The EMBO Journal vol.14 no.24 pp.6157–6163, 1995

Evidence for a role of MEK and MAPK during signal transduction by protein kinase C ζ

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Protein kinase C ζ (ζPKC) is critically involved in the control of a number of cell functions, including proliferation and nuclear factor κB (NF-κB) activation. Previous studies indicate that ζPKC is an important step downstream of Ras in the mitogenic cascade. The stimulation of Ras initiates a kinase cascade that culminates in the activation of MAP kinase (MAPK), which is required for cell growth. MAPK is activated by phosphorylation by another kinase named MAPK kinase (MEK), which is the substrate of a number of Ras-activated serine/threonine kinases such as c-Raf-1 and B-Raf. We show here that MAPK and MEK are activated *in vivo* by an active mutant of ζPKC, and that a kinase-defective dominant negative mutant of ζPKC dramatically impairs the activation of both MEK and MAPK by serum and tumour necrosis factor (TNFα). The stimulation of other kinases, such as stress-activated protein kinase (SAPK) or $p70^{S6K}$, is shown here to be independent of ζPKC. The importance of MEK/MAPK in the signalling mechanisms activated by ζPKC was addressed by using the activation of a κB-dependent promoter as a biological read-out of ζPKC.

Keywords: MAPK/MEK/protein kinase C ζ/signal transduction

Introduction

Protein kinase C ζ (ζPKC) is an atypical PKC whose mechanism of regulation differs from that of other members of its family. Thus, it is not activated by Ca^{2+} or diacylglycerol, and is insensitive to phorbol esters (Ono *et al.*, 1989; Ways *et al.*, 1992). However, ζPKC is activated by important lipid intracellular mediators like phosphatidic acid (PA; Nakanishi and Exton, 1992), phosphatidylinositol 3,4,5-trisphosphate [PI (3,4,5)P_3; Nakanishi *et al.*, 1993] and ceramide (Lozano *et al.*, 1994). PA is produced as a consequence of the activation of phosphatidylcholine-phospholipase D or the concerted action of phosphatidylcholine-phospholipase C and diacylglycerol kinase (Exton, 1994). Both phospholipases have been implicated in critical cellular functions, including cell proliferation and tumour transformation (Larrodera *et al.*, 1990; Lopez-Barahona *et al.*, 1990; Xu *et al.*, 1993). PI (3,4,5)P_3 is the product of PI 3-kinase which is a decisive step during mitogenic signal transduction (Valius and Kazlauskas, 1993). Ceramide, on the other hand, is generated following the activation of sphingomyelin hydrolysis by inflammatory cytokines, such as interleukin-1 (IL-1) or tumour necrosis factor (TNFα; Hannun, 1994; Kolesnick and Golde, 1994). Taken together, all these observations pinpoint ζPKC as a target of important lipid second messengers and support its role in cell signalling. In this regard, ζPKC has been shown to be involved in the control of a number of cellular functions, including maturation of *Xenopus* oocytes (Domínguez *et al.*, 1992), cell proliferation (Berra *et al.*, 1993), neuronal (Wooten *et al.*, 1994) and leukaemic cell differentiation (Ways *et al.*, 1994), and the maintenance of long-term potentiation (Sacktor *et al.*, 1993). Recent data also demonstrate the pivotal role played by ζPKC in the activation of nuclear factor κB (NF-κB) in *Xenopus laevis* oocytes and murine fibroblasts (Díaz-Meco *et al.*, 1993, 1994a; Domínguez *et al.*, 1993). This is in good agreement with the fact that ζPKC is activated by inflammatory lipid mediators generated by sphingomyelin hydrolysis (Lozano *et al.*, 1994), since κB-dependent promoter activation is a landmark of the TNFα mechanism of action.

In a number of instances, cell stimulation activates Ras which triggers a number of important serine/threonine kinases that have MAP kinase kinase (MEK) as substrate, such as MEK kinase, c-Raf-1 and B-Raf (Lange-Carter *et al.*, 1993; Avruch *et al.*, 1994; Vaillancourt *et al.*, 1994), and that culminates in the activation of MAP kinase (MAPK). ζPKC is also a downstream target of Ras, which has been shown to be critically involved, like ζPKC, in the activation of NF-κB (Arenzana-Seisdedos *et al.*, 1989; Devary *et al.*, 1993; Domínguez *et al.*, 1993; Finco and Baldwin, 1993). Therefore, it appears that the mechanism whereby ζPKC controls cell signalling could at least in part implicate the channelling of Ras signals in the activation of MAPK. Here, we evaluate the participation of ζPKC *in vivo* in the regulation of MAPK, and establish the critical role of MAPK in κB-dependent promoter activation by ζPKC and TNFα.

Results

Involvement of ζPKC in the activation of MAPK

In order to determine whether ζPKC is involved in the activation of MAPK *in vivo*, Cos cells were transfected with an influenza haemagglutinin (HA) protein epitope-tagged p44 MAPK (HA-MAPK) together with either control plasmid (pRcCMV) or with plasmids harbouring wild-type *X.laevis* (pRcCMVζPKC_{XL}) or rat (pRcCMVζ-PKC_{RAT}) ζPKC, or their kinase-defective mutants (pRcCMVζ$PKC^{MUT}{}_{XL}$ or pRcCMVζ$PKC^{MUT}{}_{RAT}$). We have previously shown that expression of the *Xenopus*

