Modulation of the components involved in mitogenic signaling cascades is critical to the regulation of cell growth. GTP-binding proteins and the stimulation of phosphatidylcholine (PC) hydrolysis have been shown to play major roles in these cascades. One of the enzymes involved in PC hydrolysis, a PC-specific phospholipase C (PC-PLC) has received relatively little attention. In this paper we examined the role of a particular heterotrimeric GTP-binding protein, Go, in the regulation of cell growth and PC-PLC-mediated hydrolysis of PC in IIC9 fibroblasts. The Go α-subunit was ablated in IIC9 cells by stable expression of antisense RNA. These stably transfected cells acquired a transformed phenotype as indicated by: (a) the formation of multiple foci in monolayer cultures, (b) the acquisition of anchorage-independent growth in soft agar; and (c) an increased level of thymidine incorporation in the absence of added mitogens. These data implicate Go α as a novel tumor suppressor. Interestingly, PC-PLC activity was constitutively active in the Go α-ablated cells as evidenced by the chronically elevated levels of diacylglycerol and phosphorylcholine in the absence of growth factors. In contrast, basal activities of PC-phospholipase D, phospholipase A₂, or phosphoinositol-PLC were not affected. These data demonstrate, for the first time, a role for Go in regulating cell growth and provide definitive evidence for the existence of a PC-PLC in eukaryotic cells. The data further indicate that a subunit of Go α is involved in regulating this enzyme.

Defects in signal transduction cascades involved in the regulation of cell growth often lead to pathological conditions, including the development of neoplasms. Heterotrimeric GTP-binding proteins (G proteins)¹ and induced lipid metabolism are important components in growth factor-coupled cellular signal transduction pathways. Heterotrimeric G proteins are a family of membrane-bound proteins composed of α, β, and γ subunits, which, in response to receptor activation, dissociate into free α subunits and βγ dimers. Both the GTP-bound α subunits and βγ dimers have been shown to play roles in a variety mitogenic signal transduction cascades (1), including those involving induced lipid metabolism (2, 3).

There is now strong evidence indicating that G proteins play crucial roles in the regulation of mitogenic signals and that specific defects in these proteins lead to the development of transformed phenotypes. In addition to the observation that the activation of certain growth factor receptors stimulates the dissociation of G proteins into GTP-bound α subunits and βγ dimers, mutations that reduce the intrinsic GTPase activity in specific α-subunits transform these G proteins into oncoproteins. For example, mutations in the Go α gene result in an oncogene (gsp), the protein product of which is a Go α with substitutions at amino acids 201 (R201C/H) and 227 (Q227R/H) which have been found in growth hormone-secreting pituitary tumors (4). Similarly, mutations in the Gα α gene yield another oncogene (gip2) characterized by a substitution of amino acid 179 in Gα α (R179C/H) which has been found in ovarian sex cord stromal tumors and adrenal cortical tumors (5). These data provide strong support for the notion that these G proteins are important components involved in the regulation of mitogenic signal transduction cascades and represent potential targets for oncogenic mutations in human tumors.

In addition to G proteins, agonist-induced lipid metabolism also plays a central role in mitogenic signaling cascades. While induced hydrolysis of phosphoinositides (PIs) has long been recognized as playing such a role (6), it is now well recognized that induced PC metabolism is often just as, if not more, important (7). Although PI and PC hydrolysis are induced by a variety of mitogens, PI hydrolysis is often transient while PC hydrolysis is usually sustained in the continuous presence of growth factors. In this regard, PC hydrolysis correlates with the requirement for the prolonged presence of growth factors for full mitogenic responses (8).

Depending on the cell type and specific mitogen, three enzymes have been implicated in mitogen-induced PC metabolism: PLA₂, PC-PLD, and PC-PLC. PLA₂ removes the fatty acid esterified at sn-2 of the glycerol backbone in PC resulting in the liberation of a free fatty acid, often arachidonic acid, and a lysophospholipid. PLD-mediated hydrolysis of PC results in the production of phosphatidic acid (PA) and free choline. This generated PA is often, but not always, hydrolyzed by phosphatidic acid phosphohydrolase (PAPH) leading to the production of diacylglycerol (DAG). Alternatively, in some systems (9–15), PC-derived DAGs, in addition to phospholipase, are produced from a PC-PLC-mediated hydrolysis of PC. Most studies have focused on PLA₂ and PLD while very little attention has
been given to PC-PLC. Indeed, the existence of a eukaryotic PC-PLC remains somewhat controversial.

