Suppression of Phospholipase C β, γ, and δ Families Alters Cell Growth and Phosphatidylinositol 4,5-Bisphosphate Levels

Canan G. Nebigil*

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892

Received July 15, 1997; Revised Manuscript Received September 17, 1997[⊗]

ABSTRACT: Phosphatidylinositol-specific phospholipase C (PLC) activity reflects a summation of the activities of three families, β, γ, and δ, each of which is regulated differently. In order to understand the contribution of each family to cell proliferation signaling, expression of each family was suppressed by use of an inducible expression vector for antisense PLC sequences in a single cell line, FTO-2B rat hepatocytes. Activation of second messengers of PLC [diacylglycerol (DAG) and inositol 1,4,5-tris-(phosphate) (IP_3)] was dramatically reduced, providing a strategy for probing the consequences of PLC deficiency on cell function. Importantly, while one PLC family was suppressed, the other PLCs actively responded to specific stimuli, suggesting parallel and independent signaling pathways for each PLC family in FTO-2B cells. Selective suppression of each PLC family altered cell growth markedly and differentially. The rank order for suppression of cell growth by loss of a PLC family was $\gamma > \delta > \beta$. Exploration of down-stream growth regulators revealed that loss of β and γ, but not δ, families was associated with markedly reduced basal *ras* and protein kinase C activity. Moreover, suppression of each of the three PLC families caused remarkably reduced basal and stimulated MAP kinase activities. Interestingly, cellular levels of PIP_2 were increased and dramatically correlated with growth inhibition rate in the clones with suppressed PLC activity, suggesting that PIP_2 itself can serve as a second messenger of cell growth regulation.

Hormones, neurotransmitters, and growth factors induce the rapid hydrolysis of PIP_2[1] via their cognate receptors (*1*, *2*). The hydrolysis of PIP_2 is catalyzed by an inositol lipid-specific PLC and is one of the most commonly used signal transduction pathways in a wide range of cell types. PLCs have been grouped into three major families (PLCβ, PLCγ, and PLCδ) based on immunological and structural differences (*3*). Each family has been further classified into subgroups (β1−4, γ1−2, δ1−4) that are encoded by separate genes (*4*). All PLC enzymes hydrolyze PIP_2 and generate two second messenger molecules, DAG and IP_3. IP_3 mobilizes calcium from intracellular stores. DAG activates protein kinase C (PKC), resulting in the phosphorylation of a number of intracellular proteins. An additional level of diversity exists among the PLC families in regulating their activation. PLCβ is activated by GTP-bound αq family (G α11, 14, 16) and $\beta\gamma$ subunits of receptor activated G$\alpha\beta\gamma$ (*5*−*8*), whereas PLCγ is activated by receptor-associated and nonreceptor tyrosine kinases (*9*−*12*). The molecular mechanism of activation of PLCδ remains obscure.

Although the role of PLC has been widely investigated, it is not clear whether activation of PLC isozymes is sufficient to trigger a mitogenic response. Different experimental strategies and different cell systems may explain some discrepancies concerning the role of PLC isozymes in mitogenesis. One strategy used to dissect the role PLC isozymes has been to create constitutively active receptors or to overexpress receptors that constitutively activate PLC. A correlation between cell transformation and PLCβ activation was observed in Rat-1 and NIH3T3 cells overexpressing Gq protein coupled receptors, specifically, adrenergic α-1B (*13*), muscarinic (*14*), and serotonin 5HT-1C receptors (*15*) that couple to PLCβ isozymes. The transforming potential of PLCβ was also correlated with a constitutively activated Gq mutant in NIH3T3 cells (*16*). However, expression in Rat-1 cells of a constitutively activated Gq resulted in increased PLC activity but no cell-transforming activity (*17*). In contrast, expression of a Gα16 mutant in Swiss 3T3 cells resulted in constitutive PLCβ activation and significant inhibition of cell proliferation induced by bombesin, PDGF, and serum (*18*). Experimental strategies using loss of function receptor mutants have also created confusion regarding the role(s) of PLC isozymes in controlling cell growth. Mutant FGFs receptor unable to activate PLCγ1 could still mediate FGF- stimulated DNA synthesis (*19*). In contrast, a PDGF receptor mutant expressed in HepG2 cells failed to activate PLCγ and was unable to trigger DNA synthesis (*20*). When spontaneously hypertensive rat aorta was compared to normal rat aorta, increased PLCδ activity was observed, suggesting that constitutive activation of PLCδ might contribute to the hyperplasia observed in this disease model (*21*).

An other approach used to study the role of PLC in cell growth is overexpression of PLC enzymes. Overexpression of PLCγ did not alter mitogenesis induced by growth factors (*22*) and was not sufficient to stimulate DNA synthesis in Swiss 3T3 cells (*23*). Increased intracellular concentrations

* To whom correspondence should be addressed at his present address: Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), 1 laurent Fries, B.P. 163-67404, Illkirch Cedex, France. Phone number: 33-388-65-33-87. Fax number: 33-388-65-32-01. Email: cNebigil@titus.u-stasburg.fr.

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1997.

[1] Abbreviations: Phosphatidylinositol-specific phospholipase C, PLC; diacylglycerol, DAG; inositol 1,4,5-tris(phosphate), IP_3; phoshatidylinositol 4,5-bisphosphate, PIP_2; protein kinse C, PKC; mitogen activating protein, MAP; or 8-[4-(chlorophenyl)thio]-cyclic AMP, CPT-cAMP; hepatocyte growth factor, HGF/SF; epinephrine, EPI; sense, S; antisense, AS.

S0006-2960(97)01721-2 CCC: $14.00

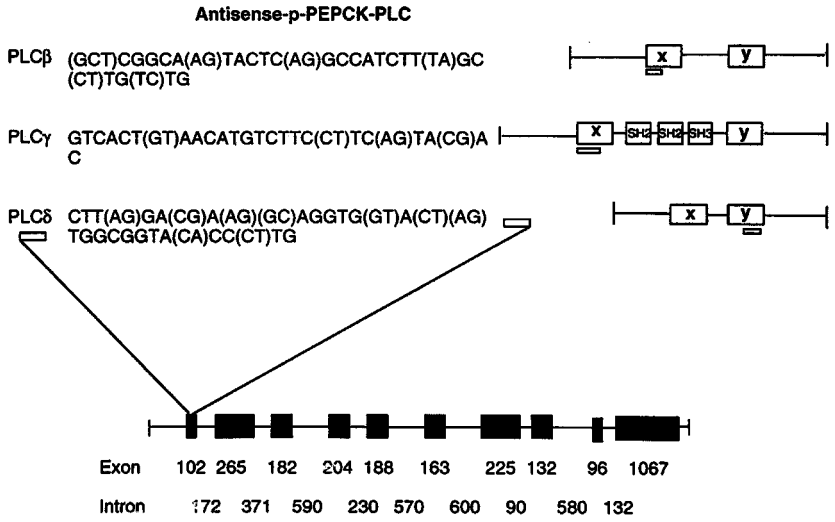

FIGURE 1: Construction of sequences to express RNA antisense to mRNA of PLCβ, -γ, -δ families. The antisense RNA target sequences were chosen on the basis of the least homology among each PLC family (β, γ, and δ) the greatest homology among the PLC family isozymes (e.g. 1, 2, 3). Both sense and antisense sequences with *Bcl*I and *Sal*I restriction sites were prepared with deoxyinosine substitution on both strands of the construct within a first exon of pPCK-AS construct. The ds DNA fragment was ligated in a *Hin*dIII/*Bam*H fragment to the 5.8 kb PEPCK gene and inserted into the PGM-72F vector (sense or antisense-p-PEPCK-PLC).

of PLC isozymes also have been achieved by microinjection of purified protein. Microinjection of either PLCβ1 or PLCγ1 stimulated DNA synthesis in growth-arrested fibroblast cells (*24*). Microinjection has also been used to introduce PLC-specific antibodies to inhibit PLC activity. Microinjection of antibodies to PLCγ into serum-stimulated cells arrested cell growth (*25*). Coinjection of PLCγ1 antibodies together with PLCγ1 into NIH3T3 cells resulted in inhibition of PLCγ1-induced DNA synthesis (*25*). Smith *et al*. (*26*) found that PLCγ1-induced mitogenesis was inhibited by an antibody that did not inhibit the enzymatic activity of PLCγ.

