Reversion of Ras- and Phosphatidylcholine-hydrolyzing Phospholipase C-mediated Transformation of NIH 3T3 Cells by a Dominant Interfering Mutant of Protein Kinase C \( \lambda \) Is Accompanied by the Loss of Constitutive Nuclear Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Activity*

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The transformed phenotype of v-Ras- or *Bacillus cereus* phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC)-expressing NIH 3T3 cells is reverted by expressing a kinase-defective mutant of protein kinase C \( \lambda \) (APKC). We report here that extracellular signal-regulated kinase (ERK)-1 and -2 are constitutively activated in v-Ras- and PC-PLC-transformed cells in the absence of added growth factors. Interestingly, the activated ERKs were exclusively localized to the cell nucleus. Consistently, the transactivating potential of the C-terminal domain of Elk-1, which is activated upon ERK-mediated phosphorylation, was strongly induced in serum-starved cells expressing v-Ras or PC-PLC. Reversion of v-Ras- or PC-PLC-induced transformation by expression of dominant negative APKC abolished the nuclear ERK activation suggesting APKC as a novel, direct or indirect, activator of mitogen-activated protein kinase/ERK kinase in response to activated Ras or elevated levels of phosphatidylcholine-derived diacylglycerol. Transient transfection experiments confirmed that APKC acts downstream of Ras but upstream of mitogen-activated protein kinase/ERK kinase. We found both the v-Ras- and PC-PLC-transformed cells to be insensitive to stimulation with platelet-derived growth factor (PDGF). No detectable receptor level, autophosphorylation, or superinduction of DNA synthesis could be observed in response to treatment with PDGF. Reversion of the transformed cell lines by expression of dominant negative APKC restored the receptor level and the ability to respond to PDGF in terms of receptor autophosphorylation, ERK activation, and induction of DNA synthesis.

A growing body of evidence suggests that the induction of the mitogen-activated protein kinase (MAPK) pathway leading to activation of extracellular signal-regulated kinase (ERK)-1 and -2 is essential for mitogenic signal transduction (1–4). ERK1 and -2 are rapidly activated after growth factor stimulation or overexpression of constitutively active Ras, Raf, or MAPK/ERK kinase (MEK) (2, 4). Following activation the ERKs translocate to the nucleus to phosphorylate their nuclear substrates (5–8). Results obtained by the use of dominant interfering mutants and antisense RNA suggest that activation of the ERKs may be required for proliferation of fibroblasts (9). The only direct activators of the ERKs identified so far are MEK1 and -2 (2, 4, 10). These dual specificity kinases activate ERK1 and -2 by phosphorylating both the threonine and the tyrosine residue in the sequence motif TEY (3). MEK1, but not MEK2, forms a ternary complex with Ras and Raf-1 (11). The Raf-1 kinase has been regarded as the major MEK1 activator in most cell systems (2, 12). However, it is now evident that several proteins may contribute to MEK1 activation (10, 13–16). Of particular relevance to this study is the finding that the atypical protein kinase C subtype \( \lambda \) (APKC) is activated by tyrosine kinase receptors (17) and is shown to phosphorylate and activate MEK1 in vivo as well as in vivo (6, 18, 19). We (20, 21) and others (22, 23) have previously used the notion \( \Delta PKC \) for this PKC subspecies cloned from *Xenopus laevis*. However, the more recent description of \( \lambda \)APKC (24, 25) identified this atypical PKC subspecies originating from *Xenopus* as \( \lambda \)APKC (19).

Polypeptide-derived growth factors and activation of Ras or Src cause an increase in the hydrolysis of phosphatidylinositol 3-phosphate (PtdIns(3)P) resulting in a sustained elevation in intracellular levels of phosphatidylinositol 3,4-diphosphate (PtdIns(3,4)P2) (26–32). Chronic stimulation of PC hydrolysis by stable expression of the gene (pc) encoding Bacillus *cereus* phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC) causes severe growth deregulation and morphological transformation of NIH 3T3 fibroblasts without activating Ras (20, 21). Moreover, expression of *B. cereus* PC-PLC was able to release NIH 3T3 cells from a block to proliferation imposed by expression of dominant negative N-17 Ras but not so when the block was due to expression of dominant negative Raf-1 (33, 34). Consistently, expression of dominant negative Raf-1 reverted the transformed phenotype induced by *pc* expression (20). Furthermore, addition of purified bacterial PC-PLC to quiescent NIH 3T3 cell cultures induced Raf-1 kinase activity, whereas an inhibitor of endogenous PC-PLC activity

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blocked Raf-1 activation in response to serum (33–35). These findings suggest that the generation of DAG by PC hydrolysis is located downstream of Ras but upstream of Raf in the mitogenic signal transduction pathway. Thus, PC-derived DAG may directly or indirectly be involved in the poorly defined activation of the Raf kinase family members (12).

Several proteins, including λPKC, bind to Ras in a GTP-dependent manner suggesting that several pathways are involved in relaying Ras-mediated signals (20, 36–42). λPKC (the human homolog is named λPKC) constitutes together with ζPKC, the atypical PKCs. These PKC subtypes contain only one cysteine-rich zinc finger and is not activated by phorbol esters or Ca2+ ions (43). A requirement for functional λPKC in the Ras-mediated insulin-induced maturation pathway in Xenopus oocytes and for serum-activated DNA synthesis in murine fibroblasts has been demonstrated (22, 23). Consistent with the notion of PKC as a downstream target of activated Ras, a dominant negative mutant of λPKC, as well as dominant negative Raf-1, was found to revert both v-ras- and plc-induced transformation (20). The atypical PKCs have been reported to be involved in several signaling pathways activating different downstream components. λPKC is important for NFκB activation in different cell lines, including NIH 3T3, probably through activation of an IκB kinase (18, 19). Furthermore, λPKC plays a critical role during stromelysin promoter activation by PDGF in fibroblasts (44). Recent experiments, using antisense oligonucleotides against different PKC subtypes, have shown that ζPKC acts as a mediator of PDGF-induced α2-integrin gene expression in human dermal fibroblasts (45). Both ζPKC and λPKC have been suggested as downstream components for PI 3-kinase-mediated signaling (17, 46). Thus, atypical PKCs clearly play critical roles in several signaling pathways.

