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Transcriptional activation of c-*fos* by oncogenic Ha-Ras in mouse mammary epithelial cells requires the combined activities of PKC-λ, ε and ζ

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The implication of protein kinase C (PKC) isoforms cPKC-α, nPKC-ε, aPKC-λ and aPKC-ζ in the transcriptional activation of a c-*fos* promoter-driven CAT-reporter construct by transforming Ha-Ras has been investigated. This was achieved by employing antisense constructs encoding RNA directed against isoform-specific 5′ sequences of the corresponding mRNA, and expression of PKC mutants representing either kinase-defective, dominant negative, or constitutively active forms of the PKC isoforms. The data indicate that in HC11 mouse mammary epithelial cells, transforming Ha-Ras requires the activities of the three PKC isozymes: aPKC-λ, nPKC-ε and aPKC-ζ, not, however, of cPKC-α, for the transcriptional activation of c-*fos*. Co-expression of oncogenic Ha-Ras with combinations of kinase-defective, dominant negative and constitutively active mutants of the various PKC isozymes are in agreement with a tentative model suggesting that, in the signaling pathway from Ha-Ras to the c-*fos* promoter, aPKC-λ acts upstream whereas aPKC-ζ functions downstream of nPKC-ε.

Keywords: antisense RNA expression/c-*fos*/Ha-Ras/mitogenic signal transduction/protein kinase C isoenzymes

Introduction

It is well-documented that the mitogenic activity of Ha-Ras is protein kinase C (PKC)-dependent (Lacal *et al*., 1987; Wolfman and Macara, 1987; Morris *et al*., 1989; Gauthier-Rouvière *et al*., 1990; Hsiao *et al*., 1990; Beckman, 1992; Überall *et al*., 1994). Evidence for an activation of PKC in Ha-Ras-transformed cells has been presented (Morris *et al*., 1989; Chiarugi *et al*., 1990; Gauthier-Rouvière *et al*., 1990; Überall *et al*., 1994). However, which PKC isoforms are employed by Ha-Ras and what their intracellular targets are, has remained insufficiently understood.

PKC represents a family of structurally related serine/threonine protein kinases presently comprising 11 isotypes. The various PKC isoforms are classified into three major subgroups: the classical or conventional PKC isotypes (cPKCs) are Ca^{2+}- and diacylglycerol (DAG)-dependent and consist of the isozymes cPKC-α, cPKC-β1, cPKC-β2 and cPKC-γ; novel PKCs (nPKCs) are Ca^{2+}-independent but DAG-responsive and comprise the isoforms nPKC-δ, nPKC-ε, nPKC-η and nPKC-θ; the PKC isozymes aPKC-λ/ι and aPKC-ζ require neither Ca^{2+} nor DAG for activation and have been classified as atypical PKC isoforms (aPKCs) (Nishizuka, 1992, 1995; Genot *et al*., 1995). In contrast to c- or n-type PKCs, aPKC isoforms also do not respond to phorbol ester treatment (Stabel, 1994).

The PKC isoforms cPKC-α (Dean *et al*., 1996), cPKC-β2 (Sauma and Friedman, 1996; Sauma *et al*., 1996), nPKC-ε (Perletti *et al*., 1996), aPKC-ζ (Powell *et al*., 1996) and aPKC-λ (Bjorkoy *et al*., 1997) have been correlated to Ha-Ras-mediated signaling or transformation. As not all of these PKC isoforms are expressed in all cellular systems, cell type-specific differences are to be expected. Little is known with regard to the role of PKC in Ha-Ras transformed mouse mammary epithelial cells. Results of studies performed with this system will be presented here. Furthermore, with respect to the function of the various PKC isotypes in Ha-Ras transformed cells, all studies presented so far have focussed on single PKC isozymes. Whether Ha-Ras requires the cooperative activity of several PKC isoforms has not yet been described and is addressed in this paper. As a read-out system for Ha-Ras activity, we employed the Ha-Ras-mediated transcriptional activation of a c-*fos* promoter driven chloramphenicol acetyltransferase (CAT) reporter.

The Raf-1/mitogen-activated protein (MAP) kinase pathway is considered to be a major route for the transmission of signals from Ha-Ras to the c-*fos* promoter (Bos, 1995; Treisman, 1995). The central role of the Raf-1/MAP kinase pathway in Ha-Ras-mediated transformation of fibroblasts is supported by observations indicating that kinase-deficient mutants of Raf-1, MAPK/ERK kinase (MEK) and MAP kinase inhibit Ha-Ras signaling and transformation (Kölch *et al*., 1991; Schaap *et al*., 1993; Khosravi-Far *et al*., 1995; Okazaki and Sagata, 1995; Qiu *et al*., 1995).

An implication of PKC isozymes in the regulation of the Ha-Ras–MAP kinase pathway has been demonstrated in a variety of systems ranging from yeast to higher eukaryotes (Marshall, 1995; Morrison *et al*., 1996; Liao *et al*., 1997). It is well-established that the association of Raf to GTP-charged Ha-Ras is insufficient for Raf-1 activation and that additional phosphorylations on tyrosine and serine/threonine residues are required (Jelinek *et al*., 1996; Zou *et al*., 1996; Cai *et al*., 1997). In mammalian cells, activation of PKC by phorbol ester or bryostatin 1 stimulates Raf phosphorylation and activation. *In vitro*, cPKC-α and nPKC-ε have been shown to activate Raf-1 (Cai *et al*., 1997). In a cell-free system the activation

of MAP kinase by a PKC, Raf, and MEK-dependent mechanism has been demonstrated (Marquardt *et al*., 1994). Evidence for an implication of the aPKC-λ and aPKC-ζ in the regulation of the MAP kinase pathway has also been presented (Berra *et al*., 1995; Bjorkoy *et al*., 1997; Liao *et al*., 1997).

Stimulation of the MAP kinase pathway leads to the phosphorylation of TCF/Elk-1 and SAP-1 and the induction of c-*fos* (Treisman, 1995). We and others had previously demonstrated that transforming Ha-Ras employs a phorbol ester-sensitive PKC for the transcriptional activation of c-*fos* and circumstantial evidence indicated that this phorbol ester-sensitive PKC might be nPKC-ε (Überall *et al*., 1994). An implication of nPKC-ε would be in accordance with the putative function of this isoform in the activation of Raf-1 obtained in *in vitro* studies (Cai *et al*., 1997). The mouse mammary epithelial cells employed in our studies express the phorbol ester-sensitive isotypes cPKC-α, nPKC-ε, and in traces, nPKC-δ (Marte *et al*., 1994; Überall *et al*., 1994). It was, therefore, decided to further identify the phorbol ester-sensitive PKC isozyme which is employed by Ha-Ras for the transcriptional activation of c-*fos*. With regard to published data supporting a role of aPKC-ζ and aPKC-λ in the regulation of the MAP kinase pathway and Ha-Ras-mediated signaling, the implication of these phorbol ester-non-responsive PKC isotypes in transcriptional activation of c-*fos* by transforming Ha-Ras has also been addressed. Both atypical PKC isoforms ζ and λ/ι are expressed in HC11 cells. In view of the remarkable sequence homology of aPKC-ζ and aPKC-λ (Akimoto *et al*., 1994; Diaz-Meco *et al*., 1996), it also appeared to be of interest to determine whether only one or both isoforms are required for the transmission of signals from Ha-Ras to the c-*fos* promoter or whether one can substitute for the other. Furthermore, if Ha-Ras requires a combination of several PKC isozymes for the transcriptional activation of c-*fos*, this raises the question of whether the different isotypes act in separate but cooperating pathways or whether they function in a hierarchically ordered sequence. By employing a combination of mRNA antisense constructs, kinase-defective, dominant negative (DN) and constitutively active (CA) PKC mutants, for the first time evidence is presented that in HC11 cells, transforming Ha-Ras employs nPKC-ε, aPKC-λ and aPKC-ζ for the transcriptional activation of c-*fos*. Furthermore, our data support a tentative model suggesting a hierarchical sequence Ha-Ras–atypical aPKC-λ–novel nPKC-ε and atypical aPKC-ζ.

