PHOSPHOLIPASE C-δ1 EXPRESSION IS LINKED TO PROLIFERATION, DNA SYNTHESIS AND CYCLIN E LEVELS

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Running title: Suppression of Phospholipase C-δ1 Alters Cell Cycle.

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We previously reported that phospholipase C-δ1 (PLC-δ1) accumulates in the nucleus at the G1/S transition, which is largely dependent on its binding to phosphatidylinositol 4,5-bisphosphate (1). Here, using small interfering RNA (siRNA) that specifically target rat PLC-δ1, we investigated whether this enzyme plays a role in cell cycle control. Inhibiting expression of PLC-δ1 significantly decreased proliferation of rat C6 glioma cells and altered S phase progression. [3H]-thymidine labeling and FACS analysis indicated that the rates of G1/S transition and DNA synthesis were enhanced. On the other hand, knockdown cultures released from the G1/S boundary were slower to reach full G2/M DNA content, consistent with a delay in S phase. The levels of cyclin E, a key regulator of the G1/S transition and DNA synthesis, were elevated in asynchronous cultures as well as those blocked at the G1/S boundary. Epifluorescence imaging showed that transient expression of human phospholipase C-δ1, resistant to these siRNA, suppressed expression of cyclin E at the G1/S boundary despite treatment of cultures with rat-specific siRNA. While whole cell levels of phosphatidylinositol 4,5-bisphosphate were unchanged, suppression of PLC-δ1 led to a significant rise in the nuclear levels of this phospholipid at the G1/S boundary. These results support a role for PLC-δ1 and nuclear phospholipid metabolism in regulating cell cycle progression.

Phosphoinositides (PI) are metabolized by a multifaceted and highly regulated set of PI-specific enzymes (2-4). Kinases sequentially phosphorylate the inositol headgroup of phosphatidylinositol, and phosphatases reverse this process (5-7); both can generate phosphatidylinositol 4,5-bisphosphate (PIP2), the principle substrate of phospholipase C (PLC) (2-4). PLC cleaves PIP2 to generate key second messengers, inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG) (2-4) that mobilize internal calcium stores (8) and activate protein kinase C (9), respectively. In mammals, the PLC family consists of at least thirteen isoforms that fall into six subtypes: β, γ, δ, ε (2-4), ζ (10) and η (11,12). PIP2 hydrolysis is vital to a wide range of cellular responses, including cytoskeletal remodeling (13), membrane trafficking (14), gene transcription (15), proliferation and differentiation (3,4). PIP2 metabolism and PLC activity (particularly in the nucleus) play prominent roles in cell cycle progression and ultimately influence global decisions, such as differentiation and proliferation (6,16-18). Indeed, homozygous deletion of PLCβ3 (19) or PLCγ1 (20) is embryonic lethal. Although PLCδ1 is not essential, homozygous deletion in mice results in aberrant expression of terminal differentiation markers in several types of skin cells, as well as the development of alopecia and spontaneous skin tumors (21). These effects appear to result from increased expression of pro-inflammatory cytokines (22). Mice that lack both PLCδ1 and PLCδ3, however, die between embryonic day 11.5 and 13.5 due to abnormal cellular proliferation and apoptosis in placental trophoblasts (23). Saccharomyces cerevisiae that lack PLC1-1, a homolog to mammalian PLCδ1, mis-segregate chromosomes (24), exhibit osmotic and temperature sensitivity, and defects in
metabolism and growth (25,26). The extents to which these phenotypes are displayed depend on the genetic background of each yeast strain, suggesting that \textit{plc1-1} modulates complex multigene processes having significant redundancies. Transformation of these yeast mutants with rat \textit{PLC}\textsubscript{δ1} can rescue growth defects (26), consistent with a high degree of functional conservation. Furthermore, over expression of cyclin dependent kinase inhibitors, \textit{SPL1} or \textit{SPL2}, rescues these same defects (27), suggesting that \textit{PLC1} is some how linked to cell cycle regulation.

Yagisawa and coworkers first reported that \textit{PLC}\textsubscript{δ1} harbored both nuclear export and import sequences that contribute to its shuttling between the cytoplasm and nucleus (28). We have demonstrated that \textit{PLC}\textsubscript{δ1} accumulates in the nucleus at the G\textsubscript{1}/S boundary in NIH-3T3 fibroblasts and C6 glioma (1); and many of these observations have been confirmed (29). Here, we set out to determine whether this protein plays a role in the cell cycle. We find that suppression of \textit{PLC}\textsubscript{δ1} increases cyclin E levels, alters S-phase progression, and inhibits cell proliferation.