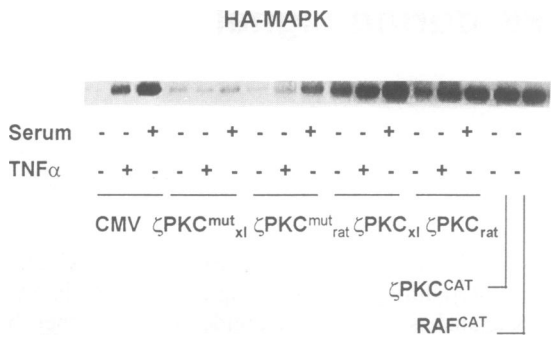

Fig. 1. ζPKC is involved in the activation of MAPK. Cos cells transfected with pCDNA-HA-MAPK were co-transfected with either 20 μg of pRcCMV (CMV) or 10 μg of pRcCMV plus 10 μg of the following plasmids: pRcCMVζPKC$_{XL}$ (ζPKC$_{XL}$), pRcCMVζPKC$_{RAT}$ (ζPKC$_{RAT}$), pRcCMVζPKC$^{MUT}{}_{XL}$ (ζPKC$^{MUT}{}_{XL}$), pRcCMVζPKC$^{MUT}{}_{RAT}$ (ζPKC$^{MUT}{}_{RAT}$), pRcCMVζPKC^{CAT} (ζPKC^{CAT}) or pRcCMVRAF^{CAT} (RAF^{CAT}). Cells were stimulated for 15 min with either TNFα (500 U/ml) or fetal calf serum (FCS; 10%). The tagged kinase was immunoprecipitated and its activity determined as described in Materials and methods. Essentially identical results were obtained in three other independent experiments.

ζPKC mutant severely impairs reinitiation of DNA synthesis in mouse fibroblasts (Berra *et al.*, 1993). The addition of serum or TNFα to transfected quiescent Cos cells provokes a dramatic activation of HA-MAPK, as measured by kinase assay (Figure 1). Transfection of both kinase-defective ζPKC mutants severely reduced the effect of serum, as well as that of TNFα, whereas the wild-type enzymes produced a significant stimulation of this parameter. The inhibition of MAPK activation by the kinase-inactive ζPKC mutants was more apparent when the *X.laevis* enzyme was used as compared with the rat ζPKC. Therefore, ζPKC appears to be required and sufficient for the activation of MAPK. To confirm that ζPKC stimulation is sufficient to activate the MAPK cascade, Cos cells were transfected with HA-MAPK together with plasmids harbouring permanently active ζPKC (pRcCMVζPKC^{CAT}) and c-Raf-1 (pRcCMV-RAF^{CAT}) mutants, obtained by deletion of their respective regulatory domains. It must be noted that the catalytic domain of ζPKC from *X.laevis* is identical to that from rat. Interestingly, transfection of both active mutants led to comparable stimulation of HA-MAPK (Figure 1). These results suggest that ζPKC is critically involved in the activation of MAPK. All ζPKCs, the c-Raf-1 mutant and the HA-MAPK were expressed at similar levels, as determined by immunoblotting (not shown).

ζPKC is not involved in the SAPK pathway

The above results are in keeping with the notion that ζPKC is not only involved in the regulation of NF-κB (Díaz-Meco *et al.*, 1993, 1994a), but that it is also critically implicated in the activation of MAPK. Recently, a parallel cascade to this of MAPK has been described, which culminates in the stimulation of a novel member of the MAPK family, termed stress-activated protein kinase (SAPK) or c-Jun N-terminal kinase (JNK), that phosphorylates c-Jun in its activation domain (Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994) and that is activated by the dual SAPK/ERK kinase (SEK; Sánchez *et al.*, 1994; Dérijard *et al.*, 1995). In order to determine whether ζPKC

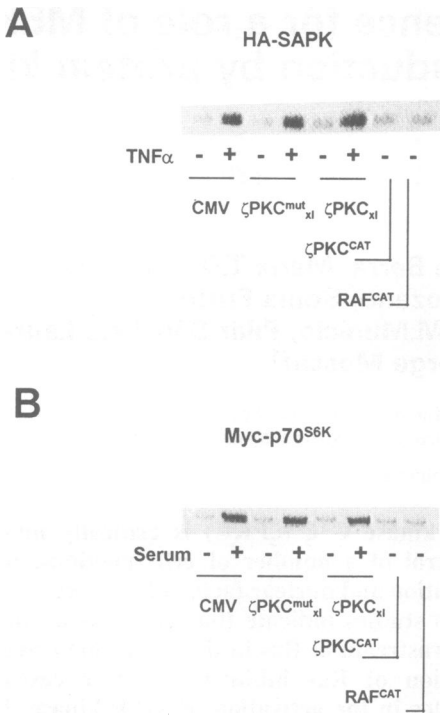

Fig. 2. ζPKC is not involved in the activation of SAPK or p70^{S6K}. Cos cells transfected with either pMT-HA-SAPK (**A**) or Myc-p70^{S6K} (**B**) were co-transfected with either 20 μg of pRcCMV (CMV) or 10 μg of pRcCMV plus 10 μg of the following plasmids: pRcCMVζPKC$_{XL}$ (ζPKC$_{XL}$), pRcCMVζPKC$^{MUT}{}_{XL}$ (ζPKC$^{MUT}{}_{XL}$), pRcCMVζPKC^{CAT} (ζPKC^{CAT}) or pRcCMVRAF^{CAT} (RAF^{CAT}). Cells were stimulated for 15 min with either TNFα (500 U/ml) or FCS (10%). The tagged kinases were immunoprecipitated and their activity determined as described in Materials and methods. Essentially identical results were obtained in three other independent experiments.

participates in this pathway, Cos cells were transfected with HA-SAPK along with either control plasmid (pRcCMV) or pRcCMVζPKC$_{XL}$, pRcCMVζPKC$^{MUT}{}_{XL}$, pRcCMVζPKC^{CAT}, pRcCMVRAF^{CAT} (Figure 2A), or pRcCMVζPKC$_{RAT}$ and pRcCMVζPKC$^{MUT}{}_{RAT}$ (not shown), after which they were stimulated or not with TNFα, and the HA-SAPK activity was determined in immunoprecipitates by kinase assay using GST–c-Jun as substrate. Addition of TNFα dramatically activated HA-SAPK, whereas transfection of pRcCMVζPKC^{CAT} or pRcCMVRAF^{CAT} produced no effect (Figure 2A). Interestingly, transfection of wild-type or kinase-defective *Xenopus* (Figure 2A) or rat (not shown) ζPKC did not affect the stimulation of HA-SAPK by TNFα. These results indicate that ζPKC does not appear to play a relevant role in the SAPK pathway, and strengthen the specificity of its involvement in the activation of MAPK. All ζPKCs, c-Raf-1 mutant and the HA-SAPK were expressed at similar levels, as determined by immunoblotting (not shown).