The diversity of structure and regulation of PLCs has made analysis of specific functions of families or individual members a formidable task. The main goal of this study was to create stably transfected cell lines in which the expression of all PLC isozymes in a single family was suppressed and to use these loss-of-function mutants to determine the role of each PLC family in cell proliferation. To this end, the antisense RNA sequences were designed to ensure that PLC suppression was family specific but complete within each family. The extent of the suppression achieved for PLC families was maximized by using a strong, inducible, liver specific promoter from the PEPCK gene (*27*) in a rat hepatoma (FTO-2B) cell line. This cell line provided a particularly useful system for screening, being competent for inducible and tissue-specific activation of the PEPCK gene promoter. Moreover, these cells offered the advantage of expressing members of all three PLC families, thus providing an excellent model system for comparing the influence of suppression of each PLC family on cell growth. Using this system, successful creation of loss-of-function mutants for each of the three PLC families was achieved, and marked differences in the influence of each family on PLC-linked signaling pathways and FTO-2B cell growth were observed.

EXPERIMENTAL PROCEDURES

Cell Culture. FTO-2B cells obtained from Dr. Y. Hod (Department of Physiology at SUNY/Stony Brook) were cultured in 100 mm dishes in DMEM medium supplemented with 10% FBS and 1% penicillin/ streptomycin. The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2.

Design and Construction of Vectors Expressing Antisense RNA. The selectivity of PLC knock out were optimized by designing RNA sequences from catalytic domains (X or Y) common to members within each PLC family (for example, among PLCβ1, -2, -3, and -4), but diverse enough among the families (β, γ, δ, see Figure 1) to ensure family-selective suppression. Complimentary oligos of 35-40 bases in length were synthesized on both strands, and the hybridized ds DNA was then force-cloned into the first exon of the pPKC-AS construct originally described and provided by Moxham *et al.* (*27*). This construct utilizes the 5.8 kb PEPCK gene to express sense or antisense in a cAMP-inducible manner. Fragments harboring the antisense (or sense) sequences were inserted into the pGEM-72F vector (designated pPEPCK-PLC with AS and suffixes denoting antisense and sense configurations, respectively). pPEPCK-PLC-AS (or S) was then subcloned, and primary bacterial colonies were selected by colony hybridization using specific antisense oligonucleotide probes for each individual PLC family member. The selected colonies were amplified and purified, and mixtures containing antisense (or sense) constructs to individual members of each PLC family were used to stably transfect rat hepatoma FTO-2B cells.

Transfection of FTO-2B Cells. Cells was transfected using lipofectin (BRL), according to the manufacturer's protocol. Antisense or sense constructs was cotransfected with a plasmid that provided neomycin resistance as a selectable marker. After transfection for 24 h, the cells were maintained with the medium containing 10% fetal bovine serum (FBS) and the neomycin analogue G-418 (1.5 mg/mL). Clones were visible 10 days after the addition of selection medium and were maintained in long-term culture in 0.5 mg/mL G-418 sulfate.

Northern Blot Analysis. Total RNA was prepared from cells in culture and Northern blot analyses were performed using labeled isozyme specific antisense oligonucleotides as

probes (*28*). Northern analysis of PLC-specific RNA from FTO-2B cells probed with oligonucleotides specific for PLCβ, PLCγ, or PLCδ revealed major hybridizing transcripts with approximate sizes of 28S, 28S, and >18S, respectively. The amount of RNA in each of the samples was determined by phosphorImager analysis.

Immunoprecipitations and Western Blotting. FTO-2B cells at a density of 10^6 cells/dish were lysed and then incubated with a 2 μg of a PLC-specific antibody overnight, followed by a 1h-incubation with protein G-coupled Sephrose at 4 °C. Monoclonal antibodies specific for PLCβ1, PLCγ1, and PLCδ were purchased from UBI. Polyclonal antibodies specific for PLCβ2−3 and PLCδ1 were purchased from Santa-Cruz. Immune complexes were collected by centrifugation, washed twice with lysis buffer, and then subjected to polyacrylamide gel electrophoresis on 7.5% acrylamide gels. The separated proteins were transferred to nitrocellulose for immunobloting and autoradiography. The nitrocellulose blots were blocked using 10% BSA and then incubated with primary antibodies for 1 h at room temperature. Immune complexes of PLCs were detected by autoradiography or color reaction after sequential staining with either ^{125}I-labeled or calf alkaline phosphatase-conjugated goat antimouse IgG for 1 h. Immunoblotting of immunoprecipitated cell extracts of FTO-2B clones with PLC isozyme-specific antibodies or antisera revealed that wild-type FTO-2B cells express PLCβ1 (M_r, 150 kDa), PLCγ1 (145 kDa), and PLCδ (85 kDa). Other PLC isozymes were not detected in the FTO-2B cells.

Measurement of Total Inositol Phosphate (IP) and Inositol 1,4,5-Tris(phosphate) (IP_3) Formation. Total IP levels were measured by the technique of Martin, as modified (*29*). Total inositol phosphates or IP_3 were isolated by chromatography on Dowex AG1-X8 resin in the formate phase. The amounts of radioactivity incorporated into IP and IP_3 was quantified by scintillation counting. The mass of IP_3 was determined by a competitive protein binding assay of ^{3}H IP_3 using rabbit cerebellar membrane as the IP_3 receptor (*30*).

Measurement of Intracellular Diacylglycerol (DAG) Content. Intracellular DAG content was quantified by the method of Preiss (*31*), based on the generation of [^{32}P]-phosphatidic acid label of intracellular DAG in lipid extracts of the cells by [^{32}P]ATP and DAG kinase. Briefly, quiescent cells were stimulated with epinephrine (for 30 s) or HGF/SF (for 1 min) and then extracted with chloroform/methanol (1:2). The samples from the chloroform phase of the cell extraction or vehicle containing a known amount of DAG were evaporated under N_2 in glass tubes. The lipid film was solubilized in octyl β-D-glycoside/DOPG solution (7.5%:25 mM) followed by incubation with reaction buffer (0.5 M imidazole, pH 6.6, 0.21 g of $LiCl_2$, 0.1 M EGTA, 1 mM DTT, and 3.8 μg/ μL of DAG kinase) at room temperature for 15 min. The reaction was started by the addition of 2 μM [^{32}P]ATP. After mixing, the reaction proceeded for 30 min at room temperature. The reaction was stopped with the addition of chloroform/methanol (1:2) solution. After two washes with chloroform, 0.5 mL of the chloroform phase was removed and dried under N_2. The lipid film was dissolved in 5% methanol/95% chloroform and spotted on a thin-layer chromatography plate. The plate was developed with chloroform:acetone:methanol:acetic acid:water mixture (10:4:3:2:1), air-dried, and subjected to autoradiography. The radioactive spots corresponding to phosphatidic acid standards were collected and transferred into scintillation vials for counting. The protein remaining at the interphase of step one was dried, solubilized in 100 μL of 1 M NaOH, and assayed for protein content.

Proliferation Assay. The doubling time of each clone was determined as an index of cell growth. Briefly, either wild-type or FTO-2B cells stably transfected with the expression vector pPEPCK-AS-PLC (both sense and antisense versions) were seeded a density of 3000 cells/well in medium DMEM and 10% FBS. The following day the cells were washed twice with phosphate-buffered saline (PBS) and the medium replaced with 0.1% BSA−DMEM. The cells were maintained in this medium for 48 h, after which the media was changed to DMEM with 10% serum, and CPT-cAMP was added for every 24 h. Cells were harvested and counted each day with the use of a hemocytometer.

Tritiated-Thymidine Incorporation. Cells were seeded on 12-well plates at a density of $5 \times 10^4-10^6$ cells/well in DMEM containing 10% FBS and grown for 2 days. After being washed with PBS, confluent cell layers were serum-starved for 36 h in DMEM, then stimulated with drugs or serum for 20 h in DMEM containing 0.5% FBS, and pulsed for 4 h with 0.5 μCi of [^{3}H]thymidine. The incorporation of radioactive thymidine was terminated by placing the dishes on ice. The cells were washed twice with PBS, and the free radioactivity released in a 0.5 mL extraction with 10% trichloroacetic acid for 20 min on ice, followed by another wash with the same solution. Precipitates containing incorporated label were then hydrolyzed in 0.2 mL of 0.2 N NaOH, 0.2% SDS, and later neutralized with 0.2 N HCl. The incorporated radioactivity was quantified by scintillation counting.

