Evidence for a Bifurcation of the Mitogenic Signaling Pathway Activated by Ras and Phosphatidylcholine-hydrolyzing Phospholipase C*

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NIH 3T3 cells stably transfected with the gene encoding phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC) from Bacillus cereus display a chronic elevation of intracellular diacylglycerol levels and a transformed phenotype. We have used such PC-PLC-transformed cells to evaluate the roles of the cytoplasmic serine/threonine kinases Raf-1, a protein kinase C (PKC) and protein kinase A (PKA) in oncogenesis and mitogenic signal transduction elicited by phosphatidylcholine hydrolysis. We demonstrate here that stable expression of dominant negative mutants of both PKC and Raf-1 lead to reversion of PC-PLC-transformed cells. Interestingly, expression of kinase defective PKC also reverted NIH 3T3 cells transformed by the v-Ha-ras oncogene. Activation of PKA in response to elevation of cAMP levels also lead to reversion of PC-PLC-induced transformation, implicating PKA as a negative regulator acting downstream of PC-PLC. On the other hand, inhibition or depletion of phorbol ester responsive PKCs attenuated but did not block the ability of PC-PLC-transformed cells to induce DNA synthesis in the absence of growth factors. These results clearly implicate both Raf-1 and PKC as necessary downstream components for transduction of the mitogenic/oncogenic signal generated by PLC-mediated hydrolysis of phosphatidylcholine and suggest, together with other recent evidence, a bifurcation in the signaling pathway downstream of PC-PLC.

Recently, evidence for a crucial role of phospholipase C-mediated hydrolysis of phosphatidylcholine (PC) in mitogenic signaling in different mammalian cells and in the maturation of Xenopus oocytes has accumulated (1–11). In fact, exogenous addition of a phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC) from Bacillus cereus is able to mimic both a significant portion of the mitogenic response to PDGF in Swiss 3T3 fibroblasts and the constitutive activation of protein kinase C (PKC) in v-ras- or v-src-transformed NIH 3T3 cells (5, 12). Furthermore, constitutive expression of the gene (plc) encoding B. cereus PC-PLC leads to a chronic elevated level of intracellular DAG and oncogenic transformation of NIH 3T3 cells (13). Both in Xenopus oocytes and in murine fibroblasts, a variety of experimental approaches have revealed that PLC-mediated hydrolysis of PC, elicited either by the endogenous activity or by the exogenous addition of the bacterial enzyme, is located downstream of Ras (3, 8, 12, 14, 15). It has also been shown that PC-PLC may be involved in coupling Ras to activation of Raf (16). However, the mechanism whereby PC-PLC transduces mitogenic signals conveyed by P31H Ras remains to be elucidated. But, since PC-PLC generates the second messenger diacylglycerol (DAG) capable of activating PKC isoform (17) and downstream targets may include one or more specific PKC isoforms (12, 18, 19) or perhaps other hitherto undetected DAG-regulated kinases. A direct activation of Raf-1 by PC-derived DAG may also be possible since the enzyme contains a reactive thiol in the regulatory domain similar to the DAG binding motifs of PKC and DAG kinase (20). The role of the atypical PKC subtype in mitogenic signal transduction is particularly interesting since it resembles Raf-1 both in terms of structural organization, insensitivity to Ca2+ and phorbol esters, and a ubiquitous expression pattern (21, 22). Furthermore, a requirement for the PKC isotype in the Ras-mediated maturation pathway in Xenopus oocytes and for serum-activated DNA synthesis in mouse fibroblasts has been documented (18, 19), suggesting an important role for PKC in mitogenic signaling. Also, PKC, but not Raf-1, is required for stimulation of the stromelysin promoter via a PDGF/Ras/PC-PLC-responsive element (23).

In the study presented here, we show that dominant negative mutants of both Raf-1 and PKC revert the transformed phenotype of cells stably transfected with the PC-PLC gene (13). Consistent with a downstream location of PC-PLC relative to Ras, we also found that v-Ha-ras-transformed cells acquired a normal, nontransformed phenotype following transfection with a dominant negative mutant of PKC. As recently demonstrated for both v-ras- and v-raf-transformed fibroblasts (24, 25), PKA activation blocked both transformation, and the mitogenic signal elicited by chronic PLC-catalyzed hydrolysis of PC. Taken together, these results clearly show that PC-derived DAG acts upstream of Raf-1 and establish PKC as a novel downstream mediator of Ras/PC-PLC signaling.

