In the present study, we examined the role of PLCδ1 (phospholipase C δ1) in the regulation of cellular proliferation. We demonstrate that RNAi (RNA interference)-mediated knockdown of endogenous PLCδ1, but not PLCβ3 or PLCζ, induces a proliferation defect in Rat-1 and NIH 3T3 fibroblasts. The decreased proliferation was not due to an induction of apoptosis or senescence, but was associated with an approx. 60% inhibition of [H]thymidine incorporation. Analysis of the cell cycle with BrdU (bromodeoxyuridine)/propidium iodide-labelled FACS (fluorescence-activated cell sorting) demonstrated an accumulation of cells in G0/G1-phase and a corresponding decrease in cells in S-phase. Further examination of the cell cycle after synchronization by serum-starvation demonstrated normal movement through G1-phase but delayed entry into S-phase. Consistent with these findings, G1 cyclin (D2 and D3) and CDK4 (cyclin-dependent kinase 4) levels and associated kinase activity were not affected. However, cyclin E-associated CDK2 activity, responsible for G1-to-S-phase progression, was inhibited. This decreased activity was accompanied by unchanged CDK2 protein levels and paradoxically elevated cyclin E and cyclin E-associated CDK2 levels, suggesting inhibition of the cyclin E–CDK2 complex. This inhibition was not due to altered stimulatory or inhibitory phosphorylation of CDK2. However, p27, a Cip/Kip family CKI (CDK inhibitor)-binding partner, was elevated and showed increased association with CDK2 in PLCδ1-knockdown cells. The result of the present study demonstrate a novel and critical role for PLCδ1 in cell-cycle progression from G1-to-S-phase through regulation of cyclin E–CDK2 activity and p27 levels.

Key words: cell cycle, cyclin E, cyclin-dependent kinase 2 (CDK2), p27, phosphoinositide-specific phospholipase C, Rat-1 fibroblast, RNA interference.

INTRODUCTION

Eukaryotic cell-cycle progression is driven by the interactions of two types of proteins: the cyclins and their catalytic subunit, the CDKs (cyclin-dependent kinases) [1,2]. The cell cycle is divided into four phases: the G1-phase, where the cell prepares for DNA synthesis; the S-phase, where DNA is replicated; the G2-phase, where the cell prepares for division; and the M-phase, where the cell is divided into two identical daughter cells. Each phase of the cell cycle is characterized by the temporal expression of distinct families of cyclins. Entry into G1 from the quiescent (G0) state and progression through G1 is dependent on the D-type cyclins and CDKs 4 and 6; G1-to-S-phase transition is controlled by cyclin E and CDK2; cyclin A and CDK2 direct S-phase progression; and G2-to-M-phase cycling is B-type cyclin- and CDK1-dependent. CDK levels remain fairly constant throughout the cell cycle and their activity is primarily controlled by changes in cyclin levels, which increase at specific cell-cycle stages. In addition, the activity of the cyclin–CDK complex is regulated by post-translational phosphorylations and association with inhibitory regulatory proteins, CKIs (CDK inhibitors) [3,4]. CKIs are divided into two families, the INK4 family that includes p15, p16, p18 and p19, and the Cip/Kip family that includes p21, p27 and p57. The INK4 CKIs specifically inhibit CDK4 and CDK6, whereas the Cip/Kip members broadly interact with most cyclin–CDK complexes and some non-CDKs but potently inhibit cyclin E- and cyclin D-associated CDK2 [3,4].

