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# NIH 3T3 Cells Stably Transfected with the Gene Encoding Phosphatidylcholine-Hydrolyzing Phospholipase C from *Bacillus cereus* Acquire a Transformed Phenotype

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**In order to determine whether chronic elevation of intracellular diacylglycerol levels generated by hydrolysis of phosphatidylcholine (PC) by PC-hydrolyzing phospholipase C (PC-PLC) is oncogenic, we generated stable transfectants of NIH 3T3 cells expressing the gene encoding PC-PLC from *Bacillus cereus*. We found that constitutive expression of this gene (*plc*) led to transformation of NIH 3T3 cells as evidenced by anchorage-independent growth in soft agar, formation of transformed foci in tissue culture, and loss of contact inhibition. The *plc* transfectants displayed increased intracellular levels of diacylglycerol and phosphocholine. Expression of *B. cereus* PC-PLC was confirmed by immunoperoxidase and immunofluorescence staining with an affinity-purified anti-PC-PLC antibody. The NIH 3T3 clones expressing *plc* induced DNA synthesis, progressed through the cell cycle in the absence of added mitogens, and showed significant growth in low-concentration serum. Transfection with an antisense *plc* expression vector led to a loss of PC-PLC expression accompanied by a complete reversion of the transformed phenotype, suggesting that *plc* expression was required for maintenance of the transformed state. Taken together, our results show that chronic stimulation of PC hydrolysis by an unregulated PC-PLC enzyme is oncogenic to NIH 3T3 cells.**

Phospholipid degradation is potently activated following stimulation of cells with polypeptide growth factors and has been proposed to constitute a critical step in mitogenic signal transduction (3, 14, 28). Most of the work has focused on phosphatidylinositol (PI) turnover (4), but many recent studies have demonstrated the involvement of phospholipase C (PLC)-mediated hydrolysis of phosphatidylcholine (PC) in mitogenic signalling in different mammalian cells as well as in maturation of *Xenopus* oocytes (5, 11, 14, 16, 24, 26, 28, 35, 38, 42, 45). In fact, the exogenous addition of PC-hydrolyzing PLC (PC-PLC) from *Bacillus cereus* is able to cause mimicking of both a significant portion of the mitogenic response to platelet-derived growth factor (PDGF) in Swiss 3T3 fibroblasts and the constitutive activation of protein kinase C (PKC) in *ras*- or *src*-transformed NIH 3T3 cells (9, 26). Furthermore, in *Xenopus* oocytes PLC-mediated hydrolysis of PC was found to be both necessary and sufficient for induction of maturation by insulin or p21$^{ras}$ (16). Activation of PC-PLC has been shown to be a relatively late event located downstream of p21$^{ras}$ in the signalling cascade in both fibroblasts and *Xenopus* oocytes (6, 9, 16, 29, 38). The recent demonstration that PC-PLC is able to bypass the block to proliferation of NIH 3T3 cells resulting from expression of a dominant negative Ha-*ras* mutant indicates PC hydrolysis as a crucial step during transduction of growth factor-initiated mitogenic signals (6). The mechanism whereby PC-PLC transduces mitogenic signals conveyed by p21$^{ras}$ remains to be elucidated. However, since PC-PLC generates the second messenger diacylglycerol (DAG) capable of activating PKC isozymes (34), possible downstream targets may include one or more specific PKC isozymes (2, 9, 12).

In light of the above-mentioned findings, the consequences of unregulated expression of PC-PLC on cell growth and cell transformation are of interest. Here, we report that stable expression of the gene encoding PC-PLC from *B. cereus* causes chronic elevation of the cellular DAG level, leading to transformation of NIH 3T3 cells. Antisense experiments demonstrate that expression of the bacterial PC-PLC gene is both necessary and sufficient for induction and maintenance of the transformed phenotype. Our findings strongly suggest a critical role for PC-derived DAG in mitogenic signalling and lend support to the notion that PC-PLC enzymes are important signal transducers following growth factor stimulation and p21$^{ras}$ activation.

## MATERIALS AND METHODS

**Plasmid constructions.** In order to direct PC-PLC to the endoplasmic reticulum, whose membrane is a rich source of PC, the 24-amino-acid signal peptide and the 14-amino-acid propeptide preceding the mature PC-PLC exoenzyme were removed and replaced with a 21-amino-acid signal peptide from the *ompA* gene of *Escherichia coli*. This signal peptide has the same characteristics as mammalian signal peptides. This was achieved by first introducing a *Bst*NI site at the junction between the propeptide and mature enzyme coding regions by site-directed mutagenesis (22) of pPLC181H (21a) and then inserting a 1.23-kb *Bst*NI (end-filled)–*Eco*RI fragment into *Hin*dIII (end-filled)–*Eco*RI-cut pIN-III*ompA*-*Hind*-(41), generating pES89. pES89 expresses active PC-PLC in *E. coli* (21). A 1.31-kb *Xba*I-*Eco*RI fragment from pES89 was then blunt end cloned into the *Nhe*I (end-filled) site of pMAMneo (Clontech, Palo Alto, Calif.), generating

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pOPLCmam. pRSV-PLC was then constructed by ligating a 2.57-kb *Hin*dIII (end-filled)–*Aat*II fragment from pRSV-CAT (17) to a 5.67-kb *Nhe*I (end-filled)-*Aat*II fragment from pOPLCmam. The antisense vector pMTAS-PLC was constructed by cloning a 692-bp *Nhe*I-*Pvu*II fragment from pOPLCmam into *Nhe*I–*Pvu*II-cut pMEP4 (Invitrogen Corp., Abingdon, United Kingdom). pOPLCmam and pRSV-PLC contain the *neo* gene and pMTAS-PLC harbors the hygromycin resistance gene for selection of stable G418- and hygromycin-resistant clones, respectively.

