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Phosphatidylcholine Hydrolysis and c-*myc* Expression Are in Collaborating Mitogenic Pathways Activated by Colony-Stimulating Factor 1

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Stimulation of diglyceride production via phospholipase C (PLC) hydrolysis of phosphatidylcholine was an early event in the mitogenic action of colony-stimulating factor 1 (CSF-1) in the murine macrophage cell line BAC1.2F5 and was followed by a second phase of diglyceride production that persisted throughout the G_1 phase of the cell cycle. Addition of phosphatidylcholine-specific PLC (PC-PLC) from *Bacillus cereus* to the medium of quiescent cells raised the intracellular diglyceride concentration and stimulated [^{3}H]thymidine incorporation, although PC-PLC did not support continuous proliferation. PC-PLC treatment did not induce tyrosine phosphorylation or turnover of the CSF-1 receptor. The major protein kinase C (PKC) isotype in BAC1.2F5 cells was PKC-δ. Diglyceride production from PC-PLC did not target PKC-δ, since unlike phorbol esters, PC-PLC treatment neither decreased the electrophoretic mobility of PKC-δ nor increased the amount of GTP bound to Ras, and PC-PLC was mitogenically active in BAC1.2F5 cells in which PKC-δ was downregulated by prolonged treatment with phorbol ester. PC-PLC mimicked CSF-1 action by elevating c-*fos* and *junB* mRNAs to 40% of the level induced by CSF-1; however, PC-PLC induced c-*myc* mRNA to only 5% of the level in CSF-1-stimulated cells. PC-PLC addition to CSF-1-dependent BAC1.2F5 clones that constitutively express c-*myc* increased [^{3}H]thymidine incorporation to 86% of the level evoked by CSF-1 and supported slow growth in the absence of CSF-1. Therefore, PC-PLC is a component of a signal transduction pathway leading to transcription of c-*fos* and *junB* that collaborates with c-*myc* and is independent of PKC-δ and Ras activation.

Colony-stimulating factor 1 (CSF-1) is a growth factor that is required for differentiation, proliferation, and survival of cells of the mononuclear phagocyte lineage (64). The biological effects of CSF-1 are mediated by its binding to a single class of high-affinity receptors (CSF-1R) (26). CSF-1R is encoded by the c-*fms* proto-oncogene (61) and consists of an extracellular ligand-binding domain linked to an intracellular protein tyrosine kinase domain by a single transmembrane helix (13). CSF-1R is similar to the platelet-derived growth factor (PDGF) receptor and the c-*kit* proto-oncogene in that the extracellular domain consists of five immunoglobin-like loops and the cytoplasmic tyrosine kinase domain is interrupted by a unique kinase insert sequence (69). CSF-1 binding triggers dimerization of the receptor (37, 38, 48), autophosphorylation of CSF-1R on tyrosine (54), and stimulation of its tyrosine kinase activity (19, 75). Autophosphorylation of CSF-1R promotes its interaction with cytoplasmic proteins that activate multiple signal transduction pathways (60, 70) that culminate in a wave of immediate-early gene (c-*fos*, *junB*, c-*myc*, etc.) expression (18, 28, 49, 58). In addition, CSF-1 is required throughout the G_1 stage of the cell cycle (68), indicating that subsequent CSF-1-dependent signalling pathways are required for production of key proteins later in the G_1 phase that ultimately determine the commitment to DNA synthesis.

Stimulation of phospholipid catabolism is closely associated with activation of growth factor receptors, although the importance of these events in the signal transduction cascade is enigmatic. Autophosphorylation of tyrosine residues within the kinase insert domain leads to association of the phosphatidylinositol 3′-kinase regulatory subunit with the receptor, its phosphorylation on tyrosine, and production of phosphatidylinositol 3′-phosphate (52, 53, 63, 71). However, CSF-1 induces cell proliferation in CSF-1R mutants lacking the entire kinase insert domain (66). These mutants are severely deficient in the ability to bind phosphatidylinositol 3′-kinase (52, 63), suggesting that this pathway is not required for mitogenesis. Breakdown of phosphatidylinositol 4,5-bisphosphate plays an important role in the action of a number of growth factors (6, 41). However, analyses of several CSF-1-responsive cell types have failed to detect enhanced inositol phospholipid turnover in response to CSF-1 (28, 32, 74), and CSF-1R neither associates with nor phosphorylates phospholipase Cγ1 (18). Hydrolysis of phosphatidylcholine (PtdCho) by a specific phospholipase C (PC-PLC) or phospholipase D (PC-PLD) is a potential alternate source for diglyceride generation (4, 22). CSF-1 elevates intracellular concentrations of diglyceride derived from PtdCho in human monocytes (32), bone marrow-derived macrophages (72), and a fibroblast cell line expressing CSF-1R (12). PC-PLC activity is precipitated by anti-phosphotyrosine antibodies following activation of CSF-1R, indicating that tyrosine phosphorylation is associated with PC-PLC activation in a manner similar to that observed for phosphatidylinositol 3′-kinase and phospholipase Cγ1 (11). However, deletion of the kinase insert region from CSF-1R does not diminish the extent of CSF-1-activated PtdCho hydrolysis, indicating that a different region of the receptor is involved in transducing the signal to PC-PLC (12). Enhanced PtdCho hydrolysis is not confined to CSF-1-responsive cells but is also triggered by other growth factors, such

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as PDGF (35), granulocyte-macrophage colony-stimulating factor (73), interleukin 1 (IL-1) (56), and IL-3 (21). The potential importance of PC-PLC in cell growth is supported by the finding that treatment of fibroblasts with exogenous PC-PLC mimics their mitogenic response to serum and PDGF (35). The goal of the present work was to investigate the participation of PC-PLC in the mitogenic response of a macrophage cell line to CSF-1 by using exogenous PC-PLC to mimic the stimulation of intracellular diglyceride accumulation by CSF-1 and to monitor the immediate-early responses to diglyceride.

MATERIALS AND METHODS

Materials. Sources of supplies were DuPont-New England Nuclear for [^{32}P]dCTP (3,000 Ci/mmol), [^{32}P]ATP (3,000 Ci/mmol), carrier-free $^{32}P_i$, and [^{3}H]thymidine (84.2 Ci/mmol); Amersham Corp. for ^{125}I-labelled protein A (35 mCi/mg) and 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (171 Ci/mmol); Lipidex for a diglyceride kinase assay kit; Boehringer Mannheim for *Bacillus cereus* PC-PLC (grade I), *B. cereus* phosphatidylinositol-specific PLC, cabbage PC-PLD, *B. cereus* sphingomyelinase, and a random-primer DNA-labelling kit; Bio 101 for a Gene-Clean DNA extraction kit; Sigma for dioctanoylglycerol, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), diolein, phosphocholine, and phosphoethanolamine; Oncogene Science for anti-Ras antibody Y13-259; Calbiochem for rabbit immunoglobulin G (IgG) antibodies directed against protein kinase C (PKC) isozymes PKC-α, PKC-β1, PKC-γ, PKC-δ, PKC-ε, and PKC-ζ; UBI for polyclonal anti-pan-PKC IgG (reacts with the PKC-α, PKC-β and PKC-γ isotypes); Pierce Chemical Co. for rabbit anti-mouse IgG and protein A-Sepharose beads; Hyclone for fetal bovine serum (FCS); GIBCO for Dulbecco modified Eagle medium (DMEM) and other tissue culture supplies; and Analtech for Silica Gel G thin-layer chromatography plates. Homogeneous human recombinant CSF-1 was kindly proved by Genetics Institute. L-cell-conditioned medium (LCM) was prepared as described by Stanley and Heard (65) and used as the source of CSF-1 for routine growth and maintenance of the BAC1.2F5 cell line. Antibody to the CSF-1 receptor (29) was kindly provided by Jim Downing, and polyclonal antiphosphotyrosine antibody (33) was provided by Albert Reynolds.