In this paper we report that ERK1 and -2 are constitutively activated and localized to the nucleus in both v-ras- and plc-transformed cells. Expression of a dominant negative mutant of λPKC abolished ERK activation in response to v-ras or plc expression. Consistently, v-ras- and plc-induced GAL-ElkC-transactivation was found to be dependent on functional λPKC but did not involve activation of JNK. Moreover, the dominant negative mutant of λPKC blocked v-ras but not activated MEK induction of ERK kinase activity. We also show that v-ras or plc transformation lead to PDGF receptor down-regulation leading to abolished superinduction of DNA synthesis in response to PDGF. This was also the case for a cell line stably overexpressing an activated mutant of MEK1. Reversion of the v-ras- and plc-transformed phenotype by expression of a dominant negative mutant of λPKC re-established the receptor level and PDGF-induced receptor autophosphorylation, ERK kinase activation, and induction of DNA synthesis.

**MATERIALS AND METHODS**

**Cell Cultures and Stably Transfected Cell Lines—**NIH 3T3 fibroblasts (passage 123) were purchased from the American Type Culture Collection (ATCC CRL 1658) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (HyClone, Logan, UT), penicillin (100 units/ml), and 100 μg/ml streptomycin (Life Technologies, Inc.) in a CO2 incubator (5% CO2) at 37 °C. NIH 3T3 cells transformed with the v-Ha-ras or v-src oncoproteins (26, 28) were grown in the same medium. A well characterized PC-PLC expressing clone (clone P18/20) was grown in the presence of hygromycin B (Calbiochem) at the same medium. A well characterized PC-PLC expressing clone (clone 20), were grown in the presence of both hygromycin B and g/ml G418 (Life Technologies, Inc.).

**Induction of DNA Synthesis—**Measurements of the induction of DNA synthesis were performed as described previously (21). The MEK inhibitor PD 098059 (New England Biolabs) was dissolved in dimethyl sulfoxide and added to the the cell cultures to a final concentration of 0.5% (v/v) of vehicle.

**Preparation of Whole Cell, Cytosolic, and Nuclear Extracts—**For preparation of cytosolic extracts, cell-starved or PDGF-stimulated cell cultures were rinsed twice with ice-cold phosphate-buffered saline and lysed in the dishes using ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4 for 30 min on ice. The lysates were collected with a rubber policeman, and insoluble material was removed by centrifugation in a microcentrifuge for 5 min at 13,000 rpm. The supernatant was aliquoted and stored at −70 °C. The protein concentrations in the cytosolic extracts were determined using a detergent-compatible protein assay kit (Bio-Rad DC Protein Assay) with bovine serum albumin as the standard.

Whole cell and nuclear extracts were prepared as described by Westwick and Brenner (47) and by Sjöstedt et al. (48), respectively, in the presence of the following protease/phosphatase inhibitors: 2 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin A, 20 μg μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin A, 20 μg μl of cold saline and left for 24 h. The cultures were subsequently serum-starved for 20 h in DMEM supplemented with 0.1% calf serum and either stimulated with 10 ng/ml PDGF (BB homodimer, Sigma) for 15 min or left untreated. The immunostaining was performed essentially as described in the protocol obtained from the supplier (New England Biolabs). Briefly, the cells were fixed in 3% paraformaldehyde, permeabilized using a buffer containing 0.1% Triton X-100, and preincubated for 1 h in 5.5% normal horse serum. The primary antibody was added for 48 h at 4 °C in a humidified chamber, and the staining was developed using biotinylated secondary antibodies and preformed streptavidin-peroxidase complexes in a Ni(OH)-enhanced DAB reaction (Vectastain, Vector Laboratories).

**Immunoblotting—**Phosphorylated/activated ERK1 and -2 were specifically detected using the phospho-ERK-specific antibody described above. The total level of ERK2 was determined using a monoclonal anti-ERK2 antibody (clone B9, Upstate Biotechnology Inc.) recognizing both phosphorylated and non-phosphorylated forms. The cell cultures were treated as indicated in the respective figure legends. Thrombin (100 units/ml) was purchased from Hoffman-La Roche. The cell cultures were harvested directly into 1 × SDS-PAGE gel load buffer and immediately heated to 95 °C for 5 min and sonicated briefly on ice. The extracts (20 μl, corresponding to 2 × 106 cells per lane) were separated by SDS-PAGE, electrotransferred onto polyvinylidene difluoride membranes (Millipore), and developed following protocols obtained from the respective suppliers of antibodies. Tyrosine phosphorylation of the PDGF receptor was determined using a phospho-specific monoclonal antibody following the protocol obtained from the supplier (clone 4G10, Upstate Biotechnology Inc.). To verify protein loading, some of the immunoblots were stripped for 2 h in 0.2 mM glycine, pH 2.4, 1% SDS at 65 °C, blocked, and reprobed with the anti-ERK2 antibody. The level of PDGF receptor was determined as described by Vaziri and Faller (49) using a polyclonal PDGF receptor antibody (PDGF β-specific, Santa Cruz Biotechnology). All immunoblots were developed using alkaline phosphatase-conjugated secondary antibodies and the chemiluminescent substrate CDP-Star (New England Biolabs or Boehringer Mannheim).

