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Hydrolysis of Phosphatidylcholine Is Stimulated by Ras Proteins during Mitogenic Signal Transduction

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We have used a dominant inhibitory *ras* mutant (Ha-*ras* Asn-17) to investigate the relationship of Ras proteins to hydrolysis of phosphatidylcholine (PC) in the transduction of mitogenic signals. Expression of Ha-Ras Asn-17 inhibited NIH 3T3 cell proliferation induced by polypeptide growth factors or phorbol esters. In contrast, the mitogenic activity of PC-specific phospholipase C (PC-PLC) was not inhibited by Ha-Ras Asn-17 expression. Similarly, cotransfection with a cloned PC-PLC gene bypassed the block to NIH 3T3 cell proliferation resulting from expression of the inhibitory *ras* mutant. Hydrolysis of PC can therefore induce cell proliferation in the absence of normal Ras activity, suggesting that PC-derived second messengers may act downstream of Ras in mitogenic signal transduction. This was substantiated by the finding that Ha-Ras Asn-17 expression inhibited growth factor-stimulated hydrolysis of PC. Taken together, these results indicate that PC hydrolysis is a target of Ras during the transduction of growth factor-initiated mitogenic signals.

The *ras* proto-oncogenes encode plasma membrane-associated guanine nucleotide binding proteins that function in signal transduction pathways leading to cell proliferation in response to mitogenic growth factors (4, 9, 10, 23, 34) and to neuronal differentiation induced by nerve growth factor (1, 12, 25, 35). The Ras proteins are associated with protein-tyrosine kinase growth factor receptors via GTPase activating protein (GAP), which regulates Ras activity and presumably serves to couple Ras to growth factor stimulation (15, 16, 22). In yeasts, Ras proteins act to regulate adenylate cyclase (37). However, despite the importance of *ras* oncogenes and proto-oncogenes in both neoplastic and normal cell growth, the targets of Ras in mammalian cells have not been identified.

Hydrolysis of phosphatidylinositol 4,5-bisphosphate is stimulated by a variety of growth factors, yielding diacylglycerol and inositol triphosphate, which activate protein kinase C and mobilize intracellular calcium, respectively (2, 24). In addition, a number of growth factors have been found to stimulate hydrolysis of a second phospholipid, phosphatidylcholine (PC), generating diacylglycerol and phosphocholine (8). PC hydrolysis also appears to be stimulated in cells expressing oncogenic Ras proteins (17, 21, 30, 39). Moreover, treatment of quiescent cells with exogenous PC-specific phospholipase C (PC-PLC) is itself mitogenic (18). Importantly, PC-PLC induces cell proliferation even if protein kinase C is downregulated by pretreatment with phorbol esters (18). It thus appears that PC hydrolysis may generate a novel second messenger, possibly phosphocholine or a species of diacylglycerol that acts on a target other than phorbol ester-sensitive members of the protein kinase C family.

We have used a dominant inhibitory *ras* mutant (9) to investigate the role of Ras in signal transduction pathways in mammalian cells. The protein encoded by this mutant *ras* gene (Ha-*ras* Asn-17) preferentially binds GDP versus GTP (9) and appears to specifically interfere with the function of endogenous proto-oncogene Ras proteins (33). Expression of Ha-Ras Asn-17 inhibits the proliferation of NIH 3T3 cells and the mitogenic response of these cells to a variety of polypeptide growth factors as well as to direct activation of protein kinase C by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (4). In contrast to its activity in NIH 3T3 cells, Ha-Ras Asn-17 does not affect proliferation of PC12 pheochromocytoma cells but blocks the neuronal differentiation of these cells induced by nerve growth factor (35).

We report here that PC-PLC can bypass the block to proliferation of NIH 3T3 cells resulting from Ha-Ras Asn-17 expression and, conversely, that expression of Ha-Ras Asn-17 in NIH 3T3 cells inhibits growth factor-stimulated hydrolysis of PC. Taken together, these findings indicate that PC-derived second messengers function downstream of Ras in mitogenic signal transduction.

MATERIALS AND METHODS

Cell lines. NIH(M17) cells are a transfected subclone of NIH 3T3 cells in which the Ha-*ras* Asn-17 gene is expressed from the dexamethasone-inducible mouse mammary tumor virus long terminal repeat (4). NIH 3T3 and NIH(M17) cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum. The stably transfected NIH(M17) cell line was maintained in medium containing G418 (400 μg/ml).