**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 modified Eagle medium (DMEM) supplemented with 10% calf serum (HyClone, Logan, Utah), penicillin (100 U/ml), and streptomycin (100 μg/ml) (GIBCO) in a $CO_2$ incubator (5% $CO_2$) at 37°C. NIH 3T3 cells transformed by the v-Ha-*ras* or v-*src* oncogene were grown in the same medium supplemented with G418 (GIBCO) at 400 μg/ml (9, 24).

To generate stably transfected cell lines, 50 to 70% confluent cultures in 100-mm-diameter petri dishes were transfected with 10 μg of plasmid DNA purified by using the Magic Maxiprep kit (Promega) and the calcium phosphate coprecipitation method. Two days following transfection, selective medium containing 400 μg of G418 per ml was added. G418-resistant clones were isolated by means of cloning cylinders 10 to 14 days after the addition of selective medium. Transfected clones were maintained in growth medium with G418 at 400 μg/ml (pOPLCmam and pRSV-PLC transfectants) or G418 and hygromycin B (Behring Diagnostics, La Jolla, Calif.) at 300 μg/ml (clones transfected with pMTAS-PLC).

**Soft agar cloning.** To assay anchorage-independent growth, $10^3$ cells were mixed into 1 ml of top agarose containing 0.35% SeaPlaque agarose in DMEM supplemented with 10% calf serum and seeded onto 2 ml of solidified bottom agarose (0.7% SeaPlaque agarose plus medium and serum) in triplicate 35-mm-diameter wells. The top agar was replenished every 4 days. The number of colonies per well and their size distribution (diameter) were scored after 30 days (v-*src* after 21 days). Only colonies with more than 30 cells were counted.

**Determination of intracellular levels of DAG and PCho.** Cells were grown in DMEM containing 10% fetal calf serum (HyClone) for 24 h in 150-mm-diameter petri dishes, washed with phosphate-buffered saline (PBS), and made quiescent by incubation in DMEM with 0.5% serum for 32 h before preparation of extracts for determinations of DAG and phosphocholine (PCho) levels essentially as described by Murray et al. (33). Briefly, cells were scraped in PBS and counted before being pelleted in a microcentrifuge. Then 500 μl of methanol, 250 μl of chloroform, and 200 μl of water were added to the cell pellet. Following thorough mixing, insoluble material was removed by a brief centrifugation. The monophase was transferred to new tubes and separated by the addition of 250 μl of chloroform and 200 μl of water. The DAG mass (picomoles per $10^6$ cells) in the organic phase was determined by the method of Preiss et al. (37) by using the Amersham DAG assay reagents system kit (RPN 200). PCho was extracted from the water phase and quantitated (picomoles per $10^6$ cells) as described previously (33).

**Induction of DNA synthesis and proliferation in low-concentration serum.** For measurements of de novo DNA synthesis, cells were serum starved by incubation for 24 h in 0.1% calf serum and incubated a further 18 h in the presence or absence of added mitogens with the addition of [$^3$H]thymidine (2 μCi/ml) 10 h before harvesting onto glass fiber filters and determination of [$^3$H]thymidine incorporation into DNA by gas-phase scintillation counting. Induction of DNA synthesis was also confirmed by bromodeoxyuridine incorporation and detection of labelled nuclei by immunoperoxidase staining with an antibromodeoxyuridine monoclonal antibody (kit RPN 210; Amersham). When used, PDGF (BB homodimer) (P4306; Sigma) and insulin (I-5500; Sigma) were added to final concentrations of 10 ng/ml and 5 μg/ml, respectively.

For assays of cell proliferation in low-concentration serum, 25,000 cells were seeded per well in 24-well culture plates on fibronectin on day 0 and incubated in DMEM supplemented with 0.5% fetal calf serum for 3 days without medium changes. Viable cell counts were determined by trypan blue exclusion.

**Flow cytometry.** For flow cytometry analysis, serum-starved cells (0.1% fetal calf serum in DMEM for 24 h) were fixed in 70% ethanol and stained with 50 μg of mithramycin (Pfizer, New York, N.Y.) per ml in 25% ethanol containing 15 mM $MgCl_2$ (39).

**Detection of PC-PLC expression by immunoperoxidase and immunofluorescence staining.** Immunoperoxidase staining was performed with methanol-fixed cells by using an affinity-purified antibody raised against *B. cereus* PC-PLC (21a) and developed with swine anti-rabbit immunoglobulin G by employing PAP (DAKO) and Ni(II)-Co(II) enhancement (1).

For immunofluorescence studies, cells were plated on Lab-Tek chamber slides (Nunc) coated with pronectin (Stratagene), incubated in DMEM with 0.1% calf serum for 24 h, washed with PBS, and fixed in 3.5% paraformaldehyde for 30 min at room temperature. After three washes with PBS–0.1 M glycine, the fixed cells were permeabilized for 1 h at room temperature in PBS containing 1% bovine serum albumin, 0.3% Triton X-100, and 10% normal goat serum. Thereafter the cells were incubated for 1 h at room temperature with the anti-PC-PLC antibody. Following five washes with PBS–0.3% Triton X-100, TRITC (tetramethylrhodamine B isothiocyanate)-labeled goat anti-rabbit immunoglobulin G (T-6778; Sigma) was applied at a 1:160 dilution in PBS for 40 min. The cells were then washed five times with PBS and once with water before being mounted in antifader solution (glycerol containing 10% PBS and 1 mg of *p*-phenylenediamine per ml adjusted to pH 8.0 with carbonate buffer [pH 9.9]). The immunofluorescence was analyzed with a Leica confocal laser microscope.