Given this lack of attention to eukaryotic PC-PLC, it is not surprising that the cellular components involved in its regulation have not been identified. The observation that activation of receptors, such as the thrombin receptor (16), which are known to couple to G proteins (17) lead to an increase in PC-PLC activity (15, 18) suggest that this enzyme is regulated by a G protein. That a G protein couples to PC-PLC is consistent with the fact that these proteins are known to regulate other specific phospholipases. PI-PLCβ is regulated by a pertussis toxin-sensitive G protein (19), involving both αq and βγ dimers (20–22). In a similar manner, heterotrimeric G proteins have been implicated in the regulation of a high molecular weight PLAz (23), while PC-PLD has been shown to be regulated by small molecular weight GTP-binding proteins (24). In view of these data, it is reasonable to hypothesize that PC-PLC may also be regulated by a G protein.

In this report, we demonstrate that the ablation of Gα results in a transformed phenotype. Furthermore, in these Gα-ablated cells, PC-PLC is significantly elevated providing definitive evidence for a PC-PLC and implicating Gα, Gα in particular, in the regulation of this enzyme in vivo. The relationship between Gα, the transformed phenotype and the constitutive activation of PC-PLC is discussed.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media components, Lipofectin reagents, Geneticin (G418), and calf alkaline phosphatase (1000 units/μg) were purchased from Boehringer Mannheim. Plastic culture dishes were purchased from Falcon Labware. Highly purified human thrombin (=4000 NIH units/ml) and bovine serum albumin (radioimmunoassay grade, fraction V) were purchased from Sigma. Escherichia coli diacylglycerol kinase was obtained from Lipidex or CalBiochem. AG1x8 Resin (200–400 mesh, formate form) was from Bio-Rad. TLC plates were purchased from EM Diagnostics, Analabs, and Analtech. CytoScint (200–400 mesh, formate form) was from Bio-Rad. TLC plates were purchased from Amersham. Molecular biology enzymes were purchased from Amersham. Molecular biology enzymes were purchased from Stratagene, Life Technologies, Inc., New England Biolabs, and Boehringer Mannheim.

Materials—Tissue culture media components, Lipofectin reagents, Geneticin (G418), and calf alkaline phosphatase (1000 units/μg) were purchased from Boehringer Mannheim. Plastic culture dishes were purchased from Falcon Labware. Highly purified human thrombin (=4000 NIH units/ml) and bovine serum albumin (radioimmunoassay grade, fraction V) were purchased from Sigma. Escherichia coli diacylglycerol kinase was obtained from Lipidex or CalBiochem. AG1x8 Resin (200–400 mesh, formate form) was from Bio-Rad. TLC plates were purchased from EM Diagnostics, Analabs, and Analtech. CytoScint scintillation counting fluid was obtained from ICN. Radioactive materials were purchased from Amersham. Molecular biology enzymes were purchased from Stratagene, Life Technologies, Inc., New England Biolabs, and Boehringer Mannheim. [methy1-3H]Choline (83 Ci/mmol) and [9,10-3H]myristic acid (53 Ci/mmol) were purchased from Amersham. [γ-32P]ATP (3000 Ci/mmol), [methy1-3H]thymidine (6.7 Ci/mmol), and [5,6,8,9,11,12,14,15-3H]arachidonic acid (180–240 Ci/mmol) were purchased from NEN Life Sciences Products. The transfected cells were subcultured and grown for several weeks in selection medium (complete medium supplemented with 500 μg/ml G418). G418-resistant clones were isolated with cloning cylinders and the transfected clones were maintained in complete medium supplemented with 250 μg/ml G418.

All other assays, including growth in soft agar, [3H]thymidine incorporation, Western blot analysis, and quantification of DAG mass, choline metabolites, and PLD activation were performed as described previously (8, 15, 26–29) as indicated in the figure legends.

RESULTS

Gα-ablated Cells Acquire a Transformed Phenotype—To investigate the physiological role of Gα, we stably transfected IIC9 cells with a Gα antisense construct (Fig. 1A). Western blot analysis demonstrated that Gα was absent in the transfected cells while other G protein α subunits, G1α, Gα, Gα, and Gα, were present (Fig. 1B). This has been observed in at least three independently isolated Gα-ablated clones (data not shown).

