Protein kinase C- ζ reverts *v-raf* transformation of NIH-3T3 cells

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We have identified protein kinase C- ζ (PKC- ζ) as a novel suppressor of neoplastic transformation caused by the *v-raf* oncogene. PKC- ζ overexpression drastically retards proliferation, abolishes anchorage-independent growth, and reverts the morphological transformation of *v-raf*-transformed NIH-3T3 cells. The molecular basis for this effect appears to be a specific induction of *junB* and *egr-1* expression, triggered synergistically by PKC- ζ via a Raf/Mek/MAPK-independent mechanism and *v-raf*. *junB*-promoter/CAT assays revealed that PKC- ζ directly targets the *junB* promoter. The induction of *junB* and *egr-1* is linked to the *v-raf* transformation-suppressing effect of PKC- ζ as constitutive expression of *junB* and *egr-1* but not of *c-jun* also abolishes anchorage-independent growth of *v-raf*-transformed NIH-3T3 cells. Moreover, *junB* overexpression leads to a retardation of proliferation in these cells. PKC- ζ interferes with the serum inducibility of an AP-1 reporter plasmid in *v-raf*-transformed NIH-3T3 cells, indicating that PKC- ζ antagonizes transformation and proliferation by down-modulating AP-1 function via induction of *junB*. In summary, our data suggest that PKC- ζ counteracts *v-raf* transformation by modulating the expression of the transcription factors *junB* and *egr-1*.

[Key Words: Protein kinase C- ζ ; *v-raf*; suppression of transformation; *junB* induction; *egr-1* induction; *junB* promoter]

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Protein kinase C (PKC) comprises a multigene family of serine/threonine kinases that are differentially expressed in various cell types and tissues (e.g. Mischak et al. 1991; Dlugosz et al. 1992; for review, see Hug and Sarre 1993; Goodnight et al. 1994). PKCs are intimately involved in critical physiological processes such as control of cellular proliferation and differentiation. To date, 11 different PKC isozymes have been identified by cDNA cloning (Knopf et al. 1986; Ono et al. 1988, 1989; Osada et al. 1990, 1992; Selbie et al. 1993; Akimoto et al. 1994; Johannes et al. 1994; for review, see Hug and Sarre 1993). Originally, PKC was described as a calcium-, diacylglycerol-, and phospholipid-dependent kinase (Inoue et al. 1977; Takai et al. 1979) as well as the major intracellular receptor for tumor-promoting phorbol esters (Castagna et al. 1982). Phorbol ester treatment of cells results in activation of most PKC isozymes and a concomitant rapid translocation of PKC from the cytosol to the membrane. PKC activation induces the phosphory-

lation of several different proteins (for review, see Hug and Sarre 1993; Goodnight et al. 1994), among others, Raf-1 (Sozeri et al. 1992; Kolch et al. 1993a; Carroll and May, 1994). Raf-1 is a proto-oncogene involved in the mitogenic Raf/Mek/MAPK signal transduction pathway that connects tyrosine kinase receptors at the cell membrane with the nucleus. Although the PKC subspecies share many structurally similar elements, they are clearly distinct from each other in cellular and tissue distribution (for review, see Goodnight et al. 1994). A major difference among the members of this gene family is their requirement for Ca^{2+} . Conventional PKCs (PKC- α , PKC- β I, PKC- β II, and PKC- γ) require Ca^{2+} for activation of their kinase activity (Knopf et al. 1986). The novel PKCs (PKC- δ , PKC- ϵ , PKC- η , PKC- θ , and PKC- μ) lack the putative calcium-binding domain C2 and hence can be activated by phospholipid and diacylglycerol alone (Ono et al. 1988; Osada et al. 1990, 1992; Mischak et al. 1991; Johannes et al. 1994).

PKC- ζ is an unusual member of the PKC family in that it neither requires Ca^{2+} for activation nor binds diacylglycerol or phorbol esters (Ono et al. 1989; Goodnight et

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al. 1992; Kochs et al. 1993; Selbie et al. 1993). However, PKC- ζ appears to be bifunctionally regulated by ceramide and arachidonic acid (Lozano et al. 1994; Müller et al. 1995). PKC- ζ is expressed in almost all cell types and tissues so far examined (Hug and Sarre 1993), suggesting an important role for this PKC isozyme in cell biology. Previous reports on the biological functions of PKC- ζ were conflicting. On one hand, PKC- ζ was reported to be involved in mitogenic signaling downstream of Ras (Berra et al. 1993; Bjorkoy et al. 1995), to be involved in deregulation of cell growth and in cellular transformation of NIH-3T3 cells (Berra et al. 1993), and to lead to I κ B- α phosphorylation and subsequent NF- κ B activation (Diaz-Meco et al. 1993, 1994; Dominguez et al. 1993; Folgueira et al. 1996). On the other hand, recent reports showed that overexpression of mouse PKC- ζ did not change the proliferation rate, did not induce transformation, and did not lead to NF- κ B activation in NIH-3T3 cells (Crespo et al. 1995; Montaner et al. 1995). A role of PKC- ζ in the differentiation of leukemic U937 cells including the induction of a slower proliferation rate was shown by Ways et al. (1994), and a role of PKC- ζ in the nerve growth factor (NGF)-induced differentiation and arrest of replication of PC12 cells was suggested by Wooten et al. (1994).