**Experimental Procedures**

Synchrony, flow cytometry and cell cycle analysis – Rat C6 glioma cells (American Type Culture Collection) were maintained in RPMI 1640 (Invitrogen) supplemented with 7.5% fetal bovine serum (FBS) and 1 mM penicillin and streptomycin (all supplements from Invitrogen) at 37°C in a 5% CO\textsubscript{2} humidified incubator. To synchronize cells to the G\textsubscript{1}/S boundary, adherent glioma cells were twice blocked with 2 mM thymidine (1). Briefly, cultures were washed with RPMI 1640 and incubated in growth medium supplemented with 2 mM thymidine for 12 h, washed three times with growth medium and incubated for an additional 10 h without thymidine. Cultures were again blocked with 2 mM thymidine for an additional 12 h. To release them from G\textsubscript{1}/S block, cells were washed three times in RPMI 1640 and fed normal growth medium. To analyze DNA content, cells were harvested by treatment with trypsin/EDTA, washed with phosphate buffered saline containing 1.0 mM calcium chloride and 2.0 mM magnesium chloride (PBS-CaMg), and fixed in 70% ethanol in phosphate buffered saline (PBS) with 0.1% FBS at 4°C overnight (1). To stain DNA, ethanol-fixed glia were incubated in PBS with 50 mM citrate buffer, 50 \mu g/ml propidium iodide, and 50 \mu g/ml RNase A for 30 min at 37°C (1). After washing with PBS, cell cycle analysis was immediately performed using a fluorescence-activated cell sorter (FACS\textsubscript{an}; BD Biosciences). Histogram data were analyzed using the program Cychlred and the Origin 7.5 (OriginLab Corporation) peak fit module.

siRNA , expression plasmids and cell transfection – We targeted the mRNA sequence 151-172bp (5’-ggA CCC Cag gCC gCU Cgg TT-3’) of rat \textit{PLC}\textsubscript{δ1} and designed and synthesized a corresponding duplexed siRNA (Proligo) based on previously described protocols (30,31). For these experiments, C6 glioma cells were plated on plastic tissue culture dishes or #1.0 borosilicate chambered glass cover slips (Nalge Nunc International) coated with poly-L-lysine (Sigma). Cells were transfected with \textit{PLC}\textsubscript{δ1}-specific siRNA (δ1-siRNA) or a commercially available non-specific control C-siRNA (Ambion) ranging from 0.01 to 320 nM, using FuGene6 (Roche) or RNAiMax (Invitrogen) according to manufacturer’s protocol. Three commercially available rat \textit{PLC}\textsubscript{δ1}-specific siRNA (Ambion # 200652, 49731 and 49541; designated here as siRNA1, siRNA2, and siRNA3, respectively) were also tested for their capacity to knockdown expression and the resulting phenotypes also assessed. In some experiments, siRNA was delivered with human \textit{PLC}\textsubscript{δ1} fused to enhanced-green fluorescence protein (EGFP, at ~0.28 \mu g DNA/cm\textsuperscript{2}) in the same FuGene6 transfection. These expression vectors have been previously described (1).

SiRNA transfection efficiency using the Fugene6 or RNAiMax reagents were found to be nearly 100 % as assessed using a fluorescently labeled, short doubled-stranded RNA (Block-It, AlexaFluor Red, Invitrogen). On the other hand, plasmid transfection efficiency using either Fugene6 or FugeneHD (Roche) rarely exceeded 25% whether using plasmids encoding the PLC-δ\textsubscript{1} EGFP fusion protein or EGFP itself.
Cell proliferation and viability assays – To estimate the rate of growth in the presence or absence of δ1-siRNA, C6 glioma were plated at densities of 1x10^3 to 1.5x10^5 cells/2 cm^2-well and allowed to grow for several days; cell numbers were determined every 24 h with a hemocytometer and growth rate constants were estimated with the following equation, GR = \[ \frac{\ln(n_f/n_i)}{t} \], where n_i = initial cell number, n_f = final cell number, t = time in hours. Trypan Blue (GIBCO) was used to determine whether cell membrane integrity was compromised as a result of siRNA treatment. Viability was also assessed using sodium 3′-[1-[(phenylamine)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT, Biological Industries), which is metabolized to the colored formazin product in the mitochondria (32). During these procedures, C6 glioma cells were cultured in an equivalent medium lacking phenol red. XTT (1 mg/ml) was prepared in serum free medium containing phenazine methylsulfate (PMS, 1.53 mg/ml), which stimulates mitochondrial metabolism of XTT (32). 100 µl of XTT/PMS reagent was transferred to each well containing 400 µl of medium and incubated for 1 h. The conditioned medium from each well was then collected and the absorbance was measured at 475 nm.

SDS-PAGE and Western blotting – Each 35 mm dish of monolayer cells was washed twice, with 2 ml each of warm PBS-CaMg. Soluble cellular proteins were then extracted with 0.5 ml of ice cold extraction buffer: 200 mM NaCl, 0.2% NP40, 20 mM Tris, pH 8, 1 mM DTT, 1 mM MgCl_2, 1 mM EGTA and 1% mammalian anti-protease mixture (SIGMA) and incubated for 5 min at 4°C. The cells were gently scrapped up with a rubber policeman and transferred to 1.7 ml Eppendorf tubes. Using this method, the nuclei remained intact and little of the cellular DNA was extruded. These samples were then subjected to centrifugation at 1,000 x g for 4 min at 4°C and the supernatant fluids transferred to new tubes. A portion of each sample was removed for determination of protein concentration. Protein concentration was determined via Bradford assay (BioRad) according to manufacturer protocol. An equal volume of acetone at -20°C was then added to the remaining samples which were then incubated at -20°C for at least 30 min. Following centrifugation at 12,000 x g for 5 min, the pellets were washed once with -20°C acetone:water (1:1; v/v) and dried under vacuum. The dried samples were dissolved in SDS sample buffer to a concentration of 1-2 µg protein per µl. Samples containing equal concentrations of total protein were separated in an 8% or 10% SDS-PAGE gel and transferred to PVDF membrane (BioRad) using a Trans Blot Semi-Dry Transfer apparatus (BioRad) at 14 V for 1.5 h. The blot was then blocked with Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween-20 for 60 min at room temperature. The membrane was incubated in solution containing anti-PLCδ1 S-11-2 monoclonal antibody (Upstate), or anti-PLCδ1 polyclonal (Santa Cruz), or anti-cyclin E polyclonal, or anti-cyclin A monoclonal, or anti-β-tubulin polyclonal antibody (Invitrogen). The membrane was incubated with secondary antibody solution containing either goat antimouse or anti-rabbit IgG (H+L)-HRP conjugate (BioRad). Enhanced chemiluminescence (ECL Plus, Amersham/GE HealthCare) to detect the binding of the secondary antibody following the manufacturer’s protocol. The membranes were imaged using a CCD camera (Kodak).