Previously, an important role for phosphoinositide-specific PLC (phospholipase C) in the regulation of proliferation and the cell cycle has been examined [5–12]. PLC is a critical cellular effector that hydrolyses membrane PIP2 (phosphatidylinositol 4,5-bisphosphate) to generate IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol), which mobilizes intracellular calcium stores and activates PKC (protein kinase C) respectively [12]. Six distinct families representing 13 isoforms have been identified, PLCβ1–4, PLCγ1–2, PLCζ1, PLCζ2 and PLCζ3–4, and each family is differentially regulated by a complex network of receptors, G-proteins and Ca2+ [13]. Of these isoforms, PLCβ1 and PLCγ1, PLCζ1 and PLCζ4 have been shown to affect proliferation [5–8,10,11,13]. Although the underlying mechanisms are poorly understood, studies suggest that PLC positively regulates the cell cycle. PLCβ1 has been shown to regulate IGF (insulin-like growth factor)-1-stimulated DNA synthesis [5], and overexpression of nuclear PLCβ1 increases proliferation in the absence of mitogenic stimuli [6]. Importantly, the mitogenic effects of PLCβ1 are thought to be mediated by increased expression and kinase activity of cyclin D3 and CDK4 [6]. Evidence also suggests that PLCζ and PLCζ4 regulate proliferation through cell-cycle-dependent mechanisms since PLCζ initiates egg activation and division by inducing M-phase-specific Ca2+ oscillations following fertilization of the mammalian egg [10], and PLCζ4 is primarily expressed in the nucleus in a cell-cycle-dependent manner and knockout of this isoform results in delayed DNA synthesis [11]. On the other hand, although PLCγ1 overexpression stimulates...
DNA synthesis [8], this PLC isoform also promotes cell survival by stimulating PI3K (phosphoinositide 3-kinase) via activation of the nuclear GTPase PIKE (PI3K enhancer) [7]. Interestingly, PLC1, the yeast homologue of PLC31, localizes at the centromeric loci at the G2/M checkpoint and is essential for cell growth and mRNA export from the nucleus [12].

The PLC isoform PLC31 has not been shown to affect cellular proliferation. However, studies by Yagisawa and co-workers demonstrated that PLC31 contains a NLS (nuclear localization sequence) and a NES (nuclear export signal) [14–16], and that upon mitogenic stimulation import into the nucleus is increased as opposed to export during quiescence [17]. In addition, Stallings et al. [18] showed that PLC31 accumulates in the nucleus in a cell-cycle-dependent manner at the G1/S-phase boundary and during the G2 phase. Although these structural and localization studies suggest a potential role for PLC31 in regulating proliferation, direct evidence is lacking. In the present studies, we demonstrate that endogenous PLC31, but not PLC3/3 or PLC6, controls cellular proliferation and that this effect is mediated by regulation of cyclin E–CDK2 activity and G1–S-phase transition.

**EXPERIMENTAL**

**Materials**

PLC31, PLC34, PLC3, CDK1, rabbit CDK2, goat CDK2, mouse CDK2, CDK4, rabbit cyclin A, goat cyclin A, cyclin B1, rabbit cyclin E, p-CDK2 (Thr14'Tyr15)-R, p21, p27, p53 total, p57 and β-actin antibodies were from Santa Cruz Biotechnology. Mouse p27 antibody was from BD Biosciences. Cyclin D2 and cyclin D3 antibodies were from Sigma. The PLCγ1 antibody was from Upstate Biotechnologies. The PLCγ antibody was a rabbit polyclonal antibody generated from the RA1 domain of PLCγ. Horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-goat and goat anti-mouse HRP (horseradish peroxidase) secondary antibodies were from Zymed.

**Tissue culture**

Rat-1, NIH 3T3 and HEK (human embryonic kidney) 293 cell lines were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum), except NIH 3T3 medium was supplemented with 10% (v/v) calf serum. Tetracycline-regulated Rat-1 cells were maintained in DMEM supplemented with 10% (v/v) tetracycline-free FBS (Clontech) and 500 μg/ml G418 sulfate (Mediatech).

**Plasmid constructs**

shRNAs (small-interfering hairpin RNAs) were designed for PLC31 targeting mouse and rat PLC31: shRNA PLC31#1, GGATGGCCCTTCTCATGTAAC; shRNA PLC31#2, GACCATCTGGCCAGGAATCT; shRNA Random, ACTGTCAAAAGTACCTACA.

Sense [5′-GATCCCG(shRNA)TTCAAGAGA(shRNA)TTTGGGAA] and antisense [5′-AGCTTTTTTTCAAA(shRNA)-TCTTTTGA(shRNA)GGG] oligonucleotides incorporating these shRNAs were synthesized (Sigma) and cloned into pSUPER-Retro (pSR) [19], a gift from Dr R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

To generate the tetracycline-regulated shRNA vectors, the tetracycline repressor element from pTet (donated by Dr Marc Van de Watering, Hubrecht Laboratories, Utrecht, The Netherlands) was subcloned into the pSR backbone to produce RanH1OpSR. The tetracycline operon region from pSiren RetroQ-TetH-Luc vector (Clontech) was amplified with the sense primer 5′-CCG-ATTCAAGATCTCTCTTC-3′ and the antisense primer 5′-TATT-GAATTCATATGACCGTAC-3′, and subcloned into the shRNA random pSR plasmid to produce RanTet6OH1.pSR. The tetracycline operon region of RanTet6OH1.pSR was subcloned into RanH1OpSR to produce RanOH1.OtSarT. An additional BglIII site was removed from RanOH1.OtSarT and PLC31 shRNA oligos were inserted into the 6OH1.OtSarT plasmid. All plasmids were sequenced.

