene assays. SRE driven gene activation by PKC $\mu$  is triggered by the Elk-1 ternary complex factor. PKC $\mu$ -mediated activation of SRE driven transcription can be inhibited by the MEK1 inhibitor PD98059. In contrast to the activation of the p42/ERK1 MAPK cascade, transient expression of constitutive active PKC $\mu$  does neither affect c-jun N-terminal kinase nor p38 MAPK. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Protein kinase C  $\mu$ ; Mitogen-activated protein kinase; p42; Serum response element

## 1. Introduction

The protein kinases C (PKCs) comprise a family of intracellular serine/threonine specific kinases, that are implicated in signal transduction of a wide range of biological responses including changes in cell morphology, proliferation and differentiation [1–3]. The 13 members of the family can be grouped into three major classes of Ca<sup>2+</sup>-dependent classical PKCs (cPKCs), Ca<sup>2+</sup>-independent, novel PKCs (nPKCs), and Ca<sup>2+</sup>- and lipid-independent atypical PKCs (aPKC). The fourth PKC subgroup consisting of PKC $\mu$  [4], its mouse homologue PKD [5], PKC $\nu$  [6] and PKD2 [7] share common structures like amino-terminal cysteine fingers defining the structural basis for lipid-mediated activation. They differ from the three major groups of PKC isozymes by the presence of an acidic domain [8], a pleckstrin homology (PH) domain [9] and the lack of a typical pseudosubstrate site. PKC $\mu$ /PKD is ubiquitously expressed and involved in diverse cellular functions like constitutive transport processes in epithelial cells [10], G protein-mediated regulation of Golgi organization [11] and protection from apoptosis [12].

PKC $\mu$ /PKD is activated by the growth factor PDGF [13] via a PLC $\gamma$ -mediated pathway [14] pointing to a role in the

extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade. The MAPK cascade, which involves the kinases Raf-1, MAPK/ERK kinase (MEK) and ERK/MAPK [15], is ubiquitously expressed in mammalian cells and serves to couple various cell surface stimuli to the alteration of cell function. This cascade is implicated in both regulation of cell proliferation as well as in the control of differentiation. Such actions are elicited at least in part through the translocation of activated MAPK to the nucleus, where it phosphorylates target molecules such as the transcription factors Elk-1 and SAP1, which consequently leads to alterations in gene expression [16]. Evidence for the involvement of PKCs in MAPK activation comes from the action of the tumor promotor phorbol ester leading to a rapid activation in most cell types [17]. Since PKC is the major receptor for phorbol ester, it has been implicated in the activation of the ERK/MAPK pathway and the triggering of cellular responses such as cell differentiation and proliferation. Further work proved that c- and nPKC isotypes activate the MAPK pathway on the level of Raf-1 whereas aPKCs activate MEK by an independent mechanism [18,19]. As PKC $\mu$ /PKD is activated directly by lipids [20] and by PDGF [13] similarly as other PKCs, we analyzed its function in signaling pathways leading to transcription factor activation via the MAPKs.

In this study we demonstrate that PKC $\mu$  selectively activates the ERK1/2 MAPK cascade at the level of Raf-1 kinase leading to p42 MAPK activation, which results in enhanced Elk-1-mediated transcription of serum response element (SRE) driven reporter genes.

## 2. Materials and methods

### 2.1. PKC $\mu$ expression plasmids and antibodies

The cloning of the PKC $\mu$  expression constructs used has been described previously [12,21]. PKC $\mu$  was detected with a rabbit antibody D20 (Santa Cruz). Secondary alkaline phosphatase-linked goat anti-mouse IgG and goat anti-rabbit IgG antibodies were purchased from Dianova or Sigma. Protease inhibitors and phosphatases were from Biomol. Phorbol ester (phorbol 12,13-dibutyrate, PdBu) was purchased from Sigma. HEK293 cells (ATCC) were cultured in RPMI medium supplemented with 5% fetal calf serum.