Cell culture. The cell line used in this work was BAC1.2F5, a CSF-1-dependent clone (43) derived from the BAC1 line which originated from transfection of splenic adherent cells from a (BALB/c × A.CA)F_1 mouse with simian virus 40 origin-defective DNA (59). The BAC1.2F5 line exhibits many properties of macrophages, including production of lysozyme, collagenase, and esterase. BAC1.2F5 cells also possess Fc receptors, express Ia antigen, and engage in Fc-mediated phagocytosis. The BAC1.2F5 cell line exhibits an absolute requirement for CSF-1 for both survival and proliferation (51). BAC1.2F5 cells were routinely grown in DMEM supplemented with 15% FCS, 25% LCM, and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.3. Human recombinant CSF-1 was added at a concentration of 6,000 U/ml (1 U = 0.44 fmol). The human growth factor is as effective as murine CSF-1 in BAC1.2F5 cells.

The BAC1.M2 clone was isolated by infecting BAC1.2F5 cells with the c-*myc* retroviral vector fpGV–c-*myc* and isolating a stable cell clone that expressed the c-*myc* gene independently of CSF-1 (55). BAC1.M2 cells still required CSF-1 for growth and survival. Control cells (BAC1.C2) were constructed in the same way with a control virus (fpGV) lacking the c-*myc* gene. The BAC1.M2 and BAC1.C2 cell lines were handled identically to the BAC1.2F5 line, except that G418 (0.8 μg/ml) was included in the medium.

PtdCho hydrolysis and quantitation of diglyceride mass. BAC1.2F5 cells were grown to a density of 5 × 10^6 per 60-mm-diameter dish and then cultured for 18 h in the absence of CSF-1. During the last 4 h of CSF-1 starvation, 2 μCi of 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine was added in 4 ml of medium. After the labelling period, greater than 80% of the intracellular radiolabel was in PtdCho. The medium was then aspirated and replaced with fresh medium with or without 25% LCM as a source of CSF-1. At the different time intervals, the cells were washed with cold phosphate-buffered saline (PBS) without Ca^{2+} and the lipids were extracted by the method of Bligh and Dyer (7). For phosphatidylethanol formation, 0.7% ethanol was included in the incubation (5), and phosphatidic acid phosphatase was inhibited by 600 μM propranolol (50). Neutral lipids were separated by thin-layer chromatography on Silica Gel G layers developed with chloroform-methanol-acetic acid (98:2:1, vol/vol), and phosphatidylethanol and phosphatidic acid were separated by chromatography with isooctane-ethyl acetate-acetic acid-water (110:50:20:100). Radioactivity was visualized by using the Bioscan radiochromatogram imaging system and quantified by liquid scintillation counting.

Mass measurement of cell-associated diglyceride was accomplished as described in the diglyceride kinase assay kit from Lipidex. Dishes of BAC1.2F5 cells were growth arrested as described above and stimulated by treatment with medium containing 25% LCM. Cells were washed with cold PBS without Ca^{2+}, and the lipids were extracted by using several washes of hexane-isopropanol (3:2, vol/vol). The BAC1.2F5 lipid extracts were used as a substrate for *Escherichia coli* diglyceride kinase in the presence of 0.5 mM [^{32}P]ATP (0.1 μCi/nmol). Radioactive products were separated on Silica Gel G layers developed with chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1, vol/vol), and the region corresponding to an authentic phosphatidic acid standard was scraped and quantified by liquid scintillation counting.

Mitogenesis assays. BAC1.2F5 cells were grown to 10^5/12-mm-diameter dish or 2.5 × 10^5/25-mm-diameter dish in DMEM containing 15% FCS and 25% LCM. The cells were then washed with warm PBS and growth arrested in DMEM plus 15% FCS for 18 h. Mitogens in DMEM plus 15% FCS and [^{3}H]thymidine (2 μCi/ml) were added to the cells and incubated for 24 h. The monolayers were washed three times with cold PBS, the cells were harvested, and [^{3}H]thymidine incorporation was determined by liquid scintillation counting (1). Cell cycle parameters were measured by flow cytofluorometry, and the percentage of cells in each stage of the cell cycle was calculated as described by Dean (14).

RNA analysis. Cell cultures were harvested by centrifugation, and total RNA was isolated by using a guanidine isothiocyanate lysis procedure followed by pelleting of RNA by CsCl gradient centrifugation (10). RNA pellets dissolved in 10 mM Tris-HCl (pH 7.5)–5% β-mercaptoethanol–0.5% Sarkosyl–0.5% sodium dodecyl sulfate (SDS)–5 mM EDTA were extracted with phenol-chloroform-isoamyl alcohol (24:24:1) and precipitated with 2 volumes of ethanol. RNA (10 μg, determined by A_{260}) was denatured for 10 min at 55°C in electrophoresis buffer (20 mM morpholine propanesulfonic acid [pH 7.0], 5 mM sodium acetate, 1 mM EDTA, 6% formaldehyde) containing 50% deionized formamide. The samples were then quickly chilled on ice and fractionated by

electrophoresis in 1.0% agarose gels. Blotting, prehybridization, hybridization with ^{32}P-labelled probes, and washing of blots were performed as described by Thomas (67). ^{32}P-labelled probes used for analysis of RNA levels were prepared by random priming with restriction enzyme fragments isolated by agarose gel electrophoresis. The c-*myc* probe was a 0.9-kb *Xba*I-*Bgl*I fragment of murine c-*myc* exon 2, the ornithine decarboxylase (ODC) probe was a 1.6-kb *Eco*RI-*Bam*HI fragment of the murine cDNA, the c-*fos* probe was a 3.7-kb *Sst*I fragment of murine c-*fos* cDNA, the *junB* probe was a 1.8-kb *Eco*RI fragment of the murine *junB* cDNA, and the actin probe was a 1.8-kb *Pst*I fragment of the chicken β-actin gene.

For immunoblot analysis, the cells were washed with ice-cold PBS and the monolayers were scraped in 1 ml of SDS-gel electrophoresis sample buffer and boiled for 5 min. Aliquots of the samples (90 μl) were fractionated by SDS-gel electrophoresis with an 8% separating gel. Proteins were electroblotted onto nitrocellulose membranes that were exposed to either anti-CSF-1R, antiphosphotyrosine antibody, or anti-PKC isotypes (2 μg of IgG per ml), washed, probed with ^{125}I-labelled protein A, and visualized by autoradiography.

Analysis of nucleotides bound to Ras. The method used for analysis of nucleotides bound to Ras is similar to that of Gibbs et al. (25), as detailed below. BAC1.2F5 cells were grown to a density of 2.5 × 10^6/100-mm-diameter dish, washed, and placed in phosphate-free DMEM plus 10% dialyzed FCS for 14 h. The cells were then labelled with 1 mCi of $^{32}P_i$ in 4 ml of the phosphate-free medium containing 10% FCS for 4 h. At the end of the labelling period, CSF-1 (6,000 U/ml), TPA (5 × 10^{-7} M) in dimethyl sulfoxide, or PC-PLC (5-U/ml final concentration) was added and the cells were incubated for 15 min. The cells were washed three times with ice-cold PBS and scraped into 1 ml of lysis buffer (150 mM NaCl, 20 mM $MgCl_2$, 20 mM Tris [pH 7.5], 1% Nonidet P-40, 1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin) containing 2 μg of Ras antibody Y13-259 per ml. The extracts were transferred to 1.5-ml microcentrifuge tubes, mixed thoroughly, and then incubated at 0°C for 30 min with constant mixing. The lysates were centrifuged to remove debris, and the supernatants were removed to new tubes containing 40 μl of protein A-Sepharose beads (50% suspension) and 10 μg of rabbit anti-mouse IgG. The mixtures were incubated and mixed at 0°C for another 30 min. Immunoprecipitates were collected by centrifugation and washed twice with cold lysis buffer and three times with cold PBS. The resulting pellets were resuspended in 25 μl of 0.75 M KH_2PO_4 (pH 3.4)–5 mM EDTA–0.1 mM GDP–0.1 mM GTP and heated at 75°C for 5 min. Equal counts of the supernatants (≈10 μl) were spotted onto polyethyleneimine-cellulose thin-layer plates and developed with 1 M KH_2PO_4 (pH 4.0). After exposure of films to the plates for 12 to 24 h to obtain an autoradiograph, the spots corresponding to GDP and GTP were removed and quantified by liquid scintillation counting.