A human PLC31–GFP (where GFP is green fluorescent protein) fusion construct was a gift from Dr Mario Becchi (Department of Anesthesiology, Stony Brook University, Stony Brook, NY, U.S.A.). Four silent mutations in the region targeted by shRNA PLC31#1 of human PLC31 were introduced by site-directed mutagenesis (QuikChange® mutagenesis kit; Stratagene) using the sense primer 5′-GATGACACAGGGAGCTTCTATGGTCG-3′ [mutations shown in lowercase compared with wild-type (CTTCTCT)]. The resulting construct was then subcloned into pQCXIH to generate hPLC31WT-shRNA.PLC31#1-pQCXIH.

**Retroviral transduction**

Mooney retroviruses were generated, titred and used to transduce Rat-1 and NIH 3T3 fibroblasts as previously described [20]. Viruses were used at concentrations of 105–106 viral colonies/ml. The pSR viral cell lines were selected with 3 μg/ml puromycin and the pQCtTS-IN viral cell lines were selected with 500 μg/ml G418 sulfate. No puromycin selection reagent was employed for the 6OH1.OtSarT viral cell lines.

**Clonal Rat-1 pQCtTS-IN cells**

Rat-1 cells were transduced with retrovirus containing the pQCtTS-IN plasmid (Clontech). Clonal populations of Rat-1 cells were transiently transfected with the Luciferase4/TO pcDNA3.1 plasmid and screened using the Promega luciferase assay kit. The clonal cell with the largest fold-change of luminescence expression with 1 μg/ml doxycycline treatment was then used for all subsequent transductions that utilize the tetracycline-inducible RNAi (RNA interference) system described in this section.

**Immunoblotting**

Proteins were harvested in lysis buffer [20 mM Tris/HCl (pH 7.5), 0.137 M NaCl, 2 mM EDTA, 1% Nonidet P40, 1 mM PMSF; 5 μg/ml aprotonin, 5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor] and supernatants were cleared of cell debris by centrifugation for 10 min at 15,800 g at 4°C. Protein concentrations were measured and normalized (Bio-Rad DC protein assay kit). Proteins were separated by SDS/PAGE and transferred to Immobilon nitrocellulose transfer membrane (Millipore). ECL (enhanced chemiluminescence) was performed using Super Signal West Pico chemiluminescent substrate (Pierce).

**Cell viability assay**

For quantification of cell viability, tetracycline-regulated PLC31 shRNA Rat-1 cells were treated ±100 ng/ml doxycycline for 48 h. Cells were then seeded at 2000 cells per well in a 96 well plate, ±100 ng/ml doxycycline. WST-1 reagent (Roche Applied Science) was added to each well, incubated at 37°C for 60 min in 5% CO2, and then plates were shaken for 1 min. Plates were analysed optically at 450 nm with a reference

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wavelength at 630 nm using a microplate bio-kinetics reader (BioTek Instruments).

For reconstitution studies, cells expressing tetracycline-regulated shRNA PLCδ1#1 were transduced with the retrovirus expressing hPLCδ1WT-shRNAPLCδ1#1-pQCXIIH or pQCXIIH vector. Cell viability was determined as outlined above except that cells were not pre-incubated with doxycycline prior to seeding.

[^H]Thymidine incorporation proliferation assay
Tetracycline-regulated PLCδ1 shRNA Rat1 cells were treated with 10 ng/ml doxycycline for 48 h. Cells were then seeded at 20000 cells per well in a 24 well plate, ± 10 ng/ml doxycycline. At 6 h prior to the end of the experiment, 0.5 μCi of methyl[^H]thymidine (PerkinElmer) was added to each well. Thymidine incorporation was determined by TCA (trichloroacetic acid) precipitation.

DAPI (4',6-diamidino-2-phenylindole) staining
Cells were seeded at 3000 cells per well in a 24-well plate on sterile glass cover slips, ± 100 ng/ml doxycycline for 72 h. Coverslips were fixed with 3.7% (v/v) formaldehyde in 1 × PBS, permeabilized (0.2 % Triton X-100 in PBS), and stained with 300 nM DAPI (Invitrogen). Coverslips were attached to glass slides using gelvatol/2.5% Dabco. Slides were visualized by fluorescence microscopy. Apoptotic cells were identified by the presence of condensed pyknotic nuclei.