MATERIALS AND METHODS

Plasmid Constructs—A PC-PLC expression vector allowing selection of hygromycin-resistant stable transfectants and expression of PC-PLC...
from the metallothionein IIa promoter was made by inserting a 1350-base pair NheI-Xhol fragment from pOPLCmm (13) into NheI-XhoI-cutt pMPE4 (Invitrogen) generating pMT-PLC. For expression of a dominant negative mutant of Raf-1, a 187-base pair EcoRI-Sall fragment from p627 (ATCC 41050), encoding amino acids 1–257 of human c-Raf-1, was cloned into the KpnI and XhoI sites of pMT-hyg yielding pMTΔnfRaf. pMT-hyg was made by deleting a 4134-base pair EcoRV-Stul fragment containing the EBNAl-or p region from pMPE4. In pMTΔnfRaf, the N-terminal regulatory domain of Raf-1 is expressed from the human metallothionein IIa promoter, and the vector also contains a hygromycin resistance gene for selection of stable transfecants. The expression vector for a kinase defective dominant negative mutant of PKC (pRCMV-kmmt) has been described previously (13). The CAT reporter plasmids for assaying AP-1 and NF-kB-mediated transactivation were obtained from A. S. Kekulé and P. H. Hofschneider and contain three copies of a consensus AP-1 binding site or two NF-kB binding sites, respectively, inserted upstream of a minimal herpes simplex virus thymidine kinase promoter (27).

Cell Culture and Generation of 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 supplemented with 10% calf serum (HyClone, Logan, Utah), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc.) in a CO2 incubator (5% CO2) at 37 °C. NIH 3T3 cells transformed by the v-Ha-ras oncogene (4, 12) were grown in the same medium. The cell line representing v-Ha-ras transformed NIH 3T3 cells was established using a gene (plc) encoding PC-PLC from B. cereus, which has been described previously (13). Stably transfected cell lines were established as described previously (13). To establish v-Ha-ras-transformed NIH 3T3 cell lines expressing the dominant negative mutant of PKC, transfection was performed with 3.56 μg of pRCMV-kmt and 0.44 μg of pMT-hyg (91 molar ratio) using Lipofectamine according to the supplier’s (Life Technologies, Inc.) instructions, and G418- and hygromycin-resistant clones were isolated as described previously (13).

Soft Agar Cloning—To assay anchorage-independent growth, 104 cells were mixed into 1 ml of top agarose containing 0.35% SeaPlaque-agarose in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (HyClone, Logan, Utah), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc.) in a CO2 incubator (5% CO2) at 37 °C. NIH 3T3 cells transformed by the v-Ha-ras oncogene (4, 12) were grown in the same medium. The cell line representing v-Ha-ras transformed NIH 3T3 cells was established using a gene (plc) encoding PC-PLC from B. cereus, which has been described previously (13). Stably transfected cell lines were established as described previously (13). To establish v-Ha-ras-transformed NIH 3T3 cell lines expressing the dominant negative mutant of PKC, transfection was performed with 3.56 μg of pRCMV-kmmt and 0.44 μg of pMT-hyg (91 molar ratio) using Lipofectamine according to the supplier’s (Life Technologies, Inc.) instructions, and G418- and hygromycin-resistant clones were isolated as described previously (13).

Determination of Intracellular Levels of DAG—The DAG mass levels of subconfluent, serum-starved (0.5% calf serum for 32 h) cell cultures were determined by using the Amersham DAG assay reagents system kit (RPN 200) as described previously (13).