[^3H]-thymidine incorporation assay – C6 glioma cells were transfected with either 160 nM control C-siRNA or PLCδ1-specific siRNA and grown in 24-well plates. 48-72 h post-transfection, cultures were washed with PBS-CaMg and incubated in 0.5 ml of RPMI 1640 (7.5% FBS, 1% PS) containing 1 µCi/ml [^3H]-thymidine for 2.5 h (a measure of the number of cells synthesizing nascent DNA, (33)). Alternatively, cultures were transfected and synchronized to the G1/S boundary as described above, and then labeled with [^3H]-thymidine following their release from G1/S block (rate of thymidine incorporation). In each experiment, a portion of each culture was used to determine cell number. Incorporated [^3H]-thymidine was precipitated with 500 µL of 10% trichloroacetic acid (TCA) on ice for 20 min. The precipitate was washed twice with 500 µL each of 10% TCA. Finally, pellets were dissolved with 200 µl of 0.1 N NaOH for 15 min and transferred to
a vial containing 4 ml of scintillation fluid and counted in a liquid scintillation spectrometer.

**Epi-fluorescence microscopy and indirect immunofluorescence** – Cell monolayers were rinsed once in PBS-CaMg then fixed with freshly prepared 3.7% (w/v) formaldehyde solution (Fisher-Scientific) in PBS for 10 min at room temperature. Samples were then washed three times in TBS for 5 min and permeabilized with 0.5% NP-40 (Sigma) in TBS for 5 min at room temperature. The detergent solution was replaced with blocking solution (TBS, containing 5% goat serum (GS) (Pierce)) for 30 min at room temperature and then replaced with primary antibody solution (1:200, rabbit anti-cyclin E in TBS with 1% GS) overnight at 4°C. Samples were washed three times in 1 ml of TBS for 7 min each and then incubated at 37°C for 1 h in goat anti-rabbit IgG (H+L) conjugate Texas Red (Molecular Probes) diluted 1:3000 in TBS containing 1% GS. Each well was then washed three times in TBS for 7 min each. Indirect immunofluorescence of PLCδ1 was performed as previously described (1). In some experiments, cells incubated with 4',6-diamidino-2-phenylindole (DAPI, 5 µg/ml) for 5 min to assess the percentage of nuclei that appeared apoptotic. Images were captured with an AxioCam 330mA 12-bit CCD camera (Zeiss) and viewed with Carl Zeiss Axovision 3.1 software. Alternatively, fixed cells were visualized by epifluorescence microscopy (Olympus IMT-2 inverted microscope with 100W Mercury arc lamp) and images taken with Nikon Plan Fluor 40x oil objective (N.A. 1.3) and Olympix AstroCam (LSR). These images were processed and analyzed with Esprit imaging software (LSR). To assess the fraction of cells (scored positive or negative) having nicked DNA as a result of siRNA treatments, an *in situ* TUNEL Assay Cell Death Detection kit (Roche) was used according to manufacturers directions.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)** – Total RNA was extracted from siRNA treated C6 cultures using an RNeasy extraction kit (Qiagen, MD) as per the manufacturer’s instructions. Following purification, 0.1 to 1 µg of total RNA was first heated to 70°C in the presence of random hexanucleotide primers. The RNA was then transferred to Ready-to-Go RT-PCR beads (GE, UK) and incubated at 42°C for 30 min. Following the RT step, cyclin E primers (5’-GTGAAAAGCGAGGATAGCAG-3’; 5’TGTTGTGATGCCATGTAAACG-3’) or GAPDH primers were added and the reactions cDNA amplified (18-26 cycles) in a Gene AMP PCR System 2000 thermocycler (Perkin Elmer) with each cycle programmed for 95°C melting for 0.5 min, 55°C annealing for 0.5 min and 72°C extension for 1 min. The reaction products were separated on a 2% agarose gel that was subsequently stained with SYBR Green I (Molecular Probes, OR). Images were recorded using a Kodak Gel Imager system and the fluorescent bands quantified using the Kodak gel analysis software. The cyclin E mRNA levels were normalized to expression of the GAPDH amplicon in each sample.