Protein Kinase C (PKC) Assay. Activation of PKC was performed as described by Haesley and Johnson (*32*). PKC activity was assayed by measuring the transfer of [^{32}P]-phosphate from [^{32}P]ATP to the PKC substrate peptide acylated myelin basic protein, Ac-MBP (*4*−*14*), by the method of Yashuda *et al*. (*33*). Samples were assayed in duplicate in the presence and absence of PKC inhibitor peptide (*19*−*36*) or lipid mixture [100 μM phorbol 12-myristate 13-acetate (PMA), 2.8 mg/mL phosphatidyl serine, Triton X-100 mixed micelles] to determine the total PKC activity.

Assay of ras Activation. This assay was used to measure the GTP/GDP binding ratio to *ras* (*34*). Briefly, cells in 100 mm dishes were serum-starved for 12 h and then labeled for 4 h with 60 μCi/mL [^{32}P]orthophosphate in the phosphate-free DMEM. The cells were lysed and incubated with 200 ng/mL monoclonal *ras* antibody (Santa Cruz Biotech., Inc.). The immune complexes were collected by centrifugation. The eluted nucleotides were separated on a polyethylenimine-impregnated cellulose plates by thin-layer chromatography in 0.75 M K_2HPO_4 (pH 3.4). Nonradioactive GDP and GTP standards were used to identify the labeled products.

Assay of Mitogen-Activated Protein (MAP) Kinase Activity. This assay was adapted from previously described methods (*35*). Briefly, quiescent cells were incubated with epinephrine or HGF/SF at 37 °C for different time periods or left untreated. After stimulation, the cells were lysed and then incubated with monoclonal mouse anti-MAPK antibody (ERK1+2, Zymed, Lab. Inc., San Francisco, CA) at 4 °C overnight. The cell lysates were mixed with Agarose beads coupled to goat anti-mouse IgG and rotated for 2 h at 4 °C.

The immune complexes were collected by centrifugation. Twenty microliter aliquots were mixed with 20 μL of 50 mM glycerophosphate, pH 7.2, 100 μM sodium vanadate, 20 mM $MgCl_2$, 200 μM [^{32}P]ATP, 50 μg/mL PKA inhibitor peptide (IP20, TTYADFIASGRTGRRNAIHD), 1 mM EGTA, and 400 μM EGFR-(662−681)-peptide (RRELVEPLTPSGEAPNQALLR). After 15 min at 30 °C, 10 mL of 20% trichloracetic acid was added and 45 μL of the reaction mix was spotted onto 2 cm squares of P-81 phosphocellulose paper (Whatman). The papers were washed in 0.75 M phosphoric acid and allowed to dry, and radioactivity was counted by liquid scintillation counting.

Measurement of Phosphatidylinositol Bis(phosphate) (PIP_2). PIP_2 and PI were assayed as described elsewhere (*36*). Briefly, cells were maintained in 2 mL of RPMI (without inositol supplement) and 8 μCi (0.5 MBq) of *myo*-[^{3}H]-inositol for 24 or 48 h prior to studies. No significant difference was observed in the ratio of total mass of PI or PIP_2 comparing 24 and 48 h incubations of the cells with *myo*-[^{3}H]inositol. The phospholipids from FTO-2B cells were extracted in 2 mL of chloroform:methanol (1:1) and 0.5 mL of 10 mM EDTA, pH 7.4, was added. The lower phase was separated and dried under N_2 at room temperature. For phopholipid separation, TLC chromatography was used with a solvent system consisting of chloroform:methanol: acetic acid:water (25:15:4:2) which separated PI from LPI. PIP_2 and PI spots corresponding to standards were collected (R_f for PI is 0.66). PI was isolated by chromatography on formaldehyde-treated paper with a solvent system consisting of the upper phase of butanol:acetic acid:water:diethyl ether (32:8:40:1) which separated PI from other phospholipids including PS. Lipid phosphorus was assayed according to the method of Rebecchi et al. (*36*) with slight modifications. Briefly, the phospholipid spots were separated on TLC using chloroform:methanol:acetic acid:water (25:15:4:2), visualized by iodine staining, scraped, and extracted with several volumes of chloroform:methanol:water (5:5:1), and the combined extracts were dried under N_2. For the phosphate release, the samples were digested with 70% of perchlorate. After addition of color reagent (6 N H_2SO_4, 2.5% ammonium molybdate, 2.5% ascorbate, and water to 50 mL) to the digested samples, the standards and samples were assayed with a spectrophotometer at 800 nm. The assay was linear up to 50 nmol of phosphate. The lower limit of sensitivity was 0.015 μg of phosphorus/mL.

RESULTS AND DISCUSSION

Establishment of Transfectants Stably Expressing S or AS PLC Construct. The selectivity of suppression was optimized by designing RNA sequences common to all three PLC families but diverse enough among families to ensure suppression was family specific (Figure 1). The maximum suppression for PLC families was achieved by using a strong, inducible, liver-specific promoter (PEPCK) in the rat hepatoma FTO-2B cell line. The PEPCK gene promoter is inducible by cAMP or 8-[4-(chlorophenyl)thio]-cyclic AMP (CPT-cAMP), a stable analogue of cAMP (*27*). CPT-cAMP (250 nM) induces expression of the PEPCK gene promoter without altering properties of wild-type FTO-2B cell morphology and growth (doubling time, 28−32 h in either the absence or the presence of CPT-cAMP). For each construct, stable transfectant clones were isolated that harbored either sense or antisense RNA sequences. Two or more independently isolated clones were analyzed for each construct, yielding essentially identical results and eliminating possible clonal aberrations from the results presented.

Expression of RNA Antisense, but Not Sense, to PLC Families Suppressed Targeted RNAs. The ability of antisense RNA expression to suppress mRNA levels of targeted PLCs was evaluated in cultures of stable transfectant clones challenged with 250 nM CPT-cAMP for 3−12 days (Figure 2A). Northern analysis with probes specific to PLCβ, -γ, or -δ families revealed a time-dependent loss of mRNA of the PLC targeted. Quantitation of the suppression of steady-state mRNA levels in FTO-2B clones demonstrated reductions of 70−80% after 12 days of induction for all PLC mRNAs evaluated. cAMP-induced loss of mRNA was most rapid and profound for PLCγ and least for PLCβ. For PLCγ, cAMP induced a 25% loss in mRNA levels within 3 days and an 85% suppression by 12 days. PLCβ mRNA levels, in contrast, were unaffected at day 3 and suppressed 70−80% by day 12 of induction. PLC mRNA levels in FTO-2B clones expressing the sense counterparts, in contrast, provided the ideal control by which to measure suppression attributable to antisense RNA expression.

Expression of RNA Antisense, but Not Sense, to PLC Families Suppresses Targeted PLC Expression. To ascertain whether the attenuation PLC mRNA levels were sufficient to reduce expression at the protein level, immunoblot analysis of PLC expression was performed (Figure 2B). Expression of PLCβ1, PLCγ1, and PLCδ was analyzed in wild-type FTO-2B cells and stable transfectants harboring either the sense or antisense RNA vectors at 3, 6, or 12 days after challenge with CPT-cAMP. The expression of the M_r 150 kDa PLCβ1 was nearly undetectable by day 12 of CPT-cAMP treatment. Faster migrating, immunoreactive species likely representing proteolytic fragments of the PLC were visible in the blots and the amount of these species declined also following induction of the antisense RNA construct. The amount of PLCγ1 with M_r 145 kDa, likewise, was reduced more than 90% in clones harboring the RNA expression vector antisense, but not sense, to PLCγ. PLCγ expression in the wild-type cells or sense RNA containing stable transfectants displayed normal levels of expression following 12 days of induction with CPT-cAMP. Clones harboring the RNA expression construct antisense to the PLCδ family displayed less than 10% of the cellular complement of the PLCδ1 following a 12-day challenge with CPT-cAMP, while clones harboring the sense construct displayed a level of PLCδ1 indistinguishable from that of wild-type FTO-2B cells. These data demonstrate the success of the strategy to target PLC families through the use of inducible RNA vectors designed to express antisense RNA sequences to the entire family.