Activation of Protease—DNA synthesis was determined as described previously (13). PDGF (BB homodimer), 8-bromo-cAMP, activated v-Ha-Ras, and shorterdoublingtimes than our previously described P clones acquired an even more transfection phenotype than our previously described M clones described before (see Figs. 2 and 3, and Table I for specific data). The fact that the P clones closely mimicked the activated v-Ha-Ras—We have recently demonstrated that stable expression of the gene (plc) encoding PC-PLC from B. cereus is oncogenic to NIH 3T3 cells (13). In this study, G418-resistant cell lines expressing the plc gene either from the murine mammary tumor virus long terminal repeat (M clones) or the Rous sarcoma virus long terminal repeat (R clones) promoters were used. To increase our ability to conveniently select doubly transfected cell lines, we have now established NIH 3T3 cell lines transfected with pMT-PLC (see “Materials and Methods”) containing the plc gene under the control of the human metallothionein IIa promoter and carrying a hygromycin resistance gene. This allows the introduction of other genes into the plc transformed cells using expression vectors containing either G418 or hygromycin resistance markers. When PC-PLC was expressed from the metallothionein IIa promoter in pMT-PLC, the cell lines isolated (P clones) acquired an even more transformed phenotype than our previously described plc-transformed cell lines (13). These new cell lines displayed a highly transformed phenotype, similar to v-Ha-ras-transformed cells, as evidenced by increased colony sizes in soft agar, a more potent induction of DNA synthesis in the absence of serum growth factors, and shorter doubling times than our previously reported plc expressing cell lines. This correlated with higher expression levels of PC-PLC as visualized by immunostaining of cells with an affinity-purified antibody raised against B. cereus PC-PLC as described previously (13). For the detection of PKC by immunoblotting, a rabbit anti- Xenopus αPKC antiseraum was used. The C-terminally truncated dnRaf protein was detected with a rabbit polyclonal antibody (K-153) raised against an N-terminal peptide of human Raf-1 (Santa Cruz Biotechnology). Cell extracts were made by adding lysis buffer containing 10 μm Tris-HCl (pH 7.5), 150 m M NaCl, 5 μ M EDTA, 2% Triton X-100, and 50 μ M dithiothreitol, 0.2% SDS, 0.5 mM GTP, 0.5 mM GDP. Eluted nucleotides were separated by thin layer chromatography using PEI-cellulose plates containing fluorescent indicator (Merck) with 1 μ M NaPO4 (pH 3.4) as the running phase. The plates were briefly prewashed in methanol and air dried. Migration of the cold carrier was visualized by UV light (254 nm), and radioactive nucleotides were quantitated using a PhosphorImager (Molecular Dynamics). The results were expressed as (GTP/(GDP + GTP)) × 100.

RESULTS

The Oncogenic Potential of PC-PLC Is Similar to That of Activated v-Ha-Ras—We have recently demonstrated that stable expression of the gene (plc) encoding PC-PLC from B. cereus is oncogenic to NIH 3T3 cells (13). In this study, G418-resistant cell lines expressing the plc gene either from the murine mammary tumor virus long terminal repeat (M clones) or the Rous sarcoma virus long terminal repeat (R clones) promoters were used. To increase our ability to conveniently select doubly transfected cell lines, we have now established NIH 3T3 cell lines transfected with pMT-PLC (see “Materials and Methods”) containing the plc gene under the control of the human metallothionein IIa promoter and carrying a hygromycin resistance gene. This allows the introduction of other genes into the plc transformed cells using expression vectors containing either G418 or hygromycin resistance markers. When PC-PLC was expressed from the metallothionein IIa promoter in pMT-PLC, the cell lines isolated (P clones) acquired an even more transformed phenotype than our previously described plc-transformed cell lines (13). These new cell lines displayed a highly transformed phenotype, similar to v-Ha-ras-transformed cells, as evidenced by increased colony sizes in soft agar, a more potent induction of DNA synthesis in the absence of serum growth factors, and shorter doubling times than our previously reported plc expressing cell lines. This correlated with higher expression levels of PC-PLC as visualized by immunostaining of cells with an affinity-purified antibody raised against B. cereus PC-PLC and a higher level of the intracellular DAG mass than the M clones described before (see Figs. 2 and 3, and Table I for specific data). The fact that the P clones closely mimicked the behavior of v-ras-transformed NIH 3T3 cells may indicate that constitutive expression of PC-PLC leads to activation of critical downstream targets of Ras. Thus, the hygromycin-resistant P18 clone and the previously characterized G418-resistant M1...
clone were selected for further studies aimed at identifying downstream effectors of the mitogenic signal elicited by chronic hydrolysis of PC.