In contrast to wild type IIC9 cells which are flat and extended (Fig. 2A), Gα-ablated cells appear round, retracted, and form multiple foci in confluent monolayer cultures (Fig. 2B). This morphology, observed in three independently isolated clones, suggests that the Gα-ablated cells have lost contact inhibition and acquired a transformed phenotype.

An important characteristic of transformed fibroblasts is their ability to grow in an anchorage independent manner. In view of this and the above data, we assessed the ability of the Gα-ablated cells to grow in soft agar. As shown in Table I, Gα1 cells formed 20–30 fold more colonies in soft agar than wild type cells and this has been observed in a second, independently isolated clone (data not shown). Furthermore, each of the colonies formed by the ablated cells were much larger and more dense (Fig. 3, B and D) than the colonies formed by the wild type cells (Fig. 3, A and C). Cells transfected with control vectors (vectors without inserts) formed colonies similar to those seen with wild type cells (data not shown).

To further investigate the possibility that the ablated cells...
In view of this and the above observations regarding the transformed phenotype of the G\(_\alpha\)-ablated cells, we measured the mass of DAG in the ablated cells. Subconfluent wild type and ablated cells were incubated in serum-free medium for 2 days and DAG levels were quantified. Interestingly, the basal DAG level in the serum-starved G\(_\alpha\)-ablated cells was twice that of quiescent wild type cells (Fig. 5A). Furthermore, while the addition of \(\alpha\)-thrombin to the wild type cells resulted in a 2-fold increase in DAG mass level, addition of \(\alpha\)-thrombin to the ablated cells did not induce a significant further increase in DAG levels (Fig. 5A). These results, observed in two independently isolated clones, indicate that the DAG level in G\(_\alpha\)-ablated cells was constitutively elevated even in the absence of any added mitogens.

The Increased DAG Is Due to a Constitutively Activated PC-PLC—We have shown that PC hydrolysis is the major, if not exclusive, source of mitogen-induced DAGs in IIC9 cells (8, 15, 26, 33–35). In view of these data, we examined the possibility that an increase in PC hydrolysis contributed to the elevated DAG level in the G\(_\alpha\)-ablated cells.

To determine if PC hydrolysis was affected in the G\(_\alpha\)-ablated cells, the cells were radiolabeled to isotopic equilibrium with \([\text{3H}]\)choline chloride in serum-free medium for 48 h and the intracellular \([\text{3H}]\)choline and \([\text{3H}]\)phosphorylcholine level were quantified (15). TLC analysis of water-soluble head groups indicated that the phosphorylcholine level in the G\(_\alpha\)-ablated cells was 5–10-fold higher than that found in wild type cells. The level of choline in the ablated and wild type cells, however, was identical (Fig. 5B). To ensure that the increased level of radiolabeled phosphorylcholine was not due to contaminant which co-migrated with the phosphorylcholine, the radioactivity in the region of the TLC plate containing phosphorylcholine was recovered, subjected to alkaline phosphatase hydrolysis, and the products were identified by TLC. All of the radioactivity that migrated with phosphorylcholine was converted to choline indicating that co-migrating contaminants were not present (data not shown). These data indicate that both phosphorylcholine and DAG, the two products of PC-PLC, are elevated in ablated cells and strongly suggest that PC-PLC is constitutively activated in G\(_\alpha\)-ablated cells. These results have been observed in three independently isolated clones.

The Increased PC Metabolism Is Not Due to PLD/PAPH/CK Activity—An alternative explanation for the above results is that PC is hydrolyzed via a PLD and the resulting PA is dephosphorylated to DAG, via PAPH, while the free choline is phosphorylated via CK. As a result of the combined action of all three enzymes, PLD, PAPH, and choline kinase (CK), an apparent PC-PLC activity would be detected similar to that observed in v-ras transformed cells (36–38).