Suppression of PLC Expression Attenuates Basal and Agonist-Stimulated IP_3 and DAG Formation. Analysis at the level of RNA and protein expression demonstrated successful suppression of PLC families via the inducible antisense RNA strategy. To investigate how suppression of specific PLCs would affect cell signaling, IP_3 and DAG formation was examined in clones harboring sense and antisense RNA expression vectors for each of the PLC families. Total IP accumulation was decreased in unstimulated, stable transfectant clones expressing RNA antisense to PLCβ, -γ, or -δ (Figure 3A, right).

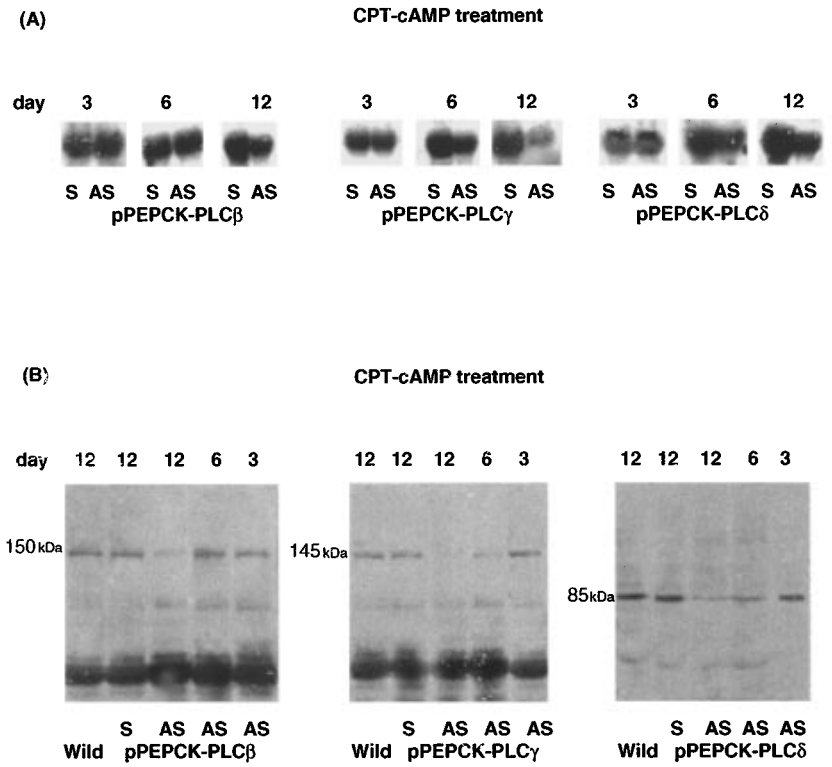

FIGURE 2: (A) Expression of RNA antisense, but not sense, to PLC families suppressed targeted RNAs. FTO-2B clones were treated with CPT-cAMP (250 nM) for different time periods (3, 6, 12 days) to induce antisense or sense RNA expression. Total RNA (15 or 30 μg/lane) from each cell clone was size fractionated by electrophoresis on 1% agarose gels containing formaldehyde and MOPS and then transferred to nitrocellulose membrane for Northern blot analysis as described in the Experimental Procedures. The results shown are representative of three independent experiments for each FTO-2B clone. Total cellular RNA was prepared from sense and antisense RNA-expressing clones at each time point shown. The samples were paired and subjected to electrophoresis and Northern analysis. The amount of RNA in each of the samples was determined by phosphorImager analysis. (B) Expression of RNA antisense, but not sense, to PLC families suppressed targeted PLC expression. FTO-2B clones were treated with CPT-cAMP (250 nM) for different time periods (3, 6, 12 days) to induce antisense or sense PLC expression. Steady state levels of specific PLC isozymes were determined by immunoblot analysis as described in the Experimental Procedures. Briefly, cultured FTO-2B cells (10^6 cells/dish) were washed twice with cold PBS and harvested. Cell lysates were prepared and incubated with PLC antibodies (2 μg) overnight followed by a 1 h incubation with protein-G−Sephrose at 4 °C. Immune complexes were collected and washed two times with solubilization buffer and subjected to SDS−PAGE on 7.5% acrylamide gels. The separated proteins were transferred to nitrocellulose and subjected to immunobloting and autoradiography. The results shown are representative of three independent experiments for each FTO-2B clone.

PLCβ can be stimulated in response to epinephrine via a G-protein-coupled, α-adrenergic receptor (*13*) that is expressed in liver cells (*37*). Expression of constitutively active α-1B adrenergic receptors results in persistent activation of PLCβ and IP_3 formation (*13*). Epinephrine was selected to activate the receptor−Gq−PLCβ pathway in the FTO-2B cells. Challenge of either wild-type or sense RNA-expressing PLCγ clones with epinephrine (100 μM) stimulated IP_3 accumulation, whereas clones expressing RNA antisense to PLCβ displayed a markedly blunted response over the time-course studied (Figure 3A, left).

Hepatocyte growth factor (also known as scatter factor; HGF/SF) induces mitogenesis and cell dissociation upon binding to the protein-tyrosine kinase receptor encoded by c-met in rat and human hepatocytes (*38*, *39*). HGF/SF also activates PLCγ, *ras*, and phosphoinositol-3 kinase (*40*). Challenge of FTO-2B clones with HGF/SF (100 ng/mL) stimulated IP_3 accumulation in sense RNA-expressing PLCγ clones, as shown in the time-course in the left-hand panel of Figure 3B. IP_3 accumulation in response to HGF/SF was decreased more than 70% in the clones expressing RNA antisense to PLCγ (Figure 3B, left).

Activation of PLC generates an endogenous activator of protein kinase C, DAG, in addition to IP_3. Basal DAG accumulation was diminished in the clones expressing RNA antisense to PLCβ, PLCγ, or PLCδ as compared to wild-type FTO-2B cells (Figure 3B, right) or clones expressing the sense RNA (data not shown). Epinephrine-stimulated DAG accumulation was diminished by 70% in the clones expressing RNA antisense to PLCβ. For the clones expressing RNA antisense to PLCγ , stimulation by HGF/SF (100 ng/mL) resulted in a markedly blunted DAG accumulation, reduced to less than 30% of the wild-type response (Figure 3B, left). These data demonstrate that the loss-of-function mutants all displayed reduced basal or stimulated levels of PLC activity as measured by either IP_3 or DAG accumulation.

Experiments were designed to determine if the suppression of the one PLC family influenced the signalings linked to other PLC families naturally expressed in the FTO-2B cell line. IP_3 and DAG accumulation was assayed in clones expressing RNA sense and antisense to one of the three PLC families in response to agonists that activate two PLC (PLCβ and PLCγ) pathways. Activation of IP_3 and DAG in response to epinephrine was absent in the PLCβ-deficient clones, but not in the PLCγ- or PLCδ-deficient clones. Activation of these two molecules in response to HGF/SF was absent in the clones lacking PLCγ, but not PLCβ and PLCδ (Table 1). These data clearly shows that selective suppression of a specific PLC family did not alter the