PC-PLC. PC-PLC was purified from *Bacillus cereus* as previously described (18).

Plasmid DNAs. The *ras* expression plasmid pZIP M17 contains the Ha-*ras* Asn-17 gene inserted into the *Bam*HI site of pZIPneoSV(X) so that the mutant *ras* gene is coexpressed with *neo* (9). The PC-PLC expression plasmid

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TABLE 1. Mitogenic activity of PC-PLC

Mitogen	DNA synthesis (fold induction)[a]					
	NIH 3T3 cells			NIH(M17) cells		
	−Dex	+Dex	Ratio[b]	−Dex	+Dex	Ratio
Serum	16 ± 1	19 ± 2	1.2	17 ± 1	6 ± 1	0.4
EGF	9 ± 0.7	9 ± 0.8	1.0	10 ± 2	1 ± 0.1	0.1
TPA	10 ± 1	8 ± 0.4	0.8	9 ± 2	1 ± 0.1	0.1
PC-PLC	15 ± 0.3	15 ± 0.3	1.0	15 ± 1	15 ± 0.3	1.0

[a] NIH 3T3 or NIH(M17) cells were incubated 24 h in media containing 0.5% calf serum and dexamethasone (Dex; 5×10^{-7} M) where indicated to induce quiescence. Cells were then stimulated by addition of calf serum (10%), EGF (10 ng/ml), TPA (100 nM), or PC-PLC (0.5 U/ml). [^{3}H]thymidine incorporation was determined 16 h after mitogenic stimulation. Data are presented as the ratio of [^{3}H]thymidine incorporation in stimulated cultures to that in unstimulated controls. Results are the averages ± standard errors of the mean of two or three independent assays, each containing duplicate plates.

[b] Ratio of stimulation of DNA synthesis with dexamethasone to that without dexamethasone.

pOPLCneo$^-$ was constructed from the *B. cereus* PC-PLC gene (14). The coding region for the 24-amino-acid signal peptide and the 14-amino-acid propeptide of *B. cereus* PC-PLC was replaced by the 21-amino-acid signal peptide of the *Escherichia coli* outer membrane protein OmpA. This construct, including 19 bp upstream of the start codon, was then inserted into the mammalian expression vector pMAMneo, and then the *neo* gene was deleted. Mammalian cells transfected with this plasmid display an increased level and intracellular activity of PC-PLC with no detectable secretion of the enzyme.

Transfection assays. NIH 3T3 cells were transfected with plasmid DNAs in the presence of 20 μg of carrier calf thymus DNA (Sigma Chemical Co.) as described previously (9). Cells were subcultured into medium containing G418 (400 μg/ml) 3 days after transfection, and neomycin-resistant colonies were either stained and counted or isolated for further study.

Mitogen-stimulated DNA synthesis. NIH 3T3 or NIH(M17) cells were plated in medium containing 10% calf serum at a density of 10^5 cells per 60-mm dish. The following day, media were changed to 0.5% calf serum, and dexamethasone (5×10^{-7} M) was added where indicated. Cells were incubated for 24 h to induce quiescence and then stimulated by the addition of serum or growth factors or by treatment with PC-PLC. DNA synthesis was assayed 16 h after mitogenic stimulation by labeling with [^{3}H]thymidine as described previously (4).

Northern (RNA) blot analysis. Cytoplasmic RNAs were isolated, electrophoresed in 1% agarose-formaldehyde gels, and hybridized with ^{32}P-labeled human Ha-*ras* probe as previously described (4).

Western blot (immunoblot) analysis. Protein samples (50 μg) were electrophoresed in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, transferred to nitrocellulose filters, and analyzed with anti-Ras antibody Ras-10 (New England Nuclear). Blots were developed by using goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate.