We and others have shown previously that PKC- α activates Raf-1 by direct phosphorylation and that PKC- α and Raf-1 synergize to transform NIH-3T3 cells (Sozeri et al. 1992; Kolch et al. 1993a; Carroll and May 1994; Marquardt et al. 1994). This property seems to be confined to the cPKCs (Sozeri et al. 1992). Therefore, we set out to reinvestigate the interaction of PKC isozymes with Raf-1 and the constitutively active *v-raf* in the context of cellular transformation and proliferation. Here, we report our findings on the interaction of PKC- ζ with

the *v-raf* oncogene in NIH-3T3 cells. Although PKC- ζ overexpression had no effect on the growth properties of wild-type or Raf-1 overexpressing NIH-3T3 cells, it reverted *v-raf* transformation of NIH-3T3 cells. PKC- ζ did not affect the activity of the Raf/Mek/MAPK pathway in vitro or in vivo. However, PKC- ζ caused a constitutive and specific induction of *junB* and *egr-1* expression in NIH-3T3 cells in synergism with *v-raf*. The induction of *junB* and *egr-1* expression by PKC- ζ was linked to the suppression of *v-raf* transformation, as constitutive expression of *junB* and *egr-1* but not of *c-jun* also abolished anchorage-independent growth of *v-raf*-transformed NIH-3T3 cells. Moreover, *junB* overexpression retarded proliferation of these cells to levels of the parental NIH-3T3 cells. PKC- ζ interfered with the serum inducibility of an AP-1 reporter plasmid in *v-raf*-transformed NIH-3T3 cells suggesting that induction of *junB* by PKC- ζ leads to a down-modulation of AP-1 function, which results in the retardation of proliferation and suppression of *v-raf* transformation. These data identify *junB* and *egr-1* as nuclear target genes of PKC- ζ signaling and as dominant suppressor genes of *v-raf* transformation.

Results

Suppression of *v-raf* transformation by PKC- ζ

To investigate the interaction of PKC- ζ and Raf in the context of cellular proliferation and transformation, we overexpressed mouse PKC- ζ (Goodnight et al. 1992) in wild-type, Raf-1-overexpressing, and *v-raf*-transformed NIH-3T3 cells. In keeping with the observations recently reported by Montaner et al. (1995), overexpression of mouse PKC- ζ in NIH-3T3 cells did not alter the prolifer-

Table 1. PKC- ζ suppresses *v-raf* transformation in NIH-3T3 cells

Cell line ^a	Expressed oncogene	PKC- ζ expression vector ^b	PKC- ζ expression ^c ($\times$)	Doubling time ^d (hr)	Colony formation in soft agar ^e (%)
NIH-3T3	—	—	1	24.6	0
NIH- ζ	—	zipneo- ζ wt	7	24.4	0
3611	<i>v-raf</i>	—	1	14.6	81
3611 ζ P47	<i>v-raf</i>	zipneo- ζ wt	9	22.9	15
3611L ζ clone 12	<i>v-raf</i>	pLTR- ζ	10	25.0	2
3611L ζ clone 16	<i>v-raf</i>	pLTR- ζ	10	N.D.	5
3611 ζ clone 3	<i>v-raf</i>	zipneo- ζ wt	8	N.D.	5
3611 ζ P25	<i>v-raf</i>	zipneo- ζ wt	11	N.D.	18
3611 ζ P44	<i>v-raf</i>	zipneo- ζ wt	11	N.D.	12

^aAll PKC- ζ transfected cell lines are pools of at least 25 G418-resistant single clones, except of the cell lines indicated by 'clone,' which represent single clones.

^bNIH-3T3 and 3611 cells were stably transfected with the indicated mouse PKC- ζ expression vectors using a modified calcium phosphate precipitation technique (Chen and Okayama 1987).

^cPKC- ζ expression was quantified by densitometric scanning of immunoblots of total cell lysates (also see Fig. 1A). The endogenous expression of PKC- ζ in NIH-3T3 cells was arbitrarily set to 1.

^dDoubling times were calculated from growth curves performed as shown in Fig. 6B.

^eColony formation in soft agar (0.4% noble agar; Sigma) was scored 13 days after seeding and is given as the percentage of the total number of seeded cells per six-well plate that formed colonies in soft agar.

eration rate (Table 1) nor did it induce cellular transformation (Table 1; Fig. 2, below). Similar results were obtained when PKC- ζ was cooverexpressed with human Raf-1, where no obvious effects on the growth properties of NIH-3T3 cells could be observed (data not shown). In contrast, overexpression of PKC- ζ in *v-raf*-transformed NIH-3T3 cells (3611 cells; Häfner et al. 1994) yielded striking results (see below).

Special precautions were taken to avoid artifacts arising from clonal variation: Experiments were carried out both with bulk cultures of PKC- ζ -transfected 3611 cells and with several independently isolated cell clones (Table 1). Figure 1A shows the immunoblot analysis of *v-raf* and PKC- ζ expression in NIH-3T3, 3611, and NIH- ζ cells and in the representative PKC- ζ -overexpressing 3611 bulk culture 3611 ζ P47. All cell lines based on 3611 cells expressed similar amounts of *v-Raf* protein. The level of PKC- ζ protein was increased ninefold in 3611 ζ P47 cells compared with wild-type NIH-3T3 and parental 3611 cells as quantified by densitometric scanning of the immunoblots shown in Figure 1A. In NIH- ζ cells, the

Figure 1. Overexpression of a kinase active mouse PKC- ζ in NIH-3T3 and *v-raf*-transformed NIH-3T3 cells (3611 cells). NIH-3T3 and 3611 cells were stably transfected with expression vectors coding for mouse PKC- ζ (see Table 1). (A) Immunoblot analysis of PKC- ζ and *v-raf* expression in PKC- ζ -transfected NIH-3T3 and 3611 cells. A selection of representative cell lines is shown. (Top) The PKC- ζ protein detected by the Boehringer anti-PKC- ζ antiserum; (bottom) the *v-Raf* protein detected by the crafVI antiserum. (B) PKC- ζ kinase activity in 3611 ζ P47 and NIH- ζ cells compared with 3611 and NIH-3T3 cells. The PKC- ζ protein was immunoprecipitated using the anti-PKC- ζ antiserum K300T and subsequently assayed for MBP substrate phosphorylation as described in Materials and methods. (Inset) An autoradiograph of MBP phosphorylation signals obtained in a representative experiment. The results of three independent experiments are compiled.