Results

Depletion of novel nPKC-ε, atypical aPKC-λ and atypical aPKC-ζ by expression of isoform-specific antisense RNA constructs suppresses Ha-Ras-mediated transcriptional activation of c-fos

Selective depletion of PKC isoforms was achieved by transient transfection with vectors encoding antisense RNA directed against 5′-sequences of the corresponding PKC-isotype mRNA. Efficiency and specificity of PKC depletion was determined by selectively collecting the transfected cells. This was performed by cotransfection with a vector encoding a truncated form of the CD4 surface marker. CD4-positive cells were separated by the magnetic cell separation (MAC)-select technique employing magnetic columns and CD4-specific magnetic microbeads. PKC isozyme protein levels were determined by Western blotting. Expression of the targeted antisense constructs resulted in a significant and selective depletion of the corresponding PKC isozyme protein (Figure 1A) but did not affect the expression levels of other PKC isozymes (data not shown).

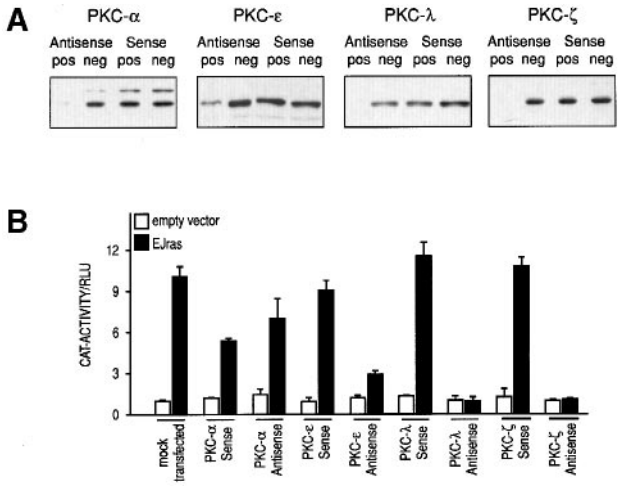

Fig. 1. Depletion of novel nPKC-ε, atypical aPKC- ζ and atypical aPKC-λ by expression of isoform-specific antisense RNA constructs suppresses Ha-Ras-mediated transcriptional activation of c-*fos*. (**A**) Isoenzyme-specific depletion of cPKC-α, nPKC-ε, aPKC- ζ and aPKC-λ. HC11 cells were cotransfected with 4 μg pMACS4 (truncated CD4 surface marker) and 8 μg of either one of the PKC isoform-specific antisense or sense constructs per 6-well plate, respectively. Thirty-six h post-transfection, CD4-positive cells (transfected cells) were separated from the negative cells (non-transfected cells) as described in Materials and methods. Total cellular extracts (corresponding to ~1.5 mg/ml protein) from both fractions were prepared, and Western blot analysis of cPKC-α, nPKC-ε and aPKC- ζ for each sample was performed as described in Materials and methods. (**B**) Suppression of Ha-Ras mediated c-*fos* induction by cPKC-α, cPKC-ε, aPKC- ζ and aPKC-λ antisense constructs. HC11 cells were transfected with 2 μg pEJ-Ha-Ras, the corresponding vector control pOPI3-RSV, 2 μg pcfos-DSE-FAP-tk-CAT, 2 μg pAG-Luc and 6 μg PKC isoform specific antisense or sense constructs as indicated per 6-well plate. Forty-eight h post-transfection, cells were harvested and CAT expression was determined as described in Materials and methods. Data are expressed as the means (± SE, $n = 9$) of at least three independent experiments done in triplicate.

After the efficacy and specificity of the antisense constructs had been established, it was investigated which antisense RNA would interfere with the Ha-Ras-mediated transcriptional activation of c-*fos*. Expression of nPKC-ε as well as aPKC-ζ and aPKC-λ antisense RNA caused a dramatic repression of the Ha-Ras-mediated transcriptional activation of the c-*fos*–CAT reporter construct (Figure 1B). Compared with aPKC-ζ and aPKC-λ antisense, which caused a complete suppression of Ras-mediated transcriptional activation of c-*fos*, nPKC-ε antisense proved to be slightly less effective. This may be explained by the observation that in contrast to aPKC-ζ/λ antisense, the antisense directed against nPKC-ε achieved only an incomplete depletion of the corresponding enzyme (Figure 1A). A marginal inhibition was also observed in cells expressing the cPKC-α antisense construct. However, in cells expressing the corresponding cPKC-α sense construct the reduction in Ras-mediated c-*fos* induction was even more pronounced. This is in marked contrast to the

behaviour of the nPKC-ε and aPKC-ζ/λ sense controls which did not interfere with the transcriptional activation of c-*fos* by Ras (Figure 1B). It appeared conceivable, therefore, that the effects observed after expression of the cPKC-α sense and antisense constructs are due to non-specific, toxic side effects. In order to confirm the conclusion that the Ras-mediated transcriptional activation of the c-*fos*–CAT construct requires the PKC isotypes ζ/λ and ε, but probably not α, it was decided to employ kinase-defective PKC mutants which had been shown to act in a transdominant fashion (Baier-Bitterlich *et al.*, 1996; Überall *et al.*, 1997). In order to exclude non-specific effects of the PKC antisense constructs we determined the effect of the antisense vectors on the transcriptional activation of a thymidine kinase (tk)-CAT, as well as a cytomegalovirus (CMV)-driven luciferase reporter. Neither the tk-CAT nor the corresponding CMV–luciferase reporter was found to be affected (data not shown).

Expression of kinase-defective, dominant negative mutants of PKC isoform nPKC-ε K436R, atypical aPKC-ζ K275W and aPKC-λ K275W, but not of cPKC-α K368R depress the transcriptional activation of c-fos by Ha-Ras

Replacement of the critical lysine at the ATP-binding site by an arginine or tryptophan has been shown to result in kinase-defective PKC mutants which compete with the endogenous wild-type enzymes and act as isoform-selective DN inhibitors (Genot *et al*., 1995; Baier-Bitterlich *et al*., 1996; Überall *et al*., 1997). The biochemical and biological properties of (DN)cPKC-α K368R, (DN)nPKC-ε K436R and (DN)aPKC-λ K275W have been described previously (Überall *et al.*, 1997). The expression of the DN PKC mutants in transiently transfected HC11 cells was determined by cotransfection with a truncated CD4 surface marker and separation of CD4-expressing cells by the MAC-select technique described above. Transfection by all vectors encoding DN mutants of PKC isoforms α, ε, ζ and λ led to a significant overexpression of the corresponding mutant isotype (Figure 2A).

In accordance with the data obtained with the PKC antisense constructs, Ras-induced transcriptional activation of c-*fos* was suppressed in cells expressing kinase-deficient, dominant negative mutants of nPKC-ε K436R, aPKC-λ K275W and aPKC-ζ K275W. Expression of the kinase-defective, dominant negative mutant of cPKC-α K368R did not interfere with Ras-mediated c-*fos* induction, supporting our supposition that the inhibitions observed in cells expressing cPKC-α sense or antisense RNA were due to non-specific side effects.

The data presented in Figures 1 and 2 suggest that in HC11 cells transforming Ha-Ras employs PKC isotypes ε, λ and ζ , but not α, for the transcriptional activation of the c-*fos*–CAT reporter construct. This conclusion was further substantiated by using constitutively active PKC mutants.