The Transformed Phenotype of NIH 3T3 Cells Constitutively Expressing PC-PLC Is Reverted by Dominant Negative Mutants of Either Raf-1 or βPKC—PC-PLC has been shown to be located downstream of Ras in the insulin-stimulated maturation pathway of Xenopus oocytes (3) and to release NIH 3T3 cells from the block to proliferation imposed by the dominant negative Ras N-17 mutant (14). Since Ras is known to function upstream of Raf-1 in the pathway leading to activation of mitogen-activated protein kinases and it has recently been suggested that activation of an endogenous PC-PLC activity couples Ras to activation of Raf (16), we asked whether Raf was required for plc-mediated transformation of NIH 3T3 fibroblasts. To answer this question, the M1 clone was transfected with a dominant negative Raf mutant (dnRaf), where only the N-terminal regulatory domain of c-Raf-1 (20, 31) was expressed from a vector containing the hygromycin resistance gene, and stable doubly transfected G418- and hygromycin-resistant cell lines were established. Expression of dominant negative mutants of Raf-1 have been shown to cause reversion of both ras- and src-transformed NIH 3T3 cells (32, 33). βPKC has been shown to be required both for maturation of Xenopus oocytes (19) and for the induction of DNA synthesis by serum in murine fibroblasts (18). To determine if this kinase also could be a necessary downstream component in the signaling pathway(s) triggered by PC-PLC action, we transfected P18 cells with a plasmid harboring a G418 resistance gene and encoding a kinase-defective mutant of βPKC (dnβPKC), previously shown to act in a dominant negative manner (18, 26), and doubly transfected cell lines were isolated. The expression of dominant negative Raf-1 and βPKC proteins was verified by immunoblot analysis (Fig. 1). The expression of dnRaf and dnβPKC in M1 and P18, respectively, lead the cells to revert to a flat, contact-inhibited, nontransformed phenotype, even though they still expressed PC-PLC as demonstrated by immunostaining (Fig. 2). Strikingly, of 12 out of 12 M1-dnRaf clones and seven out of eight P18-dnβPKC clones analyzed, all had lost the ability to reinitiate DNA synthesis following serum starvation (data not shown). One P18-dnβPKC clone, which was both G418- and hygromycin-resistant but did not display any reversion of phenotype, did not express dnβPKC (Fig. 1). As seen from Fig. 3, the ability of the plc clones (M1 and P18) to display anchorage-independent growth in soft agar was completely blocked by expression of either the dnRaf- or the dnβPKC mutants. As mentioned above, the soft agar colony size of the P18 clone was significantly larger than the M1 clone. To ensure that the expressed PC-PLC in M1-dnRaf and P18-dnβPKC, shown by immunostaining against PC-PLC (Fig. 2), had retained enzymatic activity, the DAG mass levels in these clones were determined and compared with their respective parental cell lines (Table I). Of note, the DAG levels were still elevated, and even increased from the parental plc-expressing cell lines (Table I). The P18 clone displayed a significantly higher DAG mass level than M1, as compared with the parental NIH 3T3 cells. The P18 clone also showed a shorter doubling time and grew to a higher saturation density in 10% serum than M1. However, by coexpression of either dnβPKC or dnRaf, the doubling times were increased, and the saturation densities were reduced to about the same values as for NIH 3T3 cells and vector control cell lines (Table I). As shown in Fig. 4A, the ability to induce DNA synthesis in the presence of added mitogens was reduced to background levels in the plc clones stably transfected with either of the dominant negative kinase mutants. As a control, stable expression of wild-type βPKC in plc-transformed cells had no effect on induction of DNA synthesis or growth in soft agar (data not shown). By transient transfection assays using CAT reporter plasmids containing either binding sites for the transcription factors NF-κB or AP-1 inserted upstream of a minimal herpes simplex virus thymidine kinase promoter (27), we found that in plc-transformed cells, the transactivation potential of both NF-κB and AP-1 was reduced in the absence of growth factors. However, following stable expression of dnβPKC, these parameters were reduced to background levels (Fig. 4B). Taken together, all of these data strongly suggest that both Raf-1 and βPKC are required for PC-PLC-induced transformation and that both kinases are located downstream of PLC-mediated hydrolysis of PC in the mitogenic signaling cascade.