Growth factor-stimulated PC hydrolysis. NIH 3T3 or NIH(M17) cells (10^6 cells per 60-mm plate) were labeled for 48 h with [^{14}C]choline (2 μCi per culture, 50 to 60 mCi/mmol; New England Nuclear) or [^{3}H]myristic acid (10 μCi per culture, 30 Ci/mmol; New England Nuclear). The last 24 h of labeling was performed in media containing 0.5% calf serum and 5×10^{-7} M dexamethasone (where indicated). Cells were then washed and treated with serum, growth factors, or PC-PLC. Phosphocholine and diacylglycerol were extracted and analyzed by thin-layer chromatography as previously described (32). Radioactivity present as [^{14}C]phosphocholine was quantitated by β-scanning. [^{3}H]diacylglycerol was quantitated by excising and counting the appropriate regions of the thin-layer chromatography plates in a liquid scintillation counter.

RESULTS

Expression of Ha-Ras Asn-17 does not inhibit mitogenesis induced by PC-PLC. We initially investigated the effect of Ha-Ras Asn-17 expression on the mitogenic activity of exogenous PC-PLC. NIH(M17) cells are a transfected subclone of NIH 3T3 cells in which the Ha-*ras* Asn-17 gene is expressed via the mouse mammary tumor virus promoter. These cells proliferate normally in the absence of dexamethasone, but their proliferation is reversibly inhibited by induction of the mutant *ras* gene (4). As previously reported (4), the mitogenic response of NIH(M17) cells to serum was inhibited approximately 60% by dexamethasone induction of Ha-Ras Asn-17, and the mitogenic response to epidermal growth factor (EGF) or TPA was virtually abolished by Ha-Ras Asn-17 induction (Table 1).

Addition of PC-PLC purified from *B. cereus* to the culture medium of NIH 3T3 cells was strongly mitogenic, consistent with previous results (18). Importantly, dexamethasone induction of Ha-Ras Asn-17 did not inhibit the mitogenic response of NIH(M17) cells to PC-PLC (Table 1). Thus, in contrast to serum, growth factors, and TPA, the mitogenic activity of PC-PLC did not appear to be dependent upon Ras activity, suggesting that PC-derived second messengers might function downstream of Ras in mitogenic signal transduction.

Cotransfection with a cloned PC-PLC gene overcomes Ha-Ras Asn-17 inhibition of NIH 3T3 cell proliferation. In order to ensure that the mitogenic activity of PC-PLC was not due to potential contaminants in the enzyme preparation, we tested the ability of a cloned PC-PLC gene to overcome the growth inhibitory activity of Ha-Ras Asn-17. NIH 3T3 cells were transfected with a neomycin resistance gene either alone [the pZIPneoSV(X) vector] or linked to Ha-*ras* Asn-17 (the pZIP M17 plasmid). Results of a representative experiment are illustrated in Fig. 1 and data from three independent experiments are presented in Table 2.

As previously observed (9), Ha-*ras* Asn-17 inhibited the proliferation of transfected cells, greatly reducing the number of neomycin-resistant transformants obtained with pZIP M17 compared with those obtained with the pZIPneoSV(X) vector containing *neo* alone. Cotransfection with a PC-PLC expression plasmid reversed the inhibitory effect of the

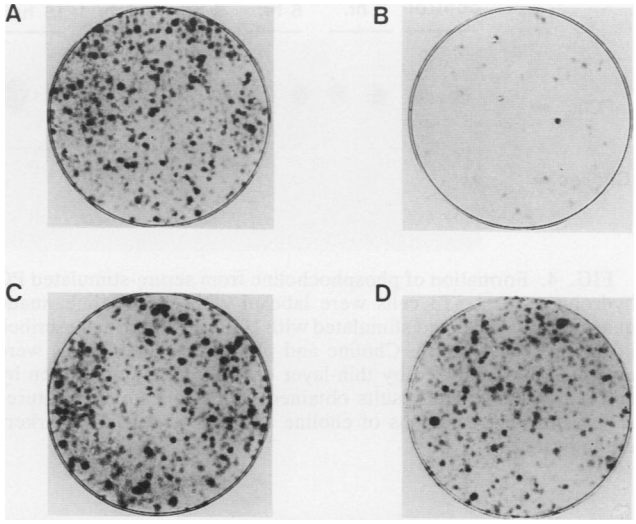

FIG. 1. Cotransfection of NIH 3T3 cells with Ha-*ras* Asn-17 and a PC-PLC expression plasmid. NIH 3T3 cells were transfected with plasmid DNAs and subcultured into medium containing G418. Colonies were stained and photographed 17 days after transfection. (A) pZIPneoSV(X) (0.1 μg); (B) pZIP M17 (0.1 μg); (C) pZIP-neoSV(X) (0.1 μg) plus pOPLCneo$^-$ (0.5 μg); (D) pZIP M17 (0.1 μg) plus pOPLCneo$^-$ (0.5 μg).