Expression of constitutively active novel nPKC-ε A159E, atypical aPKC-ζ A119E and aPKC-λ A119E, but not cPKC-α A25E results in a Ras-independent transcriptional activation of c-fos

It has previously been demonstrated that substitution of an alanine by a glutamate within the pseudosubstrate domain of PKC generates constitutively active mutants with reduced cofactor requirements. Biochemical and biological properties of these mutants have been described in preceding publications (Baier-Bitterlich *et al.*, 1996; Überall *et al.*, 1997). The expression in HC11 cells of the various mutants following transient transfection with constructs encoding the different mutated PKC isozymes is illustrated in Figure 3A.

Figure 3B demonstrates that expression of the CA forms of the PKC isotypes ζ, ε and λ leads to a significant transcriptional activation of the cotransfected c-*fos*–CAT reporter construct, whereas constitutively active cPKC-α is obviously unable to induce c-*fos* in this system. In agreement with the data reported in the previous sections, the results obtained with the constitutively active PKC isoforms demonstrate that in HC11 cells, PKC isotypes ε, λ and ζ are implicated in signal transmission to the c-*fos* promoter.

Evidence that signaling from Ha-Ras to the c-fos promoter requires atypical aPKC-λ, novel PKC-ε and atypical aPKC-ζ in a hierarchically ordered sequence

The data presented so far indicate that Ha-Ras requires novel nPKC-ε, together with the two atypical isoforms λ and ζ for transcriptional activation of the c-*fos* promoter. In order to obtain further information on whether the different PKC isozymes act in separate, but cooperating pathways or whether they act in a sequential order, cells were cotransfected with combinations of kinase-defective, DN and CA PKC mutants.

The inhibition of the Ha-Ras-mediated transcriptional activation of c-*fos* by the kinase-defective, (DN)nPKC-ε K436R mutant can be overcome by (CA)aPKC-ζ A119E mutant, but not by the (CA)aPKC-λ A119E mutant, suggesting that aPKC-λ acts upstream and aPKC-ζ downstream of nPKC-ε (Figure 4A).

If the conclusion that transforming Ras transmits signals to the c-*fos* promoter through a pathway containing the PKC isoforms λ, ε and ζ in this sequential order is correct, then CA isoforms of both PKC-ε and ζ should be able to overcome a blockade of Ras-mediated c-*fos* induction exerted by (DN)aPKC-λ K275W mutant. As demonstrated in Figure 4B, this is indeed the case.

Furthermore, if PKC-ζ acts downstream of both aPKC-λ and nPKC-ε, neither (CA)nPKC-ε nor (CA)aPKC-λ should be capable of overcoming a blockade on the level of aPKC-ζ. In the absence of transforming Ras this is indeed to be seen (Figure 4C, open bars). The transcriptional activation of c-*fos* by (CA)aPKC-λ or dPKC-ε demonstrated in Figure 3B is abrogated in the presence of (DN)aPKC-ζ K275W. If transforming Ras is expressed (Figure 4C, closed bars), (DN)aPKC-ζ still blocks the activity of (CA)nPKC-ε; however, the blockade by (DN)aPKC-ζ is antagonized by (CA)aPKC-λ (Figure 4C).

This could be interpreted to indicate that in cells expressing the (CA)aPKC-λ A119E mutant, Ras may be able to induce c-*fos* by a separate, independent mechanism. However, co-expression of the (DN)nPKC-ε K436R mutant completely blocks Ras-mediated c-*fos* induction in these cells expressing (CA)aPKC-λ A119E and (DN)aPKC-ζ K275W (Figure 5A). Thus, transforming

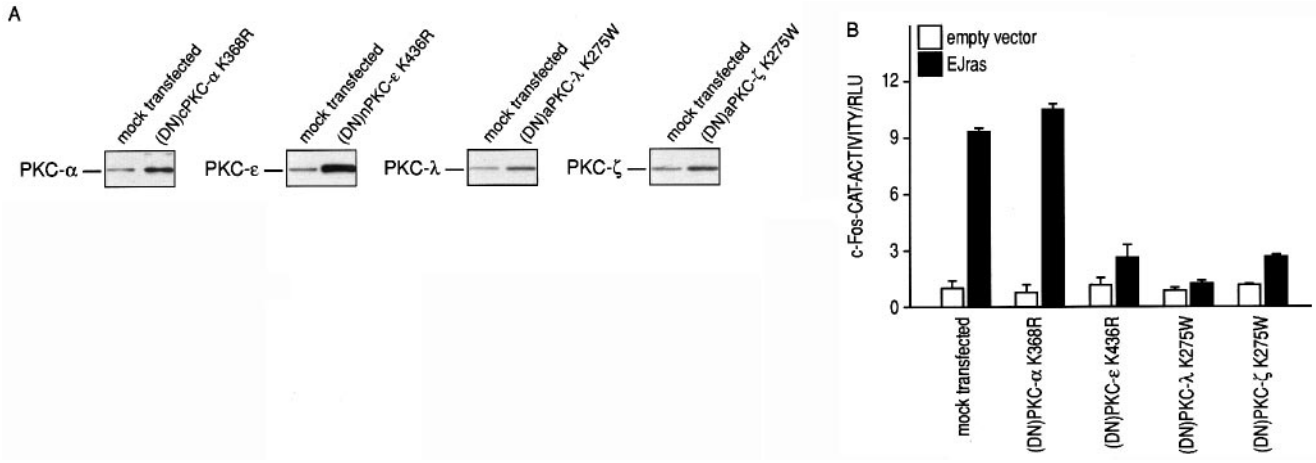

Fig. 2. Expression of kinase-defective DN mutants of PKC isoforms nPKC-ε K436R, atypical aPKC-ζ K275W and aPKC-λ K275W, but not of cPKC-α K368R depress the transcriptional activation of c-*fos* by Ha-Ras. (**A**) Expression pattern of PKC isoenzymes in cells transfected with plasmids encoding kinase-defective, DN PKC mutants. HC11 cells were cotransfected with 4 μg pMACS4 and 8 μg of one of the PKC isoform-specific DN constructs per 6-well plate, respectively. Thirty-six h post-transfection, CD4-positive cells were separated from the negative cells and analyzed by Western blotting as described in Materials and methods. (**B**) Inhibition of Ha-Ras mediated c-*fos* induction by kinase-defective PKC mutants. HC11 cells were transfected with 2 μg pEJ-Ha-Ras, the corresponding vector control pOPI3-RSV, 2 μg pc-fos-DSE-FAP-tk-CAT, 2 μg pAG-Luc and 6 μg of kinase-defective, (DN)cPKC-α K368R, (DN)nPKC-ε K436R, (DN)aPKC-λ K275W and (DN)aPKC-ζ K275W expression vectors per 6-well plate. Forty-eight h post-transfection, cells were harvested and CAT expression was determined as described in Materials and methods. Data are expressed as the means (± SE, $n = 9$) of at least three independent experiments done in triplicate.