**Subcellular fractionation and lipid analysis** – Nuclei were purified as previously described (1). Following siRNA treatment for 24 h, cultures were synchronized to the G1/S boundary, and labeled with 10 µCi/ml of [3H]myo-inositol for at least 24 h. In some cases, cultures were released from G1/S block for 3 h prior to lipid extraction. Labeled cultures were rinsed with ice-cold PBS-CaMg and then treated briefly with PBS containing 1 mM EDTA, to release them from the plastic dishes. The released cells were then subjected to centrifugation at 600 x g for 5 min at 4°C. Cells were promptly resuspended in 500 µl of pre-chilled hypotonic re-suspension buffer (RSB: 10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH7.4) on ice for 7 min. Swollen cells were then transferred to a Dounce homogenizer and lysed by 20 strokes of the glass pestle. Nuclei and debris were then layered onto a sucrose cushion: 320 mM sucrose, 7.7 mM MgCl2, 2.1 mM EGTA and 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), and centrifuged at 300 x g for 3 min at 4°C. The pellet was washed twice with 0.5 ml of RSB and resuspended in 750 µl methanol: 0.1 M HCl (v/v, 1:1), placed in silianzed borosilicate glass tubes, and mixed vigorously for 30 s. The lipids were subsequently extracted as previously described (1). Lipid extracts were applied to pre-scored Linear KD silica plates (Whatman) that had been pre-treated with 40% methanol,
1% potassium oxalate and 1 mM EGTA in water and heat activated. The solvent system used was chloroform: methanol: water: concentrated ammonium hydroxide (v/v/v/v, 60:47:11.3:2) (1). Areas corresponding to migration of PI, PI(4)P, and PI(4,5)P₂ standards were scrapped into vials containing 100 µl of a mixture of methanol and 10% NP-40 in water (v/v, 1:1). 4 ml of scintillation fluid (EcoLite) was added to each vial, mixed and the vials were counted in a liquid scintillation spectrometer. CPM values were normalized to total lipid phase extractable phosphorus.

Statistical analysis – All statistical analyses were performed in GraphPad Prism. To determine significance of the differences between mean values, one-way ANOVA with Newman-Kuels post-test, or student t-test was used where appropriate (**p<0.01, *p<0.05). Fisher’s Exact test was used to analyze the frequency data obtained from indirect immunofluorescence images.

RESULTS
siRNA-mediated knockdown of PLCδ₁ in Rat C6 glioma inhibits proliferation – RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism that suppresses synthesis of a specific protein by degrading the mRNA encoding a target protein (30,31,34). We identified a candidate site within the rat PLCδ₁ mRNA coding sequence (152-172) and designed a specific RNA duplex (δ₁-siRNA). In addition, we purchased three unique siRNA duplexes predicted to suppress expression of rat PLCδ₁.

Treatment of C6 glioma cultures with δ₁-siRNA reduced the levels of PLCδ₁ by >80% by 72 hrs compared to control siRNA or untreated cultures (Figure 1A). This was associated with reduced cell numbers. Under our transfection conditions, the significant changes in the apparent growth rates (Figure 1B, inset) were well correlated with reduced expression of PLCδ₁ (see Figure 1A, δ₁-siRNA1, and see Supplemental Figure 1A).

Commercially available siRNA1 and siRNA3, greatly reduced expression of rat PLCδ₁, whereas siRNA2 was less effective (Figure 1C). Suppression of cell proliferation mirrored their effects on PLCδ₁ levels (Figure 1D, see Supplemental Figure A1), supporting the idea that slower growth is a specific effect of PLCδ₁ suppression. As the knockdown experiments progressed, it became evident that siRNA3 rapidly reduced PLCδ₁ levels, and profoundly and rapidly reduced the fraction of cells synthesizing DNA by 48 h following treatment. In order to provide a sufficient time window for measuring the relevant cell cycle variables, we compared δ₁-siRNA and siRNA1 in further studies, since these siRNA took longer to suppress PLC-δ₁ expression.

To address the possibility that the apparent decrease in proliferation was due to increased rate of cell death, we investigated the cytotoxicity of the various siRNA and whether treatment with these reagents induced apoptosis. Trypan Blue staining revealed no significant differences between controls and δ₁-siRNA, treatments up to 72 hrs post-transfection (Figure 2A). The ability of treated C6 cell mitochondria to metabolize XTT, another indicator of cell viability, was also unchanged (Figure 2B). Similar results were found using the siRNA 1 and 3 (see Supplemental Figure 1B and C).

Another possible explanation for the decrease in growth rate was that the fraction of cells undergoing apoptosis had increased. 72 h post-transfection, the fraction of TUNEL positive cells was similar between control δ₁-siRNA (Figure 2C). Furthermore, there were no signs of nuclear fragmentation (visualized by DAPI staining) in C6 cells treated with any of the siRNA (see Supplemental Figure 1D). Taken together, these data suggest that neither cytotoxicity nor increased apoptosis accounts for the decrease in cellular proliferation.