**PCR.** Primers designed to amplify the complete coding region of *plc* were used in PCR with genomic DNA isolated as described previously (18). The following primers were used: 5′-CAAGCTTGGTCTGCTGAAG-3′ and 5′-ACGATCTCCGTACGTATCA-3′. PCR was carried out in 50-μl reaction mixtures containing 100 ng of genomic DNA as a template and 50 pmol of each primer by using a commercially available PCR kit (Perkin-Elmer Cetus) and an MJ Research MiniCycler. An initial denaturation at 94°C for 1 min was followed by 25 cycles with denaturation at 94°C for 20 s, annealing at 58°C for 20 s, elongation at 72°C for 1 min, and a final extension at 72°C for 1 min. PCR products were analyzed on 1.6% MetaPhor (FMC BioProducts, Rockland, Maine) agarose gels.

## RESULTS

**NIH 3T3 cells transfected with a PC-PLC gene acquire a transformed phenotype.** We have previously shown that the

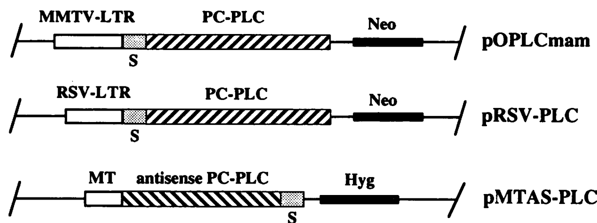


FIG. 1. Schematic representation of expression vectors for PC-PLC (pOPLCmam and pRSV-PLC) and the antisense vector, pMTAS-PLC, for inhibition of PC-PLC expression. In pOPLCmam the PC-PLC gene is placed under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR), while in pRSV-PLC the Rous sarcoma virus long terminal repeat (RSV-LTR) drives the expression of this gene. Both vectors contain the *neo* gene and pMTAS-PLC harbors the hygromycin resistance gene (*hyg*) for selection of stable G418- and hygromycin-resistant clones, respectively. The signal peptide fused to the coding region of the mature PC-PLC enzyme is indicated (S). MT denotes the human metallothionein IIa promoter.

exogenous addition of *B. cereus* PC-PLC to the culture medium of quiescent Swiss 3T3 fibroblasts leads to increased intracellular levels of DAG and PCho, with subsequent induction of DNA synthesis (26). This experimental strategy is not feasible for studies of the long-term effects of chronic stimulation of PC hydrolysis by PC-PLC. To determine whether a permanent elevation of intracellular levels of PC-derived DAG has oncogenic effects, we therefore chose to express the *B. cereus* PC-PLC gene (*plc*) (21a) in NIH 3T3 cells. We constructed two expression vectors, pRSV-PLC and pOPLCmam, containing *plc* under the control of the Rous sarcoma virus and mouse mammary tumor virus promoters, respectively (Fig. 1). The *plc* gene was modified by fusing a signal peptide to the coding region for the mature enzyme in order to ensure efficient direction of the PC-PLC enzyme to the endoplasmic reticulum, which is a rich source of PC (see Materials and Methods). Following transfection of NIH 3T3 fibroblasts with these vectors, stable G418-resistant clones were selected. Six clones transfected with pRSV-PLC (designated R1 to R6) and seven clones transfected with pOPLCmam (designated M1 to M7) were analyzed by soft agar cloning for the ability to display anchorage-independent growth (Table 1). PCR with primers designed to amplify the complete coding region of the PC-PLC gene confirmed the presence of the intact gene in all these clones (data not shown). The *plc* transfectants gave numbers of colonies in soft agar ranging from one-third as many to about the same number as given by 3T3 cells transformed by the v-Ha-*ras* oncogene. However, the maximum colony sizes were significantly smaller than for *ras*- or *src*-transformed cells, suggesting that *plc* induced a less transformed phenotype than these very potent oncogenes. The stable *plc* transfectants all displayed loss of contact inhibition and formed transformed foci in tissue culture. They also showed significantly decreased doubling times and higher saturation densities relative to those of the parent NIH 3T3 cells (Table 2). Because of its activity towards biological membranes, PC-PLC is toxic to 3T3 cells when added to cultures at concentrations above 1 U (15 ng)/ml (26). Since overexpression would be lethal, we selected clones most likely expressing only low levels of the enzyme. This could be an important factor contributing to the less transformed phenotype observed compared with those of *ras*- or *src*-transformed cells. In the M clones *plc* is controlled by a dexamethasone-inducible promoter (Fig. 1). We therefore expected the overexpression of PC-PLC in the presence of dexamethasone to reduce both the number and size of soft agar colonies. This is exactly what we observed for all seven M clones tested (Table 1). The addition of dexamethasone had no significant effect on the soft agar cloning of the noninducible R clones or the *ras*- or *src*-transformed cells (data not shown).

TABLE 1. Colony formation in soft agar of NIH 3T3 clones transfected with pOPLCmam or pRSV-PLC[a]