RESULTS

Activation of cellular PtdCho hydrolysis and diglyceride accumulation. Previous work showed that CSF-1 triggers a rapid 1.5 to 2.0-fold elevation in radiolabelled diglyceride in murine bone marrow-derived macrophages prelabelled with ^{3}H-labelled fatty acids (72), in human peripheral monocytes prelabelled with [^{3}H]glycerol (32), and in [^{3}H]glycerol-prelabelled NIH 3T3 fibroblasts expressing the CSF-1 receptor (12). We employed two different experimental approaches to determine whether CSF-1 stimulation correlates with diglyceride accumulation in the BAC1.2F5 cell line (Fig. 1). First, the PtdCho pool in CSF-1-deprived BAC1.2F5 cells was labelled with 1-*O*[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine, and then the cells were stimulated with CSF-1. In these experiments, an increase in ^{3}H-labelled diglyceride derived from [^{3}H]PtdCho was observed (Fig. 1A), consistent with enhanced PtdCho hydrolysis as a major source of cellular diglyceride following CSF-1 stimulation. The second approach was to measure the mass of diglyceride that accumulated following CSF-1 stimulation (Fig. 1B). This technique revealed a two-fold increase in total diglyceride. In both cases, maximum accumulation of diglyceride occurred at 15 min following addition of CSF-1, indicating that PtdCho breakdown is an immediate-early event in the action of CSF-1. The kinetics and amount of diglyceride accumulation in BAC1.2F5 cells (Fig. 1) were the same as those observed in macrophages (72), monocytes (32), and a fibroblast cell line expressing CSF-1R (12).

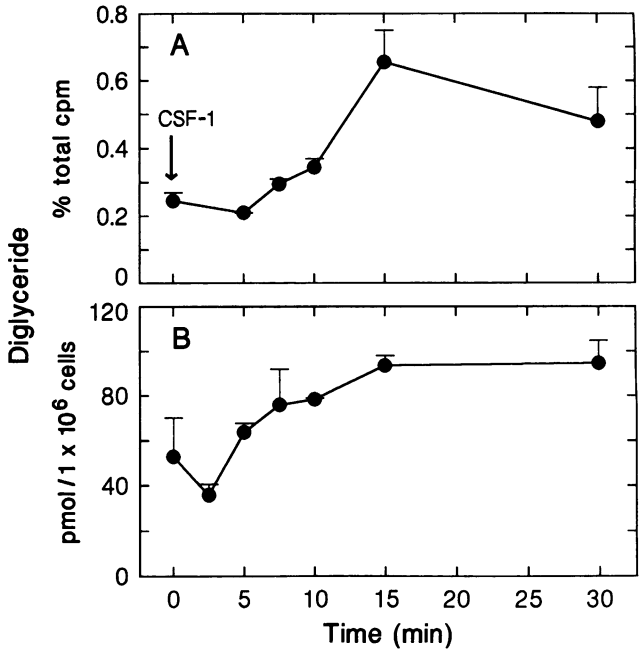

FIG. 1. CSF-1-induced [^{3}H]PtdCho hydrolysis and elevation in cellular diglyceride. (A) Growth-arrested BAC1.2F5 cells that had been prelabelled with 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine were treated with CSF-1 for the indicated times, and the total lipids were extracted and analyzed by thin-layer chromatography as described in Materials and Methods. The percentages of the incorporated counts migrating as diglyceride were determined, and the data are presented as the averages of duplicate dishes from three separate experiments. (B) Growth-arrested BAC1.2F5 cells were treated for the indicated times with CSF-1, the cellular lipids were extracted, and the cell-associated diglyceride mass was quantitated by its conversion to [^{32}P]phosphatidic acid with diglyceride kinase as described in Materials and Methods.

PDGF initiates diglyceride formation beginning at 2 h and increasing throughout the G_1 stage of the cell cycle (35). Therefore, we examined an extended time course for accumulation of diglyceride in CSF-1-stimulated cells (Fig. 2). There was an initial increase in diglyceride, as we found in the experiment described by Fig. 1, followed by a decrease

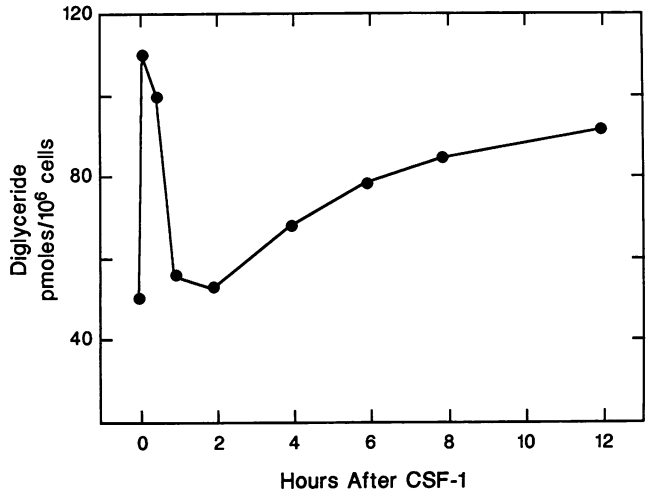

FIG. 2. CSF-1 elevates diglyceride levels throughout the G_1 phase. Growth factor-arrested BAC1.2F5 cells were treated for the indicated times with CSF-1, the cellular lipids were extracted, and the cell-associated diglyceride was quantitated by its conversion to [^{32}P]phosphatidic acid with diglyceride kinase as described in Materials and Methods.

and a slow increase in diglyceride through the rest of G_1 (Fig. 2).

Diglyceride can be generated by the breakdown of PtdCho by either PC-PLC or PC-PLD. We examined the hydrolysis of PtdCho in CSF-1-stimulated cells in the presence of ethanol to assess the relative contributions of PC-PLC and PC-PLD to diglyceride accumulation. BAC1.2F5 cells were prelabelled with 1-*O*-[^{3}H]octadecyl-*sn*-glycerol-3-phosphocholine for 3 h, incubated with 0.7% ethanol, and stimulated for 15 min with either CSF-1 or TPA. In cells stimulated with CSF-1, phosphatidylethanol accounted for 1.3% of the total label, compared with 0.6% in control nonstimulated cells. In cells exposed to 5×10^{-7} M TPA, phosphatidylethanol rose to 2.4% of the total counts. These data show that PC-PLD activity was higher in TPA-stimulated cells than in CSF-1-activated cells. To assess the contribution of PC-PLC to diglyceride formation following CSF-1 stimulation, propranolol was used to inhibit phosphatidic acid phosphatase (Table 1). The presence of propranolol neither blocked diglyceride accumulation in CSF-1-stimulated cells nor increased the amount of phosphatidic acid detected. TPA-stimulated cells also accumulated diglyceride, but in addition, an increased cellular content of phosphatidic acid was detected in the presence of propranolol. These data are consistent with the higher levels of phosphatidylethanol generated by TPA stimulation than with CSF-1 stimulation. These data suggest that PC-PLC is the most significant contributor to CSF-1-stimulated diglyceride formation in cells prelabelled with 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine, although some PC-PLD activity was detected.

Mitogenic effect of *B. cereus* PC-PLC. *B. cereus* PC-PLC was employed as an approach to investigate the role of PtdCho hydrolysis in CSF-1 mitogenic signalling in BAC1.2F5 cells. Addition of PC-PLC to the culture medium of cells arrested in G_1 by CSF-1 deprivation stimulated [^{3}H]thymidine incorporation in a dose-dependent manner, reaching a maximum at 46% of the [^{3}H]thymidine incorporation stimulated by CSF-1 (Fig. 3). Addition of higher concentrations of PC-PLC was inhibitory, and the cells were

TABLE 1. Formation of phosphatidic acid and diglyceride by BAC1.2F5 cells treated with CSF-1, PC-PLC, or TPA[a]

Stimulus	% of total cpm	
	PtdOH[b]	DG[c]
None	1.1	5.0
CSF-1	1.2	8.4
CSF-1 + propranolol	0.7	7.1
TPA	0.7	6.8
TPA + propranolol	3.0	8.3
PC-PLC	1.7	22.3

[a] BAC1.2F5 cells were grown in 60-mm-diameter dishes and deprived of CSF-1 for 18 h. During the final 3 h of starvation, the cells were labelled with 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine. The prelabelled cells were then stimulated with either CSF-1 or TPA for 15 min. Propranolol (600 μM) was added 5 min before the stimulus. The cells were then extracted, and their phospholipid composition was analyzed as described in Materials and Methods.