Knockdown of PLCδ₁ changes cell cycle distribution – Since knockdown of PLCδ₁ did not appear to enhance cytotoxicity or apoptosis, we suspected that a delay or blockage in a particular stage of the cell cycle could account for our observations. FACS analysis was used to measure the distribution of DNA content within the cell population. At 72 h post transfection, it was evident that treatment of cultures with δ₁-siRNA altered cell cycle
distribution (Figure 3) compared to untreated cultures and those transfected with C-siRNA. While no significant changes were found among cultures in their G1-DNA or S-phase DNA content, analysis indicated a significant decrease in the number of G2/M-DNA content cells compared to control conditions (Figure 3, inset), suggesting a delay in S phase progression.

**Knockdown of PLCδ1 alters S phase progression** – In addition to FACS analysis, we pulse labeled cells with [3H]-thymidine to measure the number of S phase cells in asynchronous cultures. At 72 h post-transfection, cultures were washed and incubated in normal growth media with 1 µCi/ml [3H]-thymidine for 2.5 h (Figure 4A). Under these conditions, cultures transfected with δ1-siRNA incorporated more [3H]-thymidine, indicating that either a greater number of cells synthesizing new DNA were present or that a similar proportion of S phase nuclei were present synthesizing new DNA at a faster rate or both. To determine whether the rate of DNA synthesis was altered, cells were synchronized to the G1/S boundary and released in the presence of 1 µCi/ml [3H]-thymidine (Figure 4B). DNA synthesis was measured in 2 h intervals after release from G1/S phase block. In cultures treated with PLCδ1-specific siRNA, DNA synthesis was significantly faster during the first 4 h.

S phase progression was also assessed by FACS analysis in synchronized cultures. Cultures blocked to the G1/S boundary were released and their DNA content was measured 3-5 h later (Figure 5). Suppression of PLCδ1 altered S phase progression. At 3 and 5 h a greater proportion of cells had progressed into S phase from the G1/S block (Figure 5A), in agreement with our [3H]-thymidine incorporation results (see Figure 4B, 2-6 h after G1/S release). Cultures transfected with δ1-siRNA however, were significantly delayed in their progression to G2/M (Figure 5B, see arrow). In each histogram, the distance that the S phase peak migrated from the G1 peak was determined and calculated as a fraction of the distance between the G1 and G2/M peaks (Figure 5C). In cultures treated with δ1-siRNA, the S phase peak migrated further compared to controls, yet significantly less G2/M cells were observed. An S phase peak was still observed at 5 h (Figure 5D) supporting the idea of a delay in the completion of S phase and transition into G2/M. This delay, however, is preceded by accelerated DNA synthesis. Taken together, these results may explain the overall reduction in proliferation observed in our asynchronous culture experiments.

**Knockdown of PLCδ1 elevates the levels of cyclin E** – Prior studies have shown that PLCβ and PLCγ are linked to cyclin D and cdk-4/6, important regulators of G1 progression (35,36). One explanation for the changes in DNA synthesis and distribution of S phase cells is that PLCδ1 alters factors that regulate progression through this phase. Since both initial onset of DNA synthesis and exit from S phase were affected, we focused on an important regulator, cyclin E (37,38). Treatment of cells with PLCδ1-specific siRNA substantially elevated the levels of cyclin E in both asynchronous (Figure 6A) and synchronized cultures (Figure 6B) compared to controls. On the other hand, cyclin A levels remained unaltered (data not shown). Peak expression of cyclin E occurred sooner and persisted throughout the later stages of S phase compared to controls. Moreover, elevated cyclin E levels were observed with other PLCδ1-specific siRNA, including siRNA 1 (Figure 6C). These results suggest that higher cyclin E levels are not the result of off-target effects of the siRNA’s used here.

To address whether an increase in cyclin E mRNA levels could account for the elevated cyclin E protein levels observed, the C6 cultures were treated with control or PLCδ1-specific siRNA 1 or 3. Under conditions where PLCδ1 levels were suppressed and the protein levels of cyclin E elevated, no significant changes in the levels of cyclin E transcript were observed when cultures were treated with siRNA 1 or 3 (79 +/- 26 and 93 +/- 21% of control, respectively (mean +/- SD from two independent experiments performed in triplicate)).

**Transient expression of human PLCδ1 reduces levels of cyclin E in cells treated with siRNA and synchronized to the G1/S boundary** – We co-transfected C6 glioma cells with human
PLCδ1EGFP and the rat-specific δ1-siRNA to determine if we could reverse the observed cyclin E phenotype; δ1-siRNA was not predicted to suppress human PLCδ1EGFP expression (Figure 7). Cultures were synchronized to the G1/S boundary during the transfection interval of 72 h and fixed at the G1/S boundary. Most cells expressing detectable levels of PLCδ1EGFP had lower levels of cyclin E as measured by indirect immunofluorescence intensity and many of these lacked cyclin E in the nucleus (Panels A and B). When the overall intensities were measured and compared, cotransfection with PLCδ1EGFP caused a 33% suppression of total cellular indirect immunofluorescence, whereas cotransfection with EGFP caused an apparent fall of 12% that was not statistically significant (Panel C). Analysis of the fractions of cells having high (above mean) intensity levels indicated that coexpression of PLCδ1EGFP with rat-specific siRNA directed towards endogenous PLCδ1 caused a 2.5 fold decrease in the population frequency in this group, whereas no significant fall was noted with EGFP (Panel D). These results suggest that moderate overexpression of active human PLCδ1 counteracts the effects of the δ1-siRNA treatment, further demonstrating the specificity of the siRNA’s used here, and supporting the idea that PLCδ1 is an important regulator of cyclin E expression.