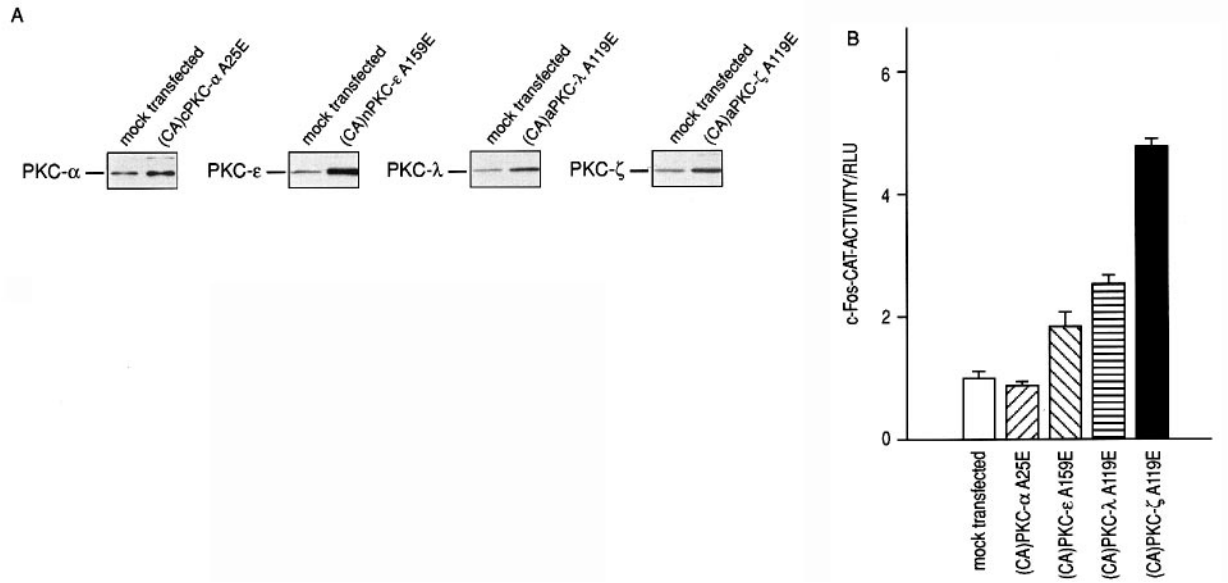

Fig. 3. Expression of CA novel nPKC-ε A159E, atypical aPKC-ζ A119E and aPKC-λ A119E, but not of conventional cPKC-α A25E results in a transcriptional activation of c-*fos* independent of oncogenic Ras. (**A**) Expression pattern of PKC isoenzymes in cells transfected with constructs encoding CA PKC mutants. HC11 cells were cotransfected with 4 μg pMACS4 and 8 μg of one of the PKC isoform-specific (CA) constructs per 6-well plate, respectively. Thirty-six h post-transfection, CD4 positive cells were separated from the negative cells and analyzed by Western blotting as described in Materials and methods. (**B**) Transcriptional activation of c-*fos* by CA PKC mutants. HC11 cells were transfected with the corresponding vector control pEF-neo (mock), 3 μg pcfos-DSE-FAP-tk-CAT, 3 μg pAG-Luc and 6 μg of PKC (CA)cPKC-α A25E, (CA)nPKC-ε A159E, (CA)aPKC-λ A119E and (CA)aPKC-ζ A119E expression vector per 6-well plate. Forty-eight h post-transfection, cells were harvested and CAT expression was determined as described in Materials and methods. Data are expressed as the means (± SE, $n = 9$) of at least three independent experiments done in triplicate.

Ras transmits signals to the c-*fos* promoter via nPKC-ε which apparently acts downstream of aPKC-λ. The conclusion previously reached that aPKC-λ operates upstream and aPKC-ζ downstream of nPKC-ε was independently confirmed by studies demonstrating that Ras-mediated c-*fos* induction in cells expressing the (CA)nPKC-ε A159E mutant was not affected by the kinase-defective, (DN)aPKC-λ K275W isoform, but was abrogated by the kinase-defective, (DN)aPKC-ζ K275W mutant (Figure 5B).

However, if aPKC-ζ acts downstream of aPKC-λ and nPKC-ε, why does a DN mutant of aPKC-ζ not inhibit Ras-mediated c-*fos* induction in cells expressing the CA mutant of PKC-λ? In view of the marked homology between the two atypical PKC isoforms (Akimoto *et al*., 1994) it appeared conceivable that the expressed CA

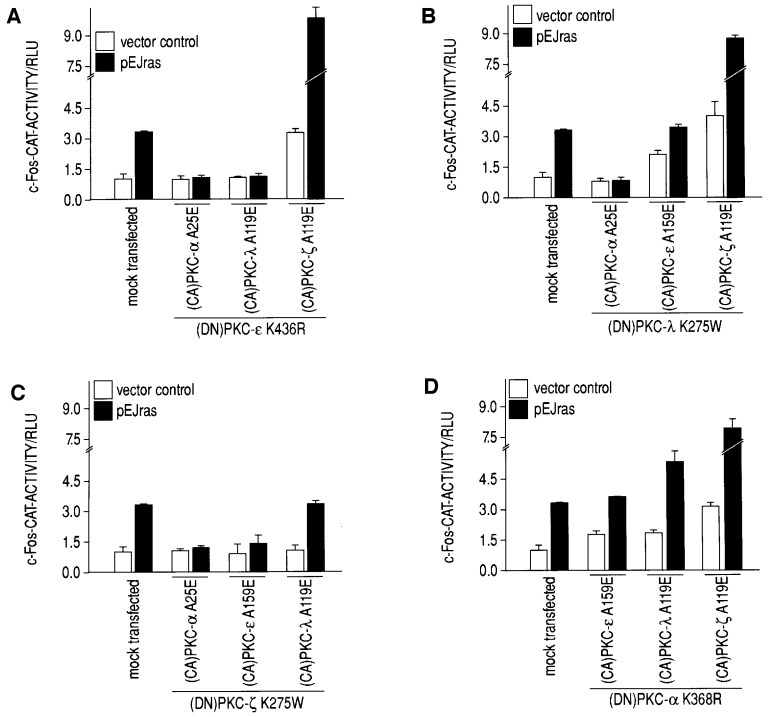

Fig. 4. Transcriptional activation of c-*fos* mediated by oncogenic Ras in HC11 cells requires the combined activities of aPKC-λ, nPKC-ε, and aPKC-ζ. (**A**) Blockade of Ha-Ras mediated transcriptional activation of c-*fos*–CAT by a kinase-defective, (DN)nPKC-ε K436R mutant can be overcome by (CA)aPKC-ζ not, however, by (CA)aPKC-λ or (CA)cPKC-α. Shown are co-expressions of kinase-defective, (DN)nPKC-ε K436R with (CA)cPKC-α A25, (CA)aPKC-λ A119E or (CA)aPKC-ζ A119E in the absence (open bars) or presence (closed bars) of transforming Ha-Ras. HC11 cells growing in 6-well plates were cotransfected with the corresponding vector control pEF-neo, 1 μg pEJ-Ha-Ras, 1 μg pcfos-DSE-FAP-tk-CAT, 1 μg pAG-Luc, and 4.5 μg (DN)nPKC-ε K436R plus (CA)nPKC-α A25E, (CA)cPKC-λ A119E or (CA)aPKC-ζ A119E, respectively. Forty-eight h post-transfection, cells were harvested and CAT expression was determined as described elsewhere (Überall *et al*., 1994). (**B**) CA isoforms of both nPKC-ε and aPKC-ζ are able to overcome a blockade of Ras-mediated c-*fos* induction exerted by kinase-defective, (DN)aPKC-λ K275W. Demonstrated are the results obtained after co-expression of kinase-defective, (DN)aPKC-λ K275W with (CA)cPKC-α A25E, (CA)nPKC-ε A159E or (CA)aPKC-ζ A119E in the absence (open bars) or presence (closed bars) of transforming Ha-Ras. Transfection was performed as described for Figure 4A employing equivalent concentrations of vector DNA. (**C**) The transcriptional activation of c-*fos* by (CA)aPKC-λ or (CA)nPKC-ε demonstrated in Figure 3B is abrogated in the presence of (DN)aPKC-ζ K275W. If transforming Ras is expressed (closed bars), (DN)aPKC-ζ still blocks the activity of (CA)nPKC-ε, however, the blockade by (DN)aPKC-ζ is antagonized by (CA)aPKC-λ. Shown are co-expression studies of kinase-defective, (DN)aPKC-ζ K275W with (CA)cPKC-α A25E, (CA)nPKC-ε A159E or (CA)aPKC-λ A119E in the absence (open bars) or presence (closed bars) of transforming Ha-Ras. Transfection was performed as described for Figure 4A employing equivalent concentrations of vector DNA. (**D**) Expression of kinase-defective, (DN)cPKC-α K368R does not affect the transcriptional activation of c-*fos*. Shown are co-expression studies of kinase-defective, (DN)cPKC-α K368R with (CA)nPKC-ε A159E, (CA)aPKC-λ A119E, or (CA)aPKC-ζ A119E in the absence (open bars) or presence (closed bars) of transforming Ha-Ras. Transfection was performed as described for Figure 4A employing equivalent concentrations of vector DNA. Data in Figure 4A–D are expressed as the means (± SE, $n = 9$) of at least three independent experiments done in triplicate.