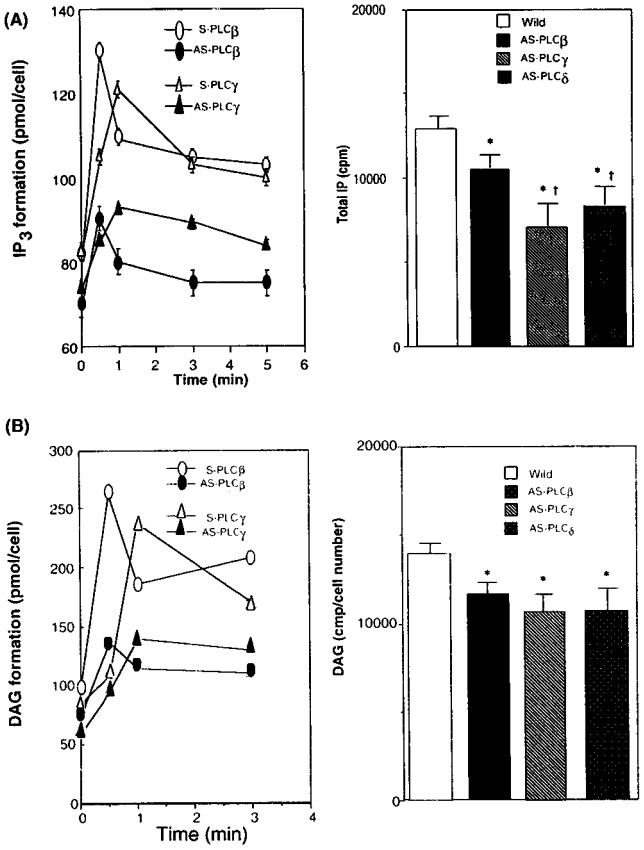

FIGURE 3: (A) Suppression of PLC expression attenuates basal and agonist-stimulated IP_3 accumulation. Cells were maintained in 2 mL of RPMI (without inositol supplement) with 0.5% FBS and 8 μCi (0.5 MBq) of *myo*-[^{3}H]inositol for 24 h prior to studies. Total IP and IP3 accumulation were detected and quantified as described in the Experimental Procedures. Basal total IP (right) and agonist-stimulated IP_3 accumulation (left) were attenuated in the all antisense RNA clones. The agonists used were epinephrine (100 μM) for antisense/sense PLCβ clones and HGF/SF (100 ng/mL) for antisense/sense PLCγ clones. The results were representative of three independent experiments obtained from each group of cell clone (mean ± SEM). Asterisks (*) indicate a significant difference from wild FTO-2B cells ($p < 0.05$). (B) Suppression of PLC expression attenuates basal and agonist-stimulated DAG accumulation. DAG accumulation was detected and quantified as described in the Experimental Procedures. Basal DAG (right) and agonist-stimulated DAG formation (left) were attenuated in the all cell clones transfected with antisense constructs. The agonists used were epinephrine (100 μM) for antisense/sense PLCβ clones and HGF/SF (100 ng/mL) for antisense/sense PLCγ clones. The results were representative of three independent experiments obtained from each group of cell clone (mean ± SEM). "Tee" (t) indicates a significant difference from antisense PLC clone ($p < 0.05$). Asterisks (*) indicate a significant difference from wild FTO-2B cells ($p < 0.05$).

signaling molecules (IP_3 and DAG) of the other PLC families.

Suppression of PLC Expression Inhibits Cell Growth. In stably transfected rat hepatoma cell clones, the loss of PLCβ, -γ, and -δ families resulted in a profound effect on cell growth (Figure 4). The doubling time for wild-type cells and those clones stably expressing the sense version of the vector was about 26 h. Induction of the expression vector with 250 nM CPT-cAMP did not alter the doubling time of these clones, whereas induction of the expression vectors which suppress PLC families by antisense RNA sequences significantly increased the cell doubling times. Decreased cell growth rates by day 12 of induction were not uniform among the PLC families targeted for suppression. The

Table 1. Suppression of Targeted PLC Does Not Alter IP_3 or DAG Formation Induced by Activation of Other PLC Isozymes[a]

cell clones	basal	epinephrine (100 μM)	HGF/SF (100 ng/mL)
	IP_3 Accumulation (pmol/10^6 cells)		
PLCβ			
sense	85 ± 5	135 ± 8	125 ± 10
antisense	69 ± 5*	88 ± 8*	120 ± 8
PLCγ			
sense	83 ± 7	128 ± 8	128 ± 8
antisense	68 ± 7*	130 ± 5	90 ± 10*
PLCδ			
sense	86 ± 4	125 ± 8	117 ±10
antisense	69 ± 10*	132 ± 8	123 ± 8
	DAG Accumulation (pmol/10^6 cells)		
PLCβ			
sense	98 ± 8	262 ± 8	230 ± 10
antisense	67 ± 5*	85 ± 8*	225 ± 8
PLCγ			
sense	80 ± 10	255 ± 8	237 ± 10
antisense	69 ± 7*	257 ± 5	131 ± 10*
PLCδ			
sense	92 ± 10	260 ± 8	225 ± 10
antisense	69 ± 10*	255 ± 8	220 ± 10

[a] IP_3 or DAG formation were detected and quantified as described in Experimental Procedures. The results are representative of three independent experiments obtained from each group of cell clone (mean ± SEM). t, significant difference from basal ($p < 0.05$). *, significant difference from sense ($p < 0.05$).

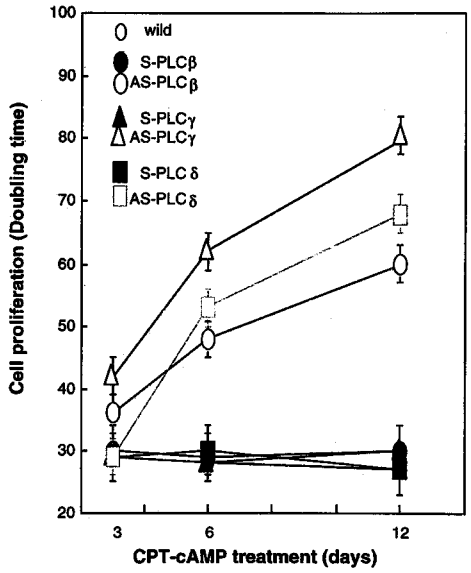

FIGURE 4: Suppression of PLC expression inhibits cell growth. Cell clones were seeded at 3000 cells/well in DMEM supplemented with 10% serum for 24 h. CPT-cAMP (250 nM) was replenished and cells were counted every day. The doubling time of cells [wild type cells and sense (S) and antisense (AS) clones] was determined by cell counting.

greatest increase in cell doubling time, from 26 to 80 h, was observed in the cells deficient in PLCγ. Loss of PLCβ increased doubling time the least (58 h). Loss of PLCδ yielded an intermediate doubling time of 65 h.

DNA synthesis was also measured independently using the incorporation of tritiated thymidine as an index (Figure 5). Basal levels of thymidine incorporation were reduced significantly ($p < 0.05$) for the cells deficient in PLCβ, PLCγ, or PLCδ. Epinephrine (100 μM) or HGF/SF (100 ng/mL) was used to stimulate DNA synthesis in wild-type FT0−2B cells. The epinephrine response was antagonized by the α1-antagonist prazosin (1 μM), whereas the HGF/SF

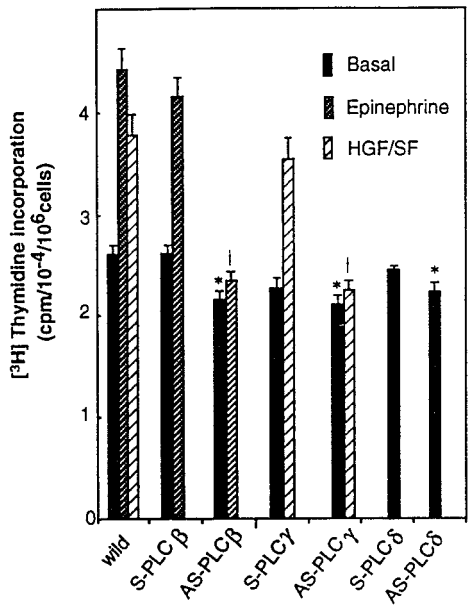

FIGURE 5: DNA synthesis was reduced in PLC-deficient cells. [^{3}H]-Thymidine incorporation was assayed in the wild FTO-2B cells and cell clones. The confluent cell layers were starved for 48 h in DMEM containing 0.1% BSA, stimulated with drugs for 20 h in DMEM containing 0.1% BSA, and pulsed for 4 h with 0.5 μCi of [^{3}H]thymidine. The agonists used were epinephrine (100 μM) for antisense/sense PLCβ expressing cell clone and HGF (100 ng/mL) for antisense/sense PLCγ expressing cell clone. [^{3}H]Thymidine incorporation was detected and quantified as described in the Experimental Procedures. The results shown are representative of four independent experiments obtained from wild type FTO-2B cells and sense (S) and antisense (AS) clones (mean $\pm$ SEM).