In view of the above, PLD activity was quantified in G\(_\alpha\)-ablated and wild type IIC9 cells by taking advantage of the unique transphosphatidylation activity of PLD and the ability to preferentially label PC by acute labeling with \([\text{3H}]\)myristate (15). In the transphosphatidylation reaction, a small molecular

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**TABLE I**

| Cell type | Number of colonies |
|-----------|--------------------|
| WT        | 10 ± 4             |
| Gao1      | 365 ± 39           |

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**FIG. 2. Morphology of G\(_\alpha\)-ablated cells and wild type IIC9 cells in serum-containing and serum-free medium.** Wild type IIC9 cells (A and C) and G\(_\alpha\)-ablated cells (B and D) were grown in complete medium containing 5% FCS in 100-mm culture dishes. Cells were grown for 1 week (A and B) or shifted to serum-free medium after 3 days and incubated in serum-free medium for an additional 9 days (C and D). Serum-free medium was replaced with fresh serum-free media every 3 days. Magnification, × 100.
weight alcohol such as ethanol is used as the nucleophile in lieu of water resulting in the generation of phosphatidylethanol instead of PA. As shown in Fig. 5C, PLD activity is indistinguishable in the ablated and wild-type cells in the absence of thrombin or FCS. Furthermore, the addition of thrombin to both cell types results in comparable increases in PLD activity. These data indicate that both basal and thrombin-activated PLD activity are unaltered in G\(\alpha\)-ablated cells and have been observed in three independently isolated clones.

To further examine the possible involvement of PLD/PAPH/CK activities, CK activity was quantified in wild type and G\(\alpha\)-ablated serum-deprived cells. These cells were incubated with \(^{[3]H}\)choline for 15 and 30 min and the level of radiolabeled phosphorylcholine was quantified. As shown in Fig. 5D, the conversion of choline to phosphorylcholine was essentially identical in both cell types demonstrating that the CK activity was not elevated in the G\(\alpha\)-ablated cells. These results have been observed in two independently isolated clones.

We should note that sphingomyelinase was also not contributing to the increased phosphorylcholine levels in G\(\alpha\)-ablated cells. If this choline metabolite was generated from a sphingomyelinase-mediated hydrolysis of sphingomyelin ceramide, in addition to phosphorylcholine, would be generated. Ceramide levels were quantified, therefore, in wild type and G\(\alpha\)-ablated cells and was found to be identical in both cell types (data not shown). Taken together, the above data eliminate the involvement of PLD/PAPH/CK as a mechanism for the chronic elevation of DAG and phosphorylcholine levels in G\(\alpha\)-ablated cells. In addition, they indicate that G\(\alpha\) ablation-induced transformation is different from v-ras-induced transformation, since the later involves PLD/PAPH/CK activities (36–38).

The Increased PC Metabolism Is Not Due to PLA\(_2\) Activity—Another mitogen-activated PC hydrolyzing enzyme is PLA\(_2\). In IIC9 cells, thrombin and FCS stimulate PLA\(_2\) activity which hydrolyze PC to lysophosphocholine and arachidonic acid (27), both of which have been implicated in mitogenic signaling cascades (7). Basal and \(\alpha\)-thrombin-induced PLA\(_2\) activities in G\(\alpha\)-ablated and wild type cells was assessed by quantifying the release of arachidonic acid and its metabolites. As observed for PLD, basal and \(\alpha\)-thrombin-activated PLA\(_2\) activity was not affected by the ablation of G\(\alpha\) (Fig. 6A). Consistent with these data, glycerolphosphocholine, another metabolite produced by the hydrolysis of PLA\(_2\) generated lysosphospholipid, was also at similar levels in both cell types (data not shown). These data, observed in three independently isolated clones, indicated that PLA\(_2\) activity is unaffected in the G\(\alpha\)-ablated cells.

PI Hydrolysis Is Suppressed in G\(\alpha\)-ablated Cells—Induced PI hydrolysis has been observed in response to a wide variety of mitogens and defects in this metabolism have been implicated in cellular transformation. \(\alpha\)-Thrombin induces the hydrolysis of PIIs and contributes to some of the thrombin-induced increase in DAG in IIC9s at early times (39). We therefore examined PI metabolism in G\(\alpha\)-ablated cells to determine if this hydrolysis contributed to the increased level of DAGs in the ablated cells. PI hydrolysis was quantified in cells radiolabeled with myo-\(^{[3]H}\)inositol as described previously (27). As
**FIG. 5. DAG and PC metabolism in G_o-ablated cells.**

**A.** DAG level in G_o-ablated and wild type cells in 35-mm dishes were incubated in the presence (open bars) or absence (closed bars) of 2 NIH units/ml (1.4 nM) thrombin for the indicated times. DAG mass level was then quantified as described previously (8). Each value represents the average of duplicate samples and is representative of at least three experiments. Error bars represent the range of values and are present on all bars.