ζPKC is not involved in the activation of p70^{S6K}

The kinase p70^{S6K} is important in mitogenesis and has recently been shown to lie on a pathway distinct from the Ras/MAPK cascade (Ming *et al.*, 1994), probably downstream of PI 3-kinase (Chung *et al.*, 1994). Since ζPKC has been reported to be activated *in vitro* by the products of that lipid kinase, it would be of interest to determine the effect of the blockade of ζPKC in the

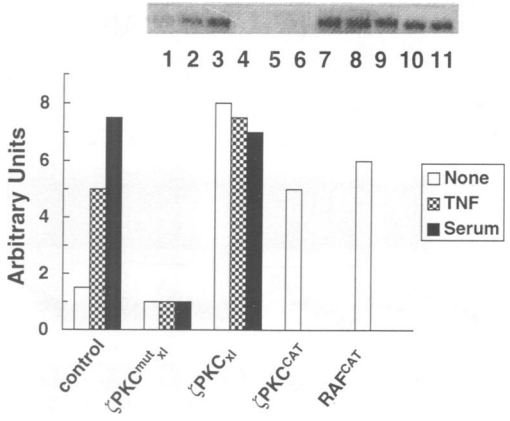

Fig. 3. MEK is downstream of ζPKC *in vivo*. Cos cells transfected with pEBGMEK1 were co-transfected with either 20 μg of pRcCMV (control; lanes 1–3) or 10 μg of pRcCMV plus 10 μg of the following plasmids: pRcCMVζPKC$_{XL}$ (ζPKC$_{XL}$; lanes 7–9), pRcCMVζ-PKC$^{MUT}{}_{XL}$ (ζPKC$^{MUT}{}_{XL}$; lanes 4–6), pRcCMVζPKC^{CAT} (ζPKC^{CAT}; lane 10) or pRcCMVRAF^{CAT} (RAF^{CAT}; lane 11). Cells were stimulated for 15 min with either TNFα (500 U/ml) (lanes 2, 5 and 8) or FCS (10%) (lanes 3, 6 and 9). The tagged kinase was precipitated and its activity determined as described in Materials and methods. Results are InstantImager quantitations of the substrate phosphorylation, and were very similar to those obtained in three other independent experiments.

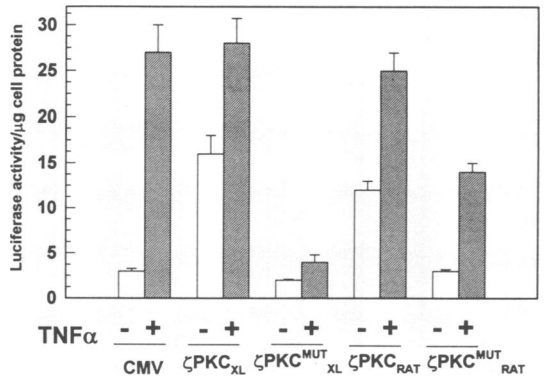

Fig. 4. Role of ζPKC in κB-dependent promoter activation. Cos cells were transfected with 500 ng of the κB enhancer-directed luciferase reporter plasmid along with 3 μg of either pRcCMV (CMV), pRcCMVζPKC$_{XL}$ (ζPKC$_{XL}$), pRcCMVζPKC$_{RAT}$ (ζPKC$_{RAT}$), pRcCMVζPKC$^{MUT}{}_{XL}$ (ζPKC$^{MUT}{}_{XL}$) or pRcCMVζPKC$^{MUT}{}_{RAT}$ (ζPKC$^{MUT}{}_{RAT}$). After 4 h, the DNA-containing medium was removed and cells were incubated with low (0.2%)-serum medium for 36 h. Afterwards, cells were either untreated or stimulated with 500 U/ml of TNFα for 6 h, after which extracts were prepared and luciferase activity was determined. A control plasmid with the minimal promoter without enhancer sequences was not affected by any treatment. Results are the mean ± SD of four independent experiments with incubations in duplicate.

activation by serum of p70^{S6K}. Thus, Myc-tagged p70^{S6K} was transfected into Cos cells either with control plasmid (pRcCMV) or plasmids pRcCMVζPKC$_{XL}$, pRcCMVζ-PKC$^{MUT}{}_{XL}$, pRcCMVζPKC^{CAT} or pRcCMVRAF^{CAT} (Figure 2B), or pRcCMVζPKC$_{RAT}$, and pRcCMVζ-PKC$^{MUT}{}_{RAT}$ (not shown). Transfected cells were stimulated or not with serum, Myc-p70^{S6K} was immunoprecipitated with an anti-tag antibody and the kinase activity towards S6 was determined. Interestingly, transfection of any of the ζPKC constructs or the Raf mutant produced no effect on p70^{S6K} activity (Figure 2B), indicating that ζPKC is not located in the p70^{S6K} pathway. Stimulation by TNFα produced little or no effect on p70^{S6K} activity (not shown). Again, all ζPKCs, the c-Raf-1 mutant and Myc-p70^{S6K} were expressed at similar levels, as determined by immunoblotting (not shown).

MEK is an intermediary in the ζPKC-MAPK pathway

Altogether, these results indicate that MAPK is a downstream step in the ζPKC-activated cascade. Because MEK is the upstream activator of MAPK (see Introduction), conceivably ζPKC will be critically involved in the activation of MEK by serum or TNFα. To address this possibility, pEBG-MEK1 was transfected into Cos cells either with control plasmid or expression vectors for ζPKC and Raf as above, after which expressed MEK1 was purified by glutathione–agarose chromatography and its kinase activity toward its substrate MAPK^{KR} was determined. Results from Figure 3 show that stimulation with serum or TNFα leads to a significant activation of MEK that is dramatically impaired by transfection of the ζPKC kinase-inactive mutant, but not by transfection of the wild-type enzyme which, consistent with the results from the MAPK experiment, provokes a significant stimulation of MEK in the absence of any stimulus. The catalytic permanently active mutant of ζPKC also significantly activated MEK1 in the absence of stimulus and that activation was comparable with that produced by the permanently active mutant of Raf (Figure 3).