**Kinase Assays—**ERK activities in cytosolic or nuclear extracts were determined as described by Sale et al. (50). The activity of hemagglutinin (HA)-tagged ERK1 in extracts from transient transfected cell lines was measured by an immune complex kinase assay using MBP as the substrate. Subconfluent cell cultures were transfected using lipofectamine (Life Technologies, Inc.) according to the instructions of the manufacturer. Following 4 h of incubation with DNA, the cells were incubated for 24 h in the presence of 10% serum and then starved in 0.1% serum for another 24 h. Preparation of cell extracts and immunoprecipitation of HA-ERK2 or HA-ERK1 using a monoclonal antibody against the HA epitope (12CA5, Boehringer Mannheim) was performed as described (6). The immune complexes were washed three times with cell lysis buffer containing 0.5 mM NaCl and twice with MBP kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, 0.1 mM Na3VO4). The complexes were resuspended in kinase buffer containing 1 mM dithiothreitol and 0.3 mM MBP, and kinase reactions were initiated by adding [γ-32P]ATP.
producing an in-frame fusion with glutathione S-transferase (GST). The fusion protein (GST-Jun 5–115) was expressed in Escherichia coli (51). GST-Jun 5–115 was coupled to glutathione-agarose beads 5 mALK-2 and 40 ml of phosphate-buffered saline (PBS) and boiled immediately for 5 min. The phosphorylated proteins were separated on a 12.5% polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (Millipore). The phosphorylated proteins were detected by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics).

The activation status of the c-Jun N-terminal kinase (JNK) in the different cell lines was measured by a solid-phase kinase assay. To make GST-Jun5–115 a sequence encoding amino acids 5–115 of c-Jun was amplified by polymerase chain reaction (5'-GAATTC-GATCCATGGAAACGACCTTCTATGAC-3' and 5'-GAATTCTCGAGTGCTCATCTGTCACGTTCTTG-3') and inserted into the BamHI and XhoI sites of pGEX-4T-3 (Pharmacia Biotech Inc.) producing an in-frame fusion with glutathione S-transferase (GST). The fusion protein (GST-Jun5–115) was expressed in Escherichia coli LE392 (51). GST-Jun5–115 was coupled to glutathione-agarose beads and stored as a 20% suspension in NETN buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 0.5% (v/v) Nonidet P-40, 2 mM dithiothreitol, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml peptatin A). The kinase assays were performed as described by Westwick and Brenner (47). Phosphorylated proteins were resolved on 10% SDS-polyacrylamide gels that were dried and subjected to autoradiography at -70 °C with intensifying screens. Equal protein loading was verified by immunoblotting with an antibody against JNK1 (C-17, Santa Cruz Biotechnology).

Transactivation Assays—To measure the activity of the C-terminal transactivation domain of Elk-1 in the different cell lines, pGAL4-ElkC was made by inserting a BglII-XhoI fragment from pBS-Elk1 into the BamHI and XhoI sites of pSG424 (52). Subconfluent cultures of the different cell lines in 100-mm diameter Petri dishes were transfected with 2 μg of pGAL4-ElkC or pSG424 (vector control), 1.2 μg of the reporter vector pG5E1bTATA-CAT (53), and 2 μg of salmon sperm DNA using the calcium phosphate coprecipitation method. The gels that were dried and subjected to autoradiography at -70 °C were normalized by determination of the total ERK2 level, using an antibody recognizing both non-phosphorylated and phosphorylated ERK2.

RESULTS

ERK1 and -2 Are Constitutively Activated and Translocated to the Nucleus in v-ras- and plc-transformed Cells in a ΠPKC-dependent Manner—We have previously shown that chronic stimulation of PC hydrolysation by expression of B. cereus PLC-PLC caused induction of DNA synthesis, enhanced proliferation in the absence of added growth factors, and led to transformation of NIH 3T3 cells (21). Expression of a dominant negative mutant of APKC (dnAPKC) led to reversion to a non-transformed, normal phenotype of both v-ras- and plc-transformed cells (20).

Here, we wanted to further evaluate the signal mediators downstream of PC-derived DAG. Recently, APKC was found to activate MEK1 both in vitro and in vivo (6). To begin elucidating if the MEK/ERK pathway is involved in transduction of the mitogenic signal generated by PC-derived DAG, we used a novel inhibitor, PD098059, that specifically interferes with the MEK/ERK pathway (54). The ability of plc- and v-ras-expressing cells to induce DNA synthesis in the absence of added growth factors was completely lost in the presence of PD098059 (Fig. 1A). Also, prolonged treatment of the plc- and v-ras transformed cell lines with this inhibitor caused complete reversion to a normal, non-transformed phenotype (data not shown), indicating that MEK is a necessary downstream component for mitogenic signaling and transformation mediated by PC-derived DAG and v-ras. However, we found no significant increase in cytosolic ERK activity in serum-starved cell cultures of v-ras- or plc-transformed cells (Fig. 1B). Furthermore, treatment of these cell lines with PDGF did not induce increased ERK activity. Consistently, immunoblotting analyses.
of whole cell extracts using an antibody specifically recognizing phosphorylated and activated ERK1 and -2 did not reveal any potent ERK activation in response to stable expression of either v-ras or plc (Fig. 1C). Interestingly, unlike their parental transformed cells, the cell lines reverted by expression of dnAPKC induced phosphorylation of myelin basic protein in response to PDGF (Fig. 1B). The reverted cell lines also consistently showed a pronounced PDGF-induced phosphorylation of ERK1 and -2 (Fig. 1C). The activation of ERK1 and -2 in response to serum was indistinguishable between the different cell lines, suggesting that the differential activation of ERK in transformed and reverted cell lines was specific for PDGF-induced signaling.