**B.** Basal choline and phosphorylcholine levels: G_o-ablated (filled bars) and wild type (open bars) cells (3.5 X 10^6 cells/35-mm dish) were serum deprived and labeled with [3H]choline as described (15, 26, 34). Choline and phosphorylcholine, identified by comparison to known standards, were isolated by TLC and quantified as described previously (15, 26, 34). Each value represents the average of duplicate samples and is representative of at least three experiments. Error bars represent the range of values and are present on all bars.

**C.** Basal and thrombin-induced PLD activity: PLD activity was quantified as the production of phosphatidylethanol (PEt) (15). Briefly, wild type (open bars) and G_o-ablated (filled bars) cells (3.5 X 10^6 cells/35-mm dish) incubated in serum-free medium supplemented with 5 µCi/[3H]myristate for 2 h at 37 °C. The cells were then incubated in fresh serum-free medium alone (Control) or serum-free medium supplemented with 100 mM ethanol in the presence or absence of 2 NIH units/ml (1.4 nM) thrombin for 30 min at 37 °C. Reactions were terminated by aspirating the medium and immediately adding ice-cold methanol. Radiolabeled phosphatidylethanol was then isolated and quantified as described previously (15). Each value represents the average of duplicate samples and is representative of at least three experiments. Error bars represent the range of values and are present on all bars.

**D.** Assessment of choline kinase activity in wild type and G_o-ablated cells: serum-deprived G_o-ablated and wild type cells (3.5 X 10^6 cells/35-mm dish) were washed once and incubated in the serum-free medium supplemented with [3H]choline (5 µCi/ml). At the indicated times, cultures were washed three times in serum-free medium supplemented with 20 mM choline chloride and incubated for an additional 5 min at 37 °C. Total choline metabolites, [3H]phosphorylcholine ([3H]PCho), [3H]choline ([3H]Cho), and [3H]glycerolphosphorylcholine ([3H]GPC) were then isolated by TLC and quantified as described previously (28). Choline kinase activity was measured as the amount of choline converted to phosphorylcholine at the indicated times. Data are presented as the ratio of...
shown in Fig. 6B, basal PI metabolism remains unaffected in the ablated cells. As in wild type cells, thrombin induced PI hydrolysis in the Gα-ablated cells. Interestingly, however, the level of PI metabolism, assessed as IP_{3} production, is attenuated in the ablated cells (11-fold increase in wild type cells and 2.5-fold in Gα-ablated cells). Similar results were obtained for the production of IP_{2} and IP_{4} and have been observed in two independently isolated clones (data not shown).

α-Transducin Reverses the Increase in PC-PLC in Gα-ablated Cells—The above data demonstrate that PC-PLC is constitutively active in Gα-ablated cells. To determine if this activation results from an increase in βγ dimers, Gα1 cells were transfected with the α subunit of transducin (Gα1) (see Ref. 66) which has been used to sequester these dimers (40, 41). As shown in Fig. 7, transfecting Gα1 cells with Gα reduces the PC-PLC activity, indicated by the phosphorylcholine level, to that observed in wild type IIC9s. These data suggest that an increase in βγ dimers is involved in the increased PC-PLC activity observed in Gα-ablated cells and support the notion that these dimers are involved in the receptor-mediated regulation of this enzyme.

Discussion

The data in this paper demonstrate for the first time that the α subunit of the Gα, GTP-binding protein is involved in regulating cell growth and PC-PLC activity. Absence of this protein in IIC9 cells results in: (a) a transformed phenotype, and (b) a 10-fold increase in basal PC-PLC activity without increasing PLD, PLA_{2}, CK, sphingomyelinase, and PI-PLC activities. These results indicate that Gα selectively regulates PC-PLC and is an important component involved in the regulation of cell growth.

While some G proteins (e.g. Ras, Gi, and Gs) are known to be involved in regulating cell growth (1, 42, 43), the data in this work are the first to demonstrate that Gα plays a role in mitogenesis. Ablation of Gα results in a transformed phenotype. This is indicated by anchorage-independent growth (Table I and Fig. 4), formation of foci in confluent cultures (Fig. 2), and increased level of thymidine incorporation when cells are serum-deprived (Fig. 4). We should note that the Gα is selectively ablated in this cell. Other homologous G proteins, including Gα whose coding sequence shows the greatest homology to Gα, are not affected (Fig. 1). This is likely due to the fact that the antisense construct included the 5′- and 3′-untranslated region of Gα which does not possess significant homology to other G proteins, including Gα (Basic Local Alignment Search Tool (BLAST) data base, NCBI, Dec. 1996).