MAPK and κB-dependent promoter activation

We have previously shown that ζPKC (Díaz-Meco *et al.*, 1993; Domínguez *et al.*, 1993; Lozano *et al.*, 1994), like Ras (Arenzana-Seisdedos *et al.*, 1989; Devary *et al.*, 1993; Domínguez *et al.*, 1993; Finco and Baldwin, 1993), is actively involved in the control of κB-dependent promoter activation. Since ζPKC and Ras are also important for the activation of MAPK, it is conceivable that MAPK could be a critical intermediary step between ζPKC and κB-dependent promoter activity. The fact that ζPKC is crucial for κB-dependent promoter activation is confirmed in Figure 4. Thus, transfection of the rat and *Xenopus* kinase-inactive mutants of ζPKC severely impaired κB-dependent transactivation by TNFα of a luciferase reporter plasmid harbouring three copies of the κB enhancer from the LTR of HIV (Arenzana-Seisdedos *et al.*, 1993). As in the results of Figure 1 for MAPK, the kinase-inactive mutant of *Xenopus* ζPKC was significantly more efficient than the rat enzyme. To determine whether MAPK is required for κB-dependent promoter activation, cells were transfected with the κB-luciferase reporter plasmid either with control plasmids (pCDNA and pEBG) or with plasmids that expressed wild-type MAPK, MEK or a MAPK dominant negative mutant (Pagés *et al.*, 1993), after which transfected cells were stimulated or not by TNFα and the κB-dependent promoter activity was determined thereafter. Interestingly, expression of the wild-type MAPK or MEK by itself promotes a significant stimulation of the κB-dependent promoter activity (Figure 5) and synergistically co-operates with TNFα (not shown) in the stimulation of this parameter. It is of great relevance that expression of the MAPK dominant negative mutant severely impairs

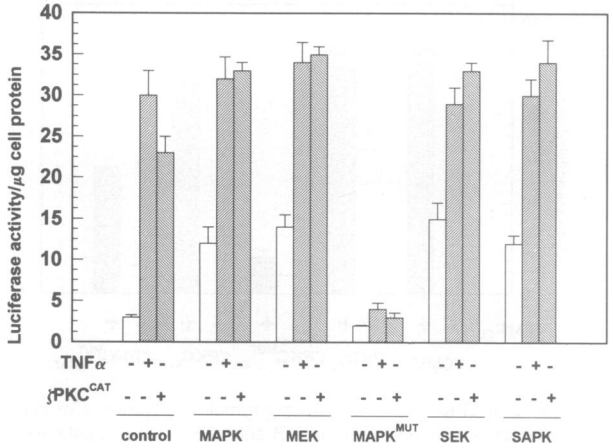

Fig. 5. Role of different kinases in κB-dependent promoter activation. Cos cells were transfected with 500 ng of the κB enhancer-directed luciferase reporter plasmid along with 3 μg of either pRcCMV, or pRcCMVζPKC^{CAT} (ζPKC^{CAT}) with 3 μg of either pCDNA, pMT or pEBG (control), pCDNAHAMAPK (MAPK), pCDNAHAMAPK^{MUT} (MAPK^{MUT}), pMT-HA-SAPK (SAPK), pEBGMEK-1 (MEK) or pEBGSEK-1 (SEK). After 4 h, the DNA-containing medium was removed and cells were incubated with low (0.2%)-serum medium for 36 h. Afterwards cells were either untreated or stimulated with 500 U/ml of TNFα for 6 h, after which extracts were prepared and luciferase activity was determined. A control plasmid with the minimal promoter without enhancer sequences was not affected by any treatment. Results are the mean ± SD of four independent experiments with incubations in duplicate.

κB-dependent promoter activation by TNFα (Figure 5). All the transfected kinases were expressed at similar levels, as determined by immunoblotting (not shown). Since ζPKC and MAPK are both required and sufficient for κB-dependent promoter activation, and ζPKC is necessary and sufficient for the stimulation of MAPK, this latter enzyme could be an intermediary step in the activation of κB promoters by ζPKC. Results from Figure 5 show that transfection of pRcCMVζPKC^{CAT} leads to κB-dependent promoter activation in the absence of TNFα, in keeping with previously published results (Díaz-Meco *et al.*, 1993). Interestingly, transfection of the MAPK dominant negative mutant, but not the wild-type enzyme or wild-type MEK, severely impaired κB-dependent promoter activation by ζPKC. In consideration of all these results, it can be concluded that MAPK is an important intermediary step, downstream of ζPKC, in the activation of κB-dependent promoters by TNFα.

Role of the SEK/SAPK pathway in κB-dependent promoter activation

The above results indicate that ζPKC does not appear to play any role in the activation of SAPK. In order to test whether the SEK/SAPK pathway could be involved in the activation of κB promoters, Cos cells were transfected with the κB-luciferase reporter plasmid along with expression plasmids for either SAPK or SEK, after which cells were stimulated with TNFα and the κB-dependent promoter activity was determined thereafter. Interestingly, transfection of SAPK or SEK stimulated by themselves the κB promoter activity (Figure 5) and significantly co-operate with TNFα to activate this parameter (not shown). Therefore, although ζPKC does not activate SAPK, this enzyme appears to be involved, like MAPK, in the control of κB-dependent promoters.

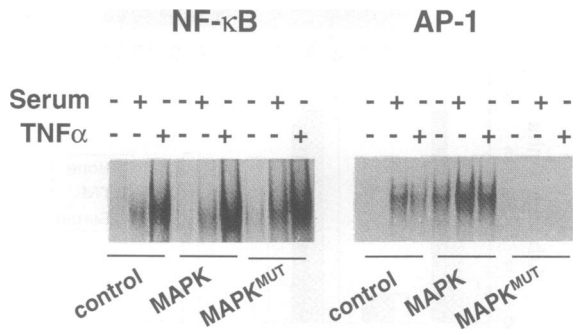

Fig. 6. Role of MAPK in NF-κB and AP-1 activation. Cos cells were transfected with either pCDNA (control), pCDNAHAMAPK (MAPK) or pCDNAHAMAPK^{MUT} (MAPK^{MUT}). After 4 h, the DNA-containing medium was removed and cells were incubated with low (0.2%)-serum medium for 36 h. Afterwards, cells were either untreated or stimulated with 500 U/ml of TNFα or 10% FCS for 1 h, after which nuclear extracts were prepared and either NF-κB or AP-1 activity was determined in band shift assays as described in Materials and methods. These are InstantImager images and essentially identical results were obtained in three other independent experiments.