Suppression of PLCδ1 increases nuclear PIP2 levels – We and others have previously reported a marked rise in nuclear PIP2 and PIP in cells synchronized to the G1/S boundary (1). To determine if suppression of PLCδ1 alters nuclear phosphoinositides, C6 glioma cells were treated with δ1-siRNA. Twenty-four hours later, cultures were labeled with [3H]-myoinositol for 24 h and blocked at G1/S boundary. Suppressing PLCδ1 significantly increased levels of nuclear phosphoinositides, particularly PIP2 and PIP, compared to control cultures (Figure 7A). By contrast, no significant changes in whole cell PIP2 and PIP, however, were evident (Figure 7B). These data support a role for PLCδ1 in the metabolism of nuclear phosphoinositides at the G1/S boundary.

**DISCUSSION**

Suppression of endogenous PLCδ1 in rat C6 glioma cultures significantly reduced cellular proliferation. FACS analyses and [3H] thymidine labeling suggest that S phase progression and exit were delayed resulting in a reduced population of G2/M cells, which may account for the overall decrease in growth rate. In an attempt to explain this phenomenon, we investigated whether siRNA-mediated suppression of PLCδ1 altered key regulators of S phase progression. (37,38).

In addition, we found that increased expression of cyclin E could be prevented by expression of human PLCδ1 in G1/S-blocked cells treated with rat-specific δ1-siRNA. On the other hand, cotransfection with EGFP failed to show a similar effect. Taken together our results demonstrate an important and specific role for this PLC in cell cycle regulation.

We previously demonstrated that PLCδ1 accumulates in the nucleus at the G1/S transition and suggested that this protein modulates the levels of nuclear PIP4,5P2 at this transition (1). Here, we also demonstrate that suppression of PLCδ1 leads to a significant increase in the levels of nuclear PIP2 and PIP, supporting the idea that PLC regulates metabolism of nuclear phospholipids at the G1/S boundary. Indeed, cotransfection with active PLCδ1 of C6 cells treated with δ1-siRNA reverses the increase in cyclin E levels at a point in the cycle where PLCδ1 is mainly localized to the nucleus (ref 1).

While our previous work (1) and results here demonstrate that most of PLCδ1 is localized to the nucleus at G1/S, we have yet to directly address whether the changes in cyclin E levels or the effects on cell cycle are due to nuclear PLCδ1. Nonetheless, our results are consistent with a role for the nuclear-localized form of this PLC in S phase progression, hydrolysis of nuclear PIP4,5P2 and modulation of cyclin E expression.

The simplest explanation for an accelerated entry into S-phase and altered DNA synthesis, is a perturbation of S phase regulators such as cyclin E, cyclin A and their common regulatory kinase cdk-2 (37,38). Cyclin E levels are regulated by E2F transactivation of transcription (39) and ubiquitin-mediated
destruction (40,41). Our results show that PLCδ1 does not modulate cyclin E transcription; therefore, it is likely that the rate of degradation of cyclin E is reduced leading to an increase in protein level. Interestingly, genetic evidence has linked PLC1, a homolog to PLCδ1, to proteolytic degradation of C-type cyclins in yeast (42).

There appear to be two pathways that regulate cyclin E degradation: a phosphorylation-independent mechanism that regulates rapid turnover of a free pool of cyclin E and a phosphorylation-dependent mechanism that appears to contribute to the ubiquinilation of the more stable cyclin E in complex with cdk-2 (43).

Since cdk-2 associates with both cyclins A and E, suppression of PLCδ1 could alter the ratio of cdk-2 bound to cyclins E and A. Thus, increasing cyclin E levels and presumably its complex with cdk-2 might result in less cdk-2 available to bind cyclin A for appropriate S phase exit (44). Over expression of cyclin E in BK cells, however, was not found to alter the amount of cdk-2 associated with this cyclin (44). Other questions that remain are whether PLCδ1 plays a direct role in the degradation of cyclin E, whether it occurs through a phosphorylation dependent or independent mechanism, and whether metabolism of nuclear PIP2 is required.

Since we find that suppression of PLCδ1 leads to an increase in the levels of nuclear PIP2, these levels could alter transcription, and thereby affect cyclin E degradation, albeit indirectly. Previous studies have demonstrated an important role for PI(5)P-binding proteins that regulate transcription in the nucleus (reviewed in Jones and Divecha (45)). PIP2 and enzymes that metabolize this lipid have been localized to the nuclear matrix, envelope, nucleoli, and nuclear speckles, the latter involved in mRNA splicing and RNA modification (46-49).

Increased nuclear levels of this lipid could also effect chromatin remodeling or histone modifications that alter transcription. In vitro studies have shown that PI(4,5)P2 binds to actin related proteins that are part of the Brg- or Brm- associated factors (BAF) complex (50) and others demonstrated that PIP2 is sufficient to target the BAF complex to chromatin in vitro (51). PI(4,5)P2 also binds to the C-terminal tails of histones H1 and H3 (52), suggesting a role in histone regulation. The hydrolysis of nuclear PIP2 associated with histones could modulate transcription (45,52). Consistent with this idea, in vitro studies have also shown that the presence of P(4,5)P2 counteracts H1-mediated basal transcription by RNA polymerase II (52).