Activation of the MAPK pathway is accompanied by translocation of ERK1 and -2 to the nucleus (4–8). Thus, we next analyzed the ERK activity in nuclear extracts from cells expressing v-ras or plc alone or together with dnAPKC (Fig. 2). The basal nuclear ERK activity was increased in both v-ras- and plc-expressing cells compared with quiescent NIH 3T3 cells. However, the increased basal ERK activity displayed by the transformed cells was inhibited by expression of dnAPKC. To further analyze this, serum-starved or PDGF-stimulated cell cultures were immunostained with an antibody specifically recognizing phosphorylated ERK1 and -2 (Fig. 2). These experiments confirmed that ERK1 and -2 were constitutively activated both in v-ras- and in plc-transformed cell lines. Moreover, phosphorylated ERKs were exclusively localized to the nuclei. Treating the transformed cell lines with PDGF for 15 min did not further increase the intensity of the nuclear staining (data not shown). Reversion of the v-ras- or plc-induced transformation by expression of dnAPKC abolished the chronic activation of the nuclear ERKs. However, the phosphorylation of ERK1 and -2 in the reverted cell lines could be induced by PDGF. These data suggest that expression of v-ras as well as increased intracellular levels of PC-derived DAG caused by expression of B. cereus PC-PLC both result in activation and nuclear translocation of ERK1 and -2. Furthermore, the activation of ERKs in response to Ras and PC-PLC is dependent on functional APKC.

Expression of dnAPKC Blocks Both v-ras- and plc-induced Activation of Elk-1—We have previously shown that dnAPKC abolished the Ras- or PC-PLC-induced activation of both NF-xB and AP-1 (20). However, since we found constitutive activation and nuclear translocation of ERKs in Ras- and PC-PLC-transformed cell lines, the activation status of the transcription factor Elk-1 was of interest. For this purpose, a chimeric transcription factor composed of the DNA binding domain of yeast GAL4 and the transactivating domain of Elk-1 (GAL4-ElkC) was constructed. The transactivation potential of this nuclear fusion protein is strongly enhanced by specific MAPK phosphorylation within the transactivation domain of Elk-1 (55). Consistent with the nuclear localization of activated ERKs in the transformed cell lines, we found that the transactivating potential of GAL4-ElkC was strongly induced in response to transformation by v-ras or plc (Fig. 4A). Reversion of the transformed cell lines by expression of dnAPKC coincided with a large decrease in the transactivation potential of GAL4-ElkC down to the background level seen in quiescent NIH 3T3 cells. Thus, both v-ras and plc induced the transactivating potential of Elk-1 in a λPKC-dependent manner. Recently, it was reported that another subclass of the MAPK family, termed the c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases, phosphorylates and activates the transactivation domain of Elk-1 (56–58). To determine the activity of the JNKs in the transformed and reverted cell lines, whole cell extracts were used in a solid phase kinase assay using GST-Jun5–115 as a substrate (Fig. 4B). GST-Jun5–115 was extensively phosphorylated when treated with extract from UV-stimulated NIH 3T3 cells. However, we found no increase in JNK activity in ras-transformed cells and only a slight increase in plc-transformed cells. The same results were obtained by carrying out

![FIG. 2. The ERK activity is constitutively elevated in nuclear extracts from serum-starved v-ras or plc-transformed cells. Expression of dnAPKC in the transformed cell lines is accompanied by an attenuation of the MBP phosphorylation in response to v-ras or plc. Serum-starved (0.1% serum for 20 h) cell cultures were either left untreated or treated with PDGF (10 ng/ml) for 15 min as indicated. The ERK activity in the nuclear extracts was determined as described in Fig. 1B. The data shown are representative of three other independent experiments.](image)
immune complex kinase assays using an antibody specifically recognizing JNK1 (data not shown). Prolonged treatment of ras- and plc-transformed cells with the specific MEK inhibitor PD 098059 totally abolished the GAL4-ElkC activity (data not shown). Altogether, these results clearly suggest that the increased transactivation potential observed for GAL4-ElkC is due to activation by nuclear ERK1 and -2 with very little, if any, contribution by JNKs.

Expression of dnAPKC Does Not Affect MEK-induced ERK Activation—The expression of dnAPKC blocked ras- and plc-induced activation of ERK as well as Elk-1 transactivation suggesting that APKC acts downstream of Ras and PC-PLC but upstream of ERK. To more firmly localize APKC with respect to MEK, we performed transient transfections measuring the activation of HA-tagged ERK1 (59) in response to Ha-Ras V-12 (22) or an activated mutant of MEK1 (aMEK) (60), in the presence or absence of dnAPKC (19). The activation of ERK1 induced by Ras was blocked by cotransfection with the dnAPKC expression vector. However, dnAPKC did not affect MEK1-induced activation of ERK1 in NIH 3T3 cells (Fig. 5). Immunoblot analysis of cell extracts from the different transfected cell cultures showed that the differences in ERK activity were not due to differences in the expression of HA-tagged ERK1 (data not shown). Collectively, these results suggest that APKC is located downstream of Ras but upstream of MEK in the MEK/ERK pathway. To further evaluate the effect of dnAPKC on MEK-mediated signaling in NIH 3T3 cells, we first made a cell line that stably overexpressed aMEK. The aMEK expressing cells displayed a transformed phenotype, induced DNA synthesis in the absence of added growth factors, showed a constitutively increased nuclear ERK activity and an increased GAL-ElkC transactivation potential (data not shown). However, several attempts to establish clones that stably coexpressed dnAPKC and aMEK failed. Of more than 50 clones analyzed from two experiments performed with two different expression vectors for dnAPKC, none expressed dnAPKC. All of them had retained the transformed phenotype (data not shown). This indicates that dnAPKC interferes with the sur-