Lack of involvement of MAPK in the translocation of NF-κB to the nucleus

Cell stimulation by activators of κB-dependent promoters display increased translocation of NF-κB to the nucleus (Dimitris and Maniatis, 1995). Previous data from this laboratory have shown that ζPKC plays a pivotal role in this process (Díaz-Meco *et al.*, 1993, 1994a). In order to determine whether MAPK regulates the levels of nuclear NF-κB, Cos cells were transfected with either control plasmid or expression plasmids for either wild-type or the dominant negative mutant of MAPK, and they were either untreated or stimulated with TNFα or serum. Afterwards, nuclear extracts were prepared and the NF-κB and AP-1 activities were determined. Activation by TNFα induces both nuclear NF-κB as well as AP-1 (Figure 6). Interestingly, transfection of wild-type MAPK did not produce any effect on NF-κB nuclear levels, although it increased the effects of TNFα and serum to activate AP-1. Conversely, transfection of the dominant negative mutant of MAPK dramatically inhibited AP-1 nuclear levels, but produced little or no effect on NF-κB. Therefore, all the above observations indicate that the critical involvement of MAPK in the control of κB-promoter activity appears to be independent of the ability of TNFα to activate the nuclear translocation of NF-κB.

Discussion

Recent results strongly implicate ζPKC in mitogenic signalling. Thus, microinjection of a peptide with the sequence of its pseudosubstrate dramatically inhibits the induction of maturation in *Xenopus* oocytes by insulin and Ras, but not by progesterone or by PMA-sensitive PKC isoforms (Domínguez *et al.*, 1992). The same specific blockade of this pathway was achieved by depletion of ζPKC with antisense RNA or oligonucleotides (Domínguez *et al.*, 1992). A third piece of evidence of the involvement of ζPKC in Ras-mediated maturation of oocytes was obtained by microinjection of a kinase-inactive ζPKC dominant negative mutant that completely abrogated the Ras pathway with little or no effect on the

progesterone route (Domínguez *et al.*, 1992). Therefore, these three independent strategies strongly indicated that ζPKC must be considered a critical step downstream of Ras in the control of maturation in *Xenopus* oocytes. A number of pieces of evidence implicate ζPKC in the mitogenic activation of somatic cells as well. Thus, microinjection of the pseudosubstrate inhibitor peptide for ζPKC into mouse fibroblasts, or the expression of its kinase-inactive mutant, severely impairs reinitiation of DNA synthesis by serum in quiescent cells (Berra *et al.*, 1993). Also of note is the fact that ζPKC physically interacts with Ras *in vitro* and *in vivo* (Díaz-Meco *et al.*, 1994b), which appears to be functionally relevant because inhibition of the Ras function with the dominant negative mutant Asn-17 severely impairs the activation of ζPKC in NIH-3T3 fibroblasts (Díaz-Meco *et al.*, 1994b). Taken together, all of these observations indicate that ζPKC could be considered a critical target of Ras-activated mitogenic signals.

Ras triggers a number of kinases that through MEK activate MAPK (see Introduction). Since ζPKC appears to be located downstream of Ras (Domínguez *et al.*, 1992; Díaz-Meco *et al.*, 1994b; Bjorkoy *et al.*, 1995), it seemed conceivable that ζPKC could be critically involved in the activation of MAPK. We show here, for the first time, that activation of ζPKC dramatically stimulates MAPK, whereas a kinase-inactive dominant negative mutant of ζPKC severely impairs MAPK activation in response to serum and TNFα. Therefore, MAPK appears to be a downstream target of ζPKC during mitogenic signalling and also in signal transduction pathways activated by inflammatory cytokines such as TNFα. Since MEK is the upstream activator of MAPK, conceivably MEK could be a direct substrate of ζPKC. Although recent data suggest that MEK is a relatively poor *in vitro* substrate for ζPKC, as compared with active c-Raf-1 (Díaz-Meco *et al.*, 1994a), from the results shown here it seems clear that, *in vivo*, ζPKC is efficiently coupled to MEK activation. Reasons for this apparent paradox could be 2-fold: (i) ζPKC activates some still unidentified downstream kinase that directly phosphorylates MEK (our preliminary results indicate that, in any event, this hypothetical kinase is not c-Raf-1); (ii) some still uncharacterized adapter couples ζPKC *in vivo* to efficiently phosphorylate and activate MEK. Of these two possibilities, we favour the second because the first one does not fit with the dominant negative phenotype of the kinase-dead ζPKC mutant. Thus, if ζPKC does not communicate directly with MEK, transfection of ζPKC^{MUT} should not block MEK/MAPK, at least during the mitogenic response, because the c-Raf-1 pathway would be functionally intact. It could be argued that ζPKC^{MUT} is chelating the upstream activator(s) of not only ζPKC, but also of c-Raf-1, consequently leading to the blockade of the MEK/MAPK cascade. However, very recent data from our laboratory (not shown) do not support this notion because transfection of a kinase-dead mutant of ζPKC that lacks the whole regulatory domain is as potent as the full-length mutant in inhibiting MEK/MAPK. This, together with the fact that the kinase-active catalytic ζPKC mutant efficiently activates MEK *in vivo*, strongly indicates that ζPKC most probably interacts directly with MEK. This would be consistent with a model whereby ζPKC and c-Raf-1 constitute two branches of a signalling cascade both initiated by Ras and converging at MEK. This model is in keeping with recently published data that indicate the existence of a bifurcation of signalling downstream of Ras (Díaz-Meco *et al.*, 1994b; Bjorkoy *et al.*, 1995). However, an important difference between c-Raf-1 and ζPKC is that whereas Raf efficiently phosphorylates MEK *in vitro* and *in vivo*, ζPKC is only able to do so *in vivo*, where the putative adapter would be available. Evidence for the existence of such adapters is found in *Saccharomyces cerevisiae*, where the STE5 protein promotes the formation of a signalling complex among the homologues of MEKK, MEK and MAPK, and is absolutely required for efficient signalling through this molecule (Choi *et al.*, 1994). The role for the adapters in mammalian cells has recently been invoked to explain the intriguing result that MEKK activates MEK *in vitro* and *in vivo*, but cannot induce the activation of MAPK *in vivo* (Xu *et al.*, 1995).