The above data suggest that the presence of Gα in quiescent cells is involved in suppressing cell growth and supports the notion that this protein acts as a tumor suppressor (44). Consistent with this hypothesis, Gα was selectively deficient in two pituitary tumors (7315a, MTW15) (45). In this regard, it is interesting to note that the human Gα gene maps to the chromosomal region (16q13) (Online Mendelian Inheritance in Man, OMIM\textsuperscript{[8]}) associated with the loss of heterozygosity identified in a variety of tumors (46–51). Furthermore, because dominant negative variants of tumor suppressor genes often result in the overexpression of their proteins, our data suggest that the increased levels of Gα protein observed in many neuroendocrine tumors (52, 53) and in Merkel cell carcinoma (54) may result from a dominant negative defect in this G protein, consistent with its role in suppressing cell prolifera-

tion. Taken together, these data demonstrate that Gα is an important component of a mechanism involved in the regulation of cell growth. The suppression of mitogenesis by Gα is a novel observation in that most defects in G proteins resulting in cell transformation involve expression of a constitutively active form (GTPase deficient) of G proteins (4, 5).

In addition to a transformed phenotype, a phospholipase associated with the regulation of cell growth (11, 15, 55–58), PC-PLC, is constitutively active in the Gα-ablated cells. The basal levels of other phospholipases involved in signal transduction cascades, PLA_{2} (Fig. 6A), PLD (Fig. 5C), and PI-PLC (Fig. 6B) are not affected in the ablated cells. Thrombin-induced levels of PLA_{2} and PLD are also unaffected. Interestingly, induced levels of PI-PLC appears to be blunted (Fig. 6B), which may be due to a protein kinase C-mediated inhibition of this enzyme resulting from the elevated levels of DAG as a result of constitutive PC-PLC activation. Importantly, however, the suppression of PI hydrolysis cannot account for the increase in DAG levels observed in the Gα-ablated cells.

The DAG elevation is significant in that the mass amount of this lipid is strictly regulated in wild type cells reflecting its importance in initiating cell proliferation. It is noteworthy that the amount of DAG present in serum-deprived Gα-ablated cells is similar to that observed in wild type cells stimulated with a maximal concentration of mitogen (2 NIH units/ml thrombin or 10% FCS). It is conceivable, therefore, that the elevated level of DAG in the ablated cells is at least partly involved in generating the transformed phenotype. Consistent with these data, comparable DAG levels were observed in NIH 3T3 cells stably transfected with Bacillus cereus PC-PLC which, interestingly, also displayed a transformed phenotype (29).

In the accompanying article (66), we present data demonstrating that Ras and ERK are constitutively active, as well as elevated expression of cyclin D1 and constitutively active cyclin D1-CDK complexes, in the Gα-ablated cells. In this regard, it is interesting to note that PC-PLC has been suggested to play a role in this pathway. Transfection of 3T3 with bacterial PC-PLC reversed the inhibition of cell growth mediated by a dominant negative Ras, but not dominant negative Raf, constructs (55, 59). Consistent with this notion, transfection of cells with bacterial PC-PLC results in a transformed phenotype (60). Although Ras also activates choline kinase that confuses the identification of a PC-PLC (30, 61), there is direct evidence for a Ras-mediated activation of PC-PLC (57). Homogenates of Ras-transformed cells contain a higher PC-PLC activity than their non-transformed counterparts, that appears to be accompanied by an increase in membrane-associated PC-PLC protein (57). Together, these data support the notion that the increased PC-PLC activity observed in the Gα-ablated cells reflects the constitutive activation of the Ras/Raf/ERK signaling pathway.