PKC- ζ protein level was elevated sevenfold compared with parental NIH-3T3 cells. To confirm that the transduced mouse PKC- ζ retained enzymatic activity, the PKC- ζ kinase activity in the representative pool 3611 ζ P47 and in NIH-3T3, 3611 and NIH- ζ cells was assayed. PKC- ζ kinase activities were measured in anti-PKC- ζ immunoprecipitates as substrate phosphorylation using myelin basic protein (MBP) as an exogenous substrate (Fig. 1B). As expected, significantly increased levels of PKC- ζ kinase activity could be found in 3611 ζ P47 and NIH- ζ cells compared with 3611 and NIH-3T3 cells. PKC- ζ immunoprecipitates of the 3611 ζ P47 cells exhibited strong substrate phosphorylation, representing a 7.5-fold higher activity than in parental 3611 and NIH-3T3 cells. In NIH- ζ cells, the PKC- ζ kinase activity was elevated sixfold compared with that in NIH-3T3 cells.

Overexpression of PKC- ζ in *v-raf*-transformed NIH-3T3 cells (3611 cells) produced striking effects on the morphology and growth characteristics of these cells. PKC- ζ drastically reduced the proliferation rate and reverted the *v-raf* transformation of 3611 cells (Table 1; Fig. 2). As shown in Figure 2A, 3611 cells in monolayer cultures were morphologically transformed, whereas the representative 3611 ζ P47 cells appeared similar to NIH-3T3 and NIH- ζ cells. The PKC- ζ -overexpressing 3611 cells were flat rather than spindle shaped and did not exhibit the crisscross growth pattern of the parental 3611 cells. When seeded in soft agar, 81% of the seeded 3611 cells formed large colonies (Fig. 2B; Table 1). In contrast, PKC- ζ overexpression in 3611 cells resulted in a drastically reduced anchorage-independent growth: Only 15% of the seeded 3611 ζ P47 cells formed colonies in soft agar at all, which, in addition, were much smaller than the soft agar colonies of the parental 3611 cells. These findings could be reproduced with additional PKC- ζ -overexpressing 3611 cell pools and single-cell clones (Table 1). NIH- ζ and NIH-3T3 cells did not show any anchorage-independent growth at all. Furthermore, proliferation of 3611 cells was severely affected by PKC- ζ overexpression (Table 1). PKC- ζ reduced the proliferation rate of 3611 cells to the levels of wild-type NIH-3T3 cells.

PKC- ζ does not affect the Raf/Mek/MAPK pathway

It has been shown previously that *v-raf* activates the Mek/MAPK pathway (Dent et al., 1992; Howe et al. 1992; Kyriakis et al. 1992) and that the constitutive activation of this pathway suffices for transformation of NIH-3T3 cells (Cowley et al. 1994). A possible mechanism by which PKC- ζ might antagonize *v-raf* transformation would be down-regulation of the Raf/Mek/MAPK pathway. To evaluate this hypothesis, we first investigated whether PKC- ζ has any impact on the Raf kinase activity itself. The purified mouse PKC- ζ (Goodnight et al. 1992; Kazanietz et al. 1993) used in the in vitro experiments described in this paper displays high specific activity toward several substrates (Kazanietz et al. 1993). Using purified recombinant enzymes for in

Figure 2. PKC- ζ reverts the morphological transformation and drastically reduces the soft agar growth of *v-raf*-transformed NIH-3T3 cells. (A) Morphology of 3611, 3611 ζ P47, NIH-3T3, and NIH- ζ cells grown to confluence in monolayer in the presence of 10% serum. (B) Colony growth of 3611, 3611 ζ P47, NIH-3T3, and NIH- ζ cells after 13 days in 0.4% soft agar in the presence of 10% serum. PKC- ζ overexpression drastically reduced the formation of soft agar colonies in *v-raf*-transformed NIH-3T3 cells (see 3611 ζ P47 cells). PKC- ζ overexpression in wild-type NIH-3T3 cells did not result in anchorage-independent growth.

vitro kinase assays, we found that Raf-1 was a very poor substrate for PKC- ζ and that PKC- ζ did not induce or down-regulate Raf-1 activity in vitro (data not shown). Consistent with these in vitro results, we could not detect any significant effect of PKC- ζ on the kinase activity of glutathione *S*-transferase (GST)-tagged Raf-1 (G-Raf) or BXB (GNX, a version of Raf-1 rendered transforming by deletion of the amino-terminal regulatory domain; Heidecker et al. 1990) upon coexpression in the baculovirus/Sf9 cell system (Fig. 3A). As shown in Figure 3B, neither Mek nor MAPK were phosphorylated signifi-

cantly by PKC- ζ in vitro. Whereas Raf-1 drastically induced Mek activity, PKC- ζ failed absolutely to activate Mek in in vitro assays (Fig. 3B).

Although these data suggested strongly that PKC- ζ does not modify directly the activity of the individual components of the Raf/Mek/MAPK pathway in vitro, we examined the effect of PKC- ζ on Raf and MAPK activity in the 3611 cell context. Figure 3C shows the data obtained on the kinase activity of Raf and MAPK proteins immunoprecipitated from 3611 and 3611 ζ P47 cells after stimulation with 10% serum at the time points indicated. The anti-Raf antiserum crafVI used for the immunoprecipitations recognizes both Raf-1 and *v-raf* (Häfner et al. 1994). Thus, the majority of the kinase activity in the Raf immunoprecipitates is contributed by *v-raf*, which is expressed at 10-fold higher levels than Raf-1 in 3611 cells (Häfner et al. 1994). In three independent experiments we could not detect any significant differences in the Raf and MAPK kinase activities between the PKC- ζ -overexpressing 3611 ζ P47 cells and 3611 cells during a stimulation time course ranging from 2 min to 6 hr. Raf kinase activities were almost identical in serum-starved 3611 and 3611 ζ P47 cells. In both cell lines, serum superinduced Raf kinase activity about twofold in a transient fashion, peaking between 2 and 10 min after stimulation. Similar results were obtained when the MAPK kinase activity was determined in 3611 ζ P47 and 3611 cells (Fig. 3C). These in vivo data validate the in vitro experiments showing that PKC- ζ does not impinge on the constitutive or induced activity of the Raf/Mek/MAPK pathway in these cells.