In any case, and whatever the mechanism, the findings reported here that MEK/MAPK are critical downstream targets of ζPKC help us to understand how this kinase may control cell function. In this regard, the fact that a dominant negative mutant of MAPK severely impairs the activation of a κB-dependent promoter (a biological read-out of ζPKC) by ζPKC and TNFα indicates that regulation of the MEK/MAPK cascade by ζPKC is functionally relevant. Previous data from Finco and Baldwin (1993) demonstrated that a permanently active mutant of c-Raf-1 was sufficient to activate a κB site reporter plasmid, and that transfection of a c-Raf-1 dominant negative significantly impaired the activation of such a reporter by TNFα. Since c-Raf-1 activates MAPK, this suggests that ζPKC and c-Raf-1 signalling to κB enhancers functionally converge at MAPK. This would be consistent with the fact that both kinases are downstream effectors of Ras, and that Ras is critically involved in the activation of κB-dependent promoters (Arenzana-Seisdedos *et al.*, 1989; Devary *et al.*, 1993; Domínguez *et al.*, 1993; Finco and Baldwin, 1993). The fact that neither ζPKC nor c-Raf-1 permanently active mutants are able to affect SAPK [this paper and Sánchez *et al.* (1994)] is in good agreement with the notion that both ζPKC and c-Raf-1 lie in a cascade completely independent of SAPK which is activated by MEKK/SEK-1 (Minden *et al.*, 1994; Sánchez *et al.*, 1994; Yan *et al.*, 1994; Dérijard *et al.*, 1995). However, we show here that SEK and SAPK also appear to be important for the activation of κB-dependent promoters. Since MEKK is the upstream activator of SEK and has been shown to be located downstream of Ras (Lange-Carter and Johnson, 1994), this suggests that Ras activates multiple signalling cascades, one of which triggers MEKK to activate SAPK, and another one initiated by either c-Raf-1 or ζPKC that culminates in the activation of MAPK. Both, in turn, modulate κB-enhancer elements.

A critical question that arises from the results presented here is the mechanism whereby MAPK and SAPK regulate κB-promoters. ζPKC activation is an important step in the actual translocation of NF-κB to the nucleus of stimulated cells as well as in the activation of AP-1 promoters (Bjorkoy *et al.*, 1995). MAPK does not appear to be involved in the control of the nuclear translocation of NF-κB (see above). Therefore, the mechanism whereby MAPK, and SAPK, modulate κB-dependent promoters

most probably does not involve the regulation of NF-κB nuclear translocation. A feasible explanation for the importance of MAPK in κB-dependent promoter activity may rely on the recent demonstration of the existence of cross-coupling between AP-1 and NF-κB transcription factors. Thus, Stein *et al.* (1993) have reported the physical interaction of c-Fos and c-Jun with p65 NF-κB which potentiates its trans-activating function. Therefore, it is conceivable that the role of MAPK in κB-dependent promoter activity could be mediated by its actions on AP-1. This would be consistent with the fact that both MAPK, possibly through c-Fos, and SAPK, possibly through c-Jun, are important regulators of the activation of κB-dependent promoters. Undoubtedly more work is necessary to unveil the precise mechanisms involved in the processes depicted here, but our results strongly suggest the importance of the MEK/MAPK cascade in the mechanisms whereby ζPKC participates not only in mitogenic signalling, but also in inflammatory pathways.

Materials and methods

Plasmids

pRcCMVζPKC$_{RAT}$ and pRcCMVζPKC$^{MUT}{}_{RAT}$ were generating by subcloning the *Eco*RI–*Xba*I fragment from pBluescriptζPKC$_{RAT}$ and pSelectζPKC$^{MUT}{}_{RAT}$, respectively, into *Hin*dIII–*Xba*I-pRcCMV (Invitrogen). pSelect ζPKC$^{MUT}{}_{RAT}$ was derived by directed mutagenesis from pSelectζPKC$_{RAT}$ and the following mutagenic oligo: 5′-GATTTA-CGCCATGTGGGTGGTGAAGAAGGAGC-3′. pSelectζPKC$_{RAT}$ was obtained by subcloning the *Eco*RI fragment containing full-length ζPKC$_{RAT}$ from pBluescriptζPKC$_{RAT}$ into pSelect (Promega). Plasmids 3EconAluc, pRcCMVζPKC$_{XL}$, pRcCMVζPKC$^{MUT}{}_{XL}$, pRcCMVζ-PKC^{CAT}, pRcCMVRAF^{CAT}, pCDNA-HA-MAPK, pCDNA-HA-MAPK^{MUT}, pMT2-HA-SAPK, Myc-p70^{S6K}, pEBG-MEK1 and pEBG-SEK1 have been described previously (Berra *et al.*, 1993; Pagès *et al.*, 1993; Díaz-Meco *et al.*, 1994a,b; Kyriakis *et al.*, 1994; Ming *et al.*, 1994; Yan *et al.*, 1994). Plasmid pCDNA-HA-MAPK^{MUT} harbours a Thr to Ala substitution at position 192 (Pagès *et al.*, 1993). The ζPKC kinase-defective mutants are mutations to tryptophan of lysines 281 and 275, respectively, for the rat and *Xenopus* dominant negative mutants. Recombinant GST–c-Jun-(5-89) and MAPK^{KR} kinase defective mutant were expressed in *Escherichia coli* as described elsewhere (Díaz-Meco *et al.*, 1994a; Kyriakis *et al.*, 1994).