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**Fig. 4.** Activation and nuclear translocation of ERKs in response to v-ras and plc is accompanied by APKC-dependent induction of Elk-1 transactivation. A, following serum deprivation, plc- or v-ras-transformed cells display constitutive activation of Elk-1, whereas stable expression of dnAPKC completely blocked this growth factor-independent activation. The chloramphenicol acetyltransferase activities determined for the parental NIH 3T3 cells were set to 1.0. The data are expressed as the mean ± S.E. for two independent experiments performed in triplicate. B, the JNK family of MAP kinases is not significantly activated by either v-ras- or plc-mediated transformation. The transformed and the reverted cell lines were serum-starved for 20 h and harvested. As a control serum-deprived NIH 3T3 cells were treated with UVC (40 J/m²) or PDGF (10 ng/ml) for 15 min. Whole cell extracts (400 μg of total protein) were allowed to complex to GST-Jun5–115 (10 μg). The kinase reaction was initiated by adding [γ-32P]ATP and proceeded for 20 min at 30 °C. Following separation by SDS-PAGE, the GST-Jun5–115 phosphorylation was determined after subjecting the dried gels to autoradiography. The data are representative for three independent experiments with similar results. Protein loading was determined by immunoblotting with an antibody against JNK1.
Fig. 5. Coexpression of dnAPCK blocks the activation of ERK induced by Ha-RasV12 but not the activation caused by an activated mutant of MEK1. Subconfluent cell cultures were cotransfected with 2 μg of pCDNA-HA-ERK1 and 5 μg of either pZipHRasVal12 (lanes 1 and 2) or pExV5MAPKK1 (lanes 3 and 4) together with 10 μg of either pCDNA3-HA (lanes 1 and 3) or pCDNA3-HA-APCKmut (lanes 2 and 4). Following transfection, the cells were incubated in 10% serum for 24 h and then serum-starved for 24 h prior to cell lysis. HA-ERK activity in the lysates (300 μg of total cell protein) was determined by an immunocomplex kinase assay using MBP as the substrate. The fold activations of HA-tagged ERK1 as determined by PhosphorImager analysis were 3.6 for RasV12 (lane 1), 1.2 for RasV12-dnAPCK (lane 2), 5.9 for aMEK (lane 3), and 6.1 for aMEK-dnAPCK (lane 4), respectively. The background activity following cotransfection of the HA-ERK1 expression vector with the various empty expression vectors was set to 1.0. The results shown are representative of two other independent experiments. All expression vectors have been described previously (59, 22, 60, 19).

Discussion

In this study we present evidence for chronic activation and nuclear translocation of ERK1 and -2 in NIH 3T3 cells transformed with v-ras or plc. Expression of a dominant negative mutant of θPKC blocked the activation of ERK1 and -2. A sustained activation of ERK1 and -2 has been suggested to be a prerequisite for proliferation of fibroblasts and differentiation of PC12 cells (4). Interestingly, the ERKs located in the cytosol were not phosphorylated/activated in the transformed cells. Previously, Gardner et al. (65) reported no significant activation of MEK in v-ras-transformed NIH 3T3 cells. These workers found only modest constitutive activation of ERKs in Ras-transformed NIH 3T3 cells and no persistent ERK activity in similarly transformed Rat 1a fibroblasts (66). Their conclusion was based on the use of cytosolic extracts. These previous results are therefore entirely consistent with our present findings showing no constitutive activation of ERKs in the cytosol of the transformed cells. However, we show here that both v-ras- and plc-transformed cells contain constitutively elevated nuclear ERK activity. This may suggest that the mechanisms for terminating ERK activation by dephosphorylation mediated by protein phosphatases are probably operating normally in the cytosol, whereas the regulation of nuclear ERK activity is subverted in the transformed cells. Our results together with the recent demonstration that Ras transformation is inhibited by coexpression of kinase-defective mutants of ERK1 and -2 (38) indicate that constitutive nuclear ERK activity may be a prerequisite for transformation by v-ras or plc. Thus, it seems logical to assume that the transformed phenotype is dependent on persistent ERK-mediated phosphorylation of transcription factors. Consistent with this notion we found a potent activation of the chimeric transcription factor GAL4-ElkC, which contains the DNA binding domain and nuclear localization signal from yeast GAL4 and the transactivation domain of Elk-1, in the transformed cells. Intriguingly, the ERKs do not contain any known nuclear localization signals, and mutants that lack the TEF motif (T192A, Y194F) can be translocated to the nucleus (5, 7, 67). Therefore, although sustained ERK activation always seems to be associated with nuclear translocation, transient activation does not lead to nuclear translocation (4), and activation is not required for nuclear localization.