### 2.2. Raf-1 and p42 activation assay

Raf-1 kinase activity was measured as previously published [22]. In brief Raf-1 was immunoprecipitated and incubated with 50 ng of kinase competent MEK1 and 150 ng of kinase negative MAPK (ERK1, Upstate Biotechnology). Phosphorylation was started by adjusting to 2  $\mu$ M ATP and 5  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP. The reaction was incubated for 30

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min at 25°C and terminated by adding sodium dodecyl sulfate (SDS) sample buffer. Samples were fractionated on a 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and subjected to overnight autoradiography and phosphoimaging. p42/44 and p38 activity was estimated by measuring phosphorylation using phospho-p42/44 and phospho-p38 specific antibodies (New England Biolabs) according to the manufacturer's instructions. Lysates of  $3 \times 10^5$  HEK293 cells per lane were analyzed by Western blot detection. Activation of c-jun N-terminal kinase (JNK) was measured as described previously [23] using GST-jun (glutathione *S*-transferase) fusion proteins as a substrate. Expression of PKC $\mu$  and PKC $\mu$  mutants was verified in all cases by Western blot detection.

### 2.3. Transfections and luciferase reporter gene assays

$2 \times 10^4$  HEK293 cells per well (96 well plate) were transfected with 1  $\mu$ l Superfect (Qiagen) for 2 h. 50 ng of a  $\beta$ -galactosidase reporter gene construct (pCH110, Amersham-Pharmacia), 50 ng of a 4 $\times$ SRE-Tk-luciferase reporter construct and 150 ng of PKC $\mu$ /pCDNA3 expression constructs or the respective amount of vector DNA were used. For inhibition of MAPK activity cells were preincubated for 1 h with the MEK1 inhibitor PD98059 (40  $\mu$ M). 48 h after transfection cells were stimulated with 100 nM phorbol ester for 6 h and harvested in 50  $\mu$ l lysis solution (Galacto-light, TROPIX). Lysates were analyzed for firefly luciferase expression (20  $\mu$ l, Promega) and for  $\beta$ -galactosidase expression (10  $\mu$ l) using a Lucy2 luminometer (Anthos). Normalized transfection efficiencies are indicated by the quotient of relative luciferase units and relative  $\beta$ -galactosidase units. All luciferase reporter gene assays were carried out in triplicates and were performed 3–5 times. Gene transcription was measured using a 4 $\times$ SRE-Tk-luciferase or a Tk-luciferase construct [24]. To measure Elk-1-mediated gene activation pGAL4-luc and pGAL4DB-Elk-1 were used. All reporter gene constructs used were gifts of Hans van Dam, Leiden University, The Netherlands and Bernd Baumann, University of Ulm, Germany. Transient expression of PKC $\mu$  in HEK293 cells was carried out in 6 well plates as previously described [21]. Expression levels of PKC $\mu$  were verified by Western blot analysis.

## 3. Results and discussion

### 3.1. PKC $\mu$ activates Raf-1

PKD, the murine homologue of PKC $\mu$ , has been reported to be activated by the growth factor PDGF and several neuro-peptides via a PKC-mediated pathway [13,25]. This prompted us to analyze in detail the function of PKC $\mu$  in growth factor-