Detection of apoptosis by annexin V staining
Subconfluent shRNA PLCδ1 Rat-1 cells ± 100 ng/ml doxycycline for 72 h were stained with annexin V conjugated to FITC (fluorescein isothiocyanate) and propidium iodide with a TACS annexin V-FITC staining kit (R&D Systems) according to the manufacturer’s protocol. Briefly, cells were suspended in 100 μl of annexin V binding buffer and incubated with 1 μl of annexin V and 10 μl of propidium iodide for 15 min in the dark. Annexin V binding buffer (400 μl) was then added and cells were counted by flow cytometry as described below. The percentage of cells undergoing apoptosis was assessed via dual-colour analysis. Cells treated with 50 ng/ml doxycycline and 10 ng/ml doxycycline were used as positive controls.

BrdU (bromodeoxyuridine) pulse labelling and flow cytometry
Cells were prelabelled with 10 μM BrdU (Sigma) for 1 h, harvested via trypsinization, fixed in ice-cold 70 % ethanol, and stored at −20°C until the day of staining. Cells were incubated in freshly prepared 2 M HCl for 30 min, and then incubated in 0.1 M sodium borate (pH 8.5) for 2 min. Cells were then resuspended in dilution buffer (1 × PBS, 0.5 % Tween 20 and 0.5 % BSA) with 0.3 μg of anti-BrdU antibody (Pharmigen) and incubated at 4°C for 1 h in the dark, followed by an incubation with 0.125 μg of FITC-conjugated goat anti-mouse IgG (BD Biosciences) in the dark at 4°C for 1 h. Finally, cells were incubated with 10 μg/ml propidium iodide/1 × PBS for 15 min in the dark. Samples were analysed by flow cytometry using a LSR-II Flow Cytometer and FACS DiVa 4.0 software (BD Biosciences).

Synchronized cell cycle release
Rat-1-shRNA-expressing cells were seeded at 150000 cells per T75 flask in complete medium ± 100 ng/ml doxycycline. At 32 h later, cells were serum-starved ± 100 ng/ml doxycycline. Cells were released from serum starvation 30 h later with the addition of 10 % (v/v) tetracycline-free FBS. Flasks were harvested at indicated time points, following a 1 h 10 μM BrdU pulse as described above. Cells were analysed via FACs analysis. Cell lysates were also collected for each time point and expression levels of cell-cycle regulatory proteins were examined.

Immunoprecipitation/kinase assay
Using samples harvested in lysis buffer, proteins were immunoprecipitated as follows. Lysates were incubated with primary antibody overnight at 4°C and Protein A–agarose beads (Amersham) or Trueblot IP beads (eBioscience) for 1 h at room temperature (22°C). Lysate–bead complexes were washed three times with lysis buffer and twice with kinase buffer [50 mM Tris/HC1 (pH 7.5), 10 mM MgCl2, and 2.5 mM EGTA]. One-third of each sample was reserved for Western blot analysis by adding 2 × sample buffer (0.05 M Tris base, 25 % glycerol, 4 % SDS, 0.04 % 2-mercaptoethanol and 0.03 % Bromophenol Blue) and boiling. The remaining two-thirds of the sample were equilibrated for 30 min at 37°C with kinase buffer containing 1 mM dithiothreitol, 0.1 mM ATP, 1 μCi [γ-32P]ATP and the appropriate substrates [0.5 μg of pRb fragment (Santa Cruz Biotechnology) for CDK4 and 50 ng of histone H1 (Upstate Biotechnology) for CDK2, cyclin E and cyclin A]. The reaction was stopped with the addition of sample buffer and proteins were boiled from beads, separated by SDS/PAGE, dried and exposed to film.

SA β-gal (senescence-associated β-galactosidase)
PLCδ1-shRNA-expressing Rat-1 cells were seeded at 7000 cells per well in 12 well plates ± 100 ng/ml doxycycline. After 72 or 96 h, the activity of SA β-gal was determined using a Senescence β-Galactosidase Staining Kit from Cell Signaling Technology. SA β-gal-positive cells were identified by light microscopy and five random fields of view were examined to estimate the proportion of senescent cells. Staining was preformed on subconfluent cells.