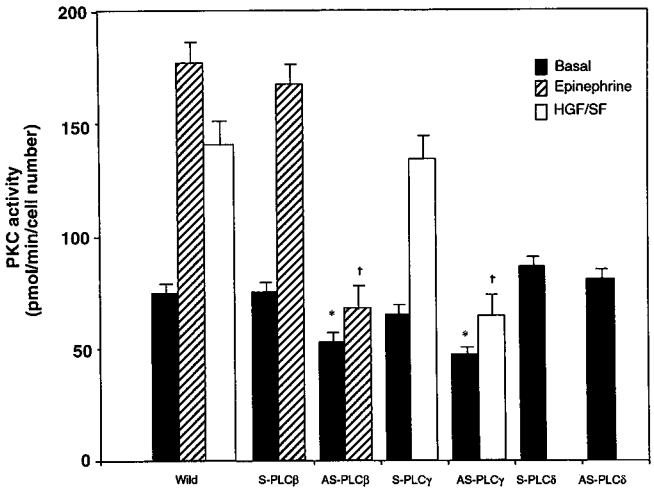

FIGURE 6: Activation of PKC is attenuated by suppression of PLCβ and PLCγ, but not PLCδ. PKC activity was assayed as described in the Experimental Procedures. The agonists used were epinephrine (100 μM for 30 s) for antisense/sense PLCβ expressing cell clone and HGF (100 ng/mL for 1 min) for antisense/sense PLCγ expressing cell clone. The results were representative of four independent experiments obtained from wild type FTO-2B cells and sense (S) and antisense (AS) clones (mean $\pm$ SEM).

response was blocked by genistein (100 μM), demonstrating the contribution from the G-protein-mediated and tyrosine kinase receptor-mediated pathways, respectively. Suppression of PLCβ (AS-PLCβ) abolished the response to epinephrine (Figure 5), as was noted for the IP_3 and DAG response to epinephrine (Figure 3). The response to HGF/SF was abolished in the PLCγ-deficient cells (Figure 5), much like the IP_3 and DAG response to HGF/SF. Serum (10%)-induced DNA synthesis (1.75-fold over control in wild type) was reduced in clones deficient in PLCβ (40%), PLCγ (54%), or PLCδ (45%) as compared with their sense or wild-type cell lines, suggesting that signals occur through all three families in response to serum. This data clearly indicate that suppression of specific families of PLC has distinct and profound effects on cell growth.

Activation of PKC Is Attenuated by Suppression of PLCβ and PLCγ but Not PLCδ. Signaling pathways implicated in cell growth were explored in an effort to define the link between the loss-of-function of a PLC family and the associated growth reduction. PLC activation generates DAG, thereby activating PKC. PKC has been shown to contribute cell growth (*41*, *42*) in many cells. In view of its position as a down-stream element in PLC action, PKC and its role in FTO-2B cell growth was investigated. Direct measurement of PKC activity in stably transfected clones and wild-type FTO-2B cells was informative (Figure 6). In wild-type cells, epinephrine (100 μM at 30 s) and HGF/SF (100 ng/mL at 1 min) stimulated peak activation of PKC activity (data not shown). Suppression of PLCβ (AS-PLCβ) resulted in a reduction in the basal PKC activity as well as a dramatic blunting of the epinephrine-induced activation. Suppression of PLCγ (AS-PLCγ) resulted in a similar reduction in basal and HGF/SF-induced PKC activity. Interestingly, the loss of PLCδ (AS-PLCδ) did not alter the basal level of PKC activity in these cells. Thus, PLCβ and PLCγ families appear to contribute to basal levels of PKC activity in unstimulated cells, since their suppression was associated with the decrease in basal PKC activity. For PLCδ, loss of activity does not appear to influence basal level of PKC activity. PKC activity measured in the presence of PMA was equivalent for wild-type and stably transfected clones (200$-$230 pmol/min per 10^6 cells), irrespective of whether RNA sense or antisense to PLCβ, -γ, or -δ families was expressed. Northern analysis of PKCα mRNA and immunoblotting of PKC protein (data not shown) indicated that PKCα expression was unaffected by altered PLC expression. Reduction of PKC activity by suppression of specific PLC families did not correlate with the cell growth inhibition in each of the three PLC family deficient cells.

Suppression of PLCβ and PLCγ, but Not PLCδ, Blunts ras Signaling. In light of the central role of *ras* in regulating cell growth (*43*), we explored the status of *ras* signaling in the clones whose growth was severely blunted by PLC suppression (Figure 7). The activation state of *ras* was assayed by measuring GTP/GDP binding in the unstimulated basal state as well as in response to epinephrine or HGF/SF. Stimulation of *ras* GTP binding was maximal within 5 min of challenge by either agent. In the PLCβ-deficient, but not PLCγ- or PLCδ-deficient, cells, the ability of epinephrine to activate *ras* GTP binding was abolished. The HGF/SF-induced response was abolished in the PLCγ-deficient cells, but not PLCβ- or PLCδ-deficient cells. Whereas the suppression of either PLCβ or PLCγ families resulted in a marked reduction in the amount of *ras* GTP binding in the basal state, suppression of PLCδ was without effect on basal *ras* GTP binding (Figure 7, Table 2). Again, suppression of one family influenced only its associated signaling pathway, while signaling pathways stimulated by other PLC families remained unaltered. These data also show that in rat hepatoma cells, activation of *ras* via tyrosine kinase receptors like the HGF/SF receptor or via G-protein-

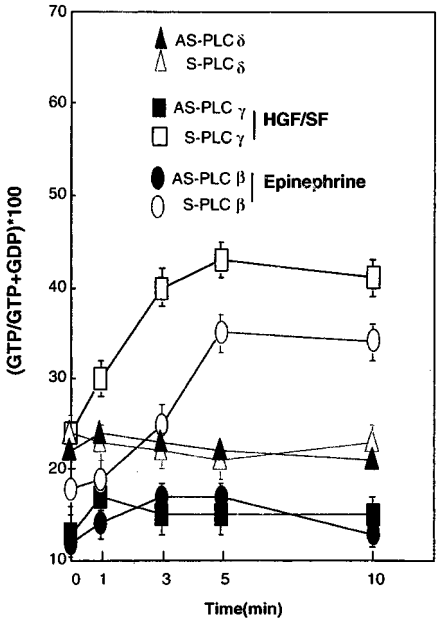

FIGURE 7: Suppression of PLCβ and PLCγ but not PLCδ blunts *ras* signaling. The time course of *ras* activity was performed in the cell clones in response to agonists. The agonists used were epinephrine (100 μM) for antisense/sense PLCβ expressing cell clone and HGF (100 ng/mL) for antisense/sense PLCγ expressing cell clone. The change in GTP/GDP ratio of bound guanine nucleotide to *ras* was determined by the method described in the Experimental Procedures. The results shown are representative of four experiments obtained from sense (S) and antisense (AS) clones (mean ± SEM).

Table 2. Selective Suppression of a Specific PLC Family Did Not Alter Significantly the Signaling via *ras* and MAP Kinase by the Nontargeted PLCs[a]

	ras GTP binding (GTP/GTP + GDP) × 100			MAP kinase activity (pmol/min per 10^6 cells)		
cell clones	basal	epi	HGF/SF	basal	epi	HGF/SF
S-PLCβ	18 ± 3	35 ± 3	40 ± 3	28 ± 5	95 ± 5	110 ± 6
AS-PLCβ	12 ± 2	18 ± 2	38 ± 3	17 ± 5	40 ± 8	112 ± 8
S-PLCγ	24 ± 3	41 ± 5	39 ± 3	38 ± 5	98 ± 9	125 ± 8
AS-PLCγ	13 ± 2	35 ± 3	16 ± 3	15 ± 5	95 ± 9	37 ± 8
S-PLCδ	24 ± 3	38 ± 3	39 ± 3	35 ± 5	94 ± 8	125 ± 8
AS-PLCδ	22 ± 2	35 ± 3	42 ± 3	20 ± 5	95 ± 8	111 ± 6

[a] MAP kinase activity and *ras* GDP/GTP binding were determined as described in the Experimental Procedures. The results are representative of three independent experiments obtained from sense (S) or antisense (AS) PLC cell clone (mean ± SEM).

mediated receptors requires PLCβ and PLCγ activation, respectively.