related signaling pathways leading to signal amplification of mitogenic factors via MAPK cascades. Therefore, HEK293 cells were transfected with constitutive active PKC $\mu$ . Deletion of the PH domain of PKD and PKC $\mu$  (PKC $\mu_{\Delta PH}$ ) has been demonstrated to constitutively activate the kinase activity [26] (see Fig. 2D). As Raf-1 is a key enzyme in transmitting proliferative, developmental and oncogenic responses from growth factors via the MAPK pathway [27–29], we first analyzed potential activation of Raf-1 kinase by PKC $\mu$ . Raf-1 activation was monitored in a coupled kinase assay (Fig. 1): Raf-1 immunoprecipitates from HEK293 cells upon stimulation with EGF were used as a positive control to phosphorylate in vitro kinase competent MEK, which in turn phosphorylates MAPK (Fig. 1, left lanes). Raf-1 activation of vector-transfected control cells was compared to immunoprecipitates from cells transfected with the constitutive active PKC $\mu_{\Delta PH}$  expression construct [21]. PKC $\mu_{\Delta PH}$  displays constitutive kinase activity (see Fig. 2D). As shown in Fig. 1 (upper panel, right lanes) Raf-1 immunoprecipitates from HEK293 cells transiently expressing PKC $\mu_{\Delta PH}$  (Fig. 1, lower panel) showed significant enhancement of MAPK phosphorylation compared to Raf-1 immunoprecipitates from vector-transfected cells (Fig. 1, upper panel, right lane). Our data point to PKC $\mu$ -mediated activation of Raf-1 similarly as reported for the c- and nPKCs [18,19]. The regulation of Raf-1 appears to be very complex [30,31] triggered by several mechanisms including activation by GTP-bound ras, binding of 14-3-3 proteins [32] and activation by PKC $\alpha$  [33]. In contrast to PKC $\alpha$ , PKC $\mu$  does not directly phosphorylate Raf-1 (unpublished observations). Furthermore Raf-1 could not be co-immunoprecipitated with PKC $\mu$  (data not shown). Therefore indirect activation via unknown mediators is most likely.

### 3.2. PKC $\mu$ selectively activates p42 MAPK

As constitutive active PKC $\mu$  enhances Raf-1 kinase activity we analyzed in detail downstream signaling pathways. Activation of the p42 MAPK which is triggered by Raf-1 can be demonstrated upon overexpression of constitutive active PKC $\mu$  directly by an independent technique. Using antibodies specific for phospho-Thr202/Tyr204, the activation of p42/p44 MAPK was measured upon PKC $\mu$  overexpression. As shown in Fig. 2A activation of p42/p44 kinase is not detectable in vector-transfected control cells, but enhancement of p42 activity can be demonstrated upon phorbol ester stimulation. Transient expression of PKC $\mu$  wild-type leads to a weak enhancement of p42 and p44 phosphorylation. In contrast however, upon phorbol ester stimulation a strong enhancement of p42 and to a lower degree of p44 phosphorylation was detectable. Expression of PKC $\mu_{\Delta PH}$  leads to an enhancement of p42 phosphorylation which was completely inhibited upon incubation with the MEK1 inhibitor PD98059. Expression of kinase dead PKC $\mu_{K612W}$  did not show any significant inhibition of p42 phosphorylation. Expression of p42/p44 (Fig. 2A, lower panel) as well as transgene expression were analyzed by Western blot analysis (see below).

Although PKC $\mu$ -mediated Raf-1 activation leads via activation of the p42/p44 MAPK cascade to gene activation (see Fig. 3), a role of PKC $\mu$  in activation of p38-MAPK and SAP/JNK was conceivable. Therefore we analyzed in HEK293 cells whether PKC $\mu$  overexpression affects these kinase cascades. As shown in Fig. 2B neither expression of constitutive active PKC $\mu_{\Delta PH}$  nor expression of the kinase dead PKC $\mu_{K612W}$  mu-

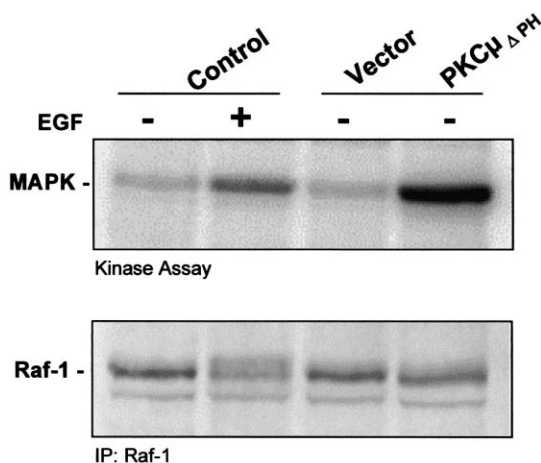


Fig. 1. PKC $\mu$  activates Raf-1. Raf-1 immunoprecipitates from HEK293 cells transfected with the indicated expression constructs were subjected to a coupled kinase assay (see Section 2) to measure MAPK phosphorylation. As a control Raf-1 immunoprecipitates from non-stimulated cells were compared to EGF-stimulated HEK293 cells. Shown is an autoradiograph upon overnight exposure (upper panel) and a Western blot detection of Raf-1 immunoprecipitates (lower panel).