| Cell line[b] | No. of colonies, mean ± SD[c] | Colony size (mm) |
|---|---|---|
| NIH 3T3 | 1 ± 1 | 0.05–0.06 |
| Neo | 9 ± 1 | 0.05–0.07 |
| v-*src* | 221 ± 7 | 0.05–1.00 |
| v-Ha-*ras* | 177 ± 6 | 0.05–0.70 |
| R1 | 84 ± 6 | 0.05–0.30 |
| R2 | 66 ± 7 | 0.05–0.40 |
| R3 | 115 ± 8 | 0.05–0.24 |
| R4 | 61 ± 3 | 0.05–0.21 |
| R5 | 133 ± 5 | 0.05–0.26 |
| R6 | 82 ± 7 | 0.05–0.26 |
| M1 | 171 ± 8 | 0.05–0.18 |
| M1 (dex.) | 77 ± 8 (55) | 0.05–0.15 |
| M2 | 101 ± 9 | 0.05–0.25 |
| M2 (dex.) | 21 ± 2 (79) | 0.05–0.12 |
| M3 | 53 ± 1 | 0.05–0.30 |
| M3 (dex.) | 21 ± 3 (60) | 0.05–0.14 |
| M4 | 57 ± 2 | 0.05–0.23 |
| M4 (dex.) | 20 ± 2 (65) | 0.05–0.12 |
| M5 | 93 ± 9 | 0.05–0.40 |
| M5 (dex.) | 68 ± 10 (27) | 0.05–0.20 |
| M6 | 60 ± 5 | 0.05–0.31 |
| M6 (dex.) | 41 ± 7 (32) | 0.05–0.25 |
| M7 | 93 ± 11 | 0.05–0.24 |
| M7 (dex.) | 58 ± 10 (38) | 0.05–0.15 |
| M1-AS6 | 0 | |
| M1-AS11 | 0 | |
| M2-AS6 | 4 ± 1 | 0.05–0.08 |

[a] The data are the results for three wells per experiment. Each experiment was repeated at least twice.

[b] All cell lines were shown by PCR to contain the complete *plc* gene. A pool of control cell lines established by transfection with pMAMneo are designated Neo. Cell lines designated M and R were established by transfection of NIH 3T3 cells with pOPLCmam and pRSV-PLC, respectively. "dex." indicates transfectants which were plated in soft agar with 0.2 μM dexamethasone. AS clones were selected as hygromycin and G418 resistant following transfection of M1 and M2 clones with the antisense vector pMTAS-PLC.

[c] The percent reduction in the number of soft agar colonies in the presence of dexamethasone is shown in parentheses for pOPLCmam transfectants. Dexamethasone showed no effect on the other cell lines.

**Cell lines stably transfected with the PC-PLC gene display increased intracellular levels of DAG and PCho.** Hydrolysis of PC by PC-PLC generates the second messenger DAG, which is capable of activating PKC (34), and the cellular metabolite PCho. An important consequence of expression of the bacterial PC-PLC gene essential for producing a transformed phenotype is a constitutive increase in the cellular DAG level. As seen from Table 3, the DAG mass levels were increased almost twofold in serum-starved *plc* transfectants compared with the levels in parent NIH 3T3 and vector-transfected cells. In fact, the DAG levels were almost as high as in *src*-transformed cells and at about the same level as in v-Ha-*ras*-transformed NIH 3T3 cells (9). As further support for expression of PC-PLC activity, the PCho levels of the *plc* transfectants were elevated three- to fourfold compared with the levels in the controls. This is essentially the same as

TABLE 2. Growth properties of cells expressing *plc*[a]

| Cell line[b] | Mean ± SD | |
|---|---|---|
| | Doubling time (h)[c] | Saturation density ($10^6$ cells)[d] |
| NIH 3T3 | 24.0 ± 0.5 | 1.1 ± 0.1 |
| M1 | 19.5 ± 1.5 | 2.2 ± 0.2 |
| M2 | 17.0 ± 1.5 | 2.3 ± 0.2 |
| M1-AS11 | 24.0 ± 0.5 | 1.0 ± 0.1 |

[a] Two independent experiments were performed in triplicate. A total of $4 \times 10^4$ cells were plated per 35-mm-diameter culture dish in DMEM supplemented with 10% fetal calf serum. Medium was changed every other day.

[b] Clones M1 and M2 displayed multilayered growth and formation of transformed foci. Incubations longer than 4 days led to a loss of cells due to detachment of cell aggregates containing viable cells for these two transformed cell lines, while the parent NIH 3T3 cells and the clone M1-AS11 (a hygromycin- and G418-resistant clone isolated following transfection of M1 cells with the antisense *plc* vector pMTAS-PLC) displayed contact inhibition with monolayer growth arrested at confluency.

[c] Doubling time was determined by counting cells every day for 4 days.

[d] Saturation density was the number of cells in culture 4 days after the cultures reached confluency.

TABLE 3. DAG and PCho levels in serum-starved cells[a]

| Cell line[b] | Mean ± SD | |
|---|---|---|
| | DAG level | PCho level[c] |
| NIH 3T3 | 1.00 | 1.00 |
| Neo | 1.06 ± 0.11 | 1.01 ± 0.21 |
| M1 | 1.78 ± 0.11 | 3.77 ± 0.20 |
| M2 | 1.99 ± 0.08 | 3.93 ± 0.16 |
| M3 | 1.56 ± 0.06 | 2.95 ± 0.14 |
| M5 | 1.62 ± 0.10 | 3.80 ± 0.07 |
| M1-AS1 | 0.90 ± 0.10 | 0.95 ± 0.21 |
| M2-AS6 | 0.99 ± 0.12 | 0.94 ± 0.31 |
| v-*src* | 2.66 ± 0.38 | ND |

[a] The data are expressed as fold increase compared with control NIH 3T3 cells in more than three independent experiments performed in duplicate.

[b] Neo represents a pool of vector-transfected cell lines. AS lines were selected as hygromycin- and G418-resistant clones following transfection of M1 and M2 clones with the antisense vector pMTAS-PLC. For the NIH 3T3 control, the mean DAG mass level was 490 pmol/$10^6$ cells and the mean PCho level was 105 pmol/$10^6$ cells.

[c] ND, not determined.

previously observed for cells transformed with the activated Ha-*ras* oncogene (29, 38).

Our PC-PLC expression constructs contained a signal peptide fused to the coding sequence of the mature enzyme in order to direct the enzyme to the endoplasmic reticulum and possibly also other intracellular membranes. Immunoperoxidase staining with an affinity-purified antibody raised against homogeneously pure *B. cereus* PC-PLC (21a) showed cytoplasmic staining of *plc*-transfected clones (Fig. 2B). Furthermore, strong perinuclear staining, indicative of localization of PC-PLC to the endoplasmic reticulum, was revealed by immunofluorescence studies using confocal laser microscopy (Fig. 2D).