High levels of cyclin E are typically associated with unregulated proliferation (53) and previous work has demonstrated that increased cyclin E leads to an accelerated progression into S phase when degradation is hindered (54-57). Under some circumstances, over expression of this protein results in both enhanced G1/S transition and delayed exit from S phase (44), comparable to the phenotype we observed here.

Other mammalian PLC isoforms have been shown to promote expression and/or activity of cell cycle regulators. FGF-2-mediated PLCγ1 activation promotes up-regulation and nuclear import of cyclin-dependent kinase (cdk)-4, and stimulates nuclear export of the cdk-inhibitor p27kip1 (36). PLCβ1α and PLCβ1b which both possess functional nuclear import sequences, promote cyclin D3/cdk-4 complex formation and hyperphosphorylation of retinoblastoma protein, a critical regulator of G1/S transition (35). Our study points to a comparable role for PLCδ1.

It is possible that generation of local second messengers, such as DAG, activate PKC (16) and modulate cell cycle progression. Indeed, nuclear localized PLCβ1 has been shown to regulate IGF-1 stimulated proliferation of Swiss 3T3 fibroblasts and the commitment of MEL cells to proliferate or differentiate through the generation of DAG and activation of nuclear PKC isoforms (18,58,59). In regenerating rat liver nuclei, PLCβ localizes to chromatin that is actively incorporating BrdU, while PLCγ1 is associated with interchromatin regions and the nuclear envelope (60). These authors have also suggested that nuclear PLCβ plays a role in DNA synthesis, while PLCγ1 is more likely involved at the G2/M transition and nuclear lamin phosphorylation (60). Recent work shows that PLCγ1 is also critical for reassembly of the nuclear envelope from PI(4,5)P2 enriched vesicles.
Our results place PLCδ₁ at an unidentified control point, somewhere in the S phase. The timing of its peak nuclear localization to the G₁/S boundary coincident with peak nuclear PI(4,5)P₂ levels (1) suggests that hydrolysis of this phosphoinositide is required for normal S phase progression, possibly through enhanced degradation of cyclin E.

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Figure 1. siRNA-mediated suppression of PLCδ1. (Panel A) Western blot analysis 24-72 h post-transfection with δ1-siRNA (δ1), compared to untreated (C) or cultures treated with FuGene6 alone (F). Lanes were normalized to cell number. (Panel B) Representative growth curve. Cell number was measured with a hemocytometer. The data points are plotted as average of triplicate determinations and the bars represent standard deviation. Suppression of PLCδ1 (δ1-siRNA) significantly reduced proliferation compared to controls untreated (Control) or transfected control siRNA (C-siRNA). (Inset) Growth rates were determined based on seven independent proliferation experiments and are calculated as fraction of the control untreated growth rate, ***P<0.001. (Panel C) Western blot analysis 48 h post-transfection with a control siRNA (Cntrl siRNA) and three commercially available siRNA (siRNA1-3) predicted to suppress expression of PLCδ1. Lanes were normalized to total protein. (Panel D) Representative phase contrast images 48 h post-transfection with a control siRNA and three different siRNA.

Figure 2. Treatment of cultures with δ1-siRNA is not cytotoxic. (A) To determine if transfection of cultures with siRNA1 resulted in cytotoxicity, cultures (n=7) were exposed to Trypan Blue. No significant changes in the fraction of Trypan Blue positive cells occurred between 24-96 h post-transfection. (B) Mitochondrial viability assay (n=8). Absorbance / 1x10⁴ cells at 475 nM was determined as described in Experimental Procedures. No significant changes occurred. (C) Cells were also stained with antibodies that recognized nicked DNA, a marker of apoptosis. Cells were scored as positive or negative, and the fraction of positive was determined.

Figure 3. Suppression of PLCδ1 changes cell cycle distribution. (A) Cultures were untreated (Control), transfected with control siRNA (C-siRNA), or transfected with siRNA specific for PLCδ1 (δ1-siRNA). After 72 h, DNA content was determined by FACS analysis. Histogram data were pooled and normalized to G1-DNA content, analysis revealed a late-S-phase peak (arrow) in cultures treated with siRNA1. (Inset) Analysis using the cell cycle algorithm (Cychlred) assigned a significant portion of this peak to G2/M. One-way ANOVA analysis was performed on the G2/M data: **P<0.01 when comparing control and siRNA1 (*P<0.05 when comparing C-siRNA and δ1-siRNA, not shown), n=3.

Figure 4. Suppression of PLCδ1 alters DNA synthesis. (A) Cultures were pulse labeled with [3H] thymidine (0.5 µCi/ml). At 72 h post transfection, there was a significant increase in radiolabel incorporation as a result of PLCδ1 knockdown (δ1-siRNA) after 2.5 h compared to control untreated (Control) or transfection with a control siRNA (C-siRNA). *P<0.05 when comparing δ1-siRNA to both control and C-siRNA. (B) Triplicate cultures were blocked to the G1/S boundary and released in the presence of [3H] thymidine (0.5 µCi/ml). As a result of knockdown (δ1-siRNA), the initial rate of DNA synthesis was enhanced compared to control untreated cells.