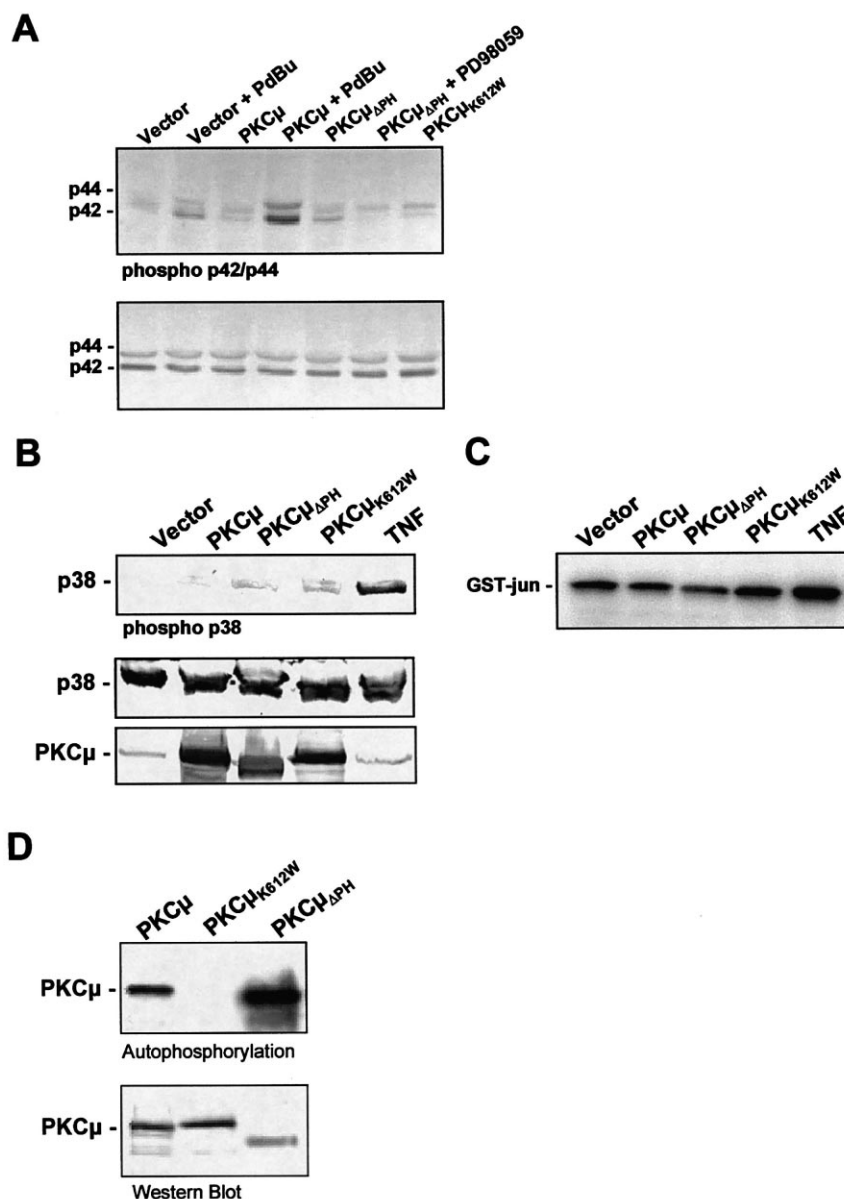


Fig. 2. PKC $\mu$  activates the p42/44 MAPK but not p38 and JNK. A: Constitutive active PKC $\mu$  activates p42/44 MAPK. HEK293 cells were transfected with the indicated constructs and subjected to Western blot analysis using either phospho-p42/44 specific antibodies (upper panel) or p42/p44 antibodies (lower panel) to estimate loads of each lane. B: Activation of the p38 MAPK. Upon transient expression of the indicated constructs, p38 activation was measured with phospho-p38 specific antibodies (upper panel). The lower panels show Western blot detection of p38 and PKC $\mu$ . C: PKC $\mu$  does not activate JNK activity. HEK293 cells were transfected with the indicated constructs and JNK activation was measured by in vitro phosphorylation of GST-jun using JNK immunoprecipitates. D: In vitro kinase assay of PKC $\mu$  expression constructs used in this study. The indicated PKC $\mu$  mutants were expressed in HEK293 cells, immunoprecipitated and subjected to in vitro kinase assays (upper panel). Western blot controls are shown in the lower panel.

tants significantly affect p38-MAPK activation as shown by Western blot detection with phospho-p38 specific antibodies. Expression levels of p38 and of PKC $\mu$  were analyzed by Western blot detection (Fig. 2B, middle and lower panel). Activation of JNK was measured upon transient expression of the indicated PKC $\mu$  mutants by in vitro phosphorylation of GST-jun using JNK immunoprecipitates. As a positive control for JNK activation cellular stimulation with TNF was performed. Expression of PKC $\mu$  was monitored by Western blot analysis (data not shown). As shown in Fig. 2C no enhancement, rather weak inhibition of JNK activity by PKCμ<sub>ΔPH</sub> could be demonstrated (Fig. 2C). JNK activation was furtheron

measured upon coexpression of PKC $\mu$  mutants with FLAG-tagged JNK. FLAG immunoprecipitates did not show significant changes in GST-jun phosphorylation (I.B., unpublished observations). PKC $\mu$  mutants used in this study were analyzed by in vitro kinase assays upon transient expression (Fig. 2D). The data presented in this study point to a rather selective role for PKC $\mu$  in the activation of the p42 MAPK.

### 3.3. Activation of transcription factors by PKC $\mu$

As PKC $\mu$  activates p42 MAPK cascade, potential downstream gene activation was expected. The SRE in the *c-fos* promoter is necessary and sufficient for growth factor-medi-