**The PC-PLC-transfected cell lines induce DNA synthesis and progress through the cell cycle in the absence of added mitogens.** We have previously shown that the exogenous addition of *B. cereus* PC-PLC to quiescent 3T3 fibroblasts elicits a potent mitogenic response and that insulin enhances this response synergistically (26). As seen from Fig. 3A, serum-starved *plc* transfectants induced DNA synthesis in the absence of added mitogens at a magnitude varying from 13 to 28% of that of 10% serum, while the parent 3T3 cells were quiescent following a 24-h incubation in 0.1% serum. The addition of insulin, which by itself is not a potent mitogen for NIH 3T3 cells (15, 26), resulted in a twofold increase in DNA synthesis for the *plc* transfectants. For example, for clone M3 this amounted to 50% of the response obtained with 10% serum. The addition of PC-PLC to the clones did not increase the number of cells initiating DNA synthesis in 0.1% serum, while it elicited a mitogenic response in control 3T3 cells similar to that of the clones in the absence of added mitogen (data not shown).

To determine whether serum-starved *plc* transfectants also progressed through the cell cycle in the absence of added mitogens, we performed flow cytometry analyses of clones M2 and M5. As seen from Fig. 3B, a significant fraction of the cell population was able to complete the cell cycle following serum starvation. Furthermore, clones expressing the PC-PLC gene showed significant cell proliferation in low-concentration serum compared with that of the parent 3T3 cells. As found for the induction of DNA synthesis, the addition of insulin stimulated this ability (Fig. 3C).

**Transfection with an antisense *plc* vector reverses the transformed phenotype.** In order to determine whether *plc* expression was necessary and sufficient for maintenance of the transformed phenotype, we transfected clones M1 and M2 with an antisense construct (Fig. 1) containing part of the *plc* gene in inverted orientation downstream of the human metallothionein IIa promoter in a vector containing a dominant selectable hygromycin resistance gene. The stably G418- and hygromycin-resistant antisense clones lost the abilities both to form colonies in soft agar (Table 1) and to induce DNA synthesis in the absence of added mitogens (Fig. 3D). These clones displayed mitogenic responses to PDGF and 10% serum of the same magnitudes as those of the parent NIH 3T3 cells, indicating no nonspecific effects of the antisense construct as such (data not shown). Strikingly, the antisense clones also reverted to a flat, contact-inhibited, nontransformed phenotype correlating with the loss of PC-PLC expression as evidenced by immunoperoxidase staining with the anti-PC-PLC antibody. Immunofluorescence studies using confocal laser microscopy also confirmed the lack of PC-PLC expression (Fig. 2C and E). This was corroborated by the fact that the DAG and PCho levels of the antisense clones were reduced to control levels (Table 3). As illustrated by clone M1-AS11, the antisense clones displayed doubling times and saturation densities similar to those of normal NIH 3T3 cells (Table 2). PCR analysis of DNA isolated from the antisense clones showed that the loss of PC-PLC expression was not due to deletion of the *plc* gene during establishment of these clones (Fig. 4). Since these clones were selected and maintained in medium containing both G418 and hygromycin, deletion of *plc* without concomitant loss of the G418 resistance gene is rather unlikely. Taken together, these results strongly suggest that the transformed phenotype is dependent on constitutive *plc* expression and is not the result of secondary events occurring during and after establishment of the stable transfectants.

## DISCUSSION

A number of recent studies suggest that PC hydrolysis is critically involved in mitogenic signal transduction in response to stimulation by polypeptide growth factors and oncogenic Ras proteins (5, 6, 11, 14, 16, 24, 26, 28, 35, 38, 45). Particularly, PC-PLC rather than PC-PLD seems to be responsible for a sustained increase in the cellular DAG level