Figure 5. Suppression of PLCδ1 alters S phase progression. (A) To assess S phase progression, cultures were blocked to the G1/S boundary as described in the experimental procedures, and then release
for 3-5 h. These 9 histograms are representative of two experiments. **(B)** Overlay of +5 h release histograms seen in (A), arrow indicates delay in S phase progression. **(C)** At 5 h release, the relative shift of the S phase peaks were determined by calculating the distances migrated of the S phase peak as a fraction of the total distance between G1 and G2/M peaks. Significance was determined by one way analysis of variance, *P<0.05 and ***P<0.001. **(D)** Fraction of G2/M cells after 5 h release from the G1/S boundary was calculated by estimating the area under the G2/M peak as a fraction of the total number of cells measured.

**Figure 6. Suppression of PLCδ1 up-modulates Cyclin E expression.** (Panel A) Asynchronous cultures (Asynch) were treated with C-siRNA (C) or δ1-siRNA (K) for the times indicated, whereas synchronous cultures were treated 24 prior to double thymidine synchronization and then released for the times indicated. Lanes were normalized to total protein. **(Panel B)** C6 cell cultures were also treated with C-siRNA or siRNA1, which targets a different region of PLCδ1 mRNA, for 72 hrs. Lanes were normalized to total protein. These samples were immunoblotted for PLCδ1, cyclin-E and the constitutively expressed protein, β-tubulin.

**Figure 7. Expression of exogenous human PLCδ1EGFP suppresses cyclin E expression.** C6 cell cultures were cotransfected with either PLCδ1EGFP plasmid and δ1-siRNA, or EGFP and δ1-siRNA. 48 hrs later, the cultures were synchronized to the G1/S boundary (additional 24 hrs), fixed and cyclin E levels imaged by indirect immunofluorescence (IMF) using a highly specific cyclin E primary antibody and Texas-Red-labeled secondary antibody. Two representative epifluorescence images of the cultures, co-transfected with PLCδ1EGFP plasmid and δ1-siRNA are shown. Image (A) was obtained in the Texas Red channel. The circles correspond to cells appearing in the EGFP channel image (B). **(C)** Intensities of indirect IMF of individual cells were binned into transfected and untransfected groups depending on detection of signal in the EGFP channel. This bar graph summarizes the images analyses of 410 and 124 cells, in 14 and 10 separate fields, in cultures cotransfected with PLCδ1EGFP plasmid and δ1-siRNA, or EGFP and δ1-siRNA, respectively (**P < 0.001; n=2). **(D)** Indirect IMF intensities were stratified and the frequencies of observing intensity levels above the untransfected mean were analyzed and expressed as the fraction of total cells in each group and the indicated confidence intervals (CI).

**Figure 8. Suppression of PLCδ1 elevated nuclear phosphoinositides at the G1/S boundary.** (A) Cultures were transfected with δ1-siRNA (open bars) and blocked to the G1/S boundary (black bar) while labeled with [3H]myoinositol. Control cultures (filled bars) were not transfected with siRNA. Nuclei were isolated by osmotic swelling and extracted as described in Experimental Procedures. The data are representative of two independent experiments; the data are plotted as average of triplicate determinations and the bars represent standard deviation, **P<0.01, *P<0.05. CPM values were normalized to lipid extractable phosphorus. (Note that the right Y-axis is ten fold greater, and refers only to phosphatidylinositol, while the left Y-axis refers to both PIP2 and PIP). **(B)** Whole cell PIP2 levels as a function of PLCδ1 suppression. Cultures were transfected with 160 nM δ1-siRNA or untreated, while being labeled with [3H]myoinositol for 72 h. Whole cell phosphoinositide levels were also determined as previously described (1) and no significant changes were observed.
Figure 1.

A

|       | 24h |      | 48h |      | 72h |      |
|-------|-----|------|-----|------|-----|------|
| C     | F   | δ1   | C   | F   | δ1  | C   | F   | δ1  |

B

![Graph showing cell number over hours](image)

C

![Western blot of PLCδ1](image)

D

![Cell morphology images](image)
Figure 3.
Figure 4.

A

[Graph showing CPM/1x10^4 cells for Control, C-siRNA, and δ₁-siRNA, with a significant asterisk (*) indicating a difference between δ₁-siRNA and Control.]

B

[Graph showing CPM/1x10^4 cells over G₁/S Release hours for Control and δ₁-siRNA, with a trend line showing a difference in cell proliferation.]
Figure 5.

A  Control  C-siRNA  δ1-siRNA

B  

Cell Number  G1/S  Cell Number

Intensity (PI-FLA2-Area)

C  Relative Shift in S-Phase

D  Fraction G2/M after 5h Release

Control  C-siRNA  δ1-siRNA  Control  C-siRNA  δ1-siRNA
Figure 6.
Phospholipase C-δ1 is linked to proliferation, DNA synthesis and cyclin E levels
Jonathan D. Stallings, Yue X. Zeng, Francisco Narvaez and Mario J. Rebecchi

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