A Dominant Negative Mutant of βPKC Is Able to Revert v-ras-transformed Cells—Having found that a dominant negative mutant of βPKC reverted plc-transformed cells, we next asked whether expression of this mutant also could cause reversion of cells transformed by v-ras. v-Ha-ras-transformed NIH 3T3 cells were cotransfected with the dnβPKC expression vector and a plasmid carrying a hygromycin resistance gene (see “Materials and Methods”). After selection of stably transfected clones, the expression of dnβPKC was demonstrated by immunoblotting (Fig. 5A). We found that expression of dnβPKC in v-ras expressing cells led to reversion to a flat, contact-inhibited, nontransformed phenotype, although these cells still contained activated Ras as verified by immunoprecipitation of Ras followed by analyses of GTP/GDP ratios in the precipitates (Fig. 5B). The cell doubling time was significantly increased (from 15 to 26 h), the saturation density decreased (2.4-fold), and the ability to induce DNA synthesis in the absence of

Fig. 1. Expression of a kinase defective mutant of βPKC (dnβPKC) and the N-terminal regulatory domain of Raf-1 (dnRaf) in transfected cell lines. A, immunoblot analysis of βPKC overexpression. Cellular proteins (5 μg) were resolved by SDS-polyacrylamide gel electrophoresis, electrophoretically transferred onto an Immobilon P membrane, and incubated with a polyclonal anti-βPKC antibody. The molecular mass of βPKC was estimated to be 65 kDa. Equal protein loading in each well was verified by reprobing the blot with an anti-β-actin antibody (not shown). Note that the P18-dnβPKC-1 cell line was included as a negative control since it showed a transformed phenotype indistinguishable from the parental P18 cell line. B, immunoblot analysis of dnRaf expression in M1. The cells were incubated for 24 h in the presence or absence of 1 μM Cd2+ prior to extraction of cellular proteins. Seventy μg of protein was loaded into each well. The molecular mass of dnRaf was estimated to be 32 kDa. M1 denotes M1 cells stably transfected with the empty expression plasmid. Cd2+ was used to increase the expression of dnRaf from the human metallo-thionein IIa promoter.
growth factors or to form colonies in soft agar was completely abolished (see Fig. 5, C and D). In fact, of 15 independent clones analyzed, all had lost the potent ability of the parental v-ras-transformed cells to induce DNA synthesis in the absence of serum (data not shown). As a separate control, we measured activation of the transcription factors AP-1 and NF-κB by transient transfection assays with CAT reporter constructs in serum-starved v-ras cells expressing dnPKC compared with parental v-ras cells. As evident from Fig. 6, transactivation of the CAT gene via AP-1 or NF-κB binding sites was completely abolished. These results are completely consistent with the notion that αPKC is located downstream of Ras and PC-PLC and serves as a necessary component in Ras-mediated mitogenic signaling and transformation.