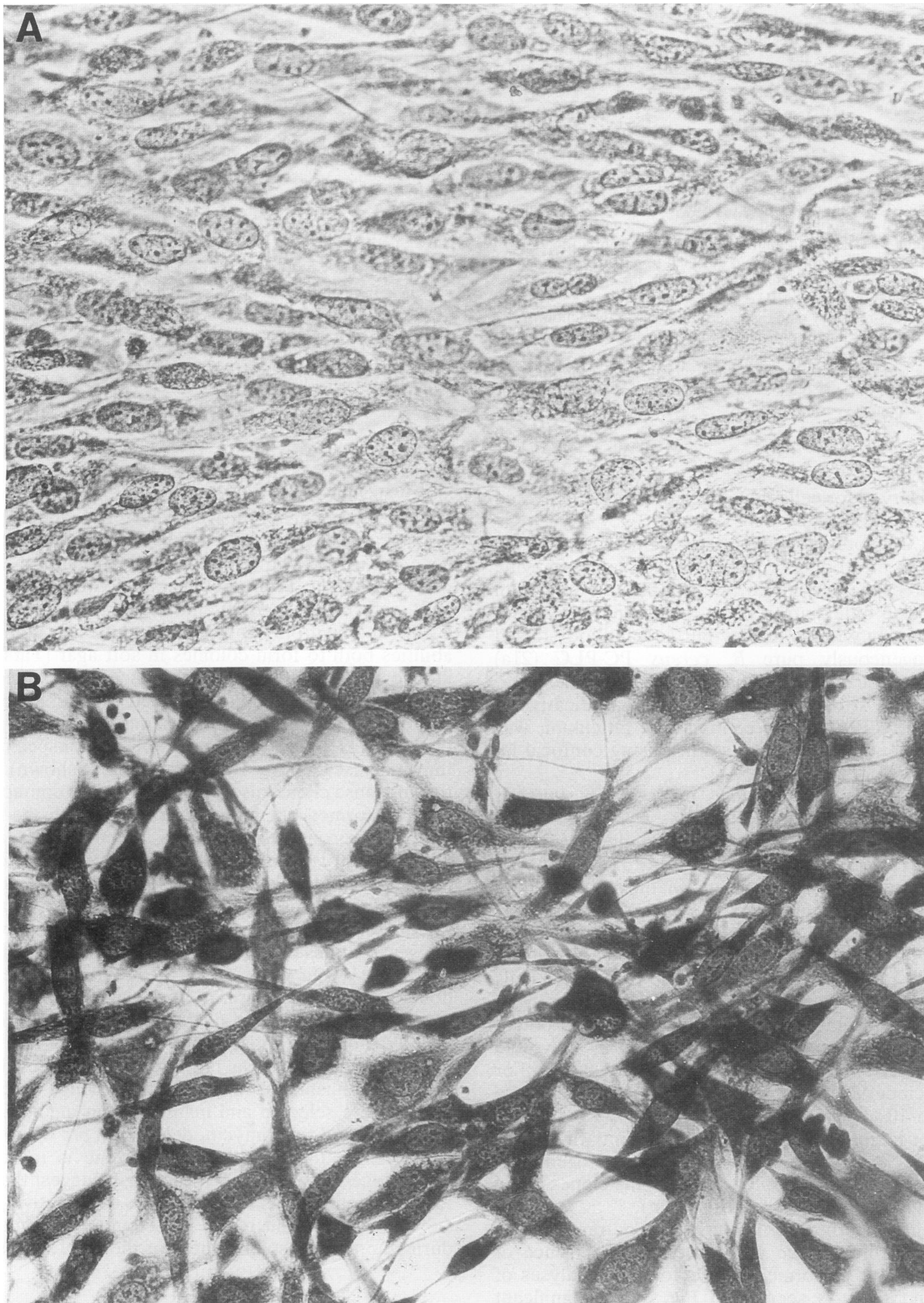


FIG. 2. Visualization of PC-PLC expression by immunoperoxidase and immunofluorescence staining with an affinity-purified anti-PC-PLC antibody. Immunoperoxidase staining of control NIH 3T3 cells (A) and NIH 3T3 cells transfected with pOPLCmam (clone M1) (B) is shown. (C) Abolishment of PC-PLC expression following introduction of the antisense vector pMTAS-PLC into clone M1. Note the transformed morphology of the PC-PLC-expressing cells versus the flat, normal morphology of the cells following introduction of the antisense vector. Magnification, ×400. (D and E) Confocal laser microscopy of clone M1 (D) and of the antisense clone (E) showing the perinuclear localization of PC-PLC in clone M1 and the lack of immunofluorescence signal after transfection of clone M1 with pMTAS-PLC. Scale bars, 10 μm.