Induction and prolongation of *junB* and *egr-1* expression by PKC- ζ

The results presented above show that PKC- ζ does not interfere directly with the transduction of transforming signals by the cytosolic components of the Raf/Mek/MAPK pathway. Previous work has identified the genes for the nuclear transcription factors *c-jun*, *junB*, and *egr-1* as the nuclear target and effector genes of *v-raf* (Qureshi et al. 1991; Kolch et al. 1993b; Coffey et al. 1994; Rapp et al. 1994; Suzuki et al. 1994). Moreover, the induction of *c-jun* is a crucial event in transforming cells by *v-raf* (Rapp et al. 1994). The transcriptional regulation of the *jun* family members and *egr-1* is complex and also involves signal transduction pathways other than the Raf/Mek/MAPK pathway (e.g., de Groot et al. 1991; Han et al. 1992; Kitabayashi et al. 1993; Coffey et al. 1994, 1995; Sakamoto et al. 1994). Therefore, it was tempting to speculate that PKC- ζ , via a Raf/Mek/MAPK independent pathway, might interfere with the transforming signals generated by *v-raf* at the nuclear level by modulating the expression of nuclear target genes of *v-raf*. To test this hypothesis, we analyzed the expression of *egr-1* and the *jun* family members *c-jun*, *junB*, and *junD* by Northern blotting of RNA from NIH-3T3 cells that coexpress PKC- ζ with *v-raf*. The salient findings of these experiments are shown in Figure 4.

Figure 3. PKC- ζ does not affect the activity of the Raf/Mek/MAPK pathway in vitro or in vivo. (A) G-Raf or GNX was expressed in the baculovirus/Sf9 system, without or together with PKC- ζ . Linked Raf kinase assays were performed with the purified G-Raf or GNX using Mek(+)/MAPK(-) as substrates. (Top) The PKC- ζ protein expressed in the Sf9 cells detected by the Boehringer anti-PKC- ζ antiserum. (Middle) The autophosphorylation signals originating from the glutathione-bound G-Raf and GNX in the kinase reactions. (Bottom) MAPK(-) phosphorylation obtained in the linked Raf kinase assays. In the negative control (middle column: -), the linked kinase assay was performed with Mek(+)/MAPK(-) alone. Similar results were obtained in two further experiments. (B) The kinase activities of Raf-1 and PKC- ζ were tested using the indicated substrates: Mek(+), kinase active Mek; Mek(-), kinase negative Mek; MAPK(-), kinase negative MAPK. The immunoblots show the Raf-1 or the PKC- ζ proteins detected by the crafVI antiserum or the Boehringer anti-PKC- ζ antiserum, respectively. (C) PKC- ζ does not affect the activity of Raf-1 or MAPK in 3611 cells. 3611 (open bars) and 3611 ζ P47 (solid bars) cells were grown to early confluence, serum starved overnight, and subsequently stimulated with 10% serum. At the time points indicated, Raf proteins were immunoprecipitated using the anti-Raf antiserum crafVI and MAPK proteins using the rabbit anti-MAPK antiserum. Raf activity was measured in linked kinase assays using Mek(+)/MAPK(-) as substrates. MAPK activity was assayed using MBP as a substrate. MAPK(-) and MBP phosphorylations, respectively, were quantified by PhosphorImager scanning and are given as relative (PhosphorImager) units. Consistent data were obtained in three independent experiments.

As shown in Figure 4A, the comparison between the PKC- ζ -overexpressing 3611 ζ P47 cells and the parental 3611 cells revealed no significant alterations of *junD* expression. In contrast, 3611 ζ P47 cells featured a constitutive expression of *egr-1*, *c-jun*, and *junB* mRNAs. In 3611 ζ P47 cells, the basal expression of *egr-1* was induced fourfold, that of *junB* twofold, and that of *c-jun* threefold compared with the parental 3611 cells (as quantified by PhosphorImager scanning of the Northern blots in Fig. 4A). Treatment with serum superinduced but did not prolong *c-jun* expression in 3611 ζ P47 cells. In contrast, the serum induction of *egr-1* and *junB* was dramatically sustained for 6 hr in the 3611 ζ P47 cells, whereas in 3611 cells, the expression of both early genes declined rapidly, starting 60–90 min after administration of serum (Fig. 4A). To evaluate whether PKC- ζ also affects the expression of *egr-1* and the *jun* family members *c-jun*, *junB*,

and *junD* in parental NIH-3T3 cells, we examined their mRNA expression in NIH-3T3 cells and in a pool of PKC- ζ -overexpressing NIH-3T3 cells (NIH- ζ cells; see Table 1; Fig. 1). *junD* expression did not differ in the pool NIH- ζ compared with parental NIH-3T3 cells (data not shown). In contrast to *v-raf*-transformed cells, PKC- ζ overexpression did not enhance the basal expression of *egr-1*, *c-jun*, or *junB* in NIH-3T3 cells (Fig. 4B). PKC- ζ , however, extended the induction of *junB* and *egr-1* by serum stimulation up to 4 hr, albeit not to the extent seen in 3611 ζ P47 cells. In summary, these results suggested that PKC- ζ does not regulate the activity of the Raf/Mek/MAPK signaling pathway. Instead, PKC- ζ prolongs the induction of the *v-raf* effector genes *junB* and *egr-1* via a Raf-independent mechanism and both pathways synergize to cause a constitutive expression and a long-lasting induction of *junB* and *egr-1*.

Figure 4. PKC- ζ and *v-raf* cooperate to induce a constitutive expression and long-term induction of *junB* and *egr-1* in NIH-3T3 cells. Northern blot analysis of immediate-early gene expression in PKC- ζ -overexpressing (A) 3611 and (B) NIH-3T3 cells. Cells were serum starved overnight and subsequently stimulated with 10% serum. At the time points indicated, total RNA was isolated. Northern blots were sequentially hybridized with the probes indicated.