PKC-λ may interfere with the inhibitory activity of DN PKC ζ resulting in an incomplete inhibition of the endogenous aPKC-ζ.

In order to test this hypothesis, endogenous aPKC-ζ was depleted by the antisense technique outlined before. Under these conditions, and in accordance with the model which places aPKC-λ upstream of aPKC-ζ, a significant, albeit incomplete, inhibition of Ras-mediated c-*fos* induction by depletion of aPKC-ζ can be registered even in presence of the (CA)aPKC-λ A119E mutant (Figure 6A). In these cells, Ras-mediated c-*fos* induction is completely abrogated by the additional expression of the kinase-defective, (DN)nPKC-ε K436R mutant, confirming the assumption that aPKC-λ acts upstream of both nPKC-ε and aPKC-ζ during signal transmission from Ha-Ras to the c-*fos* promoter.

Finally, additional support for the conclusion that aPKC-ζ acts downstream of nPKC-ε in this system was provided by depleting aPKC-ζ in cells expressing the (CA)nPKC-ε A159E. Expression of the (CA)nPKC-ε A159E mutant results in a transcriptional activation of the c-*fos*–CAT reporter construct which is further stimulated by co-expression of transforming Ha-Ras (Figure 6B). Antisense-mediated depletion of aPKC-ζ suppresses c-*fos* induction by the (CA)nPKC-ε A159E mutant in the absence as well as in the presence of transforming Ha-Ras.

Neither the kinase-defective, DN, nor the CA mutant of cPKC-α interfered with the effects of any of the other PKC isoforms investigated, confirming that the PKC-α isozyme is not involved in Ras-mediated c-*fos* induction in HC11 cells (Figure 4A–D).

Implication of the PKC isotypes in the Raf-1–ERK pathway

In order to investigate whether the effects of the various PKC isoforms on c-*fos* promoter activity are mediated through the Raf-1–extracellular signal regulated kinase (ERK)1,2 pathway, it was determined whether the kinase-defective PKC mutants are able to interfere with the transcriptional activation of c-*fos* by constitutively active

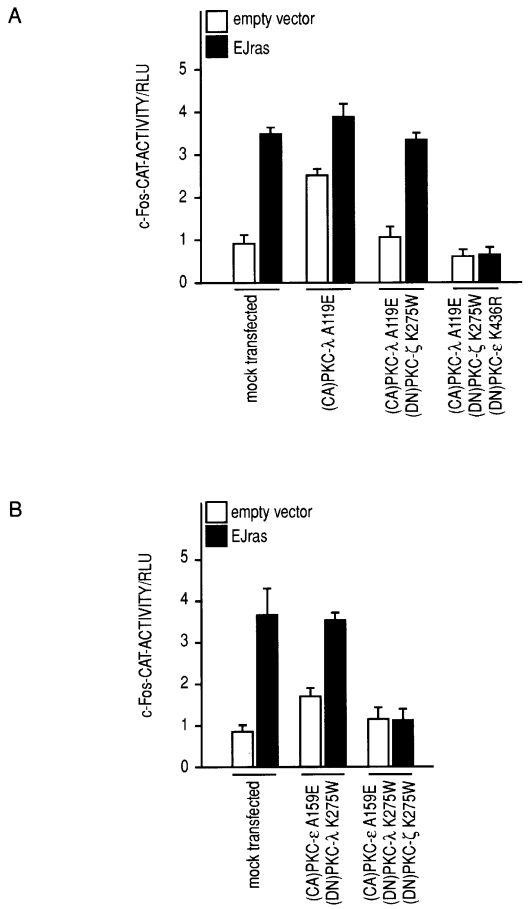

Fig. 5. (**A**) Kinase-defective (DN)nPKC-ε blocks the transcriptional activation of c-*fos* in cells expressing (CA)aPKC-λ and (DN)aPKC-ζ in the absence (open bars) or the presence (closed bars) of transforming Ha-Ras. HC11 cells growing in 6-well plates were cotransfected with the corresponding vector control pEF-neo, 1 μg pEJ-Ha-Ras, 1 μg pcfos-DSE-FAP-tk-CAT, 1 μg pAG-Luc, 4.5 μg (CA)aPKC-λ A119E, plus (DN)aPKC-ζ K275W, or in combination 4.5 μg (CA)aPKC-λ A119E, (DN)aPKC-ζ K275W and (DN)nPKC-ε K436R as described. (**B**) Kinase-defective (DN)aPKC-ζ blocks the transcriptional activation of c-*fos* in cells expressing (CA)nPKC-ε and (DN)aPKC-λ in the absence (open bars) or the presence (closed bars) of transforming Ha-Ras. Cotransfection with the corresponding vector control pEF-neo, 1 μg pEJ-Ras, 1 μg pcfos-DSE-FAP-tk-CAT, 1 μg pAG-Luc, 4.5 μg (CA)nPKC-ε A159E, plus (DN)aPKC-λ K275W or in combination 4.5 μg (CA)nPKC-ε A159E plus 4.5 μg (DN)aPKC-λ K275W and (DN)aPKC-ζ K275W, respectively. Forty-eight h post-transfection, cells were harvested and CAT expression was determined as described elsewhere (Überall *et al*., 1994). Data are expressed as the means (± SE, $n = 9$) of at least three independent experiments done in triplicate.

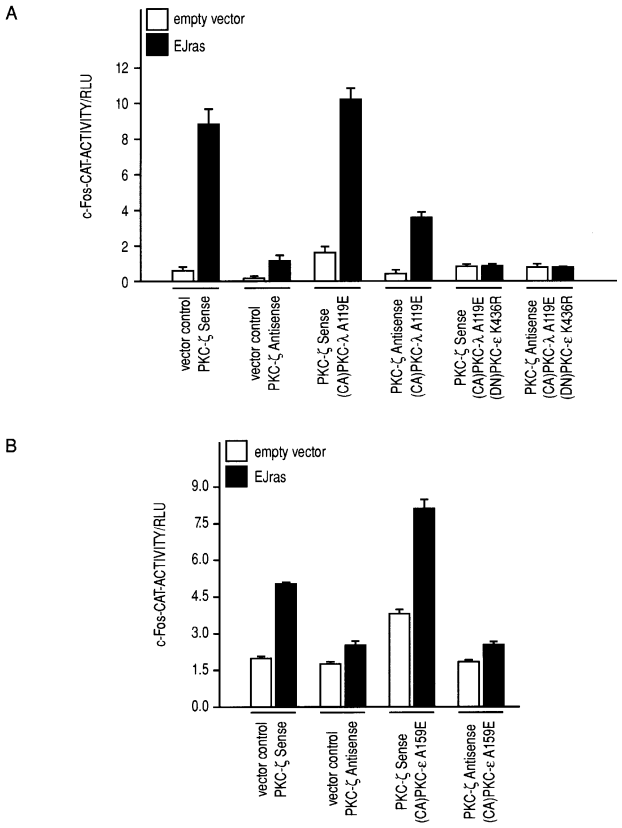

Fig. 6. Antisense mediated depletion of aPKC-ζ suppresses transcriptional activation of c-*fos* by (CA)aPKC-λ (A) or (CA)nPKC-ε (B) in the absence (open bars) or presence (closed bars) of transforming Ha-Ras. (**A**) HC11 cells growing in 6-well plates were cotransfected with the corresponding vector control pEF-neo, 1 μg pEJ-Ras, 1 μg pcfos-DSE-FAP-tk-CAT, 1 μg pAG-Luc, 4.5 μg (CA)aPKC-λ A119E, plus 3 μg antisense/sense PKC-ζ or in combination 4.5 μg (DN)nPKC-ε K436R, (CA)aPKC-λ A119E, (DN)aPKC-ζ K275W and (DN)nPKC-ε K436R as described in Materials and methods. (**B**) Cotransfection with the corresponding vector control pEF-neo, 1 μg pEJ-Ha-Ras, 1 μg pcfos-DSE-FAP-tk-CAT, 1 μg pAG-Luc, 4.5 μg (CA)nPKC-ε A159E, plus 3 μg aPKC-ζ antisense/sense vector, respectively. Forty-eight h post-transfection, cells were harvested and CAT expression was determined as described elsewhere (Überall *et al*., 1994). Data are expressed as the means (± SE, $n = 9$) of at least three independent experiments done in triplicate.