MAP Kinase Activity Is Attenuated in PLC-Deficient Cells. Analysis of the loss-of-function PLC mutants revealed roles for PKC and *ras* signaling in the cell growth noted in these clones. A common point of convergence for all three parameters is MAP kinase, which is regulated by a cascade of serine/threonine kinases and modulates transcription factor activity (*44*). MAP kinase activity was dramatically reduced in clones deficient in PLCβ, -γ, or -δ families (Figure 8); basal levels were reduced 40−60% (Table 1). The reduced basal activity of MAP kinase in response to PLCδ suppression was unexpected in view of its lack of effect on *ras* GTP binding, suggesting that PLCδ contributes to basal MAP kinase activity in FTO-2B cells. MAP kinase activity was stimulated acutely by treating FTO-2B cells with either epinephrine or HGF/SF. Activation of MAP kinase in

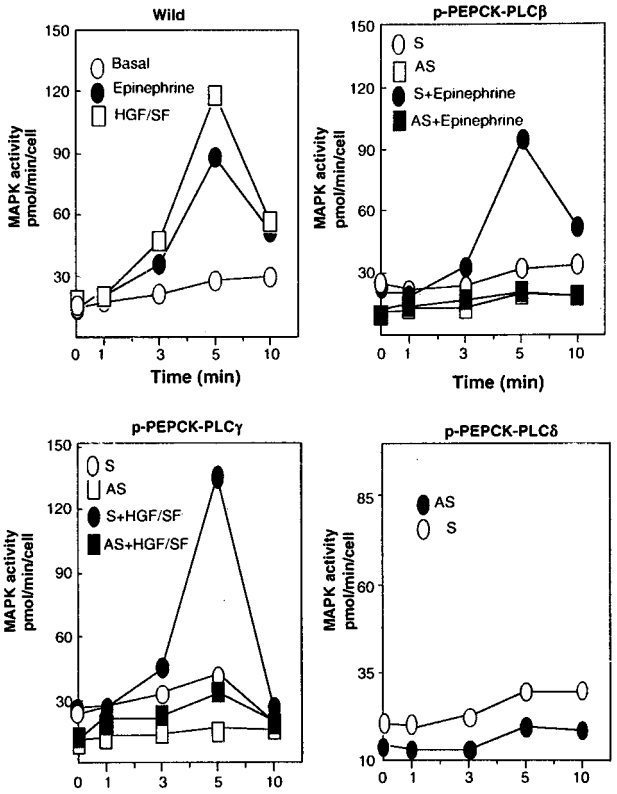

FIGURE 8: MAP kinase activity is attenuated in PLC-deficient cells. The time course of MAP kinase activity in the wild (A) and clones expressing antisense/sense RNA for PLCβ (B), PLCγ (C), and PLCδ (D) was determined as described in the Experimental Procedures. The agonists used were epinephrine (100 μM) for antisense/sense PLCβ expressing cell clone and HGF/SF (100 ng/mL) for antisense/sense PLCγ expressing cell clone. The results were representative of three independent experiments obtained from wild type FTO-2B cells and sense (S) and antisense (AS) clones (mean ± SEM).

response to epinephrine was absent in the PLCβ-deficient clones, whereas the response to HGF/SF was absent in the clones lacking PLCγ. These changes in *ras* and MAP kinase reflect changes in activity, not abundance, as shown by immunoblot analysis (data not shown). In response to stimulation, clones expressing RNA antisense to a PLC family displayed markedly reduced signaling, while not affecting signaling via other PLC families that were not targeted by the construct (Table 2). Loss of PLCβ, for example, abolished stimulation of MAP kinase in response to epinephrine, but not HGF/SF. Together these observations suggest that the mitogenic potential of the cells lacking PLCβ, -γ, or -δ families was not globally compromised by PLC deficiency and clearly indicate differential contributions of PLCβ and PLCγ to basal rates of cell growth.

Suppression of PLC Expression Elevates PIP$_2$ and PI. To explore whether the loss of a specific family of PLC would influence the levels of either PI or PIP$_2$, cells were metabolically labeled for 24 and 48 h, and a total cell lipid extraction was performed. Specific activity determinations revealed no effect of the loss-of-function mutants on PI or PIP$_2$ specific activity (data not shown). In fact, levels of PI and PIP$_2$ were significantly elevated (Figure 9), suggesting that when PLC expression is suppressed, PI or PIP$_2$ levels increase proportionally to the degree to PLC suppression. PI levels increased 25%, 37%, and 55% over controls in clones deficient in δ, β, and γ PLC families, respectively. For PIP$_2$ levels, deficiency in PLCγ yielded the most profound effect,

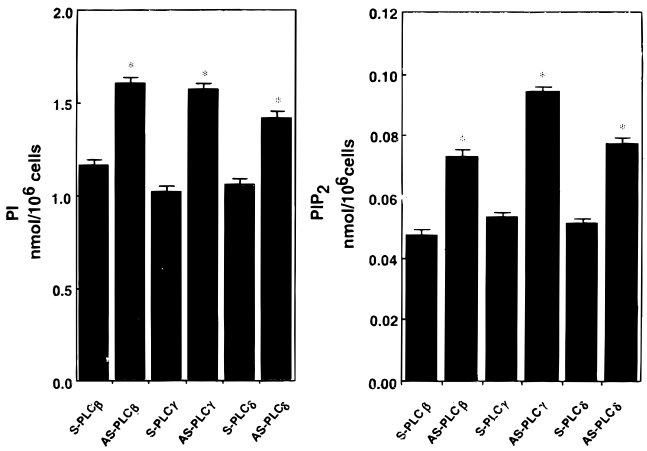

FIGURE 9: Suppression of PLC expression attenuates the metabolism of PIP_2. Cells were maintained in 2 mL of RPMI (without inositol supplement) with 0.5% FBS and 8 μCi (0.5 MBq) of *myo*-[^{3}H]inositol for 24 h prior to studies. PI and PIP_2 mass and specific activity were detected and quantified as described in the Experimental Procedures. Basal PI (left) and PIP_2 (right) were increased in the PLC-deficient cell clones. The specific activity for PI and PIP_2 was not changed in the clones expressing antisense RNA for each of PLC families (the ratio of specific activity of antisense (AS)/sense (S) clones was 1.034 $\pm$ 0.03). The results were representative of three independent experiments obtained from each group of cell clones (mean $\pm$ SEM).

increasing the complement of PIP_2 88% over controls. Both PLCβ- and PLCδ-deficiencies yielded an increase of $\sim$50% in PIP_2 levels. These data suggests PLC is rate-limiting for PI or PIP_2 hydrolysis. All three families contribute to PI or PIP_2 hydrolysis in these cells, and the suppression of one family is not compensated for by increased activity in other families. The increased levels of PI or PIP_2 suggest a lack of negative feedback mechanisms to regulate their levels. Their increased levels in the face of decreased cell growth suggest that IP_3 and DAG production by PLCβ and PLCγ families are critical in α-adrenergic and growth factor receptor pathways, respectively. Interestingly, the rank order of PLC deficiency with respect to inhibition of cell growth and elevation of PIP_2 levels were very similar.

Conclusions. This study demonstrates that suppression of each of the three PLC families leads to a disruption of a subset of cytoplasmic signals that ultimately contribute to the growth rate of FTO-2B cells. Suppression of each of the three PLC families led to decreased IP_3 and DAG, suggesting that activation of any PLC family generates these two second messenger molecules, consistent with previous studies (*3*). Prominent among the elements in growth signaling are PKC, *ras*, and MAP kinase, all of which displayed differential sensitivity to the loss of specific PLC families. Interestingly, suppression of PLCδ, unlike the other PLC families, did not alter PKC or *ras* activity in FTO-2B cells. Thus, although PKC and *ras* are critical in the control of cell growth, they were not sufficient to compensate for the decrease in basal growth rate observed in PLCδ-deficient cells. MAP kinase is activated in many cells following mitogenic stimuli (*44*), operating largely via tyrosine kinase receptors and G-protein-linked receptors (*45*, *46*). Suppression of the each of the three PLC families differentially reduced MAP kinase activity in FTO-2B cells, suggesting that attenuation of MAP kinase activity may mediate the growth inhibition of PLC-deficient cells. Interestingly, suppression of each of the three PLC family resulted in increased levels PIP_2 that directly correlated with the rank order of the inhibition of FTO-2B cell clones ($\gamma > \delta > \beta$). It is possible that PIP_2 directly suppressed MAP kinase activity, but such an effect has not been noted previously. The observation that PLC suppression had a profound effect on the intracellular levels of PIP_2 is quite surprising in light of reports that PIP_2 is synthesized on demand (*46*, *47*). The present study shows that PLC activation is important not only in the regulation of cell proliferation, but also in the regulation of basal PIP_2 levels. PIP_2 production is one of the early events stimulated by many growth factors and mitogens. Suppression of each of three PLC families resulted in significantly increased PIP_2 levels that might directly contribute to controlling the growth rate of FTO-2B cells. The fluctuation in the concentration of PIP_2 levels itself could play a messenger role, as PIP_2 is known to serve as a regulator of actin-binding proteins (*48*), as a molecule responsible for the localization of many proteins containing pleckstrin domains at the membrane surface (*49*), and as a cofactor for phosholipase D (*50*). Recently, PIP_2 has been shown to enhance the receptor-kinase-mediated β-adrenergic receptor desensitization by facilitating membrane association of the receptor kinase (*51*). Collectively, these results suggest that PIP_2 might be important for localizing proteins containing pleckstrin domains at the membrane surface, and increased levels of PIP_2 by suppression of PLC families might prevent the translocation of many proteins from the membrane surface, thereby altering FTO-2B cell growth.