PKC- ζ targets the *junB* -promoter

The induction of *junB* and *egr-1* appeared to be the germane trait linked with PKC- ζ overexpression in parental and *v-raf*-transformed NIH-3T3 cells. Therefore, the ability of PKC- ζ to activate the promoter of the *junB* gene was explored. Unfortunately, a reporter construct to also test the *egr-1* promoter was not available, so that we had to restrict our studies to the *junB* promoter. For this purpose, PKC- ζ was transiently coexpressed with pJB1 [Coffer et al. 1994], a plasmid containing the *junB* promoter fused to a CAT reporter gene. In the presence of 10% serum, PKC- ζ activated transcription of the *junB* promoter in a dose-dependent manner in NIH-3T3 cells (Fig. 5). Increasing amounts of cotransfected PKC- ζ led to an increasing activity of the *junB* promoter. Thus, these findings demonstrate that PKC- ζ has a direct impact on the transcriptional regulation of the *junB* promoter, and they corroborate the results obtained with expression analysis of mRNAs by Northern blotting in cells that stably overexpress PKC- ζ .

Suppression of *v-raf* transformation by constitutive expression of *junB* or *egr-1*

To test whether the induced expression of *junB* and *egr-1* might be sufficient for the reversion of *v-raf* transformation by PKC- ζ , we stably transfected *v-raf*-transformed NIH-3T3 cells (3611 cells) with expression vectors coding for mouse *junB* (pELB) or mouse *egr-1* (pCMVEgr-1). Several clones of 3611 cells that expressed the exogenous *junB* or *egr-1* could be established readily. As shown in Figure 6A, the cell lines overexpressed the introduced genes as well as *v-raf*, with the exception of 3611*junB* clone 14, which expressed *v-raf* but not the transfected

junB. This clone, therefore, served as an additional negative control. The cell lines were examined for reversion of cellular transformation. The overexpression of *c-jun* failed to affect *v-raf* transformation (data not shown),

Figure 5. PKC- ζ induces the transcription of the *junB* promoter. NIH-3T3 cells were transfected with 2 μ g of a CAT reporter construct containing the *junB* promoter, pJB1, or the control vector, pCAT. As indicated, different ratios of the PKC- ζ expression plasmid zipneo- ζ wt and pUC18 were cotransfected. CAT assays were quantified using a PhosphorImager and ImageQuant software. CAT activity is expressed as fold induction relative to the level obtained by cotransfection of pJB1 (solid bars) or pCAT (open bars) with pUC18. Results of three independent experiments are compiled.

Figure 6. Constitutive overexpression of *junB* and *egr-1* in *v-raf*-transformed 3611 cells reverts cellular transformation. 3611 cells were stably transfected with the pELB expression vector coding for mouse *junB* or with the pCMVEgr-1 expression vector coding for mouse *egr-1*. (A) Immunoblot analysis of *junB* or *egr-1* protein expression (top) and *v-raf* protein expression (bottom) in *junB*-transfected (left) or *egr-1* transfected (right) 3611 cell lines. (B) Growth curves of representative 3611*junB* and 3611*egr-1* clones in comparison with parental 3611 and NIH-3T3 cells. Constitutive *junB* expression in 3611 cells retarded the rate of proliferation to the level seen in NIH-3T3 cells, whereas constitutive expression of *egr-1* had no effect. (C) Colony growth in soft agar of the representative clones 3611*junB* clone 10, 3611*egr-1* clone 2, and the parental 3611 cells. *junB*- and *egr-1*-overexpressing 3611 cells did not form soft agar colonies. The control cells 3611*junB* clone 14 yielded colonies of the same size as the 3611 cells (data not shown). Photographs were taken on day 13 after seeding.

whereas *junB* and *egr-1* were able to revert transformation, but in a qualitatively different manner. Constitutive expression of *junB* but not *egr-1* reduced the growth rate of 3611 cells to the level of parental NIH-3T3 cells (Fig. 6B). Both transgenes, however, were able to abolish the growth of 3611 cells in soft agar (Fig. 6C). The 3611*junB* clone 14 control cells, lacking detectable *junB* expression, formed colonies in soft agar of essentially the same size as 3611 cells (data not shown).

The effect of PKC- ζ on AP-1 activity in 3611 cells

JunB was reported to antagonize c-Jun function in the heterodimeric transcription factor AP-1 and to act as a repressor of AP-1-dependent transcription (Chiu et al. 1989; Schutte et al. 1989). Several lines of evidence suggest that the AP-1 transcription factor is a critical nuclear effector of *v-raf* and is required for its transforming effect (Jamal and Ziff 1990; Kolch et al. 1993b; Kortengann et al. 1994; Rapp et al. 1994). Thus, PKC- ζ might retard proliferation and suppress transformation in *v-raf*-transformed NIH-3T3 cells by down-modulating AP-1 function via the induction of *junB* expression. To test this hypothesis, we transiently transfected 3611 and PKC- ζ -overexpressing 3611 ζ P47 cells with a reporter plasmid that carries the luciferase gene under the control of four AP-1 sites (pRTU14). Thirty hours after transfection, cells were serum starved overnight and subsequently stimulated with 10% serum for 0, 1, or 4 hr. The AP-1-dependent transcriptional *trans*-activation was assayed in total cell lysates as luciferase activity. As expected, a significant induction of luciferase activity was observed 1 and 4 hr after stimulation with serum in the parental 3611 cells (Fig. 7). In contrast, serum stimulation did not increase AP-1-dependent transcription at all in 3611 ζ P47 cells. Thus, PKC- ζ overexpression severely interfered with the serum inducibility of the AP-1 reporter in 3611 cells.