Raf-1 or MEK-1. (DN)aPKC-ζ K275W strongly depresses c-*fos* activation by Raf-BxB or (CA)MEK-1, whereas the (DN)aPKC-λ K275W mutant does not affect the transcriptional activation of c-*fos* by Raf-BxB or (CA)MEK-1 (Figure 7). These data indicate that aPKC-ζ acts downstream of Raf-1 and MEK-1, whereas aPKC-λ acts either upstream or independently of both Raf-1 and MEK-1. According to the data described above, atypical aPKC-ζ acts downstream of nPKC-ε and aPKC-λ, these findings indicate that the PKC effects are finally mediated to the fos promoter via the ERK pathway. The kinase-defective (DN)nPKC-ε K436R mutant also inhibits c-*fos* activation by Raf-BxB or MEK-1. However, in contrast to (DN)aPKC-ζ K275W, only a partial inhibition could be observed.

Discussion

HC11 mouse mammary epithelial cells express the PKC isotypes cPKC-α, nPKC-ε, traces of nPKC-δ, aPKC-ζ and aPKC-λ.

The data presented here demonstrate that transforming Ha-Ras employs nPKC-ε, aPKC-λ and aPKC-ζ for the transcriptional activation of c-*fos*. This conclusion is based on the observation that (i) depletion of nPKC-ε, aPKC-λ or aPKC-ζ by isotype-specific antisense constructs inhibits Ha-Ras-mediated c-*fos* induction. (ii) The effects of the antisense constructs are confirmed by expression of kinase-defective, (DN)nPKC-ε K436R, (DN)aPKC-λ K275W and (DN)aPKC-ζ K275W. (iii) Finally, the conclusions based on antisense techniques and dominant negative mutants were confirmed by

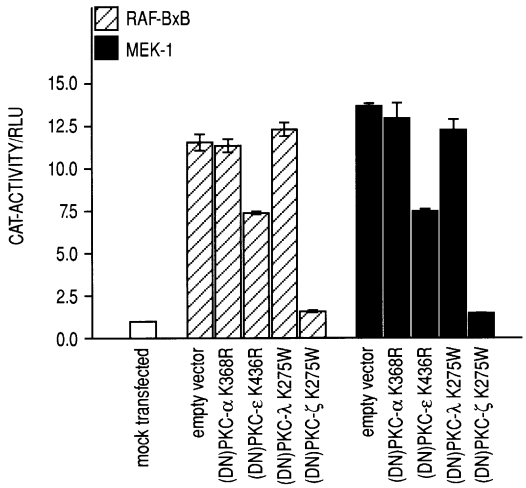

Fig. 7. Transcriptional activation of c-*fos* mediated by constitutively active Raf-BxB and MEK-1 is blocked by kinase-defective (DN)nPKC-ε and (DN)aPKC-ζ, but not (DN)aPKC-λ and (DN)cPKC-α. HC11 cells growing in 6-well plates were cotransfected with 2 μg pRaf-BxB or pFA-MEK1, the corresponding vector control pEF-neo, 2 μg pc-fos-DSE-FAP-tk-CAT, 2 μg pAG-Luc, and 6 μg of kinase-defective, (DN)cPKC-α K368R, (DN)nPKC-ε K436R, (DN)aPKC-λ K275W and (DN)aPKC-ζ K275W expression vectors per 6-well plate. Forty-eight h post-transfection, cells were harvested and CAT expression was determined as described in Materials and methods. Data are expressed as the means (± SE, $n = 9$) of at least three independent experiments done in triplicate.

employing constitutively active forms of the isotypes. These studies revealed that expression of (CA)nPKC-ε A159E, (CA)aPKC-λ A119E and (CA)aPKC-ζ A119E leads to a transcriptional activation of c-*fos* in the absence of transforming Ha-Ras.

Although, expression of cPKC-α antisense RNA caused a relatively small but significant inhibition of Ha-Ras-mediated c-*fos*-induction, an implication of cPKC-α is considered unlikely. Expression of the cPKC-α sense sequence had a similar effect as expression of cPKC-α antisense, in contrast to nPKC-ε and aPKC-ζ sense constructs which proved to behave as inert. Furthermore, expression of the kinase-deficient (DN)cPKC-α K368R did not interfere with Ha-Ras-mediated c-*fos* induction and (CA)cPKC-α A25E proved to be unable to enhance the transcriptional activation of c-*fos*. The conclusion that cPKC-α is not involved in the Ha-Ras-mediated induction of c-*fos* is also in agreement with previous studies from our laboratory in which less specific techniques for the differential depletion of cPKC-α and nPKC-ε had been employed and which mutants interfered with the transcriptional activation of a tk-CAT reporter, revealed that Ha-Ras employs nPKC-ε, but not cPKC-α for the transcriptional activation of c-*fos* in HC11 cells (Überall *et al*., 1994).

Neither PKC depletion by antisense nor expression of any of the employed PKC mutants interfered with the transcriptional activation of a tk-CAT, excluding non-specific effects on general promoter activities (data not shown).

Transformation of colonic epithelial cells by Ha-Ras has been reported to result in an overexpression of nPKC-ε (Perletti *et al*., 1996). In non-transformed colonic epithelial cells, overexpression of nPKC-ε has been shown to lead to increased saturation densities and anchorage-independent colony formation in semisolid agar, suggesting a causal relation between Ha-Ras-mediated transformation and nPKC-ε overexpression (Perletti *et al*., 1996). Although in HC11 cells oncogenic Ha-Ras does not lead to an overexpression of nPKC-ε (Kampfer, 1996), the finding that the Ha-Ras-mediated transcriptional activation of c-*fos* absolutely depends on activated nPKC-ε, underscores the essential role of this PKC isoform for the transformation by this oncogene.

A kinase-defective, dominant negative mutant of aPKC-λ has recently been shown to reverse v-Ras-mediated transformation of NIH 3T3 cells and to interfere with the activation of nuclear ERKs in v-Ras transformed cells (Bjorkoy *et al*., 1997). These findings are in accordance with our data which demonstrate that the Ha-Ras-mediated transcriptional activation of c-*fos* is depressed by kinase-defective, (DN)aPKC-λ and that a (CA)aPKC-λ enhances c-*fos* expression independent of Ha-Ras.

The requirement of aPKC-ζ was clearly demonstrated by the antisense probe directed to an aPKC-ζ mRNA sequence which shares no homology with aPKC-λ. In view of the sequence homology between aPKC-λ and aPKC-ζ it may be questioned whether the studies conclusively demonstrate that aPKC-λ is required in addition to aPKC-ζ. However, the following points support the notion that indeed both atypical PKC isoforms are necessary for the Ha-Ras-mediated induction of c-*fos*. (i) The inhibition caused by expression of kinase-defective (DN)aPKC-ε could be overcome by a (CA)aPKC-ζ, but not by a (CA)aPKC-λ. (ii) Depletion of aPKC-ζ by aPKC-ζ-specific antisense abrogates Ha-Ras-mediated c-*fos* induction. Expression of (CA)aPKC-λ in aPKC-ζ depleted cells does not compensate the depletion of aPKC-ζ.