The increase in PC-PLC is particularly interesting in that there has been some controversy regarding the existence and regulation of this enzyme (7). As mentioned, it is indeed possible that the "apparent" PC-PLC activity observed in some studies was due to the combined increase in PC-PLD, PAPH, and/or CK (30, 36, 37). The data in this work, however, provide definite evidence that a PC-PLC is present and constitutively active in the Gα-ablated cells. Phosphorylcholine and DAG, two products for PC-PLC mediated hydrolysis, are elevated in the ablated cells (Fig. 5, A and B). The increase in phosphoryl-

\[^{[H]}PCHO\text{ relative to the total amount of water-soluble choline metabolites (\[^{[H]}PCHO/[^{[H]}PCHO +[^{[H]}CHO +[^{[H]}glycerolphosphocholine]. Each ratio represents the average of duplicate samples and is representative of at least three experiments. Error bars represent the range of values and are present on all bars.}^{[8]}\]
A, basal and thrombin-induced arachidonic acid release
and inositol trisphosphate. A, basal and thrombin-induced arachidonic acid release: the release of arachidonic acid and its metabolites was quantified essentially as described previously (27). Briefly, serum-deprived wild type and Gα1-ablated cultures (3.5 × 10^5 cells/35-mm dish) were labeled with [3H]arachidonic acid for 48 h and then washed and incubated in serum-free medium supplemented with fatty acid-free bovine serum albumin for at least 30 min at 37 °C. Cells were then incubated in fresh serum free medium alone (open bars) or serum-free medium supplemented with 2 NIH units/ml (1.4 nM) thrombin (filled bars). The release of [3H]arachidonic acid and its metabolites into the media was then quantified (27). Each value represents the average of duplicate samples and is representative of at least three experiments. Error bars represent the range of values and are present on all points.

B, basal and thrombin-induced inositol trisphosphate: inositol trisphosphate levels (IP3), were quantified in myo-[3H]inositol-labeled cultures essentially as described previously (27), except the medium was supplemented with 20 mM LiCl 1 min prior to the addition of growth factors and was present for the remainder of the experiment. Briefly, Gα1-ablated and wild type cells (3.5 × 10^5 cells/35-mm dish) were labeled with myo-[3H]inositol (1 μCi/ml) for 48 h and then incubated in serum-free medium supplemented with 20 mM LiCl in the presence or absence of 2 NIH units/ml thrombin for 15 min at 37 °C. IP3 was then quantified using anion exchange columns as described (27). Each value represents the average of duplicate samples and is representative of at least three experiments. Error bars represent the range of values and are present on all bars.

Three simplified working models for the mechanism by which Gα modulates PLC. In one scheme (referred to as Scheme I), the βγ activation model, PLC-PC is activated by βγ dimers dissociated from Gα1 in response to agonist stimulation. A second model (referred to as Scheme II), the Gα-bound inhibitor model, PLC-PC is bound to an inhibitor which is in a complex with the Gα heterotrimer in quiescent cells. Receptor activation leads to a dissociation of this complex which relieves the inhibition of PLC. In both Schemes I and II, the interaction of the G protein with the enzyme may be direct or may involve an auxiliary protein. This is particularly true for Scheme I as Ras has been implicated in mediating the βγ dimer-induced activation of PLD. The third model (referred to as Scheme III), the dual G protein model, PLC-PC is regulated by two G proteins, Gα1 and another as yet unidentified G protein, Gγ. The GTP-bound form of Gα suppresses PLC-PC activity while this form of Gα stimulates activity. In the basal state, a certain proportion of the Gα subunits of each G protein is present in the free GTP-bound state (62), but the predominant influence is suppression of PLC-PC by Gα. Receptor stimulation results in an increase in GTP-Gα binding to PC-PC to stimulate its activity.

These models can be used to explain the increase in PC-PLC activity observed in the Gα1-ablated cells. In Scheme I, ablation of Gα results in an increased level of βγ dimers resulting in an increase in PC-PLC activity. In Scheme II, ablation of Gα results in an inability of the inhibitor to complex and inhibit PLC-PC. In Scheme III, the ablation of Gα removes the inhibitory G protein, resulting in the presence of the stimulatory G protein, Gα1, only. We should note that Schemes II and III are consistent with the observation that Gα1 constitutively suppresses PI-PLC. Watkins et al. (63) demonstrated that ablation of Gα1 resulted in enhanced basal PI-PLC in a βγ dimer-independent manner. The data in Fig. 7, however, lend strong support to Scheme I. This is supported in the accompanying paper (66) demonstrating that the specific growth-associated activities that are constitutively elevated in Gα1 (Ras/ERK and cyclin D1) are reduced to basal levels in the presence of Gα1. We cannot, however, completely rule out the possibility...
that $\mathrm{G}_\alpha$ functionally complements $\mathrm{G}_\alpha$ in schemes II or III. Experiments to discriminate among these three models, and to completely define the role of this G protein in regulating PC-PLC and cell growth, are in progress.

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