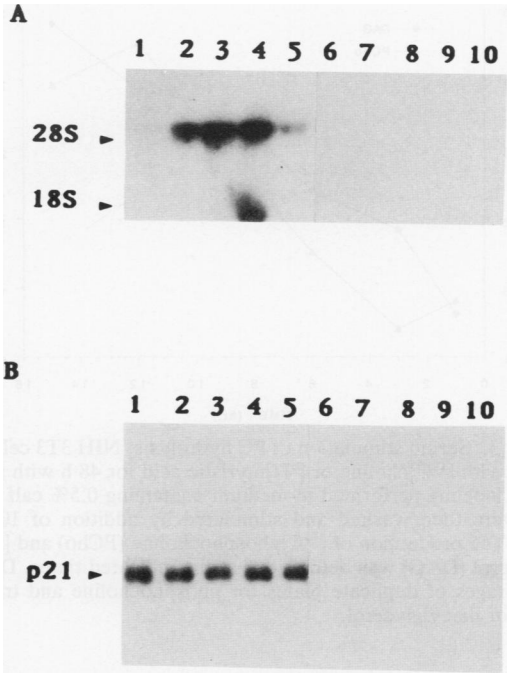

FIG. 2. Expression of Ha-*ras* Asn-17 in NIH 3T3 cells cotransfected with PC-PLC. (A) Cytoplasmic RNA (15 μg per lane) was electrophoresed in 1% agarose-formaldehyde gels and analyzed by Northern blot hybridization with ^{32}P-labeled human Ha-*ras* probe. Lanes 1 to 5, five independent lines of G418-resistant NIH 3T3 cells cotransfected with pZIP M17 plus pOPLCneo$^-$; lanes 6 to 10, G418-resistant NIH 3T3 cells isolated after transfection with pZIP M17 alone. Each lane contained similar amounts of 18S and 28S rRNAs. (B) Cell extracts (50 μg of protein) were electrophoresed in an SDS–12% polyacrylamide gel and analyzed by immunoblotting with anti-Ras antibody Ras-10. The position of the Ras protein (p21) is indicated. The lanes are as described for panel A.

mutant *ras* gene, so that the yield of neomycin-resistant transformants obtained by cotransfection with the pZIP M17 plasmid plus PC-PLC was similar to that obtained with the pZIPneoSV(X) vector containing *neo* alone (Fig. 1 and Table 2). These results are similar to those obtained after cotransfection of pZIP M17 together with plasmids expressing *raf* oncogenes (9) and confirm that PC-PLC can stimulate cell proliferation independently of normal Ras activity.

The ability of PC-PLC to relieve the growth inhibitory effect of Ha-Ras Asn-17 was further verified by analysis of expression of the mutant *ras* gene in the neomycin-resistant transformants obtained in the cotransfection assays. Expression of the transfected Ha-*ras* Asn-17 gene was detected by Northern and Western blot analysis of five neomycin-resistant cell lines obtained after cotransfection with the pZIP M17 plasmid plus PC-PLC. Each of these cell lines expressed 5.6-kb Ha-*ras* transcripts (Fig. 2A, lanes 1 to 5), as expected for expression of the mutant *ras* gene in the ZIP vector (9). In contrast, no expression of the transfected Ha-*ras* Asn-17 gene was detected in five neomycin-resistant cell lines obtained after transfection with the pZIP M17 plasmid alone (Fig. 2A, lanes 6 to 10), indicating that these rare neomycin-resistant transformants did not express detectable levels of the inhibitory *ras* mutant. Consistent with the RNA results, the level of Ras protein was increased in the five cell lines that were cotransfected with pZIP M17 plus PC-PLC (Fig. 2B, lanes 1 to 5), whereas cells transfected with pZIP M17 alone expressed low levels of Ras protein similar to that in NIH 3T3 cells (Fig. 2B, lanes 6 to 10). Expression of the Ha-*ras* Asn-17 gene in cells that were cotransfected with PC-PLC therefore indicates that PC-PLC is able to overcome the inhibitory effect of Ha-Ras Asn-17 on cell proliferation.