The activation of ERKs in response to Ras and PC-PLC was blocked by expression of a dominant interfering mutant of θPKC. Thus, θPKC may be critical for the Ras- and PC-PLC-mediated activation of MEK1. Although Raf-1 has been regarded as the major MEK1 activator in most cell systems, it is now evident that several protein kinases may serve as MEK1 activators (10, 19–15, 18, 68). In fact, Raf-1 may not be an important activator of MEK1 upon stimulation with serum or insulin (15). Also, a MEK1 mutant unable to bind to Raf-1 and B-Raf was still potently activated in response to serum, thrombin, and v-Ras (10). Of direct relevance to our present results is the finding that θPKC was able to phosphorylate and activate a MEK1 preparation from COS-1 cells in vitro (18). Furthermore, by the use of transient transfections, it was recently demonstrated that both MEK and ERK were activated in vivo by expressing activated θPKC and that the dominant negative
mutant of λPKC severely impaired the activation of both kinases following stimulation with serum or tumor necrosis factor-α (6). The N-terminal regulatory domain of λPKC has been found to bind to Ras in a GTP-dependent manner in vitro and λPKC co-immunoprecipitated with Ras-GTP in vivo (37). Therefore, the full-length construct of a dominant interfering mutant of λPKC used in this and other studies (6, 20, 22, 37) could simply function to sequester Ras from interacting productively with other downstream targets. However, cotransfection studies using a mutant construct expressing only a catalytically inactive C-terminal kinase domain of λPKC, unable to interact with Ras-GTP, inhibited MEK1 activation as potently as the full-length dominant negative mutant (6). Consistent with these observations we found that the dominant negative λPKC mutant blocked Ras- but not MEK1-induced ERK activation when assayed by transient overexpression. Together, these results place λPKC upstream of MEK in Ras- and PC-PLC-mediated signaling. However, several attempts to establish clones that coexpressed dnλPKC with aMEK failed. This is not due a blockade of the MEK/ERK pathway since transient transfections of aMEK cells with a dnλPKC expression plasmid did not abolish the GAL-ElkC activity nor did it affect the activation of HA-tagged ERK. This suggests that dnλPKC interferes with the survival of aMEK-transformed cells without affecting the activation of ERK. Interestingly, Moscat and co-workers (61) have recently reported that the atypical PKCs are clearly involved in cell survival. The zinc finger domain of both zPKC and λPKC was shown to interact with the product of the par4 gene that is involved in growth inhibition and induction of apoptosis. The interaction of Par4 with zPKC/λPKC reduced the activity of the kinases dramatically. Furthermore, overexpression of Par4 as well as dnzPKC/dnλPKC in NIH 3T3 cells induced apoptosis, whereas the cells survived when Par4 was coexpressed with the wild-type kinases. Altogether, these find-

FIG. 6. The constitutive down-regulation of the PDGF-β receptor observed in both v-ras and plc-transformed cells does not occur in the dnλPKC-reverted cell lines. A, serum-starved cell cultures were either left untreated or treated with PDGF (10 ng/ml) or thrombin (1.5 units/ml) for 15 min. Total cellular proteins (20 μg per lane) were separated in 7.5% polyacrylamide gels. Following electrophoresis, the membranes were probed with an anti-phosphotyrosine antibody. The membranes were stripped and reprobed with an anti-ERK2 antibody to verify equal protein loading (data not shown). The results are representative of three other independent experiments showing similar results. B, isolated plasma membranes (100 μg of membrane protein) from serum-deprived cell cultures were analyzed for PDGF-β receptor level using an anti-PDGF-β receptor antibody. C, only NIH 3T3 cells and the dnλPKC-reverted cell lines display increased DNA synthesis in response to PDGF. Serum-deprived cell cultures were either left untreated or stimulated with 10 ng/ml PDGF or 10% calf serum and incubated for a further 18 h, with the last 8 h in the presence of [3H]thymidine. The data are expressed as the mean ± S.E. of the counts/min (cpm) of 3 to more than 10 independent experiments.
ings suggest that atypical PKCs have a role in mediating cell survival signals. It has been reported that PI 3-kinase is required for generating survival signals in PC12 cells (69). Interestingly, the kinase activity of αPKC, which is closely related to αPKC, is stimulated in vitro by phosphatidylinositol 3,4,5-triphosphate (46). Furthermore, PI 3-kinase was recently suggested as an in vivo activator of αPKC based on measurements of AP-1 transactivation (17). We found that reversion of Ras- or PC-PLC-transformed cells by stable expression of dnαPKC was accompanied by loss of growth factor-independent AP-1- and NFκB-mediated transactivation (20). Constitutively active mutants of Ras relay signals through several parallel pathways (27, 38, 40, 70). Hydrolysis of phosphatidylinositol has also been reported to be involved in several signaling pathways (27, 28, 70). However, the ERKs are the only known substrates for MEK. Since we were unable to establish cell lines where activated MEK1 and αPKC were stably coexpressed, activation of the ERKs is by itself evidently not sufficient to overcome the apoptotic effect of overexpressing αPKC. We found that the ERK activation in response to PDGF or serum was not blocked in cells coexpressing Ras or PC-PLC and αPKC. Expression levels of dominant interfering mutants that completely block Ras-dependent signaling would clearly be lethal to the cells. Therefore, clones that stably express cytotoxic αPKC as a downstream mediator of PC-derived DAG acts via the ERK1 and -2 MAPK pathway (77), was linked to a CDC28-dependent produc- tion of PC-derived DAG most probably due to activation of a PC-PLC. These findings strongly support our notion outlined above concerning the location of Ras and PC-derived DAG in the mitogenic MAPK pathway and provide evidence that a signaling pathway generally thought of as solely stimulated by membrane-bound receptors is also employed by a cell cycle regulatory kinase in the absence of extracellular stimuli. In light of the conserved nature of MAPK modules and the cell cycle regulatory machinery, future work aimed at elucidating the functions of PC-derived DAG will clearly profit from parallel experimental approaches involving both mammalian and yeast cell systems.