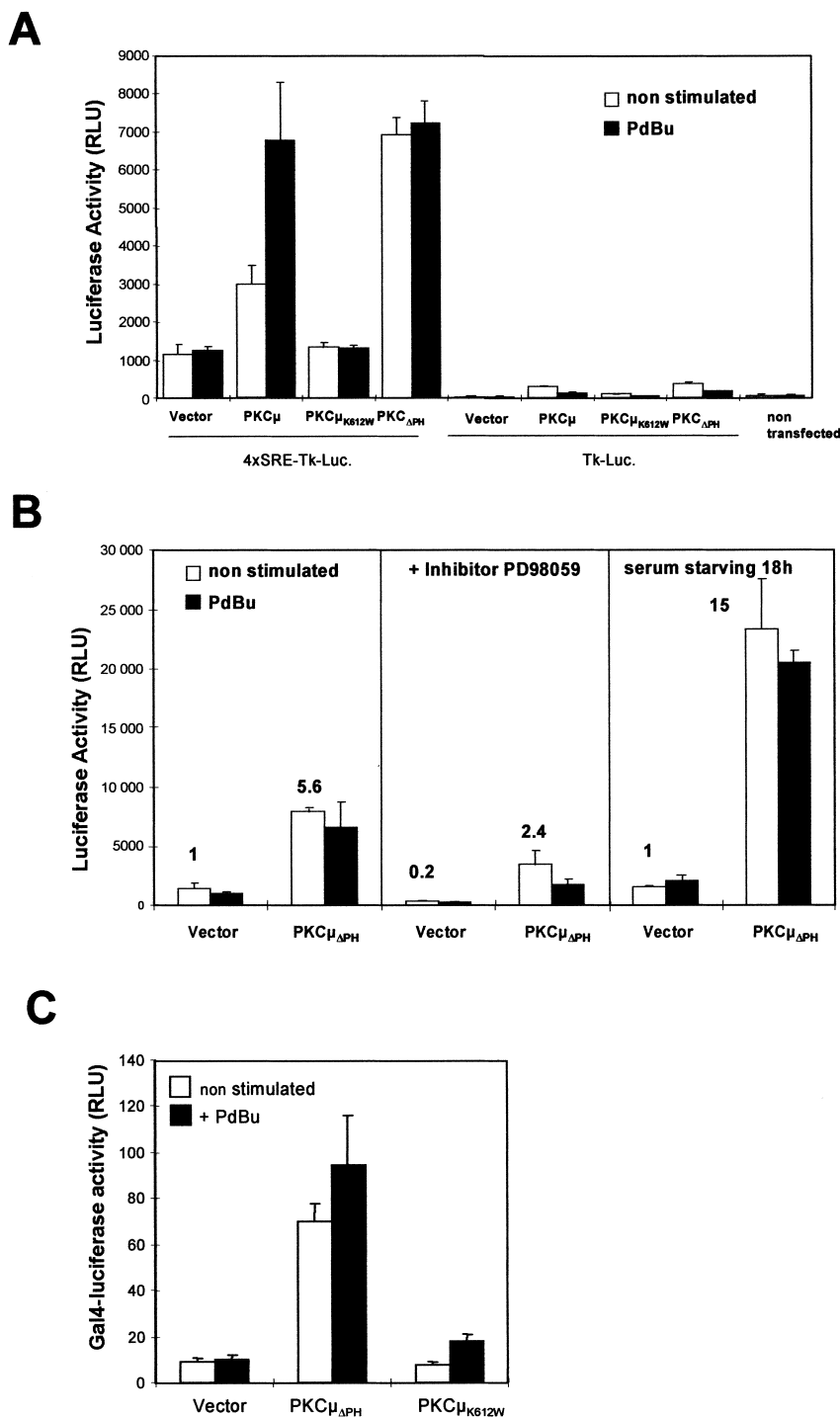


Fig. 3. Expression of constitutive active PKC $\mu$  enhances SRE driven luciferase activity. A: HEK293 cells were transfected with the indicated expression constructs and either left untreated or stimulated for 6 h with phorbol ester. Luciferase activity was normalized against transfection rates by measuring  $\beta$ -galactosidase activity (see Section 2). SRE-transfected cells were compared to cells transfected with a Tk-luciferase construct lacking any SRE sites to show specificity. B: HEK293 cells were transfected with the indicated constructs and proceeded as in A. Experiments were carried out in the presence of the MEK1 inhibitor PD98059 or upon serum starving as indicated. Relative activation factors of non-stimulated cells are indicated. C: PKC $\mu$  specifically activates TCF/Elk-1. pGAL4-luc and pGAL4DB-Elk-1 plasmids were expressed together with PKC $\mu_{\Delta PH}$  and pCH110. This allows monitoring of luciferase activity directly driven by activation of the Gal4-Elk-1 transcriptional activator.

ated transcription. It has been demonstrated previously that PKC $\alpha$  and PKC $\epsilon$  are involved in SRE-mediated transcription [34]. Therefore we tested whether PKC $\mu$  similarly activates SRE-triggered gene expression. A reporter gene construct con-

taining a basal thymidine kinase (Tk) promoter followed by four SRE recognition sites and a luciferase gene was used (4 $\times$ SRE-Tk-luc). Wild-type PKC $\mu$  or PKC $\mu$  mutants were coexpressed together with 4 $\times$ SRE-Tk-luc and pCH110 for

normalization of transfection efficiencies. As indicated in Fig. 3A luciferase activity was measured upon PKC $\mu$  expression in either non-stimulated or phorbol ester-stimulated cells (see Section 2). Phorbol ester stimulation of vector-transfected cells did not lead to a significant change in luciferase expression. Expression of PKC $\mu$  wild-type resulted in a 3-fold enhancement of luciferase activity which could be significantly amplified upon phorbol ester stimulation. Expression of the kinase dead PKC $\mu_{K612W}$  mutant did not lead to a detectable change in luciferase expression. In contrast, expression of constitutive active PKC $\mu_{\Delta PH}$  results in high basal SRE driven luciferase activity which is unaffected by phorbol ester treatment (Fig. 3A). As a control for SRE specificity PKC $\mu$  mutants were coexpressed with a Tk-luciferase reporter gene lacking the serum responsive elements. In all cases only weak background activity was obtained (Fig. 3A, right columns).