TABLE 2. Cotransfection of PC-PLC with Ha-*ras* Asn-17

Expt	No. of neomycin-resistant colonies[a]			
	ZIP	ZIP + PC-PLC	pZIP M17	pZIP M17 + PC-PLC
1	340 ± 20	328 ± 23	23 ± 3	290 ± 4
2	252 ± 13	257 ± 19	13 ± 2	238 ± 12
3	222 ± 12	234 ± 19	14 ± 4	217 ± 16

[a] NIH 3T3 cells were transfected with 0.1 μg of pZIPneoSV(X) (designated ZIP) or 0.1 μg of pZIP M17. Where indicated, cultures were cotransfected with 0.5 μg of a PC-PLC expression plasmid (pOPLCneo$^-$). Cells were subcultured into medium containing G418 3 days after transfection, and neomycin-resistant colonies were stained and counted 2 weeks later. Results of three independent experiments are shown. Data are the averages of duplicate or triplicate plates ± standard errors of the mean.

Ha-Ras Asn-17 expression inhibits PC hydrolysis in response to mitogenic growth factors. The ability of PC-PLC to bypass the Ha-Ras Asn-17 block to cell proliferation indicates that PC-PLC acts either downstream or independently of Ras in mitogenic signal transduction. To distinguish between these possibilities, we investigated the effect of Ha-Ras Asn-17 expression on the growth factor-stimulated hydrolysis of PC.

Hydrolysis of PC in response to growth factors can be catalyzed by either PC-PLC or PC-PLD (8). The former yields phosphocholine plus diacylglycerol, while the latter yields choline plus phosphatidic acid. Since choline can be subsequently converted to phosphocholine by choline kinase and phosphatidic acid to diacylglycerol by phosphatidate phosphohydrolase, the actions of PC-PLC and PC-PLD can

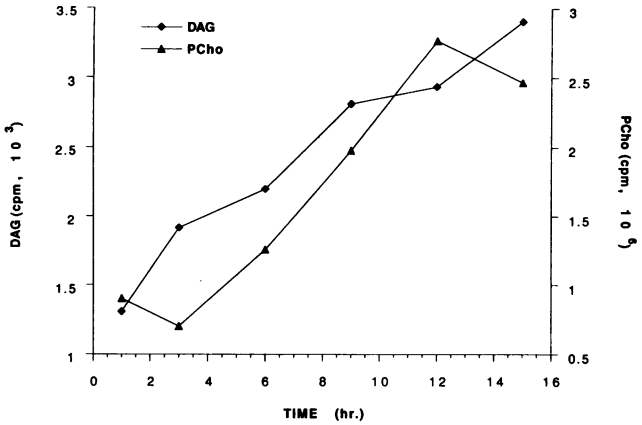

FIG. 3. Serum stimulation of PC hydrolysis. NIH 3T3 cells were labeled with [^{14}C]choline or [^{3}H]myristic acid for 48 h with the last 24 h of labeling performed in medium containing 0.5% calf serum. Cells were then washed and stimulated by addition of 10% calf serum. The production of [^{14}C]phosphocholine (PCho) and [^{3}H]diacylglycerol (DAG) was determined at the indicated times. Data are the averages of duplicate plates for phosphocholine and triplicate plates for diacylglycerol.

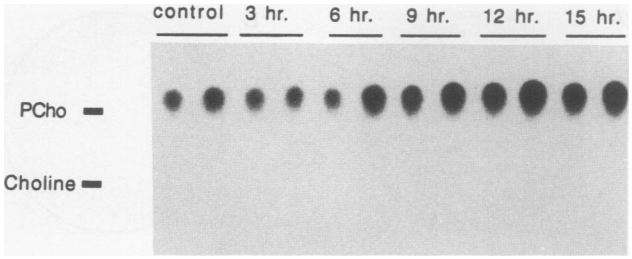

FIG. 4. Formation of phosphocholine from serum-stimulated PC hydrolysis. NIH 3T3 cells were labeled with [^{14}C]choline, made quiescent, washed, and stimulated with 10% calf serum as described in the legend to Fig. 3. Choline and phosphocholine (PCho) were extracted and analyzed by thin-layer chromatography and then by autoradiography. The results obtained with duplicate cell cultures are shown. The positions of choline and phosphocholine markers are indicated.

yield similar products. In order to characterize PC turnover, we therefore investigated the kinetics of production of choline, phosphocholine, diacylglycerol, and phosphatidic acid in response to mitogenic stimulation of NIH 3T3 cells.