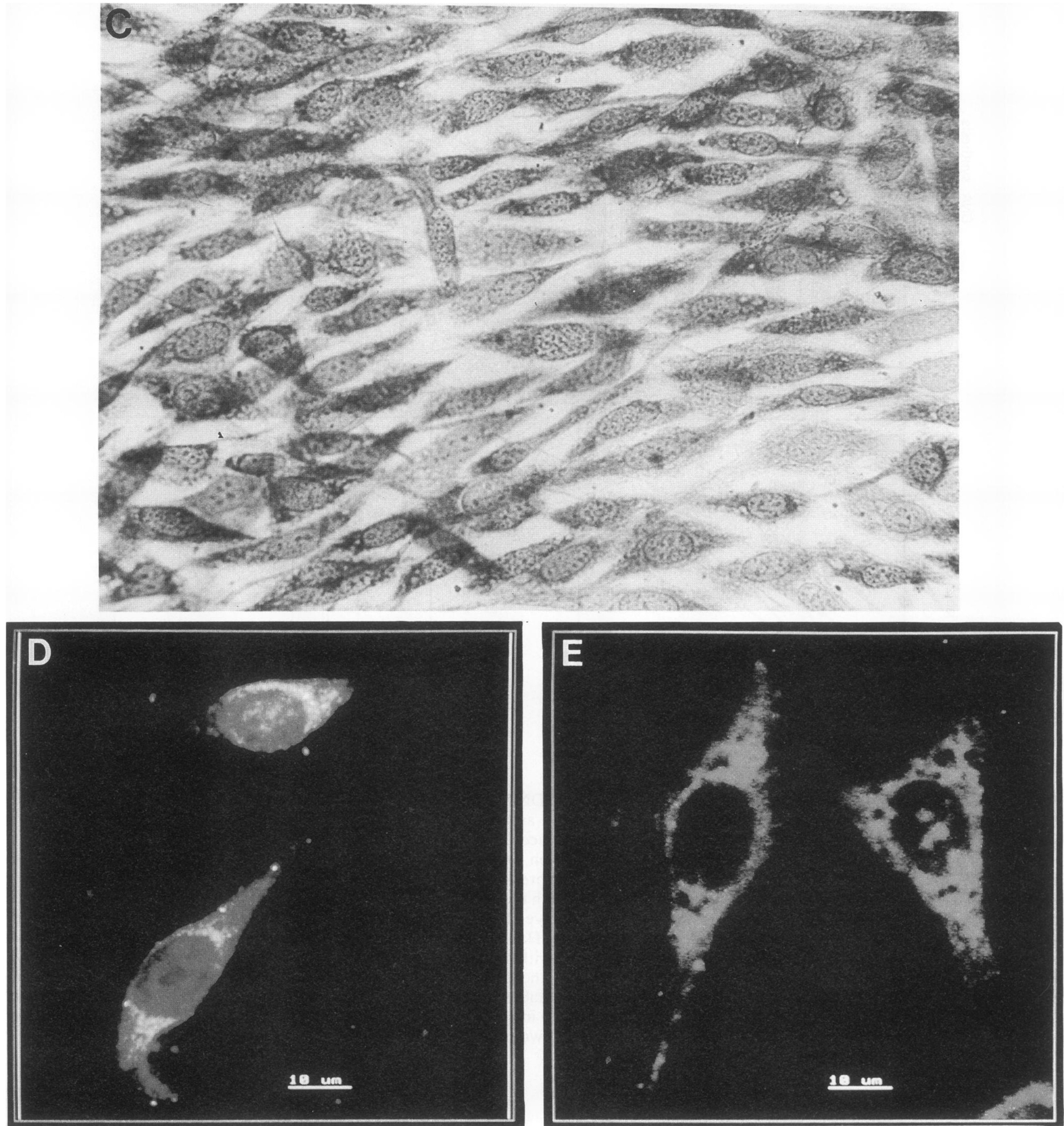


FIG. 2—*Continued.*

preceding the induction of DNA synthesis in fibroblasts and the maturation of *Xenopus* oocytes following stimulation with growth factors activating protein tyrosine kinase receptors (16, 26). The PC-PLC activity involved is poorly characterized; no enzymes have been purified and no genes have been cloned. We and others have therefore in previous studies used the very-well-characterized PC-PLC from *B. cereus* (20, 21a) to mimic the effects of PC-PLC activation in 3T3 fibroblasts, a murine macrophage cell line, and *Xenopus* oocytes (6, 10, 11, 16, 26, 45). In this report we demonstrate that stable expression of the gene (*plc*) encoding PC-PLC from *B. cereus* is oncogenic to NIH 3T3 cells. Maintenance of the transformed phenotype is dependent on expression of PC-PLC resulting in increased intracellular levels of DAG and PCho. PCho is a major cellular metabolite, which makes it unlikely to be a mediator of the oncogenic effect of PC-PLC expression. DAG, on the other hand, is an established second messenger capable of activating PKC isozymes (34) and perhaps also other protein kinases. We therefore suggest that a permanent elevation of the intracel-