Discussion

Here, we report that overexpression of mouse PKC- ζ leads to a reversion of *v-raf* transformation in NIH-3T3 cells. PKC- ζ causes a marked reduction in the proliferation rate and soft agar growth of *v-raf*-transformed NIH-3T3 cells. Furthermore, PKC- ζ leads to a reversion of the transformed cell morphology in these cells. The basis of the reversion appears to be the constitutive and prolonged expression of *egr-1* and *junB*, triggered synergistically by PKC- ζ via a Raf/Mek/MAPK-independent mechanism and *v-raf*, because reversion could be reproduced by transfection with *junB* or *egr-1* expression vectors. In this context, it is important to stress that reversion is not just a nonspecific consequence of the untimely expression of early genes in general, as the constitutive expression of c-jun failed to revert 3611 cells. The specificity of this phenomenon is also apparent from the differential effects of *junB* and *egr-1* on *v-raf* transformation. Constitutive expression of either *junB* or *egr-1* inhibited the growth of 3611 cells in soft agar,

Figure 7. High PKC- ζ activity abolishes AP-1-responsivity to serum in 3611 cells. 3611 and 3611 ζ P47 cells were transiently transfected with the AP-1 reporter plasmid pRTU14, which carries the luciferase reporter gene under the control of four AP-1 sites. Thirty hours after transfection, cells were serum starved overnight and subsequently stimulated with 10% serum for the time indicated. Luciferase activity was assayed in total cell lysates using a luminometer. The obtained data for the unstimulated cells were set to 100%. Results of three independent experiments are compiled. No induction of luciferase activity in both cell lines could be obtained in a parallel experiment with the control plasmid pRTU1, which lacks the four AP-1 sites (data not shown).

but only *junB* significantly reduced the proliferation rate, pointing to a paramount role for *junB* in the inhibition of transformation.

junB and *egr-1* were identified originally as early growth response genes, and a role as transformation suppressor genes seems counterintuitive. However, *egr-1* has previously been reported to inhibit transformation by *v-sis* (Huang et al. 1994), although the underlying mechanisms have not been elucidated yet. In a related experiment, Kharbanda et al. (1994) showed that during monocytic differentiation of U937 myeloid leukemia cells, *egr-1* expression is induced. Notably, differentiation of U937 cells can be stimulated by overexpression of PKC- ζ (Ways et al. 1994). An important observation is that *JunB* antagonizes c-Jun function by regulating the activity of the transcription factor AP-1 (Chiu et al. 1989; Schutte et al. 1989). AP-1 is targeted by *v-raf* and is required for its transforming effect (Jamal and Ziff 1990; Kolch et al. 1993b; Kortjenann et al. 1994; Rapp et al. 1994). Owing to *JunB*'s ability to compete with c-Jun for dimerization (Deng and Karin 1993), prolonged or constitutive expression of *junB* would be expected to suppress AP-1 transcriptional activity and, hence, transformation and proliferation. Here, we could show that (1) PKC- ζ causes a constitutive expression and prolonged induction of *junB* in *v-raf*-transformed NIH-3T3 cells, (2)

PKC- ζ leads to a prolonged induction of *junB* in NIH-3T3 cells, (3) the expression of PKC- ζ activates transcription from the *junB* promoter in reporter gene assays, (4) constitutive *junB* expression leads to the reversion of *v-raf* transformation in NIH-3T3 cells, and (5) PKC- ζ interferes with the serum-induction of AP-1 *trans*-activating activity in *v-raf*-transformed NIH-3T3 cells. Thus, PKC- ζ —via its modulation of *junB* expression—seems to alter AP-1 function during the critical time frame after serum stimulation of the cells, when the proliferative response is initiated, and thereby interferes with proliferation and transformation in *v-raf*-transformed NIH-3T3 cells. The amplitude and duration of the *junB* induction seems crucial for the cell's decision between proliferation and growth arrest. Although a short, transient expression of *junB* might be necessary for mitogenesis, prolonged or constitutive expression in fact inhibits cell growth and transformation.

Berra et al. (1993) previously reported that PKC- ζ is critical for the mitogenic signal transduction in NIH-3T3 cells downstream of *ras* and that PKC- ζ leads to the induction of some of the characteristics of the transformed phenotype. Furthermore, these investigators postulated that PKC- ζ activates Mek and MAPK (Berra et al. 1995). On the other hand, Crespo et al. (1995) and Montaner et al. (1995) demonstrated that overexpression of mouse PKC- ζ in NIH-3T3 cells neither affected the proliferation rate nor elicited transformation. The latter results are concordant with our observations regarding PKC- ζ overexpression in NIH-3T3 cells. Moreover, we have not been able to obtain any evidence that Mek or MAPK are activated by PKC- ζ in vitro or in vivo. These discrepancies most likely are attributable to the fact that Berra et al. (1993, 1995) used an atypical PKC derived from *Xenopus laevis*. This PKC isoform was designated as PKC- ζ by the investigators, but as the same authors stated in a recent publication (Diaz-Meco et al. 1996), it actually is not PKC- ζ but displays much higher homology to another mammalian atypical PKC, PKC- λ (Selbie et al. 1993; Akimoto et al. 1994; Diaz-Meco et al. 1996). This atypical PKC derived from *X. laevis* displays an overall 72% identity at the amino acid level to mammalian PKC- ζ , whereas the homology to the mammalian PKC- λ is 90% (Diaz-Meco et al. 1996). In contrast, we, as Crespo et al. (1995) and Montaner et al. (1995), used a mouse PKC- ζ (Goodnight et al. 1992) in mouse NIH-3T3 cells, which surely comes much closer to the physiological situation than the use of a *Xenopus* PKC(- ζ)- λ in a mouse cell.