[b] PtdOH, phosphatidic acid.

[c] DG, diglyceride.

lysed by PC-PLC concentrations higher than 15 U/ml. Heat-inactivated PC-PLC was not mitogenic. Cell cycle parameters of BAC1.2F5 cells treated with PC-PLC were measured by flow cytofluorometry at 24 h after addition of the enzyme. These data show that >90% of the PC-PLC-treated cells were in G_1 at 24 h, demonstrating that the cell population was not arrested in the S phase. Cell number was also monitored in our experiments. PC-PLC caused a 1.3-fold increase in cell number at 24 h, compared with a 1.9-fold increase in cell number caused by CSF-1. These data indicate that once cells activated by PC-PLC commit to DNA synthesis, they complete the cell cycle.

Other phospholipid-hydrolytic enzymes and products of

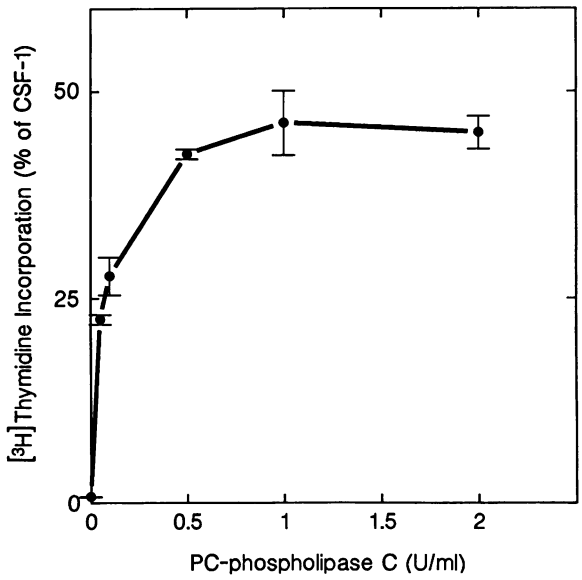

FIG. 3. Stimulation of [^{3}H]thymidine incorporation into BAC1.2F5 cells by PC-PLC. Cells were growth arrested by removal of CSF-1 for 18 h. The indicated concentration of *B. cereus* PC-PLC was then added along with [^{3}H]thymidine (2 μCi/ml), and the incorporation of label at the end of 24 h was measured. The results are expressed as percentages of the response to CSF-1 (6,000 U/ml). The values shown are averages of triplicate incubations ± the standard errors.

TABLE 2. Effects of phospholipases and their hydrolytic products on [^{3}H]thymidine incorporation into BAC1.2F5 cells[a]

Potential mitogen (concn)	[^{3}H]thymidine incorporation (% of CSF-1)
None	0.2 ± 0.02
CSF-1 (2,000 U/ml)	100.0 ± 3.0
PC-PLC (1 U/ml)	46.5 ± 2.8
Phosphatidylinositol-specific PLC (0.1 U/ml)	40.5 ± 1.7
PC-PLD (200 U/ml)	28.4 ± 4.8
Sphingomyelinase (0.1 U/ml)	26.2 ± 2.6
TPA (10^{-7} M)	87.0 ± 2.7
Dioctanoylglycerol (1–10 mM)	0.8 ± 0.2
Diolein (0.1–10 mM)	0.5 ± 0.1
Phosphocholine (10 mM)	1.6 ± 0.9
Phosphoethanolamine (10 mM)	1.7 ± 1.0
Phosphatidic acid (0.1 mM)	4.4 ± 0.6
1-Oleoyl-2-acetylglycerol (1 mM)	0.5 ± 0.1

[a] BAC1.2F5 macrophages were seeded into 24-well cluster dishes and grown to a density of about 10^5 per well. The cells were synchronized in the G_1 phase by removal of CSF-1 for 18 h. [^{3}H]thymidine incorporation was assayed as described in Materials and Methods. The concentration indicated gave maximum [^{3}H]thymidine incorporation in a dose-response experiment. The results shown are averages ± the standard errors of triplicate incubations.

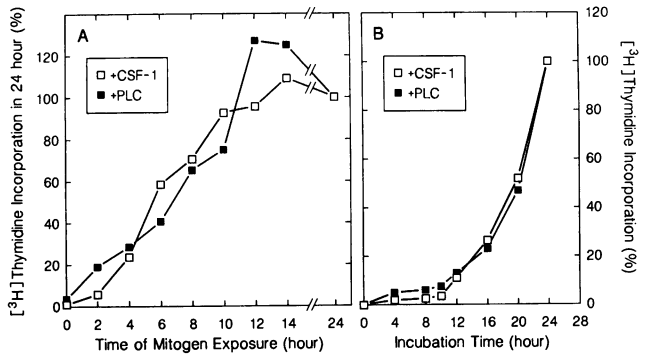

FIG. 4. PC-PLC and CSF-1 function in the G_1 stage of the cell cycle. BAC1.2F5 cells were synchronized in the G_1 phase of the cell cycle by deprivation of CSF-1 for 18 h in DMEM containing 15% FCS. (A) At time zero, either CSF-1 (6,000 U/ml) or PC-PLC (1 U/ml) was added along with DMEM plus 15% FCS and [^{3}H]thymidine (2 μCi/ml). At the indicated times, the medium was removed and the cells were washed with warm PBS, which was replaced with [^{3}H]thymidine-containing medium without CSF-1 or PC-PLC. The amount of [^{3}H]thymidine incorporated at 24 h was measured, and the data were normalized to [^{3}H]thymidine incorporation with either CSF-1 or PC-PLC exposure for the entire 24-h labelling period. The actual amount of [^{3}H]thymidine incorporated in the dishes treated with PC-PLC was 34% of the amount of [^{3}H]thymidine incorporated in the dishes treated with CSF-1. (B) At time zero, the medium was replaced with DMEM plus 15% FCS and [^{3}H]thymidine (2 μCi/ml) containing either CSF-1 (6,000 U/ml) or PC-PLC (1 U/ml). At the indicated times, dishes were harvested and the amount of [^{3}H]thymidine incorporated was measured as described in Materials and Methods. The data were normalized to 100% at 24 h for both treatments. The actual amount of [^{3}H]thymidine incorporated in the dishes treated with PC-PLC was 44% of the amount of [^{3}H]thymidine incorporated in dishes treated with CSF-1.

the PLC reaction were tested for mitogenic potency (Table 2). All phospholipases capable of generating diglyceride either directly or indirectly were also active as mitogens in BAC1.2F5 cells. Phosphatidylinositol-specific PLC was almost as potent as PC-PLC in stimulating [^{3}H]thymidine incorporation. PC-PLD and sphingomyelinase indirectly generate diacylglycerol either by forming phosphatic acid, which is converted to diglyceride by phosphatidic acid phosphatase, or by forming ceramide, which is converted to sphingomyelin by transfer of phosphocholine from PtdCho, thus generating diglyceride. Although these two enzymes were mitogenic, they were less potent than the PLCs that directly produce diglyceride. TPA was the most potent mitogen, generating a response of up to 87% of that of CSF-1. The water-soluble products of the PLC reaction were not mitogenic. Diglyceride or diglyceride analogs did not stimulate [^{3}H]thymidine incorporation; however, phosphatidic acid elicited a small but significant response. The inability of long-chain diglycerides to stimulate [^{3}H]thymidine incorporation into BAC1.2F5 cells was anticipated, since these compounds are not appreciably taken up by cells (23).