PKC $\mu_{\Delta PH}$ -enhanced activation of SRE driven luciferase activity is dependent on MAPK activation. As shown in Fig. 3B PKC $\mu_{\Delta PH}$ -induced luciferase activity could be effectively repressed by preincubation with the MEK1 specific inhibitor PD98059 (Fig. 3B, middle columns). Activation of SRE driven luciferase could be enhanced upon serum starvation of transfected cells. In this case expression of constitutive active PKC $\mu_{\Delta PH}$  led to a 15-fold enhancement of luciferase activity independent of phorbol ester stimulation (Fig. 3B, right columns). Similarly, enhancement of PKC $\mu_{\Delta PH}$ -mediated SRE driven gene activation was demonstrated in NIH3T3 cells (data not shown).

The serum responsive element is constitutively occupied by a protein complex comprising the serum responsive factor and a ternary complex factor Elk-1 [35]. Phosphorylation of Elk-1 which is the major substrate of p42/44 in mammalian cells leads to c-fos transcription. To test whether PKC $\mu$  triggers Elk-1-mediated gene activation, HEK293 cells were transfected with pGAL4-luc and pGAL4DB-Elk-1 plasmids together with PKC $\mu_{\Delta PH}$  and pCH110 for normalization. pGAL4-luc is a luciferase reporter plasmid containing five copies of the GAL4 response element. pGAL4DB-Elk-1 encodes a fusion protein consisting of the amino-terminal GAL4 DNA binding domain and a carboxy-terminal Elk-1 transactivation domain. Activation of the Elk-1 transactivation domain by phosphorylation activates transcription of the GAL4 driven luciferase gene. As shown in Fig. 3C expression of PKC $\mu_{\Delta PH}$  results in 8–10-fold enhancement of GAL4 driven luciferase activity. Expression of the kinase dead PKC $\mu_{K612W}$  mutant almost leads to basal levels of reporter gene activity.

In this study we can show that PKC $\mu$  is involved in the mitogenic pathway. Expression of constitutive active PKC $\mu$  activates Raf-1 leading to p42 MAPK activation. This in turn leads to Elk-1 phosphorylation resulting in SRE driven gene transcription.

Until now the precise function of PKC $\mu$ /PKD in growth control is unknown. Growth factor-mediated activation of PKD is considered to be mediated via PLC $\gamma$ -generated diacylglycerol [14]. Independent studies similarly showed that PKC $\mu$  is associated with PLC $\gamma$  within the activated B cell receptor complex suggesting a negative regulatory role in the phosphoinositol metabolism [36]. According to these data a receptor proximal function of PKC $\mu$  is predicted leading to Raf-1 activation. PKD has been reported to be activated by oxidative stress [37] which also activates p42 MAPK

(I.B., unpublished observations). PKD activation by oxidative stress is inhibited partially by a PLC $\gamma$  specific inhibitor which further underlines the role of PLC $\gamma$  in PKC $\mu$ /PKD-triggered cellular pathways leading to MAPK activation.

Further studies showed that PKC $\mu$  is activated by PKCs as upstream kinases identifying the isotypes  $\epsilon$  and  $\eta$  as most likely candidates [25]. Especially PKC $\eta$  seems to be most important to activate PKC $\mu$ /PKD as it is physically associated with PKD [38]. PKC $\mu$ -induced Raf-1 activation is most likely not dependent on PKC $\eta$ . Initial studies coexpressing constitutive active PKC $\eta$  together with wild-type PKC $\mu$  in NIH3T3 cells did not lead to enhanced p42/p44 MAPK activation (I.B., unpublished observations). Further studies to unravel the precise role of PKC $\mu$ /PKD in PLC $\gamma$ -triggered cellular pathways have to be performed, especially to characterize pathways leading to Raf-1 activation and downstream transcription factor activation.

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