The Induction of DNA Synthesis in PLC-transformed Cells Is Largely Independent of Phorbol Ester-responsive PKC—PC-PLC action generates DAG, a potent activator of both classical and novel PKC subtypes (17). In NIH 3T3 cells, only the α subtype of classical PKCs, the δ and ε subtypes of novel PKCs, and the atypical γPKC subtype are expressed. Other PKC isoforms are not expressed at all or at very low levels (34, 35). γPKC is not activated by either phorbol esters or short chain DAGs (22, 36). The αPKC has been shown to phosphorylate Raf-1 both in vitro and in vivo (37, 38), but this does not stimulate the activity of Raf-1 toward its natural substrate mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (39). To investigate the possible contribution of DAG-responsive PKC to the mitogenic signal elicited by PC-PLC, we measured the induction of DNA synthesis in the presence and absence of the novel PKC inhibitor GF 109203X and following depletion of PKC by long term treatment with the phorbol ester TPA (5). GF 109203X is reported to be completely nontoxic and acts as a highly selective PKC inhibitor able to block both classical PKC subtypes and the novel δ and ε subtypes but not γPKC (19, 40–42). As seen from Fig. 7A, blockade of phorbol ester-responsive PKC (αPKC), by either down-regulation or direct inhibition, attenuated somewhat the magnitude of the mitogenic response (25–35% inhibition) of v-ras and plc-transformed cells, indicating that PKC may contribute but that it is by no means essential for mitogenesis. For PDGF-stimulated cells, TPA down-regulation and direct inhibition with GF 109203X produced opposite effects, with down-regulation being somewhat stimulatory and GF 109203X giving 25% inhibition of DNA synthesis. This may reflect the fact that GF 109203X is a less potent inhibitor of the growth inhibitory δ isoform (41) than α- and εPKC (40), whereas long term treatment with TPA will down-regulate all of these three PKC isoforms (5). Together, our results indicate that both PKC-dependent and -independent mechanisms are involved but that phorbol ester-responsive PKCs are not necessary for the induction of DNA synthesis in NIH 3T3 cells elicited by PDGF, activated Ras, or overexpression of PC-PLC. This conclusion is
also supported by the fact that long term treatment of v-Ha-ras- or plc-transformed cells with GF 109203X at concentrations up to 3 μM did not change the transformed phenotype of these cells or abolish their ability to form colonies in soft agar. In fact, the number and sizes of colonies in soft agar showed a similar slight reduction of about 25% as described above for the effect on DNA synthesis (data not shown).

Protein Kinase A Is a Negative Regulator of Mitogenesis Functioning Downstream of PC-PLC—A number of research groups have recently demonstrated that activation of PKA by elevation of cAMP levels blocks the Ras-dependent activation of the mitogen-activated protein kinase pathway (28, 43, 44). Treatments with the adenylate cyclase-stimulating agent forskolin or cAMP analogues such as 8-bromo-cAMP cause reversion of both Ras- and Raf-transformed cells (24, 25). The inhibition by PKA has been shown to be due to direct phosphorylation of Raf (24, 44). On this background and as a further means to investigate the location of PC-PLC action in this pathway, we assayed the effect of forskolin and 8-bromo-cAMP on the induction of DNA synthesis following serum starvation of cells transformed by v-Ha-ras or plc (P18). As shown in Fig. 7B, both forskolin and 8-bromo-cAMP strongly inhibited the mitogenic response in all of these cell lines as well as the PDGF response of parent NIH 3T3 cells. As a control, treatment with 8-bromo-cGMP had no effect. Furthermore, forskolin or 8-bromo-cAMP caused reversion of the transformed phenotype of NIH 3T3 cells expressing PC-PLC (Fig. 7B), while stable transfection of these cells with plasmids expressing dominant negative Raf (M1-dnRaf) or dominant negative PKC (P18-dnPKC) completely abolished their ability to anchor-independent growth.

![Image](303x466 to 557x607)

**FIG. 3.** NIH 3T3 cells expressing PC-PLC form colonies in soft agar, while stable transfection of these cells with plasmids expressing dominant negative mutants of either Raf-1 (M1-dnRaf) or PKC (P18-dnPKC) completely abolished their ability to anchorage-independent growth.

**FIG. 4.** Reversion of the transformed phenotype of P18 cells by stable expression of a dominant negative mutant of PKC correlates both with the loss of ability to induce DNA synthesis in the absence of growth factors and the failure to transactivate reporter plasmids containing binding sites for the transcription factors NF-κB or AP-1 following serum starvation. A, serum-starved NIH 3T3 cells expressing PC-PLC induced DNA synthesis in the absence of added mitogens while transfection of plc clones with either dominant negative Raf-1 (M1-dnRaf) or dominant negative PKC (P18-dnPKC) made the cells quiescent. The magnitude of the mitogenic response is expressed relative to the response to 10% serum, which was set to 100%. The data are expressed as means with standard errors of from three to more than 10 other independent experiments performed in triplicate. M1-vector control denotes a clone isolated from the M1 cell line stably transfected with the empty expression vector pMT-hyg. P18-vector control is the parental P18 cell line stably transfected with the pcCMV expression plasmid and is representative of 13 different isolated clones. B, following serum deprivation, PC-PLC-transformed cells (P18) display constitutive activation of the transcription factors NF-κB and AP-1, while stable expression of a dominant negative mutant of PKC completely blocks this growth factor-independent activation. The CAT activities determined for the parental NIH 3T3 cells were set to 1.0. The data are expressed as the mean ± S.E. for one experiment performed in triplicate and are representative of two other independent experiments with similar results.