PC-PLC was necessary only during the G_1 phase to elicit a maximum mitogenic response in BAC1.2F5 cells (Fig. 4A). These data support the concept that the presence of PC-PLC, like that of CSF-1, is required throughout the G_1 stage of the cell cycle for maximal effectiveness. In addition, BAC1.2F5 cells stimulated by PC-PLC entered the S phase at the same time as cells stimulated with CSF-1 (Fig. 4B). These data show that onset of the S phase is neither hastened nor delayed in cells stimulated with PC-PLC compared with cells activated by CSF-1. The finding that stimulation of BAC1.2F5 cells with PC-PLC exhibited the same kinetics and cell cycle specificity as CSF-1 led us to determine whether PC-PLC functioned by activating the CSF-1 receptor (Fig. 5). Quiescent BAC1.2F5 cells were treated with PC-PLC, CSF-1, or TPA for 15 min. Cell lysates were prepared, and Western blot (immunoblot) analysis was employed to determine the amount of CSF-1R by using an anti-receptor antibody (Fig. 5, left panel) and the extent of tyrosine phosphorylation by using an antibody to phosphotyrosine (Fig. 5, right panel). Addition of CSF-1 caused extensive tyrosine phosphorylation of CSF-1R, the hallmark of receptor activation (19, 54). TPA caused some CSF-1R degradation during the 15 min of incubation; however, TPA did not induce tyrosine phosphorylation of CSF-1R. PC-PLC neither triggered CSF-1R degradation nor induced tyrosine phosphorylation and activation of CSF-1R. These data indicate that PC-PLC acts at a step downstream of the CSF-1 receptor in the signal transduction pathway.

PC-PLC does not act through TPA-stimulated PKC. TPA was an effective mitogen in BAC1.2F5 cells (Table 2) and is known to induce proliferation of macrophage colonies (70). Since TPA or diglyceride activates PKC, we examined whether the mitogenic properties of PC-PLC could be attributed to stimulation of PKC (Fig. 6). The response of BAC1.2F5 cells to TPA is known to be eradicated by prolonged treatment of the cells with TPA (20). When BAC1.2F5 cells were treated with TPA for 30 h, the mitogenic response to TPA was abolished, although there was a significant increase in the basal level of [^{3}H]thymidine incorporation (Fig. 6B). In contrast, PC-PLC was still capable of stimulating [^{3}H]thymidine incorporation in TPA-pretreated cells (Fig. 6B). Pretreatment of BAC1.2F5 cells for 30 h with PC-PLC had no effect on the ability of PC-PLC, TPA, or CSF-1 to stimulate [^{3}H]thymidine incorporation (Fig. 6C). PC-PLC pretreatment also increased background [^{3}H]thymidine labelling but not to the extent that TPA did. These data support the concept that TPA and PC-PLC act by different mechanisms.

The basal level of [^{3}H]thymidine incorporation in TPA-

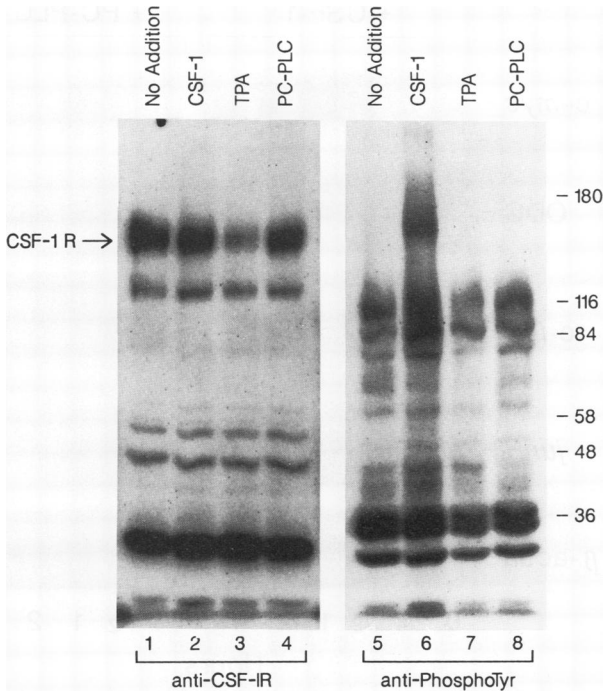

FIG. 5. Effects of PC-PLC, CSF-1, and TPA on CSF-1R activation. BAC1.2F5 cells were growth arrested by withdrawal of CSF-1 from the medium for 18 h. CSF-1 (6,000 U/ml), TPA (5×10^{-7} M), or PC-PLC (5 U/ml) was then added, and the cells were incubated for 15 min at 37°C. The cells were harvested, aliquots of the lysate were fractionated by SDS-gel electrophoresis, and the amount and the phosphorylation state of CSF-1R were determined by Western blotting as described in Materials and Methods. Panels: left, immunoblotting with antibody to CSF-1R; right, duplicate of the gel on the left blotted with antibody to phosphotyrosine. The numbers on the right are molecular sizes in kilodaltons.

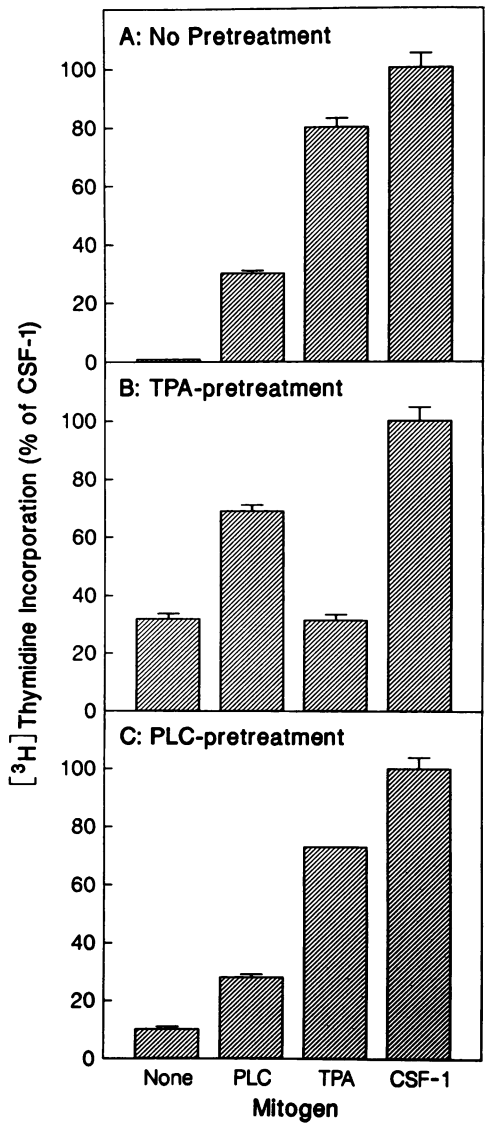

FIG. 6. Mitogenic potency of PC-PLC in cells pretreated with either TPA or PC-PLC for 30 h. BAC1.2F5 cells were preincubated in DMEM plus 15% FCS without CSF-1 in medium for 30 h (A), in medium plus TPA (10^{-7} M) for 30 h (B), or in medium plus PC-PLC (1 U/ml) for 30 h (C). [^{3}H]thymidine (2 μCi/ml) incorporation was measured in the subsequent 24 h of incubation with or without PC-PLC (1 U/ml), TPA (10^{-7} M), or CSF-1 (6,000 U/ml). The data shown are averages of triplicate incubations ± the standard errors.

treated cells was higher than that in control cells (Fig. 6B). TPA is known to stimulate production of CSF-1 by several cell lines (27, 31), so we tested whether TPA triggered autocrine production of CSF-1 by BAC1.2F5 cells. Extracts from BAC1.2F5 cells that were growth arrested for 18 h and then stimulated for 24 h with CSF-1, TPA, or PC-PLC were analyzed by Western blotting for the presence of CSF-1R and its phosphorylation on tyrosine. Cells treated with CSF-1 showed small amounts of CSF-1R but significant levels of receptor phosphorylation on tyrosine. Cells treated with TPA exhibited high levels of CSF-1R and significant levels of receptor phosphorylation on tyrosine. Cells treated with PC-PLC had high levels of CSF-1R, but there was no evidence of tyrosine phosphorylation. These experiments indicate the presence of CSF-1 ligand and receptor autophosphorylation in cells treated with TPA but not in cells treated with PC-PLC.