ACKNOWLEDGMENT

I thank Dr. Craig Malbon and various members of his laboratory for reagents and helpful discussions during the course work of this work and in the preparation of the manuscript. I am indebted to Dr. Don Bottaro for constructive critiques, invaluable support, and encouragement. Technical help from Dr. Mario Rebecchi is also gratefully acknowledged.

REFERENCES

1. Carpenter, G. (1987) *Ann. Rev. Biochem. 56*, 881−914.
2. Ullrich, A., & Schlessinger, J. (1990) *Cell. 81*, 203−212.
3. Rhee, S. G., & Choi, K. D. (1992) *Adv. Second Messenger Phosphoprotein Res. 26*, 35−61.
4. Rhee, S. G., Kim, H., Suh, P. G., Choi, W. C. (1991) *Biochem. Soc. Transact. 19* (2), 337−341.
5. Taylor, S. J., & Exton, J. H. (1991) *FEBS Lett. 286*, 214.
6. Smrcka, A. V., Hepler, J. R., Brown, K. O., & Sternweis, P. C. (1991) *Science 251*, 804−807.
7. Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., & Gilman, A. G. (1993) *J. Biol. Chem. 268*, 14367−14375.
8. van Carvon, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., & Moolenaar, W. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1257−1261.
9. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., & Pawson, F. (1991) *Science 252*, 668−674.
10. Rhee, S. G., & Choi, K. D. (1992) *J. Biol. Chem. 267*, 12393−12396.
11. Blenis, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5889−5892.
12. Eagan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., & Weinberg, R. A. (1993) *Nature 6*, 363, 15−16.
13. Allen, L. F., Lefkowitz, R. J., Caron, M. G., & Cotecchia, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11354−11358.
14. Gutkind, J. S., Novotny, E. A., Brann, M. K., & Robbins, K. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4703−4707.

15. Julius, D., Livelli, T. J., Jessell, T. M., & Axel, R. (1989) *Science 244*, 1057−1062.
16. Kalinec, G., Nazarali, A. J., Hermouet, S., Xu, N., & Gutkind, J. S. (1992) *Mol. Cell. Biol. 12*, 4684−4693.
17. Devivo, M., & Iyengar, R. (1994) *J. Biol. Chem. 269*, 19671−19674.
18. Qian, N.-X., Russel, M., Buhl, A. M., & Johnson, G. L. (1994) *J. Biol. Chem. 269*, 17417−17423.
19. Mohammadi, M., Dianne, C. A., Li, W., Li, N., Spiwak, K., Hannegger, A. M., & Schlessingeer, J. (1992) *Nature 358*, 681−684.
20. Valius, M., & Kazlauskas, A. (1993) *Cell 73*, 321−334.
21. Katsuya, T., Higaki, J., Miki, T., Kohara, K., Yagisama, H., Tanase, H., Mikomi, H., Serikawa, T., Nojima, H., Ogihara, T. (1992) *Biochem. Biophys. Res. Commun. 181*, 1359−1356.
22. Margolis, B., Zilberstein, A., Franks, C., Felder, S., Kremer, S., Ulrich, A., Rhee, S. G., Skoredki, K., & Schlessinger, J. (1990) *Science 248*, 607−610.
23. Pouyssegur, J., & Seuwen, K. A. (1992) *Annu. Rev. Physiol. 54*, 195−210.
24. Smith, M. R., Liu, Y.-L., Kim, H., Rhee, S. G., & Kung, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3654−3663.
25. Smith, M. R., Liu, Y.-L., Kim, H., Rhee, S. G., & Kung, H. F. (1990) *Science 247*, 1074−1077.
26. Smith, M. R., Liu, Y.-L., Matthews, N. T., Rhee, S. G., Sung, W. K., & Kung, H. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6554−6558.
27. Moxham, C. M., Hod, Y., Malbon, C. C. (1993) *Science 260*, 991−995.
28. Nebigil, C. G., Garnowskaya, M. N., Spurney, R. F., Raymond, J. R. (1995) *Am. J. Physiol. 268*, F122−127.
29. Garnowskaya, M., Nebigil, C. G., Arthur, J. M., Spurney, R. F., & Raymond, J. R. (1995) *Mol. Pharmacol. 48*, 230−237.
30. Worley, P. F., Baraban, J. M., Calvin, J. S., & Synder, S. H. (1987) *Nature 325*, 159−161.
31. Preiss, J. E., Loomis, C. R., Bell, R. M., & Niedel, J. E. (1987) *Methods Enzymol. 141*, 301−295.
32. Hasley, L. E., & Johnson, G. L. (1992) *Mol. Cell. Biol. 3*, 545−553.
33. Yashuda, I., Kishimato, A., Tanaka, S., Torminaga, M., Sakurai, A., & Nishizuka, Y. (1990) *Biochem. Biophys. Res. Commun. 166*, 12220−12224.
34. Winitz, S., Russell, M., Qian, N, X., Gardner, A., Dwyer, L., & Johnson, G. L. (1993) *J. Biol. Chem. 268*, 19196−19199.
35. Alverez, E., Northwood, I. C., Gonzalez, F. A., Latour, D. A., Seth, Abete, C., Curna, T., & Davis, R. J. (1991) *J. Biol. Chem. 266*, 15277−15285.
36. Rebecchi, M. J., Kolesick, R. N., & Gershengorn, M. C. (1983) *J. Biol. Chem. 258*, 227−234.
37. Borst, S. E., Oliver, R. J., & Scarpace, P. J. (1994) *J. Geront. 49*, B18−2.
38. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmiecik, T. E., Van de Woude, G. F., & Aaronson, S. A. (1991) *Science 25*, 802−804.
39. Weidner, K. M., Sachs, M., & Birchmeier, W. (1993) *J. Cell. Biol. 121*, 145−154.
40. Ponzetto, C., Bordelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., & Comoglo, P. M. (1994) *Cell 77*, 261−271.
41. Fagerstrom, S. P., Ahlman, S., Gestblom, C. N., & Anberg, E. (1996) *Cell. Growth. Differ. 7*, 775−785.
42. Zheng, W., Fink, D. W., Jr., & Groff, G. (1996) *J. Neurochem. 66*, 1868−1875.
43. Yoko-o, O. T., Matsui, Y., Yagisawa, H., Nojima, H., Uno, I., & Toh-e, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1804−1808.
44. Leevers, S. J., & Marshall, C. J. (1992) *EMBO J. 11*, 569−57416. Yoko-O, O. T., Matsui, Y., Yagisawa, H., Nojima, H., Uno, I., & Toh-e, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1804−1808.
45. Kolch, W., Heidecker, G., Koshs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., & Rapp, U. R. (1993)*Nature 364*, 249−252.
46. Thomas, G. M. H., Cunningham, A. F., Ball, A., Totty, N. F., & Cockcroft, S. (1993) *Cell 74*, 919−928.
47. Lee, S. B., & Rhee, S. G. (1995) *Curr. Opin. Cell. Biol. 7*, 183−189.
48. Yu, F. X., Johnson, P. A., Sudhof, T. C., & Yin, H. L. (1990) *Science 250*, 1411−1415.
49. Harlan, J. E., Hajduk, P. J., Yoon, H. S., & Fesik, S. W. (1994) *Nature 371*, 168−170.
50. Lee, Y. H., Kim, H. S., Pai, J. K., Ryu, S. H., & Suh, P.-G. (1994) *J. Biol. Chem. 269*, 26843−26847.
51. Pitcher, J. A., Fredericks, Z. L., Stone, W. C., Premont, R. T., Stoffel, R. H., Koch, W. J., & Lefkowitz, R. J. (1996) *J. Biol. Chem. 271*, 24907−24913.

BI971721M