Cells were prelabeled with either [^{14}C]choline or [^{3}H] myristic acid, which is incorporated specifically into PC (13, 32), and then stimulated by addition of serum. Elevated levels of both phosphocholine and diacylglycerol were detectable within 6 h after serum addition and continued to increase for at least 12 h thereafter (Fig. 3). An increase in diacylglycerol was apparent somewhat earlier than phosphocholine (3 h after serum addition), which might suggest production of [^{3}H]myristate-labeled diacylglycerol from another source at early times after mitogen stimulation. The parallel production of both diacylglycerol and phosphocholine for 6 to 12 h after serum addition is similar to previous results (18) and indicates that PC hydrolysis is a long-term response to mitogenic stimulation.

In contrast to the serum-stimulated production of [^{14}C]phosphocholine, no [^{14}C]choline was detected in either untreated or serum-stimulated cells (Fig. 4). The production of phosphocholine in the absence of any detectable choline suggests that the serum-stimulated hydrolysis of PC was catalyzed by PC-PLC rather than PC-PLD, although the possibility that choline was rapidly converted to phosphocholine by the action of choline kinase cannot be excluded.

Phosphatidic acid, as well as diacylglycerol, was produced after serum stimulation of [^{3}H]myristic acid-labeled cells (data not shown). We therefore further investigated the possibility that diacylglycerol was being generated via a PC-PLD pathway by addition of 250 μM propanolol, an established inhibitor of phosphatidate phosphohydrolase (3), at the time of serum stimulation. In contrast to its inhibition of diacylglycerol production resulting from the action of PC-PLD in other systems (3, 21, 32), propanolol had no effect on the amount of diacylglycerol detected 14 h after serum addition (data not shown). It thus appears that the action of PC-PLC, rather than PC-PLD, was primarily responsible for generation of both diacylglycerol and phosphocholine in serum-stimulated NIH 3T3 cells.

The effect of Ha-Ras Asn-17 expression on mitogen-stimulated PC hydrolysis, assayed by production of both diacylglycerol and phosphocholine, was then investigated (Fig. 5). Both serum and EGF induced PC turnover in NIH 3T3 cells and in NIH(M17) cells in the absence of dexamethasone. Expression of Ha-Ras Asn-17, however, inhibited serum stimulation of PC turnover by about 50% and completely blocked the stimulatory effect of EGF. These inhibitory effects of Ha-Ras Asn-17 on PC turnover are parallel to the effects of the mutant *ras* gene on serum and EGF-induced mitogenesis (compare with Table 1). In contrast, expression of Ha-Ras Asn-17 did not inhibit PC hydrolysis induced by treatment with exogenous PC-PLC. As previously reported (18), addition of the bacterial enzyme to the culture medium stimulated the production of intracellular phosphocholine as well as diacylglycerol, indicating uptake of the enzyme by the cells. It thus appeared that the inhibitory Ras mutant specifically interfered with serum and EGF-stimulated PC hydrolysis, indicating that normal Ras function is required for stimulation of PC turnover in response to mitogenic growth factors in NIH 3T3 cells.

DISCUSSION

Previous studies have implicated PC hydrolysis as a source of second messengers in response to stimulation by growth factors and oncogenic Ras proteins (8, 17, 18, 21, 39). In the present experiments, we have used a dominant inhibitory *ras* mutant to directly probe the relationship between PC hydrolysis and Ras in mitogenic signal transduction. In NIH 3T3 cells, treatment with exogenous PC-PLC or transfection with a cloned PC-PLC gene was found to bypass the block to proliferation resulting from expression of Ha-Ras Asn-17, indicating that PC-derived second messengers acted either downstream or independently of Ras to signal cell division. In addition, hydrolysis of PC in response to stimulation of NIH 3T3 cells by serum and EGF was inhibited by expression of the mutant *ras* gene. Moreover, the extent to which Ha-Ras Asn-17 inhibited PC hydrolysis stimulated by serum and EGF paralleled its effectiveness in inhibiting mitogenesis induced by these agents. Taken together, these results implicate PC hydrolysis as an important target of Ras in mitogenic signal transduction.