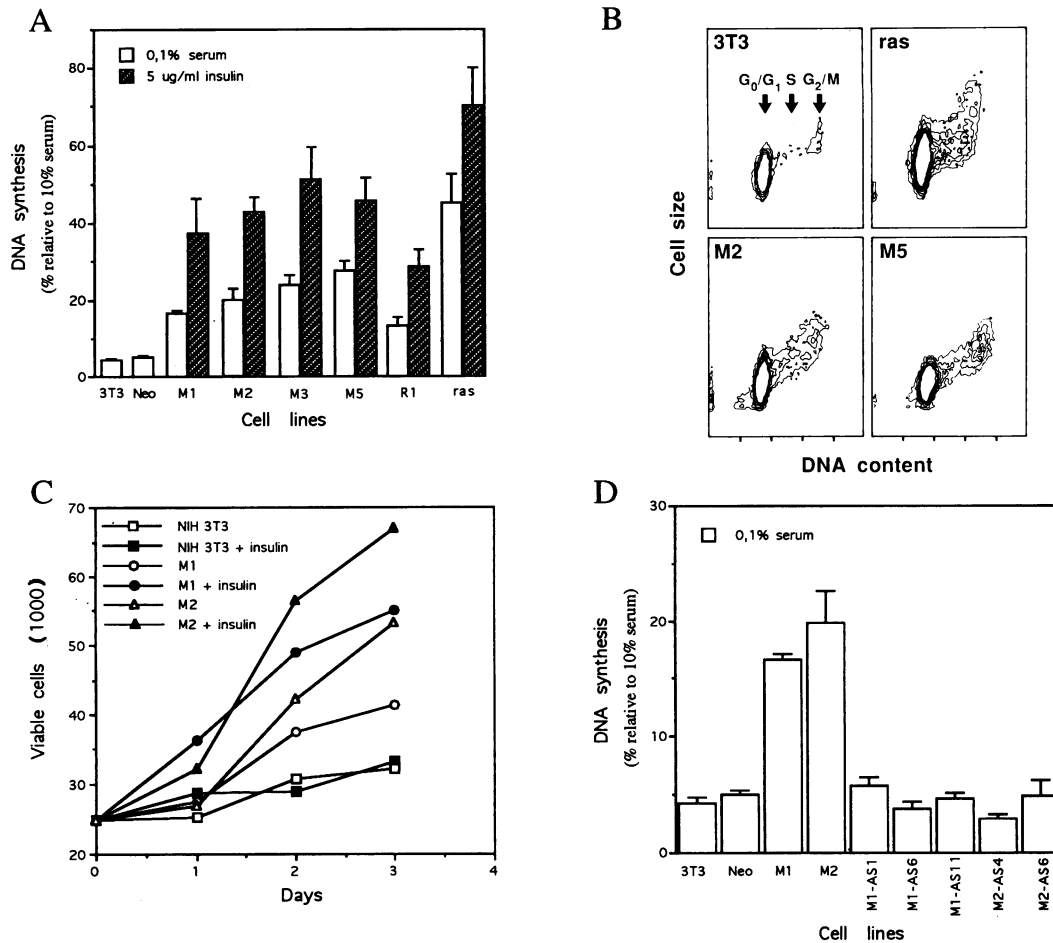


FIG. 3. Serum-starved NIH 3T3 cells expressing PC-PLC induce DNA synthesis in the absence of added mitogens, progress through the cell cycle, and grow in low-concentration serum. Transfection with an antisense vector shows that the mitogenic effect is dependent on expression of PC-PLC. (A) Induction of DNA synthesis in the absence (open bars) or presence (hatched bars) of insulin as determined by [$^3$H]thymidine incorporation in four clones established by transfection with pOPLCmam (M1, M2, M3, and M5) and one clone transfected with pRSV-PLC (R1). The magnitude of the mitogenic response is expressed relative to the response to 10% serum and compared with those of parent NIH 3T3 cells, a pool of clones (Neo) stably transfected with the vector pMAMneo, and v-Ha-*ras*-transformed NIH 3T3 cells. The data are the means for three wells per experiment and are representative of four other independent experiments. (B) Bivariate flow cytometric contour plots of the *plc* transfectants M2 and M5, NIH 3T3, and v-Ha-*ras*-transformed 3T3 cells. (C) Proliferation of *plc* transfectants in low-concentration serum (DMEM containing 0.5% fetal calf serum) with or without the addition of insulin. A total of $2.5 \times 10^4$ cells per well were plated on fibronectin on day 0 and incubated for 3 days without medium changes. The results are the means of triplicates and representative of two other independent experiments. (D) Clones established from the PC-PLC-expressing cell lines M1 and M2 following transfection with the antisense vector pMTAS-PLC lose the ability to induce DNA synthesis in the absence of added mitogens. The data are the means for three wells per experiment and are representative of two other independent experiments.

lular level of PC-derived DAG is by itself oncogenic. In this context it is particularly noteworthy that overexpression of PI-PLC-γ is not mitogenic to NIH 3T3 cells (8, 31). Furthermore, evidence suggesting that activation of PI turnover is neither necessary nor sufficient for induction of DNA synthesis is accumulating (7, 8, 13, 19, 31, 32, 36). It has been established that distinct molecular species of DAG are generated by PC-PLC and PI-PLC (14, 27, 35). It also seems clear that while PI-derived DAG is only transiently (lasting for minutes) elevated following stimulation of protein tyrosine kinase receptors, a sustained (lasting for hours) increase of PC-derived DAG can be observed (reviewed in reference 28). Lee et al. (reference 27 and references therein) have suggested that PC-derived DAGs are mainly generated from PC in internal (endoplasmic reticulum or nuclear) membranes, whereas PI-derived DAGs are generated in the plasma membrane. DAG generated from PC is not phosphorylated to phosphatidic acid by the plasma membrane-bound DAG kinase, while PI-derived DAG is rapidly phosphorylated by this enzyme (30, 44). This could contribute to the sustained increase seen for PC-derived DAG relative to PI-derived DAG following growth factor stimulation. Our strategy of targeting the bacterial PC-PLC to the endoplasmic reticulum should also then closely mimic the consequences of permanently activating the endogenous PC-PLC. In a previous study we have shown that *ras*- and *src*-transformed cells contain constitutively elevated levels of DAG and permanently activated PKC. Furthermore, the lack of downregulation of PKC seen in these cells was able to be mimicked after chronic treatment (for 24 h) of normal fibroblasts with exogenous PC-PLC, while such treatment with a PI-PLC led to downregulation of PKC (9). It thus

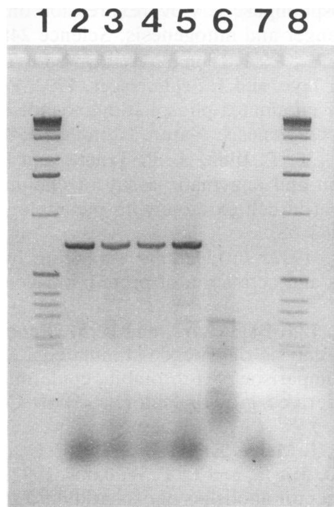


FIG. 4. PCR analyses of DNA isolated from clones M1 and M2 (lanes 2 and 3) and the corresponding antisense clones M1-AS11 and M2-AS6 (lanes 4 and 5) demonstrate the presence of the complete coding region of *plc* (741-bp PCR product) in the G418-hygromycin-double-resistant antisense clones. Results of a PCR with DNA from a pool of vector-transfected cell lines are shown in lane 6, and in lane 7 no template DNA was added. Lanes 1 and 8 contain the 1-kb ladder size marker (Bethesda Research Laboratories). The size markers migrating in front of and behind the 741-bp *plc* product are 516 and 1,018 bp, respectively. A negative image of an ethidium bromide-stained 1.6% agarose gel is shown. One-tenth of each of the PCR mixtures was loaded on the gel.

seems plausible that PC-derived DAG displays a functionality towards PKC subspecies different from that shown by PI-derived DAG. NIH 3T3 fibroblasts have been shown to contain only the $\alpha$ and $\zeta$ subspecies of PKC, and while PKC-$\alpha$ is downregulated by treatment with phorbol esters, PKC-$\zeta$ is not (43). PKC-$\zeta$ also seems to be critically involved in the maturation of *Xenopus* oocytes induced by insulin, $p21^{ras}$, or PC-PLC, as well as in the induction of DNA synthesis by serum in Swiss 3T3 fibroblasts (2, 12). The mitogenic responses to both PDGF and PC-PLC are independent of phorbol-ester-sensitive PKC, suggesting that activation of PKC-$\zeta$ is a likely downstream event following PC-PLC activation. Another potential downstream target following PC-PLC activation is the proto-oncogene product Raf-1 (40). Both PKC-$\zeta$ and Raf-1 kinase may be involved in relaying the mitogenic signal following activation of PC-PLC by $p21^{ras}$ to the mitogen-activated protein kinase pathway (23, 25). Future studies concerning the role of PC-PLC activation in mitogenic signalling triggered by growth factors stimulating protein tyrosine kinase receptors will have to address how PC-PLC is activated by $p21^{ras}$ as well as the nature of the direct downstream target(s) for activation by PC-derived DAG.

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