Transfections

Subconfluent cultures of Cos cells in 100 mm plates were transfected by the calcium phosphate method (Gibco, BRL) with 20 μg of either pCDNA-HA-MAPK, pMT2-HA-SAPK, Myc-p70^{S6K} or pEBG-MEK1 together with 20 μg of pRcCMV or 10 μg of pRcCMVζPKC, pRcCMVζPKC^{CAT}, pRcCMVζPKC^{MUT} or pRcCMVRAF^{CAT} plus 10 μg of pRcCMV. Plasmid DNA was removed 4 h later, and cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS for 16 h. Afterwards, medium was replaced with medium containing 0.5% FCS for 16 h, followed by an additional 8 h in serum-free medium. Cultures were then either untreated or stimulated with 10% FCS or 500 U/ml of TNFα, lysed and extracts prepared as described below.

For the κB-dependent promoter activity assay, subconfluent cultures of Cos cells in P60 mm were transfected by the calcium phosphate method (Promega) with 500 ng of 3EconAluc and 3 μg of the different kinase constructs for 4 h. After transfection, cells were washed and allowed to remain in culture for 36 h with 0.2% FCS. Transfected cells were induced with the different stimuli for the last 6 h of the culture period. For detection of luciferase activity, total cell extracts were prepared in a lysis cell buffer and samples were analysed, as previously described (Arenzana-Seisdedos *et al.*, 1993), in a luminometer. Data are expressed in terms of relative luciferase activity units, calculated as (light emission from experimental sample – light emission of lysis buffer alone)/micrograms of cellular protein in the sample.

Assays for MAPK, SAPK, p70^{S6K} and MEK

Extracts of Cos cells transfected with pCDNA-HA-MAPK or pCDNA-HA-SAPK were immunoprecipitated with anti-HA antibody (2 μg/mg of protein extract). For MAPK assay, phosphorylation of 3 μg of myelin basic protein was determined in the following buffer: 35 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM $CaCl_2$, 1 mM phenylphosphate. For SAPK assay, phosphorylation of 3 μg of GST–c-Jun-(5-89) was determined in the following buffer: 20 mM MOPS (pH 7.2), 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1% Triton X-100. The activity of Myc-p70^{S6K} in Cos cells transfected with the corresponding plasmid was determined using 40S ribosomal subunits as substrate (Ming *et al.*, 1994). For MEK activity, extracts of Cos cells transfected with pEBG-MEK1 were purified on glutathione–agarose and, after washing, incubated with 250 ng of recombinant MAPK^{KR} in the MAPK assay buffer.

Gel mobility shift assay

Nuclear extracts were obtained as described previously (Díaz-Meco *et al.*, 1993). Subconfluent cultures of Cos cells in 100 mm plates were transfected by the calcium phosphate method (Gibco, BRL) with 20 μg of either pCDNA-HA, pCDNA-HA-MAPK or pCDNA-HA-MAPK^{MUT} cells. Plasmid DNA was removed 4 h later, and cells were incubated in DMEM containing 10% FCS for 16 h. Afterwards, medium was replaced with medium containing 0.5% FCS for 16 h, followed by an additional 8 h in serum-free medium. Cultures were then either untreated or stimulated with 500 U/ml of TNFα or 10% FCS for 1 h. Cells were lysed for 6 min at 4°C in lysis buffer (Díaz-Meco *et al.*, 1993) and centrifuged at 6500 r.p.m. for 15 min at 4°C, and nuclear extract was prepared from the pellet. For mobility shift assays, 5–7 μg of nuclear protein extract were incubated at 22°C for 15 min with 15 000 c.p.m. of a ^{32}P-labelled double-stranded synthetic oligonucleotide probe corresponding either to the HIV enhancer containing the NF-κB binding site or to the AP-1 site as described previously (Díaz-Meco *et al.*, 1993). The binding reaction was analysed by electrophoresis in non-denaturing 6% polyacrylamide gels. DNA-binding competition was assessed by pre-incubating the extracts with a 40-fold excess of unlabelled oligonucleotide.

Acknowledgements

This work was supported by grants SAF93-0018 and SAF93-1340CE from Comisión Interministerail de Ciencia y Tecnología, PB93-180 from Dirección General de Investigación Científica y Técnica, SC1*-CT92-0769 from the Science Plan of the European Union and 94/1910-E from Fondo de Investigaciones Sanitarias. E.B. is a Fellow from Gobierno Vasco. M.T.D.-M. has a postdoctoral contract from CSIC-Fundación Ramon Areces. L.S. and J.L. are Fellows from Ministerio de Educación. M.M.M. is a Fellow from Glaxo-CSIC. We also want to thank Glaxo Spain for partially funding this work, and Fundación Ramón Areces for an Institutional Grant to the Centro de Biología Molecular (CBM). We are indebted to Jacques Pouyssegur for the HA-MAPK construct. We thank Paul Dent, Tom Sturgill, John Kyriakis, Jim Woodget and George Thomas for reagents and very hepful comments during this work. We also thank Chris Marshall for important and useful suggestions. E.B. and M.T.D.-M. contributed equally to this study.

References

Arenzana-Seisdedos,F., Israel,N., Bachelerie,F., Hazan,U., Alcamí,J., Dautry,F. and Virelizier,J.L. (1989) *Oncogene*, **2**, 1359–1362.

Arenzana-Seisdedos,F., Fernandez,B., Domínguez,I., Jacqué,J.M., Thomas,D., Díaz-Meco,M.T., Moscat,J. and Virelizier,J.L. (1993) *J. Virol.*, **67**, 6596–6604.

Avruch,J., Zhang,X.-f. and Kyriakis,J.M. (1994) *Trends Biochem. Sci.*, **19**, 325–329.

Berra,E., Díaz-Meco,M.T., Domínguez,I., Municio,M.M., Sanz,L., Lozano,L., Chapkin,R.S. and Moscat,J. (1993) *Cell*, **74**, 555–563.

Bjorkoy,G., Overvatn,A., Díaz-Meco,M.T., Moscat,J. and Johansen,T. (1995) *J. Biol. Chem.*, **270**, 21299–21306.