PKC isozymes in BAC1.2F5 cells. Western blot analysis was used to determine the number and relative levels of seven PKC isotypes in BAC1.2F5 cells. We detected no PKC-α, PKC-β1, PKC-β2, PKC-γ, or PKC-ε polypeptides with polyclonal rabbit antibodies specific for these PKC isotypes. In addition, an antibody that reacts with all group A PKCs (α, β1, β2, and γ) did not detect cross-reacting material in BAC1.2F5 extracts. Control lanes containing rabbit or rat brain extracts verified the reactivity of the PKC antibodies under our experimental conditions. PKC-δ was the only PKC isotype definitively detected in BAC1.2F5 cells (Fig. 7). When PKC-δ is activated, it is autophosphorylated, and phosphorylated PKC-δ has a slightly retarded electrophoretic mobility (47). The electrophoretic mobility of PKC-δ was retarded in cells treated with TPA for 15 min, but 15 min of exposure to either CSF-1 or PC-PLC did not alter the mobility of PKC-δ (Fig. 7A). Treatment of BAC1.2F5 cells with TPA resulted in the complete disappearance of PKC-δ protein, whereas treatment with PC-PLC for 30 h neither altered the levels of PKC-δ nor led to activation of this kinase (Fig. 7B). A faint band was detected with anti-PKC-ζ that was only 3% of the intensity of the PKC-δ band. This level of PKC-ζ did not change following

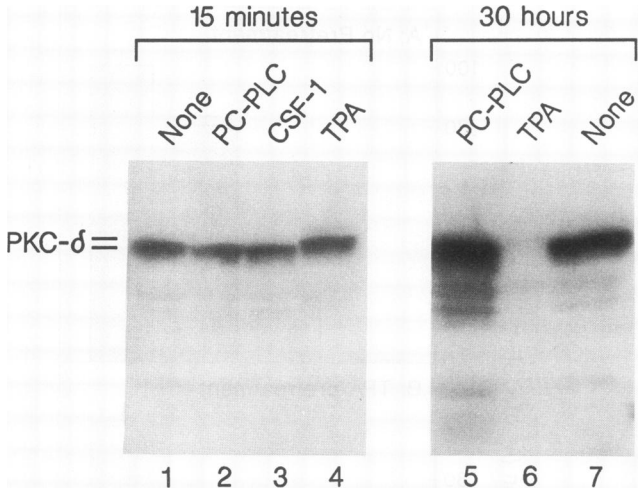

FIG. 7. Activation and downregulation of PKC-δ by TPA in BAC1.2F5 cells. (A) BAC1.2F5 cells (2×10^7) were growth arrested by deprivation of CSF-1 for 18 h. The cells were then incubated for 15 min without any addition (lane 1), with 5 U of PC-PLC per ml (lane 2), with 6,000 U of CSF-1 per ml (lane 3), or with 5×10^{-7} M TPA (lane 4). Cell lysates were prepared and analyzed by Western blotting with PKC-δ-specific IgG. (B) BAC1.2F5 cells were treated for 30 h in the absence of CSF-1 in medium containing 1 U of PC-PLC per ml (lane 5), 5×10^{-7} M TPA (lane 6), or no additions (lane 7). The presence of PKC-δ was detected by Western blotting with PKC-δ-specific IgG.

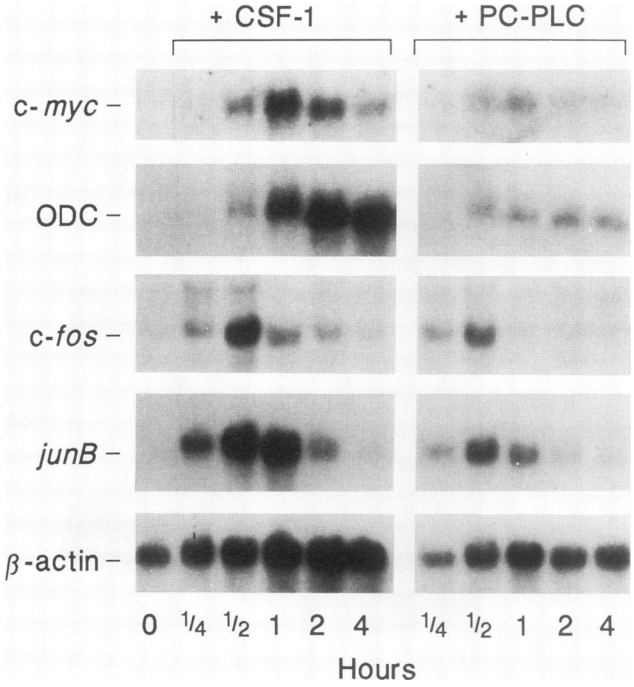

FIG. 8. Regulation of immediate-early gene expression by PC-PLC. BAC1.2F5 cells were growth arrested by withdrawal of CSF-1 for 18 h. CSF-1 (6,000 U/ml), TPA (10^{-7} M), or PC-PLC (1 U/ml) in DMEM plus 15% FCS was then added to the cultures, which were incubated for the indicated times at 37°C. At each time point, cells were harvested and total RNA was purified. Northern (RNA) analysis was performed as described in Materials and Methods, by using ^{32}P-labelled DNA probes for β-actin, c-*myc*, c-*fos*, *junB*, and ODC.

prolonged treatment with TPA. Therefore, the possibility remains that BAC1.2F5 cells have a low level of PKC-ζ that is not downregulated by TPA.

Regulation of gene expression by PC-PLC. CSF-1 induces a pattern of immediate-early gene expression characterized by an increase in c-*fos* and *junB* mRNA, followed by c-*myc* and ODC (Fig. 8; 60, 70). We found that β-actin expression was significant in quiescent BAC1.2F5 cells, but the abundance of this mRNA also increased following CSF-1 stimulation. The pattern of gene expression elicited by TPA was essentially the same as that observed with CSF-1 (data not shown). Treatment of BAC1.2F5 cells with PC-PLC triggered a rapid increase in c-*fos*, *junB*, and β-actin mRNA levels, although consistent with the lower mitogenic potency of PC-PLC, the levels of these mRNAs did not reach the levels observed in CSF-1-stimulated cells. The lanes in Fig. 8 were quantitated by densitometry, and the increases in mRNA species triggered by PC-PLC as percentages of the amount accumulated in cells stimulated by CSF-1 were as follows: c-*myc* (1 h), 4.6%; ODC (2 h), 8.9%; c-*fos* (30 min), 38%; *junB* (30 min), 39%; β-actin (1 h), 97%. Thus, accumulation of c-*myc* mRNA was selectively impaired in PC-PLC-treated cells compared with CSF-1-stimulated cells. ODC mRNA (Fig. 8), whose expression is thought to be regulated by c-*myc* (2), was also significantly reduced in PC-PLC-treated cells. These data show that PC-PLC activates a signal transduction pathway coupled to c-*fos* and *junB* expression but is less efficient at activating the signal transduction pathway(s) leading to stimulation of c-*myc* expression.

Collaboration between constitutive c-*myc* expression and PC-PLC. To determine whether the weak induction of c-*myc* accounted for the low mitogenic potency of PC-PLC relative to that of CSF-1, we examined the effect of PC-PLC on [^{3}H]thymidine incorporation in a BAC1.2F5 clone (BAC1.M2) that constitutively expresses c-*myc* because of introduction of the fpGV–c-*myc* retroviral vector (Fig. 9). Although the BAC1.M2 cell line expresses c-*myc* independently of CSF-1, it remains dependent on CSF-1 for survival and proliferation (55). As shown in Fig. 9, the mitogenic potency of PC-PLC was markedly augmented in BAC1.M2 cells. PC-PLC stimulated [^{3}H]thymidine incorporation into the cell line harboring the control vector (BAC1.C2) 26.6% (Fig. 9A), which was similar to a typical result obtained with the parental cells (Fig. 6A). In contrast, PC-PLC stimulated [^{3}H]thymidine incorporation into BAC1.M2 cells to 86.2% of the level achieved with CSF-1 (Fig. 9B). The responses of the two cell lines to TPA were not altered. We also examined an additional independent clone that constitutively expressed c-*myc* (BAC1.M4) and obtained results identical to those obtained with the BAC.M2 line (data not shown), supporting the conclusion that constitutive expression of c-*myc* was responsible for the heightened response of these cells to PC-PLC. These results indicate collaboration between PC-PLC and c-*myc* in mitogenic signalling.