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REFERENCES

1. Waskiewicz, A. J., and Cooper, J. A. (1995) *Curr. Opin. Cell Biol.* 7, 798–805
2. Seger, R., and Krebs, E. G. (1995) *FASEB J.* 9, 726–735
3. Marshall, C. J. (1994) *Curr. Opin. Genet. & Dev.* 4, 82–89
4. Marshall, C. J. (1995) *Cell* 80, 135–138
5. Blenis, J., Santi, S., and Pines, J. (1992) *Mol. Cell. Biol.* 12, 915–927
6. Berra, E., Diaz-Meco, M. T., Lozano, J., Frutos, S., Municio, M. M., Sanchez, P., Sanz, L., and Moscat, J. (1995) *EMBO J.* 14, 6157–6163
7. Lenne, A., Sardet, G., Pages, G., I. Let al., Brunet, G., Aubert, A., and Pouyssegur, J. (1993) *J. Cell Biol.* 122, 1079–1088
8. Sanghera, J. S., Peter, M., Nigg, E. A., and Pelech, S. L. (1992) *Mol. Cell. Biol.* 12, 775–787
9. Pages, G., Lenne, A., G. P., Barthes, J.-C., M. L., and Bouysegur, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 8319–8323
10. Cattin, A., Schaeffer, H.-J., Reuter, C. W. M., Reddy, G. R., and Weber, M. J. (1995) *Mol. Cell. Biol.* 15, 5214–5225
11. Jelinek, T., Catling, A. D., Jelinek, T., and Weber, M. J. (1994) *Mol. Cell. Biol.* 14, 8212–8218
12. Daum, G., Eisenmann-Troppmair, H., Tronnimp, M., and Rapp, U. (1994) *Trends Biochem. Sci.* 19, 474–477
13. Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. L. (1996) *J. Biol. Chem.* 271, 5361–5368
14. Burgers, B. M. T., de Vries-Smith, A. M. M., Medema, H. R., van Weeren, P. C., Tertoolen, L. G. J., and Bos, J. L. (1993) *Mol. Cell. Biol.* 13, 7248–7256
15. Reuter, C. W. M., Catling, A. D., Jelinek, T., and Weber, M. J. (1995) *J. Biol. Chem.* 270, 7644–7651
16. Salmeron, A., Ahmad, T. B., Carfile, G. W., Pappin, D., Narshman, R. P., and Levy, S. C. (1996) *EMBO J.* 15, 817–820
17. Akimoto, K., Takahashi, R., Moriya, S., Nishioka, N., Takayanagi, J., Kimura, K., Fuki, Y., Osada, S.-I., Mizuno, K., Hira, S.-I., Kajulauskas, A., and Ohno, S. (1996) *EMBO J.* 15, 788–798
18. Diaz-Meco, M. T., Dominguez, I., Sanz, L., Dent, P., Lozano, J., Municio, M. M., Berra, E., Hay, R. T., Sturgill, T. W., and Moscat, J. (1994) *EMBO J.* 13, 2842–2848
19. Diaz-Meco, M. T., Municio, M. M., Saneez, P., Lozano, J., and Moscat, J. (1996) *Cell Mol. Biol.* 16, 109–114
20. Bjerck, G. J., Overvarnt, A., Diaz-Meco, M. T., Moscat, J., and Johansen, T. N. (1995) *J. Biol. Chem.* 270, 21299–21306
21. Johansen, T. N., Bjerck, G. J., Overvarnt, A., Diaz-Meco, M. T., Traavi, T., and Moscat, J. (1994) *Mol. Cell. Biol.* 14, 846–854
22. Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J., Chakrapin, R. S., and Moscat, J. (1995) *Cell* 74, 555–565
23. Dominguez, I., Diaz-Meco, M. T., Municio, M. M., Berra, E., Garcia de Herre- ros, A., Cornet, M. E., Sanz, L., and Moscat, J. (1992) *Mol. Cell. Biol.* 12, 3776–3783
24. Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., and Ohno, S. (1994) *J. Biol. Chem.* 269, 12677–12683
25. Sellhe, L. A., Schmitz-Peiffer, C., Sheng, Y., and Biren, T. J. (1995) *J. Biol. Chem.* 269, 24296–24302
26. Diaz-Laviada, I., Pardo-Diez, M. T., Cortez, M. E., Gudall, P. H., Johansen, T., and Moscat, J. (1990) *EMBO J.* 9, 3907–3912
27. Eton, J. H. (1994) *Biochem. Biophys. Acta* 1212, 26–42
28. Latch, J. C., Moscat, J., and Yaffe, B. A. (1997) *Nature* 380, 417–421
29. Lopez-Barahona, M., Kaplan, P. L., Cornez, M. E., Diaz-Meco, M. T., Larr- roder, P., Diaz-Laviada, I., Municio, M. M., and Moscat, J. (1990) *J. Biol. Chem.* 265, 9023–9028
30. Nishizuka, Y. (1995) *FASEB J.* 9, 484–496
31. Price, B. D., Morris, J. D. H., Marshall, C. J., and Cooper, G. M. (1992) *Cell* 69, 1663–1664
32. Wyke, A. W., Cook, J. S., MacNulty, E. E., and Wakelam, M. J. O. (1992) *Cell Signalling* 4, 267–274
33. Cai, H., Erhardt, P., Szczepniewy, J., Diaz-Meco, M. T., Johansen, T., Moscat, J., and Cooper, G. M. (1993) *Mol. Cell. Biol.* 13, 5329–5335
34. Cai, H., Erhardt, P., Tronnimp, J., Diaz-Meco, M. T., Sithanandam, G., Rapp, U. R., Moscat, J., and Cooper, G. (1993) *Mol. Cell. Biol.* 13, 7645–7651
35. Dent, P., and Sturgill, T. W. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 9541–9548
36. Hofer, F., Fields, S., Schneider, C., and Martin, G. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 11089–11093
37. Diaz-Meco, M. T., Lozano, J., Municio, M. M., Berra, E., Frutos, S., Sanz, L.,
and Moscat, J. (1994) *J. Biol. Chem.* 269, 31706–31710