In order to obtain further information on how the PKC isozymes employed by oncogenic Ha-Ras cooperate, cells were cotransfected with combinations of constructs encoding CA and DN mutants of the various PKC isotypes. These studies suggested that aPKC-λ acts upstream whereas aPKC-ζ functions downstream of nPKC-ε. This assumption is based on the observation that the blockade by kinase-defective, (DN)nPKC-ε K436R can be overcome by (CA)aPKC-ζ A119E not, however, by (CA)aPKC-λ A119E. In accordance with the suggested sequence aPKC-λ–nPKC-ε–aPKC-ζ, the depression by kinase-defective (DN)aPKC-λ K275W could be overcome by the (CA)nPKC-ε A159E and aPKC-ζ A119E and, as should be expected if the postulated sequence is correct, (CA)nPKC-ε A159E was not able to counteract the inhibitory effect of (DN)aPKC-ζ K275W. Surprisingly, (CA)aPKC-λ A119E restored the responsiveness to Ha-Ras in cells expressing the kinase-deficient (DN)aPKC-ζ K275W which is hard to reconcile with a model in which aPKC-ζ is downstream of aPKC-λ. In view of the remarkable sequence homology between aPKC-λ and aPKC-ζ which approaches ~90% in the catalytic domains of the enzymes (Akimoto *et al*., 1994; Diaz-Meco *et al*., 1996) it appeared possible that the inhibitory effect of the (DN)aPKC-ζ K275W is reduced by the co-expressed (CA)aPKC-λ A119E mutant. In order to test this possibility, aPKC-ζ was depleted by the PKC-ζ-specific antisense construct which, as shown before, abrogates Ha-Ras-mediated c-*fos* induction. If the (CA)aPKC-λ A119E mutant is expressed in aPKC-ζ depleted cells, Ha-Ras-induced transcriptional activation of c-*fos* remains reduced in accordance with aPKC-ζ acting downstream

of aPKC-λ. Nevertheless, in aPKC-ζ depleted cells expressing (CA)aPKC-λ A119E, Ha-Ras is still able to cause a small but significant activation of c-*fos* expression. With regard to the extensive sequence homology between aPKC-λ and aPKC-ζ this may indicate that the (CA)aPKC-λ A119E mutant could act as a 'poor aPKC-ζ', i.e. phosphorylates the same substrates at a reduced rate. Alternatively, (CA)aPKC-λ A119E may be able to fully compensate the function of aPKC-ζ and the reduced activation observed in presence of (CA)aPKC-λ A119E could be due to lower expression levels of (CA)aPKC-λ A119E compared to (CA)aPKC-ζ A119E. However, no significant difference of the expression levels between the aPKC-λ- and ζ-encoding vectors could be detected.

Thus, the data reported here are in agreement with a model in which aPKC-λ, nPKC-ε and aPKC-ζ function in this sequential order. As the substrates of the PKC isoforms are not yet known, this model does, of course, not imply a direct interaction of these protein kinases. At this point it should be emphasized that results obtained by co-expression of PKC mutants have to be interpreted with extreme caution. This applies especially to the (CA)A/E mutants. Although *in vitro*, these mutants exhibit cofactor-independent activity (Baier-Bitterlich *et al*., 1996; Überall *et al*., 1997), they may intracellularly still be subject to additional activation giving rise to different *in vivo* activities. The kinase-defective DN mutants, although expressed at similar levels, are confronted with different concentrations of active endogenous enzyme which may affect their dominant inhibitory activity.

However, even if the proposed model for the location of the different PKC isozymes within the signaling cascade from Ha-Ras to the c-*fos* promoter is correct, additional pathways for the activation of nPKC-ε and aPKC-ζ by Ha-Ras have to be postulated. This conclusion refers to the observation that (CA)nPKC-ε A159E not only permits transcriptional activation of c-*fos* in the presence of (DN)aPKC-λ K275W, but that co-expression of transforming Ha-Ras leads to an additional enhancement of c-*fos* induction inspite of the block further upstream. In accordance with the model, (CA)aPKC-ζ A119E can overcome the inhibitory effect of both (DN)aPKC-λ K275W and (DN)nPKC-ε K436R.

However, co-expression of Ha-Ras caused a 'super-induction' of c-*fos* even in the presence of either (DN)aPKC-λ K275W or (DN)nPKC-ε K436R. At present the biochemical nature of these additional pathways are unknown, but several independent mechanisms for the activation of both nPKC-ε and aPKC-ζ have been described (Moriya *et al*., 1996), e.g. aPKC-ζ may be activated by a PI3 kinase-dependent pathway or by direct association with Ha-Ras (Diaz-Meco *et al*., 1994; Mizukami *et al*., 1997); nPKC-ε can be activated by a Rac-dependent mechanisms via phospholipase A_2 (PLA_2) or by diacylglycerol (Koide *et al*., 1992; Ha and Exton, 1993; Kim and Kim, 1997; Kim *et al*., 1997).

It should be emphasized, however, that these additional, Ras-activated pathways do not circumvent the proposed hierarchical sequence of PKC isoforms, otherwise it should not have been possible to obtain a complete block of Ras-mediated transcriptional activation of c-*fos* by inhibiting or depleting only one of the three implicated PKC isoforms, regardless of which isotype was affected.

The precise biological function of the PKC isozymes employed by Ha-Ras for the transcriptional activation of c-*fos* remains to be elucidated and is at present topic for further investigations.

Initial results revealed that the effects of the various PKC isotypes are, at least in part, mediated through the Raf-1/ERK pathway. As outlined above, aPKC-ζ acts downstream of the nPKC-ε and aPKC-λ isotypes. Expression of the kinase-defective, dominant negative aPKC-ζ K/W mutant abrogates the transcriptional activation of c-*fos* by Raf-BxB or MEK-1 (Figure 7), indicating that aPKC-ζ is implicated in signal transmission through ERK1,2 and acts somewhere downstream of MEK-1. Induction of c-*fos* by Raf-BxB or MEK-1 was not affected by expression of the dominant negative aPKC-λ mutant (Figure 7), suggesting that aPKC-λ acts upstream of Raf-1. However, the data available so far do not exclude an alternative scenario in which aPKC-λ transmits signals independent from Raf-1 and MEK-1 perhaps via PI3 kinase or phospholipase D (PLD). As depletion or inhibition of aPKC-ζ blocks c-*fos* induction by a constitutively active aPKC-λ mutant, this alternative pathway, if it exists, requires the cooperation of the ζ isotype. According to Bjorkoy *et al.* (1997), aPKC-λ is required for the activation of nuclear ERKs. In any case, the exact function of aPKC-λ still remains to be elucidated. Expression of the kinase-defective, DN nPKC-ε K436R mutant which depresses c-*fos* induction by oncogenic Ras or (CA)aPKC-λ A119E inhibits transcriptional activation of the c-*fos*–CAT reporter by Raf-BxB or MEK-1, indicating that nPKC-ε is implicated in the ERK pathway. However, inhibition by (DN)nPKC-ε K436R of Raf-BxB or MEK-1 induced transcriptional activation of c-*fos* was incomplete and definitely weaker than the interference with the Ras- or (CA)aPKC-λ A119E-stimulated c-*fos* induction. These results suggest that nPKC-ε may in addition to its effect downstream of MEK, also be involved in Raf-1 activation as suggested by Cai *et al*. (1997), or that nPKC-ε signals through Raf-1-/MEK-1-dependent and independent pathways.