Choi,K.Y., Satterberg,B., Lyons,D.M. and Elion,E.A. (1994) *Cell*, **78**, 499–512.

Chung,J., Grammer,T.C., Lemon,K.P., Kazlauskas,A. and Blenis,J. (1994) *Nature*, **370**, 71–75.

Dérijard,B., Hibi,M., Wu,I.-H., Barret,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) *Cell*, **76**, 1025–1037.

Dérijard,B., Raingeaud,J., Barret,T., Wu,I., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) *Science*, **267**, 682–685.

Devary,Y., Rosette,C., DiDonato,J.A. and Karin,M., (1993) *Science*, **261**, 1442–1445.

Díaz-Meco,M.T. *et al.* (1993) *Mol. Cell. Biol.*, **13**, 4770–4775.
Díaz-Meco,M.T. *et al.* (1994a) *EMBO J.*, **13**, 2842–2848.
Díaz-Meco,M.T., Lozano,J., Municio,M.M., Berra,E., Frutos,S., Sanz,L. and Moscat,J. (1994b) *J. Biol. Chem.*, **269**, 31706–31710.
Dimitris,T. and Maniatis,T. (1995) *Cell*, **62**, 529–532.
Domínguez,I., Díaz-Meco,M.T., Municio,M.M., Berra,E., Garcia de Herreros,A., Cornet,M.E., Sanz,L. and Moscat,J. (1992) *Mol. Cell. Biol.*, **12**, 3776–3783.
Domínguez,I., Sanz,L., Arenzanana,F., Díaz-Meco,M.T., Virelizier,J.-L. and Moscat,J. (1993) *Mol. Cell. Biol.*, **13**, 1290–1295.
Exton,J.H. (1994) *Biochim. Biophys. Acta*, **1212**, 26–42.
Finco,T.S. and Baldwin,A.S. (1993) *J. Biol. Chem.*, **268**, 17676–17679.
Hannun,Y.A. (1994) *J. Biol. Chem.*, **269**, 3125–3128.
Kolesnick,R. and Golde,D.W. (1994) *Cell*, **77**, 325–328.
Kyriakis,J.M., Banerjee,P., Nikolakaki,N., Dai,T., Rubie,E.A., Ahmad,M.F., Avruch,J. and Woodget,J.R. (1994) *Nature*, **369**, 156–160.
Lange-Carter,C.A. and Johnson,G.L. (1994) *Science*, **265**, 1458–1461.
Lange-Carter,C.A., Pleiman,C.M., Gardner,A.M., Blumer,J.J. and Johnson,G.L. (1993) *Science*, **260**, 315–319.
Larrodera,P., Cornet,M.E., Díaz-Meco,M.T., Lopez-Barahona,M., Díaz-Laviada,I., Guddal,P.H., Johansen,T. and Moscat,J. (1990) *Cell*, **61**, 1113–1120.
Lopez-Barahona,M., Kaplan,P.L., Cornet,M.E., Díaz-Meco,M.T., Larrodera,P., Díaz-Laviada,I., Municio,A.M. and Moscat,J. (1990) *J. Biol. Chem.*, **265**, 9022–9026.
Lozano,J., Berra,E., Municio,M.M., Díaz-Meco,M.T., Domínguez,I., Sanz,L. and Moscat,J. (1994) *J. Biol. Chem.*, **269**, 19200–19202.
Minden,A., Lin,A., McMahon,M., Large-Carter,C., Dérijard,B., Davis,R., Johnson,G.L. and Karin,M. (1994) *Science*, **266**, 1719–1723.
Ming,X.-F., Burgering,B.M.T., Wennström,S., Claesson-Welsh,L., Heldin,C-H., Bos,J.L., Kozma,S.C. and Thomas,G. (1994) *Nature*, **371**, 426–429.
Nakanishi,H. and Exton,J.H. (1992) *J. Biol. Chem.*, **267**, 16347–16354.
Nakanishi,H., Brewer,K.A. and Exton,J.H. (1993) *J.Biol.Chem.*, **268**, 13–16.
Ono,Y., Fuji,T., Ogita,K., Kikkawa,U., Igarashi,K. and Nishizuka,Y. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 3099–3103.
Pagès,G., Lenormand,P., L'Allemain,G., Chambard,J.C., Méloche,S. and Pouyssegur,J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8319–8323.
Sacktor,T.C., Osten,P., Valsamis,H., Jiang,X., Naik,M.U. and Sublette,E. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8342–8346.
Sánchez,I., Hughes,R.T., Mayer,B.J., Yee,K., Woodgett,J.R., Avruch,J., Kyriakis,J.M. and Zon,L.I. (1994) *Nature*, **372**, 794–798.
Stein,B., Baldwin,A.S., Ballard,D.W., Greene,W.C., Angel,P. and Herrlich,P. (1993) *EMBO J.*, **12**, 3879–3891.
Vaillancourt,R.R., Gardner,A.M. and Johnson,G.L. (1994) *Mol. Cell. Biol.*, **14**, 6522–6530.
Valius,M. and Kazlauskas,A. (1993) *Cell*, **73**, 321–334.
Ways,D.K., Cook,P.P., Webster,C. and Parker,P.J. (1992) *J. Biol. Chem.*, **267**, 4799–4805.
Ways,D.K. *et al.* (1994) *Cell Growth Differ.*, **5**, 1195–1203.
Wooten,M.W., Zhou,G., Seibenhener,M.L. and Coleman,E.S. (1994) *Cell Growth Differ.*, **5**, 395–403.
Xu,S., Robbins,D., Frost,J., Dang,A., Lange-Carter,C. and Cobb,M.H. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 6808–6812.
Xu,X.-X., Tessner,T.G., Rock,C.O. and Jackowski,S. (1993) *Mol. Cell. Biol.*, **13**,1522–1533.
Yan,M., Dai,T., Deak,J.C., Kyriakis,J.M., Zon,L.I., Woodgett,J.R. and Templeton,D.J. (1994) *Nature*, **376**, 798–800.

Received on July 11, 1995; revised on September 11, 1995