These data suggested that PC-PLC could support the long-term growth of BAC1.M2 cells. Therefore, we determined the viable cell numbers of BAC1.C2 and BAC1.M2 cells cultured for several days in the presence of CSF-1 or PC-PLC or in the absence of mitogens (Fig. 10). BAC1.M2 cells treated with PC-PLC survived, and the cell number slowly increased for several days in the absence of CSF-1 (>90% viable). BAC1.M2 cells underwent a morphological conversion upon PC-PLC treatment. The BAC1.M2 cells

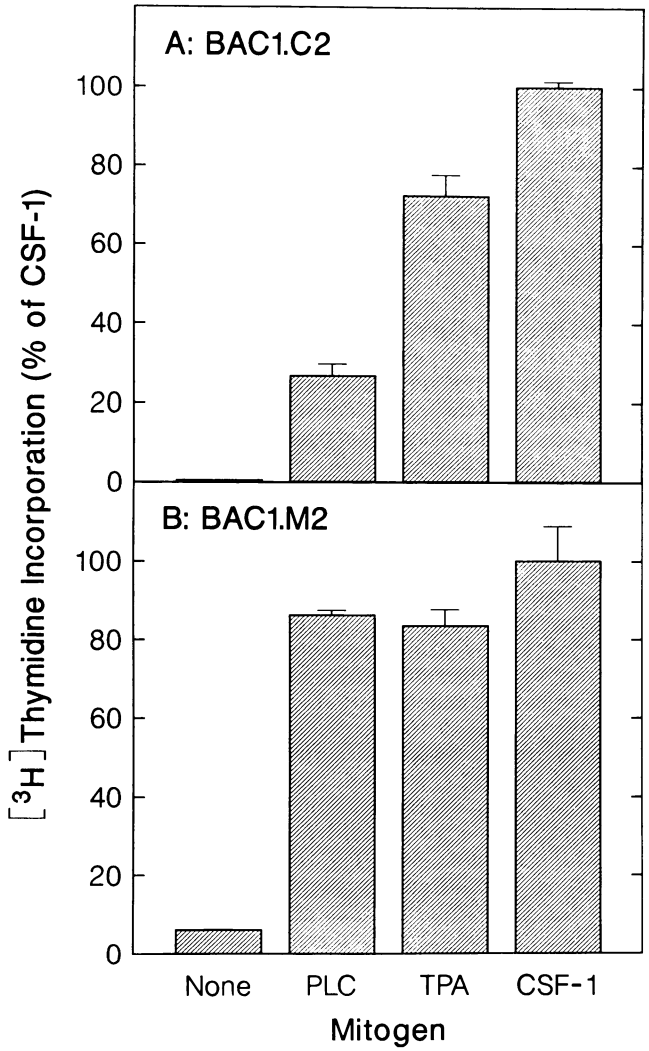

FIG. 9. Mitogenic effects of PC-PLC, TPA, and CSF-1 on BAC1.2F5 cells constitutively expressing c-*myc*. BAC1.C2 cells transfected with a control vector (A) or BAC1.M2 cells constitutively expressing c-*myc* (B) were grown to a density of 10^5/25-mm-diameter dish. After withdrawal of CSF-1 from the medium for 18 h, the cells were incubated in DMEM plus 15% FCS containing [^{3}H]thymidine (2 μCi/ml), either alone or supplemented with CSF-1 (6,000 U/ml), TPA (5×10^{-7} M), or PC-PLC (1 U/ml). At 24 h, the cells were harvested and the amount of [^{3}H]thymidine incorporated was measured. The results shown are averages of triplicate incubations with standard error bars.

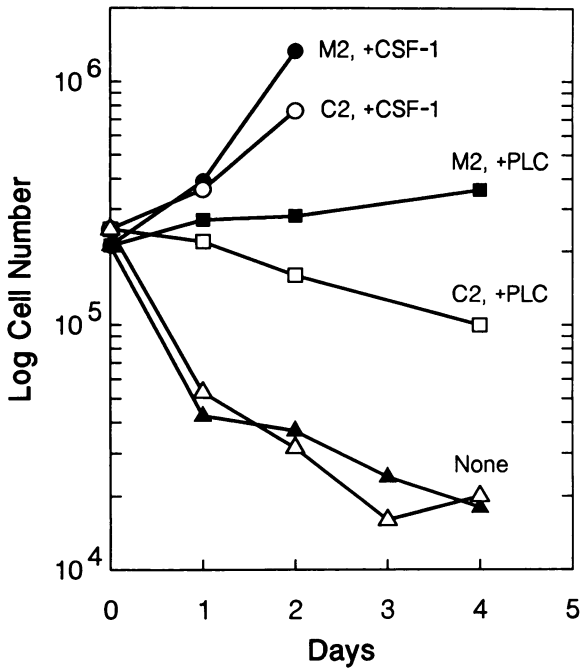

FIG. 10. Growth responses of BAC1.M2 and BAC1.C2 cells in the presence of CSF-1 or PC-PLC. Cell lines were grown in 60-mm-diameter dishes and deprived of CSF-1 for 18 h. Then the cells were placed in DMEM (None), CSF-1 (2,000 U/ml), or PC-PLC (1 U/ml). Duplicate dishes were harvested at daily intervals, and viable cells were counted.

detached from the dish, assumed a round morphology, and grew as a suspension culture after 2 days in medium containing PC-PLC. An additional *myc* clone (BAC1.M4) was examined, and the results were the same as those obtained with the BAC1.M2 line. The cell number did not increase in BAC1.C2 cells treated with PC-PLC, although they did not die as fast as cells cultured in the absence of CSF-1. These data support the concept that the PC-PLC signal transduction pathway collaborates with c-*myc*; however, the combination of these two agents was not as effective as CSF-1, as reflected by the slower growth rate.

PC-PLC did not activate Ras. An increase in the amount of GTP bound to Ras is another early response to CSF-1 stimulation (30). Therefore, we compared the abilities of CSF-1, TPA, and PC-PLC to increase the amount of GTP bound to Ras (Fig. 11). Both CSF-1 and TPA significantly increased the amount of GTP bound to Ras. In contrast, PC-PLC stimulation did not significantly change the molar fraction of GTP bound to Ras compared with that in unstimulated cells. These data show that the mitogenic action of PC-PLC is independent of Ras activation.