**Table 1**

| Cell line | Mean ± SD | Growth in soft agar
| --- | --- | --- |
| --- | --- | --- |
| NIH 3T3 | 1.00 | – |
| Vector control | 1.02 ± 0.11 | 2.2 ± 0.1 |
| M1 | 1.78 ± 0.11 | 1.2 ± 0.1 |
| M1-dnRaf | 2.15 ± 0.22 | 1.4 ± 0.1 |
| P18 | 2.75 ± 0.35 | 2.4 ± 0.2 |
| P18-dnPKC | 3.61 ± 0.27 | 1.2 ± 0.1 |

* Vector control denotes a NIH 3T3 clone stably transfected with the empty expression vector pMEP4.
* The data are expressed as -fold increase compared with control NIH 3T3 cells in more than three independent experiments performed in duplicate.
* Doubling time in 10% serum was determined by counting viable cells every day for 4 days.
* Saturation density was the number of cells in culture 4 days after the cultures reached confluency. P18 was counted 2 days after confluency due to detachment and loss of viable cells by prolonged incubation. The M1 clone started to detach after 4 days, while the parental and reverted cell lines displayed contact inhibition with growth arrest at confluency. The data are expressed as number of cells × 10⁶ and represent three independent experiments performed in duplicate.
* Colony formation in soft agar was scored after 21–28 days.
that PC-PLC is not able to bypass the block to mitogenesis and transformation imposed by PKA activation. This also corroborates the finding that PC-PLC is acting upstream of Raf, which is thought to be the target of the inhibitory action of PKA.

DISCUSSION

A number of recent reports have firmly established that PC hydrolysis is critically involved in growth factor-mediated mitogenic signal transduction (1–11, 14–16). Hydrolysis of PC can be catalyzed either by PC-PLC or PC-PLD (2, 6). However, PC-PLC action seems to be responsible for the sustained increase in cellular DAG levels observed in fibroblasts upon growth factor stimulation or following transformation by v-ras or v-src oncogenes (5, 10, 14, 15). Importantly, PC-PLC has been shown to act downstream of Ras and upstream of the serine/threonine kinase Raf-1 (3, 14–16). Consistent with a critical role of PC-PLC in mitogenic signal transduction, we have recently shown that constitutive expression of the gene (plc) encoding B. cereus PC-PLC leads to transformation of NIH 3T3 cells and that the transformed phenotype is completely dependent on plc expression, resulting in a chronic increase of the cellular DAG mass (13). In the present report, we extend upon these findings and show that there seems to be a direct relationship between the expression level of the bacterial PC-PLC, the resulting DAG levels, and the extent of oncogenic transformation achieved as evaluated by parameters such as cell morphology, growth pattern, cell doubling times, size of soft agar colonies, and induction of DNA synthesis in the absence of growth factor addition. We also show that like v-ras-transformed cells, pclc-transformed cells possess activated AP-1 and v-ras-dnPKC in Ras- and PC-PLC-induced Transformation.

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in the regulatory CR1 domain of ras-1 p74 (16, 20). Alternatively, PC-derived DAG may activate a kinase(s) distinct from phorbol ester-sensitive PKCs, which then can activate Raf by direct phosphorylation (55, 56). Whatever the mechanism, our present results and those of Cai et al. (14, 16) together strongly implicate a crucial role for PC-derived DAG in this mitogenic signaling pathway and place this step downstream of Ras but upstream of Raf. Additional support for this notion is provided by our demonstration that activation of PKA by treatment with forskolin and/or 8-bromo-cAMP reverted the transformed phenotype of plc-transformed cells as evidenced by morphological reversion, loss of ability to form colonies in soft agar and lack of induction of DNA synthesis in the absence of growth factors. Since Raf-1 has been shown to be the target of the inhibitory action of PKA (24, 44), our results with forskolin and 8-bromo-cAMP confirm the downstream location of Raf-1 relative to PC-PLC. Furthermore, we show that plc-transformed cells do not contain elevated levels of GTP-bound Ras. Together with the previous finding that expression of PC-PLC is able to relieve the block to proliferation imposed by expression of the N-17 dominant negative mutant of Ras (14), this strongly supports the conclusion that PC-PLC acts downstream of Ras.