38. Khosravi-Far, R., Solski, P. A., Clark, G. J., Kinch, M. S., and Der, C. J. (1995) *Mol. Cell. Biol.* 15, 6443–6453

39. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) *Science* 260, 1658–1661

40. Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) *Nature* 370, 527–532

41. Spaargaren, M., and Bischoff, J. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 12609–12613

42. Kikuchi, A., Demo, S. D., Ye, Z. H., Chen, Y. W., and Williams, L. T. (1994) *Mol. Cell. Biol.* 14, 7483–7491

43. Nishizuka, Y. (1992) *Science* 258, 607–614

44. Kirstein, M., Sanz, L., Quinones, S., Moscat, J., Diaz-Meco, M. T., and Saus, J. (1996) *J. Biol. Chem.* 271, 18231–18236

45. Xu, J., Zutter, M. M., Santoro, S. A., and Clarke, A. F. (1996) *J. Cell Biol.* 134, 1301–1311

46. Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993) *J. Biol. Chem.* 268, 13–16

47. Westwick, J. K., and Brenner, D. A. (1995) *Methods Enzymol.* 255, 342–359

48. Sjøttem, E., Anderssen, S., and Johansen, T. (1996) *J. Virol.* 70, 188–198

49. Vaziri, C., and Faller, D. V. (1995) *Mol. Cell. Biol.* 15, 1244–1253

50. Sale, E. M., Atkinson, P. G. P., and Sale, G. J. (1995) *EMBO J.* 14, 674–684

51. Smith, D. B., and Johnson, K. S. (1988) *Gene (Amst.)* 67, 31–40

52. Sadowski, I., and Ptashne, M. (1989) *Nucleic Acids Res.* 17, 5539

53. Lillie, J. W., and Green, M. R. (1989) *Nature* 338, 39–44

54. Alesi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* 270, 27489–27494

55. Marais, R., Wynne, J., and Teismann, R. (1993) *Cell* 73, 381–393

56. Gille, H., Strahl, T., and Shaw, P. E. (1995) *Curr. Biol.* 5, 1191–1200

57. Zinck, R., Cahill, M. A., Kracht, M., Sachsenmaier, C., Hipshkind, R. A., and Nordheim, A. (1995) *Mol. Cell. Biol.* 15, 4930–4938

58. Zipper, J.-C., Chambard, J.-C., and Pouyssegur, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 8319–8323

59. Zinck, R., Cahill, M. A., Kracht, M., Sachsenmaier, C., Hipshkind, R. A., and Nordheim, A. (1995) *Mol. Cell. Biol.* 15, 4930–4938

60. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) *Cell* 77, 841–852

61. Diaz-Meco, M. T., Munioz, M. M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L., and Moscat, J. (1996) *Cell* 86, 777–786

62. Quinones, M. A., Mundechau, L. J., Rake, J. B., and Faller, D. V. (1991) *J. Biol. Chem.* 266, 14055–14063

63. Rake, J. B., Quinones, M. A., and Faller, D. V. (1991) *J. Biol. Chem.* 266, 5348–5352

64. Tomasko, L., and Resnick, R. J. (1993) *Biochem. J.* 293, 215–221

65. Gardner, A. M., Vaillancourt, R. R., and Johnson, G. L. (1993) *J. Biol. Chem.* 268, 17896–17901

66. Gupta, S. K., Gallego, C., Johnson, G. L., and Heasley, L. R. (1992) *J. Biol. Chem.* 267, 7897–7900

67. Gonzalez, F. A., Seth, A., Raden, D. L., Bowman, D. S., Fay, F. S., and Davis, R. J. (1993) *J. Cell Biol.* 122, 1089–1101

68. Chen, M., and Cooper, J. A. (1995) *Mol. Cell. Biol.* 15, 4727–4734

69. Yao, B., and Cooper, G. M. (1995) *Science* 267, 2003–2006

70. Joneson, T., White, M. A., Wigler, M. H., and Bar-Sagi, D. (1996) *Science* 271, 510–812

71. Xu, X.-X., Tessner, T. G., Rock, C. O., and Jackowski, S. (1993) *Mol. Cell. Biol.* 13, 1522–1533

72. Wiegmans, K., Schütze, S., Machleidt, T., Witte, D., and Kronke, M. (1994) *Cell* 78, 1005–1015

73. Larrrodera, P., Cornet, M. E., Diaz-Meco, M. T., Lopez-Barahona, M., Diaz-Laviada, I., Guddal, P. H., Johansen, T., and Moscat, J. (1996) *Cell* 61, 1113–1120

74. Diaz-Meco, M. T., Dominguez, I., Sanz, L., Munioz, M. M., Berra, E., Cornet, M. E., Garcia de Herreros, A., Johansen, T., and Moscat, J. (1992) *Mol. Cell. Biol.* 12, 302–308

75. Reynolds, T., Polyak, K., Iavarone, A., and Massague, J. (1995) *Genes Dev.* 9, 1811–1845

76. Marini, N. J., Meldrum, E., Buescher, B., Hubberstey, A. V., Stone, D. E., Trayanor-Kaplan, A., and Reed, S. I. (1996) *EMBO J.* 15, 3040–3052

77. Levin, D. E., and Errede, B. (1995) *Curr. Opin. Cell Biol.* 7, 197–202