DISCUSSION

The finding that CSF-1 triggers PtdCho hydrolysis and diglyceride accumulation, coupled with the ability of exogenous PC-PLC to mimic a subset of the early events triggered by CSF-1, indicates that PC-PLC is an important component of the mitogenic signalling pathway. PtdCho hydrolysis and diglyceride formation are associated with the action of CSF-1 (Fig. 1; 12, 32, 72), IL-1 (56), IL-3 (21), and PDGF (35). PtdCho hydrolysis was initiated within minutes with CSF-1, IL-1, and IL-3, but analysis of PtdCho degradation products at later time points showed that maximum accumulation of phosphocholine occurred late in the G_1 phase in PDGF-stimulated fibroblasts, suggesting that PC-PLC activity is also involved in later events. In BAC1.2F5 cells, diglyceride accumulation was biphasic, with an early response, noted in previous work, followed by steadily increasing diglyceride levels through G_1 (Fig. 2), reminiscent of the findings obtained with PDGF (35). Our results point to the conclusion that PC-PLC plays a role throughout the G_1 phase, since the maximum mitogenic response to PC-PLC required the continuous presence of exogenous PC-PLC throughout the G_1 phase (Fig. 4B). PC-PLC cannot replace the growth factor in inducing either a complete or a sustained mitogenic response, implicating PC-PLC as a participant in

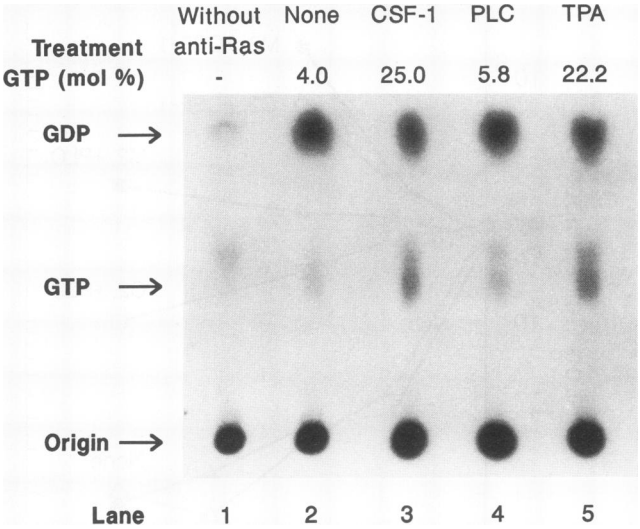

FIG. 11. Activation of Ras by PC-PLC, CSF-1, and TPA. BAC1.2F5 cells were deprived of CSF-1 in phosphate-free DMEM plus 15% dialyzed FCS for 14 h. Then the cells were labelled with 1 mCi of $^{32}P_i$ in 4 ml of the phosphate-free medium containing 15% dialyzed FCS for an additional 4 h. At the end of the 4-h labelling period, the cells were stimulated with CSF-1 (6,000 U/ml), TPA (5×10^{-7} M), or PC-PLC (5 U/ml) for 15 min. Nucleotides bound to Ras were analyzed by immunoprecipitation of Ras as described in Materials and Methods. Nucleotides were separated by thin-layer chromatography, the corresponding spots were removed from the thin-layer plate, and the levels of radioactivity in the GTP and GDP areas were quantitated by scintillation counting. The moles percent of bound GTP were calculated.

one of the several signal transduction cascades initiated by CSF-1.

Diglyceride generated by PC-PLC does not function through activation of a TPA-responsive PKC isotype. Although all molecular species of diglyceride activate PKC in vitro, physiological experiments showed that ligands stimulating diglyceride production from phosphoinositide turnover are potent activators of PKC in vivo, whereas diglyceride derived from PtdCho hydrolysis is not always associated with activation of PKC (36). PKC-δ is the only PKC isotype that is significantly expressed in BAC1.2F5 cells (Fig. 7). The selective expression of PKC-δ in BAC1.2F5 cells is consistent with the finding that PKC-δ is the major PKC isotype expressed by murine myeloid cells (42). PKC-δ belongs to the group B PKCs, which lack the calcium-binding domain present in group A enzymes (e.g., PKC-α), but retains the Cys-rich phorbol ester-binding domain (45). Accordingly, PKC-δ protein is downregulated by prolonged treatment with TPA (Fig. 4; 39). In BAC1.2F5 cells, the inability of exogenous PC-PLC to activate PKC-δ or ras, downmodulate CSF-1R, or induce c-*myc* mRNA and the retention of the PC-PLC mitogenic response following chronic TPA treatment all point to the conclusion that the action of PC-PLC is independent of PKC. Similar experiments have ruled out PKC activation as an intermediate in exogenous PC-PLC-stimulated mitogenesis in Swiss 3T3 cells (35) and in PC-PLC-mediated expression of the stromelysin gene (16). In fact, TPA is universally effective as an activator of PC-PLC- and/or PC-PLD-mediated PtdCho breakdown in every system examined (4); therefore, TPA-responsive PKC is positioned upstream of PC-PLC in the signal transduction cascade. Distantly related members of the PKC family may be candidates for diglyceride regulation. For example, *Saccharomyces cerevisiae* possesses a diglyceride-activated protein kinase that is not regulated by phorbol esters (46), and PKC-ζ has been implicated as a downstream target of PC-PLC in *Xenopus* oocytes (17).

Our data place PC-PLC and c-*myc* in separate, collaborating signal transduction pathways in BAC1.2F5 cells. PC-PLC stimulation of BAC1.2F5 cells triggers c-*fos* and *junB* mRNA accumulation (Fig. 8), showing that PC-PLC participates in a signal transduction pathway that leads to expression of the Fos and Jun families of transcription factors. Induction of these two families of genes is a universal, early event in growth factor action, and expression of both of these gene products is necessary (34), but not sufficient (8, 58), for cell cycle progression in fibroblasts. The requirement for two collaborating signal transduction pathways involving c-*fos*–*junB* and c-*myc* has been uncovered from the analysis of CSF-1R mutants. CSF-1R mutants that have Phe substituted for Tyr at position 809 are deficient in transducing a mitogenic signal, although both phosphatidylinositol 3′-kinase and expression of c-*fos* and *junB* are activated (58). However, CSF-1 binding to CSF-1R[809F] does not direct the expression of c-*myc*, and the constitutive expression of c-*myc* in CSF-1R[809F] mutants restores the ability of CSF-1 to induce proliferation (57). Similarly, IL-2 stimulation of hematopoietic cells can be divided into two pathways; one pathway leads to transcriptional activation of *fos* and *jun* genes, whereas a separate pathway leads to c-*myc* transcription (62). PC-PLC would be classified as a component of the Fos-Jun pathway activated by CSF-1, since stimulation of BAC1.2F5 cells with PC-PLC leads to expression of c-*fos* and *junB* but not c-*myc* (Fig. 8), and constitutive expression of c-*myc* significantly augments the mitogenic potency of PC-PLC (Fig. 9 and 10). The inability of exogenous PC-PLC to trigger c-*myc* expression is not universal, since *B. cereus* PC-PLC induces c-*myc* in BALB/MK keratinocytes (15). Maximum accumulation of the water-soluble product of PC-PLC, phosphocholine, occurs late in the G_1 phase, and activation of PC-PLC has previously been viewed as a relatively late event in the signal transduction cascade activated by growth factors (16, 35, 40). However, the finding that PC-PLC can activate immediate-early gene expression and the association of heightened PC-PLC activity with Ras activation (see below) suggest that the PC-PLC plays an important role in the early events of growth factor action.

Our results are consistent with the proposal that PC-PLC acts downstream of Ras. Activation of the product of the *ras* oncogene is a well-known event in growth factor signal transduction, and its pivotal role in the mitogenic cascade is established (3). Downstream targets of Ras activation have been more difficult to identify, but recent evidence supports the concept that PLC-catalyzed degradation of PtdCho is one of the events regulated by Ras. Microinjection of transforming mutants of Ras triggers both PtdCho hydrolysis and maturation of *Xenopus laevis* oocytes, whereas neutralizing Ras antibodies inhibit PtdCho breakdown and maturation induced by insulin (24, 44). Furthermore, a temperature-sensitive Ki-*ras* mutant exhibits rapid activation of PtdCho breakdown following a shift to the permissive temperature (40), and PC-PLC mimics the activation of stromelysin transcription by the *ras* oncogene via a PKC-independent mechanism (16). Furthermore, expression of dominant-negative Ras mutants blocks the mitogenic response of cells to serum, polypeptide growth factors, and TPA but does not inhibit the mitogenic response to PC-PLC (9). Our finding

that treatment of BAC1.2F5 cells with PC-PLC did not increase the amount of Ras in the GTP-bound form (Fig. 11) is consistent with the notion that PC-PLC is a target rather than an activator of Ras. Choudhury et al. (11) have reported that PC-PLC is rapidly phosphorylated on tyrosine following activation of CSF-1R and that tyrosine phosphorylation of PC-PLC is inhibited by pertussis toxin, which opens the possibility that PC-PLC activation is independent of the Ras pathway. However, tyrosine phosphorylation as a mechanism for regulating PC-PLC activity requires additional experimental verification, and the possibility remains that the putative tyrosine kinase is itself regulated by Ras.

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