As for Raf-1, the mechanism(s) of activation of the atypical ζ subtype of PKC is not completely understood. However, it is clear that the enzyme is not activated by phorbol esters or short-chain DAGs (22, 36). Recently, it was reported that in vitro ζPKC can be activated by phosphtidylinositol 3,4,5-trisphosphate, a product of PI 3-kinase (57). If this mechanism is active in vivo, a direct link between PI 3-kinase activation following binding to activated tyrosine kinase receptors and stimulation of ζPKC can be envisioned. Interestingly, Ras may contribute to the activation of PI 3-kinase by directly interacting with the catalytic p110 subunit (58, 59). This would place both activation of PI 3-kinase and ζPKC downstream of Ras. There is also evidence that ζPKC is activated in vivo by treatment of NIH 3T3 cells with sphingomyelinase C capable of generating the lipid second messenger ceramide and that ceramide can activate ζPKC in vitro (60). Thus, ζPKC may be regulated by different lipid mediators. Different lines of evidence suggest that PC-PLC is acting upstream of ζPKC (19, 26, 60). Considering the fact that ζPKC is activated by ceramide, the downstream location relative to PC-PLC is completely consistent with the prevalent model for tumor necrosis factor α signaling where an acidic sphingomyelinase C is activated by DAG generated by a membrane-bound PC-PLC (45). As previously shown for Raf-1, it has recently been demonstrated that ζPKC interacts with Ras both in vitro and in vivo and that the in vivo interaction is dependent on GTP-bound active Ras and takes place between the N-terminal regulatory domain of ζPKC and the effector domain of Ras (61). Thus, analogous to Raf, the ζPKC-Ras interaction may serve to bring ζPKC to the membrane where its kinase activity is induced by a lipid mediator(s). This activation is probably direct since, contrary to Raf-1 (56), there is no evidence for a role of phosphorylation/dephosphorylation in the activation of the kinase activity of ζPKC. Thus, the same role for PC-derived DAG proposed for Raf-1 activation above may be applicable to ζPKC, which is similar to Raf-1 in its structural organization. Hitherto, experiments directly addressing binding and activation by physiological relevant PC-derived DAG species have not been performed for these kinases. Alternatively, as outlined above, PC-derived DAG may activate a sphingomyelinase C, which in turn produces ceramide that will directly activate ζPKC.

In view of the proven direct interaction between Ras and ζPKC, our results with expression of the kinase-defective mutant of ζPKC in v-ras cells could be explained as simply due to a blockade of the ability of Ras to interact productively with both ζPKC, Raf-1, and PI 3-kinase as well as other presently unknown downstream targets that may be dependent on binding to the effector domain of activated Ras. However, this model fails to explain why dominant negative mutants of both ζPKC and Raf are able to revert plc-transformed cells since PC-PLC has been convincingly shown, by different experimental approaches, to act downstream of Ras (3, 14–16, this work). Colony formation assays have revealed that cotransfection of activated ζPKC is not able to relieve the block to proliferation of NIH 3T3 cells imposed by a dominant negative Raf-1 mutant. Likewise, an activated form of Raf-1 did not abolish the
growth inhibitory action of a dominant negative PKC mutant (61). Furthermore, expression of dominant negative PKC in NIH 3T3 cells did not inhibit PDGF-stimulated Raf-1 phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1. Also, a kinase defective mutant of PKC, but not a similar mutant of Raf-1, is able to block the activation of the stromelysin promoter mediated through a novel palindromic PDGF, Ras and PC-PLC responsive element (23). Together with our present results, these findings suggest a bifurcation of the mitogenic signaling pathway downstream of PC-PLC with Raf-1 and PKC located on separate branches. Thus, more than one signaling pathway need to be activated in order to bring about mitogenesis or oncogenic transformation.

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