There is good evidence that the transforming potential of Ras in NIH-3T3 cells depends on the activity of the Raf/Mek/MAPK pathway (Smith et al. 1986; Kolch et al. 1991; Cowley et al. 1994). Thus, a corollary of our findings is that PKC- ζ also ought to revert Ras transformation. Therefore, we investigated the effects of PKC- ζ overexpression on NIH-3T3 cells transformed by the activated T24ras oncogene (Kalinec et al. 1992). We could show that PKC- ζ reproduced some but not all effects on T24ras transformation as on *v-raf* transformation (A. Kieser, D.W. Henderson, J.F. Mushinski, W. Kolch, and

H. Mischak, unpubl.). PKC- ζ overexpression completely abolished anchorage-independent growth and retarded proliferation in T24ras-transformed NIH-3T3 cells. In contrast to *v-raf*-transformed cells, PKC- ζ had no effect on the morphological transformation of T24ras-transformed cells. The reasons for these differential effects of PKC- ζ on *v-raf* and T24ras transformation might reflect essential differences between the transduction of transforming signals generated by *v-raf* and activated Ras. Recent reports demonstrated that transforming signals generated by activated Ras are transduced by at least two distinct pathways: (1) the classical Raf/Mek/MAPK pathway and (2) a Rac- and Rho-dependent pathway. Both pathways cooperate to elicit the full morphogenic and mitogenic consequences of Ras transformation (Khosravi-Far et al. 1995; Prendergast et al. 1995; Qiu et al. 1995). In addition, Ras, Rac, and Rho regulate the structure of the actin cytoskeleton, which participates in the control of cell morphology (for review, see Ridley 1995). Thus, PKC- ζ might interfere only with the transforming signals generated by T24ras that are transduced via a Raf-dependent pathway, but not with the Rac/Rho-dependent signals, which are responsible for the effects of activated Ras on the cell morphology.

We have characterized PKC- ζ as a novel dominant suppressor gene of *v-raf* transformation, which exerts its biological effects via the transcription factors *junB* and *egr-1*. Because PKC- ζ has no effects on the growth properties of wild-type NIH-3T3 and Raf-1-overexpressing NIH-3T3 cells (Raf-1-overexpressing NIH-3T3 cells are not transformed; Stanton et al. 1989; Heidecker et al. 1990), but "resets" *v-raf* transformation to growth properties of normal NIH-3T3 cells, PKC- ζ might act as a safeguard against transforming signals transduced via the Raf/Mek/MAPK signaling pathway.

Materials and methods

Expression vectors

A HindIII–EcoRI fragment that contains the complete coding sequence of mouse PKC- ζ cDNA (Goodnight et al. 1992) was blunt-ended and inserted into the blunt-ended EcoRI sites of zipneo or pLTR (Megidish and Mazurek 1989), respectively, to yield zipneo- ζ wt and pLTR- ζ . The construction of the mouse *junB* expression plasmid pELB (Kolch et al. 1993b) has been described previously. pCMVEgr-1 coding for mouse *egr-1* (Gashler et al. 1993) was kindly provided by Vikas P. Sukhatme (Beth Israel Hospital, Boston, MA) and pRSV-c-jun by Wolfgang Hammerschmidt (Institut für Klinische Molekularbiologie und Tumorgenetik, Munich, Germany).

Cells and cell culture methods

NIH-3T3 cells and derivatives were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL) supplemented with 2 mM glutamine and 10% bovine serum (Seromed). Cells were serum starved by overnight incubation in medium without serum. 3611 cells represent a pool of 20 soft agar colonies of *v-raf*-transformed NIH-3T3 cells (Häfner et al. 1994). Expression plasmids were introduced by a modified calcium phosphate precipitation technique (Chen and Okayama 1987). pLTR- ζ carries

the *Escherichia coli* gpt gene as a selectable marker, whereas all other expression plasmids contain the neomycin resistance gene. Transfected cells were selected in complete DMEM containing 1 mg/ml of G418 (GIBCO BRL) or 20 μ g/ml of mycophenolic acid (Sigma) plus hypoxanthine–aminopterin–thymidine (Sigma). After 10–16 days of selection, resistant colonies were pooled. In addition, as indicated, single colonies were isolated. The expression of the transduced genes was analyzed by Western blotting with specific antibodies (see below). For soft agar assays, 25,000 cells per well of a six-well plate (Nunc) were seeded in DMEM supplemented with 2 mM glutamine, 10% bovine serum, and 0.4% noble agar (Sigma). Colony growth was scored 13 days after seeding of the cells. For growth curves, 10^4 cells per well of a 24-well plate were seeded. At day 1 after seeding, cells were fed with medium containing 10% serum and subsequently counted every 24 hr. Two wells were counted per time point and cell line. Doubling times were calculated from growth curves performed as described.

Immunoblotting and antibodies

Cells (2×10^7) of each cell line were lysed in 400 μ l of TBST buffer (10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml of leupeptin, 0.5 mM β -glycerophosphate, 0.5 mM sodium fluoride, 0.5 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, and 1% Triton X-100). Cellular debris was removed by centrifugation for 10 min at 15,000g. Protein content was determined using the Bio-Rad protein assay. Equal amounts of protein, diluted with SDS sample buffer (final concentration, 30 mM Tris-HCl at pH 6.8, 1 mM EDTA, 5 mM DTT, 10% glycerol, and 1% SDS), were separated by SDS–polyacrylamide gel electrophoresis and blotted onto Hybond-C membranes (Amersham). Equal loading was verified by staining duplicate gels with Coomassie Brilliant Blue R-250. The rabbit PKC- ζ antiserum was obtained from Boehringer Mannheim. The rabbit anti-PKC- ζ antiserum K300T has been described previously (Crespo et al. 1995). The Raf antiserum crafVI was raised in Chinchilla bastard rabbits immunized with a synthetic peptide corresponding to the 12 carboxy-terminal amino acids of Raf-1 (Häfner et al. 1994). It recognizes both Raf-1 and *v-raf*. The antibodies against JunB and Egr-1 were purchased from Santa Cruz Biotech., Inc. The rabbit anti-MAPK antiserum was a kind gift from Peter Shaw (Max-Planck-Institut für Immunbiologie, Freiburg, Germany) (Häfner et al. 1994). Western blots were developed using Enhanced chemiluminescence (ECL; Amersham) and quantified using a Cybertech laser scanner.