Hydrolysis of PC in response to growth factors and oncogenes can be catalyzed by either PC-PLC or PC-PLD, yielding diacylglycerol and phosphocholine or phosphatidic

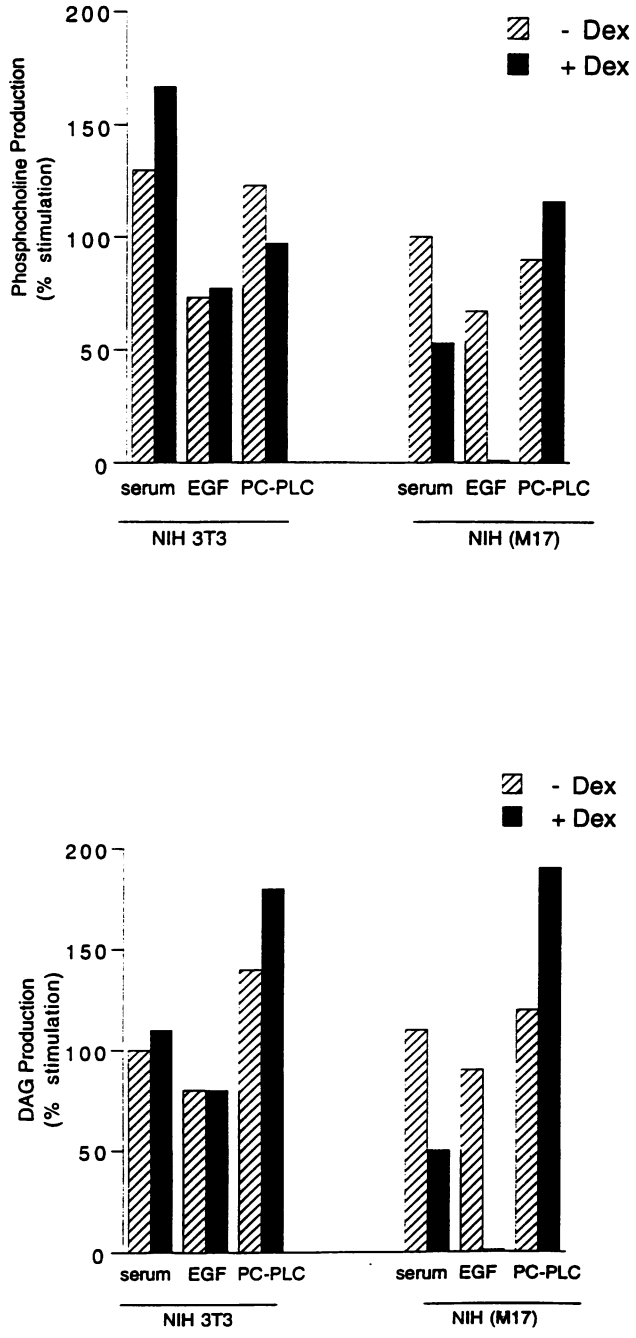

FIG. 5. The effect of Ha-Ras Asn-17 on mitogen-stimulated PC hydrolysis. NIH 3T3 or NIH(M17) cells were labeled for 48 h with [^{14}C]choline (upper panel) or [^{3}H]myristic acid (lower panel). The last 24 h of labeling was performed in medium containing 0.5% calf serum and 5×10^{-7} M dexamethasone (Dex) where indicated. Cells were then washed and treated with calf serum (10%), EGF (10 ng/ml), or PC-PLC (0.5 U/ml) for 14 h, and the amount of [^{14}C]phosphocholine or [^{3}H]diacylglycerol was determined. Data represent the averages of duplicate or triplicate plates and are presented as the percent stimulation of PC hydrolysis compared with that in untreated control cultures.