At this point it is satisfying to note that each of the three PKC isoforms which have been identified in these studies as required for the transmission of signals from Ha-Ras to the c-*fos* promoter had previously described as downstream targets of Ha-Ras and regulators of the Ha-Ras–MAP kinase pathway (Berra *et al*., 1995; Diaz-Meco *et al*., 1994; Cacace *et al*., 1996; Morrison *et al*., 1996; Zou *et al*., 1996; Cai *et al*., 1997; Liao *et al*., 1997). The novelty of this paper is the demonstration that, at least in the system studied here, a combination of all three isozymes is required which appear to cooperate in a hierarchically ordered sequence.

The data obtained in this paper were obtained by transient transfections. Thus, it cannot be determined whether the PKCs are involved in direct protein synthesis-independent signaling or whether the generation of new signaling elements is induced. In order to address this question, studies have been initiated to prepare constructs encoding steroid hormone responsive PKC–hormone-binding domain (HBD) fusion proteins following the strategy described for Raf-ER which can be activated by estrogen (Kerkhoff and Rapp, 1997).

Materials and methods

Reagents and plasmids

Dulbecco's modified Eagle's medium (DMEM), RPMI medium and restriction enzymes for molecular biological approaches were purchased from Boehringer Mannheim (Mannheim, Germany). Fetal calf serum and L-glutamine were obtained from Schoeller Pharma (Vienna, Austria). Epidermal growth factor (EGF), insulin, leupeptin, and aprotinin are products from Sigma (Vienna, Austria). Lipofectin transfection reagents and Opti-Mem I medium were purchased from Life Technologies, Inc. (Vienna, Austria). CalPhosMaximizer™ transfection kit was purchased from Clontech (Biotrade, Vienna, Austria). PCR primers used for subcloning strategy were obtained from ARK Scientific (Darmstadt, Germany). Rabbit polyclonal anti-PKC isoenzyme antibodies were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany) or Transduction Laboratories (Vienna, Austria).

To generate PKC isoform-specific antisense RNA-expressing constructs, PKC-isoform specific DNA fragments were amplified by PCR using HC11 cDNA as a template. The oligonucleotides listed below were used for the generation of the constructs:

PKC-α:
5′-CGGAGAGAGCCAGAGAGAGC-3′
5′-TTTGCGGGCGAAGCGGTTGG-3′ (PKC-α DNA fragment from positions 74–324)
PKC-ε:
5′-TGCTCCTGCTCTTCAATCCTGC-3′
5′-ATGTAGGGGTCCAGAAGGAACG-3′ (PKC-ε DNA fragment from positions 138–318)
PKC-ζ:
5′-GGACATCCTGATTACCAGCG-3′
5′-CGGTTGTTCTGGGATGCTTG-3′ (PKC-ζ DNA fragment from positions 72–309)
aPKC-λ:
5′-TTGAGGGAATTCAGTGAGGAGATGCCGACCCAGAGG-3′
5′-TTGAGGGAATTCCTGAAAGCCTCTTCTAACTCCAAC-3′
(aPKC-λ/ι DNA fragment from positions 1–281).

Polished fragments were first blunt-end ligated into the multiple cloning site of the plasmid pPoly3, then rescued by restriction with *Not*I and ligated into the *Not*I site of the plasmid pOPI3-RSV (Stratagene). Orientation of insertion was determined by restriction analysis and sequencing. Subcloning strategy, mutagenic primers, as well as selection primers for the generation of DN, as well as CA mutants of PKC isoenzymes have been described elsewhere (Baier-Bitterlich *et al*., 1996; Überall *et al*., 1997). The plasmid pRc-CMV–PKC-λ K275W and pCDNA3–PKC-ζ K275W-HA-tagged were a generous gift from J.Moscat (Madrid, Spain). For construction of (CA)PKC-ζ A119E mutant site-directed mutagenesis of a full-length PKC-ζ cDNA derived from mouse brain (donated by H.Mischak, Munich, Germany) was performed. All PKC cDNAs were subcloned into the pEFneo expression vector. The plasmids pEJrasVal12, ptk-CAT and pcfos-DSE-FAP-tk-CAT have been described elsewhere (Überall *et al*., 1994). The plasmid pRaf-BxB was a generous gift from T.Beckers (ASTA-Medica, Frankfurt, Germany). The plasmid (CA)pFAMEK-1 was obtained from Stratagene Cloning Systems (Heidelberg, Germany).

Cell culture and transient transfections

HC11 mouse mammary epithelial cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 5 μg/ml insulin and 10 ng/ml EGF at 37°C in a humidified atmosphere of 95% air with 5% CO_2. One day after plating HC11 cells into 6-well dishes, transfection of the subconfluent monolayers with the indicated expression vectors was carried out either with Lipofectin (Gibco-BRL) or CalPhosMaximizer™ as described by the manufacturer. Six h after post-transfection, the cells were washed with prewarmed DMEM and kept in RPMI medium complemented with 0.5% heat-inactivated FCS and 2 mM L-glutamine for 48 h.

Chloramphenicolacetyltransferase (CAT) assay

CAT assays were performed as described previously (Überall *et al*., 1994).

Luciferase assay

An aliquot of the harvested cell-monolayer was incubated on ice for 30 min in 200 μl lysis buffer (25 mM glycylglycine pH 7.8, 15 mM $MgSO_4.7H_2O$, 5 mM EGTA, 1% Triton X-100, 1 mM DTT). After a centrifugation step (10 000 *g*, 10 min, 4°C) 60 μl of the supernatant was mixed with 400 μl assay buffer (lysis buffer without Triton X-100 complemented with 15 mM KH_2PO_4, and 2 mM Di-Na-ATP) and the relative light units (RLU) were counted for 20 sec in a Lumat LB9501 (Berthold, Vienna) after injection of 200 μl luciferin solution (lysis buffer without Triton X-100 complemented with 20 nM luciferin-free acid).

Magnetic separation of transient transfected HC11 cells

HC11 cells cotransfected with pMACS4 (Miltenyi Biotech), and therefore expressing a truncated CD4 surface marker, were washed twice with phosphate-buffered saline (PBS) and then incubated with PBE (PBS with 5 mM EDTA and 0.5% skimmed milk powder) for 30 min at 37°C to detach cells from the cell culture dish. Suspended cells were incubated for 15 min with MACS4 magnetic microbeads (diluted 1:10 in PBE) at room temperature and the positive cells were separated from the non-transfected background over magnetic columns.

Western blot analysis

Whole-cell extracts for immunoblot analysis were prepared by freezing and thawing the washed cells in cell extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 25 μg/ml leupeptin and 25 μg/ml aprotinin). After a centrifugation step (20 000 *g*, 30 min, 4°C) the supernatant was mixed with 5× sample buffer (10 ml 1.25 M Tris–HCl, pH 6.8, 20 ml glycerine, 10 μl mercaptoethanol, 16 mg Bromphenol Blue and 4 g SDS) and boiled for 3 min. Equal amounts of protein were separated electrophoretically on a 10% SDS–polyacrylamide gel and transferred to PVDF-membranes (Millipore, Vienna). The membranes were blocked in TBST-M (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20, 1% milk powder) and incubated with PKC-isoform specific antibodies for 1 h at room temperature (anti-PKC-α, anti-PKC-λ and anti-PKC-ζ; 1:1000 in TBST-M or anti-PKC-ε; 1:400 in TBST-M; the PKC-isoenzyme-specific antibodies anti-PKC-α, anti-PKC-ε and anti-PKC-ζ were purchased from Santa Cruz, anti-PKC-λ was obtained from Transduction Laboratories), then washed extensively in TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) and incubated with anti-rabbit mouse-IgG (obtained from Amersham; 1:1250 in TBST-M) for 45 min at room temperature, respectively. Expression analysis was performed after washing the membranes several times in TBST by ECL detection as described by the manufacturer.

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