Immunoprecipitation and immunocomplex kinase assays

Cells were treated as indicated in the legend to Figure 3 (c), washed with PBS, and lysed in TBST buffer. Cleared lysates were incubated overnight at 4°C with the anti-Raf antiserum crafVI or the anti-MAPK antiserum or the anti-PKC- ζ antiserum K300T, respectively, bound to protein A–agarose beads (Boehringer Mannheim). Immunoprecipitates were washed three times in TBST and once with kinase buffer. Raf activity was measured in a linked kinase assay containing 20 mM Tris-HCl (pH 7.4), 20 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 2 μ M ATP, and 5 μ Ci of [γ -³²P]ATP together with 50 ng of purified recombinant kinase active Mek [Mek(+); Gardner et al. 1993] and 100 ng of purified recombinant kinase negative MAPK [MAPK(–); Gardner et al. 1993] as substrates. MAPK kinase assays were conducted in the same buffer with 1 μ g of MBP (GIBCO BRL) as substrate. PKC- ζ kinase reactions contained 20 mM Tris-HCl (pH 7.5), 1 mM CaCl₂, 10 mM magnesium acetate, 80 μ g/ml of

phosphatidylcholine, 20 μ g/ml of phosphatidylserine, 10 μ M ATP, and 10 μ Ci of [γ - 32 P]ATP. One microgram of MBP was included as substrate. After incubation for 30 min at 25°C, reactions were terminated by boiling in SDS-gel sample buffer, separated by SDS-gel electrophoresis, electrophoretically blotted on Hybond-C membranes (Amersham), and subsequently autoradiographed. Phosphorylation was quantitated using a Fuji BAS1000 PhosphorImager.

Protein expression and purification

G-Raf (Häfner et al. 1994), the GST-tagged Raf kinase domain BXB (GNX; Häfner et al. 1994), untagged Raf-1 (Kolch et al. 1993a), PKC- α (Kazanietz et al. 1993), and PKC- ζ (Goodnight et al. 1992; Kazanietz et al. 1993) were expressed in Sf9 cells as described (Kazanietz et al. 1993; Häfner et al. 1994). The GST-tagged Raf proteins were precipitated out of total cell lysates using glutathione-Sepharose in the presence of 1% Triton X-100 as described (Häfner et al. 1994). G-Raf and GNX kinase assays were performed under the same conditions as described above for Raf immunoprecipitates. Untagged Raf-1 was immunoprecipitated from baculo-lysates (see immunoprecipitation and immunocomplex kinase assays) using the crafVI antiserum. PKC- α and PKC- ζ were purified as described (Kazanietz et al. 1993).

In vitro Mek and MAPK phosphorylation assays

Kinase reactions with purified PKC- ζ were performed under the same conditions as described above for PKC- ζ in immunocomplex kinase assays. As indicated, 50 ng of purified kinase negative Mek [Mek(-); Gardner et al. 1993], 50 ng of Mek(+), 100 ng of MAPK(-), or in a linked kinase assay 50 ng of Mek(+) together with 100 ng of MAPK(-) were used as substrates. Raf kinase reactions were performed in Raf kinase buffer as described above. The indicated substrates were used.

Purification of total RNA and Northern blot analysis

Cells were grown to subconfluence in 10-cm tissue culture plates (Nunc), serum starved overnight, and subsequently stimulated as indicated. Cells were lysed with 7 ml of GTC buffer (4.2 M guanidinium thiocyanate, 0.025 M sodium citrate, and 0.7% 2-mercaptoethanol at pH 7.0) per dish. The lysates were layered onto a cushion of 3 ml of cesium chloride solution (5.7 M cesium chloride, 100 mM EDTA at pH 8.0) and centrifuged at 32,000 rpm in a SW 40Ti rotor (Beckman) for 20 hr. The RNA pellet was washed twice with 80% ethanol at -20°C and, finally, resuspended in DEPC-treated H₂O. Seven micrograms of each RNA were fractionated on a 1.25% agarose gel containing 6% formaldehyde. The RNA was transferred to a Hybond-N membrane (Amersham) by capillary blotting and hybridized with 32 P-labeled cDNA probes as indicated in the figures. Radioactive labeling was performed with the Random Primers DNA Labeling System (GIBCO BRL) according to the manufacturer's protocol. The membranes were hybridized overnight with 3×10^6 dpm/ml of probe, washed with $2 \times$ SSC, 0.1% SDS at 65°C, and autoradiographed overnight. Signals were quantitated with a Fuji BAS1000 PhosphorImager. For sequential hybridization of the same blots with different probes, membranes were stripped with boiling 0.1% SDS solution.

CAT assays

NIH-3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells were seeded

at 1.5×10^5 per well in 6-well plates and 24 hr later were transfected with 10 μ g of supercoiled plasmid DNAs by a calcium phosphate precipitation technique. The following day, the medium was changed and cells were grown for an additional 24 hr. CAT activity was determined as described previously using β -galactosidase expression to correct for the transfection efficiency (Coffer et al. 1994). Results were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics). *junB*-Promoter and control plasmids have been described previously (Coffer et al. 1994).

Luciferase assays

Cells (1.5×10^5) were seeded per six-well plate in DMEM containing 10% serum. After 24 hr, cells were transfected with 2.5 μ g of DNA per six-well plate using the Lipofectamine reagent (GIBCO BRL) according to the manufacturer's protocol. For luciferase assays, the pRTU14 plasmid [pRTU14 carries the luciferase gene under the control of four AP-1 sites; kindly provided by Dirk Bohmann, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany] or the control plasmid pRTU1 [pRTU1 lacks the four AP-1 sites] was transfected. Transfected cells were treated as described in the legend to Figure 7. Luciferase activity was assayed in total cell lysates as described (Henglein et al. 1994). The efficiency of transfection was monitored in parallel transfections with the β -galactosidase expression vector pRSVlacZ.

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