acid and choline, respectively (8). Phosphatidic acid can subsequently be converted to diacylglycerol by phosphatidate phosphohydrolase, and choline can be converted to phosphocholine by choline kinase. Previous studies have indicated that PC-PLC, rather than PC-PLD, is the primary catalyst of PC turnover in 3T3 cells stimulated by either platelet-derived growth factor or *ras* oncogene expression (18, 21). PC-PLD, however, also appears to be stimulated in 3T3 cells in response to platelet-derived growth factor or expression of the *src* oncogene (29, 32), potentially as a result of the activation of PC-PLD by protein kinase C (5). In the present study, hydrolysis of PC resulted in the formation of phosphocholine in the absence of any detectable choline production, suggesting that Ras-mediated PC turnover resulted primarily from the action of PC-PLC. In addition, the generation of diacylglycerol was not inhibited by propanolol, an inhibitor of phosphatidate phosphohydrolase. It thus appears that PC-PLC, rather than PC-PLD, is primarily responsible for Ras-mediated PC turnover.

In light of these findings, the consequences of unregulated expression of PC-PLC on cell growth are of interest. Cells transfected with the PC-PLC expression plasmid have a higher content of both phosphocholine and diacylglycerol as well as a detectable level of intracellular *B. cereus* PC-PLC, as observed in immunostaining experiments with an affinity-purified anti-PC-PLC polyclonal antibody (22a). In addition, expression of the *B. cereus* PC-PLC gene in NIH 3T3 cells results in a reduced serum dependence as well as the ability to form colonies in soft agar (22a). Chronic stimulation of PC hydrolysis thus appears to induce at least some properties characteristic of cell transformation.

The targets of PC-derived second messengers remain to be determined. As expected, since diacylglycerol is a product, some effects of PC hydrolysis are mediated by protein kinase C and can be blocked by downregulation of protein kinase C resulting from prolonged treatment with phorbol esters, such as TPA (6). However, both Ras and PC-PLC also appear to activate signal transduction pathways that are not blocked by phorbol ester downregulation of protein kinase C (4, 7, 18, 20). One possibility is that phosphocholine acts as a second messenger, but this seems unlikely since phosphocholine is a major cellular metabolite. The more likely candidate for a PC-derived second messenger therefore appears to be diacylglycerol. In this regard, it is noteworthy that distinct molecular species of diacylglycerol are generated by hydrolysis of PC and phosphatidylinositol 4,5-bisphosphate, so PC-derived diacylglycerols might be associated with novel second messenger activities (8, 11, 19, 28). Moreover, protein kinase C denotes a family of isozymes consisting of at least seven members, which may differ in both catalytic and regulatory properties (24, 26). In particular, the ζ isozyme of protein kinase C does not bind phorbol esters (27) and is therefore insensitive to downregulation by treatment with TPA. It is therefore possible that activation of specific isozymes of protein kinase C by PC-derived diacylglycerol is involved in the Ras and PC-PLC signaling events that are not blocked by TPA downregulation.

Alternatively, PC-derived diacylglycerols might activate other protein kinases. In this regard, recent studies have shown that expression of Ha-Ras Asn-17 blocks the activation of MAP kinase (36, 40) and the Raf proto-oncogene kinase (38, 40) in PC12 cells, as well as the activation of Raf in NIH 3T3 cells (38). In addition, Raf is able to bypass the inhibition of both NIH 3T3 cell proliferation (9, 31) and PC12 neuronal differentiation (38) resulting from interference with Ras proto-oncogene function. The Raf and MAP kinases therefore appear to act downstream of Ras in signal transduction pathways, raising the possibility that their activities are regulated by a PC-derived second messenger. However, it is also possible that stimulation of PC turnover and

activation of these kinases are distinct, independent targets of Ras. Indeed, previous studies indicated that Ras is coupled to at least two distinct signaling pathways in both the NIH 3T3 and PC12 cell systems (4, 35).

Finally, it is noteworthy that the turnover of PC induced by mitogens is a long-term response, persisting for several hours after growth factor treatment, and may thus provide a source of diacylglycerol leading to prolonged activation of protein kinase C or other targets (8, 18). Ras activity is similarly thought to be required for several hours following mitogen stimulation, since microinjection of anti-Ras antibodies 6 to 8 h after growth factor exposure still inhibits the initiation of DNA synthesis (23). The persistence of PC-derived second messengers may therefore provide long-term coupling between the growth factor-mediated activation of Ras and subsequent entry of stimulated cells into S phase.

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