RESULTS
PLCδ1-knockdown in Rat-1 fibroblasts causes a cell shape change and proliferation defect
To determine the role of PLCδ1 in Rat-1 fibroblast proliferation, shRNAs targeting PLCδ1 were cloned into pSUPER-retro [19] (see Figure 1A) and stable clonal populations were generated. Figure 1(B) shows that shRNA PLCδ1I almost completely knocked down (> 99%) protein levels of PLCδ1 but had no effect on other PLC isoforms expressed in Rat-1 cells. PLCδ1, PLCδ2, PLCδ4, PLCγ2 and PLCδ3 were not detected (results not shown). In the PLCδ1-knockdown cells, phenotypic changes were observed characterized by round cells with an increased diameter (Figure 1C). In addition, the doubling time of the cells was delayed from 18 to 70 h (Figure 1D, passage 1) indicating that PLCδ1 regulates cell proliferation. Subsequent passaging, however, resulted in increased doubling times approaching control cells (Figure 1D, passage 6). This normalization of doubling times was accompanied by increased PLCδ1 expression suggesting a selection bias for PLCδ1-expressing cells.
Figure 1  PLCδ1 shRNA transduction in Rat-1 fibroblasts

(A) Schematic representation of the shRNA pRS plasmid. (B) Western blot analysis of RNAi-mediated knockdown of PLCδ1 in Rat-1 cells stably transduced with retrovirus (pSUPER-Retro) expressing shRNA random and shRNA PLCδ1#1. Lysates stained with anti-PLCδ1, anti-PLCβ3, anti-PLCγ1, anti-PLCε and anti-PLCδ4 antibodies. (C) Representative images of shRNA random- and shRNA PLCδ1#1-expressing Rat-1 fibroblasts. Stable cells were seeded at 400,000 cells per well in complete DMEM for 2 days in a six-well plate (n = 3). (D) Effect of PLCδ1 knockdown on Rat-1 cell doubling times following stable transduction of shRNA random or PLCδ1#1 initially, passage 1, and after passage 6. Immunoblots showing knockdown of PLCδ1 at the indicated passage are shown. (E) Schematic representation of shRNA 6OH-1O pSuperRetro plasmid. (F) Western blot analysis of tetracycline-regulated PLCδ1 knockdown. Lysates from shRNA random, shRNA PLCδ1#2 and shRNA PLCδ1#1 Rat-1 cells +−100 ng/ml doxycycline (Doxycycline) for 72 h. (G) Representative images of shRNA random-, shRNA PLCδ1#1- and shRNA PLCδ1#2-expressing Rat-1 fibroblasts. Tetracycline-regulated cells were seeded at 2500 cells per well for 3 days in a 12-well plate with 100 ng/ml doxycycline (n = 3). Magnification is ×200. (H) Effect of regulated PLCδ1 knockdown on cell viability. Cells were seeded at 2000 cells per well in a 96-well plate in the presence of 100 ng/ml doxycycline (doxycycline), after 48 h pretreatment + doxycycline. WST-1 cleavage was measured at the indicated time points. Values are means ± S.D. of three experiments performed in triplicate. *P < 0.05 as determined by a Student’s t test. (I) Effect of knockdown of PLCε or PLCβ3 on cell viability. Cells were stably transduced with the indicated pSuper retroviruses as described previously [20] and the effect on WST-1 cleavage was determined. Values are means ± S.D. of three experiments performed in triplicate. (J) Effect of PLCδ1 knockdown on cell viability in NIH 3T3 cells. NIH 3T3 cells were transiently transduced with shRNA PLCδ1#1 and WST-1 cleavage was determined. A representative of three experiments performed in triplicate is shown. (K) Reconstitution of the proliferation defect. Rat-1 cells expressing shRNA PLCδ1#1 were transduced with retrovirus expressing an shRNA PLCδ1#1-resistant human PLCδ1–GFP fusion construct or vector alone and clonal populations were obtained. The cell viability was determined as described for (H), however, cells were not pretreated with doxycycline and the WST-1 assay was performed at day 4 after seeding and addition of doxycycline or vehicle. Values are means ± S.E.M. of three experiments performed in quadruplicate. *P < 0.05 as determined by a Student’s t test. An associated immunoblot demonstrating overexpression of human PLCδ1–GFP and endogenous PLCδ1 in the presence or absence of doxycycline is shown.
To circumvent this problem, in order to expand clonal populations and to perform detailed analyses, a tetracycline-regulated system was developed utilizing multiple tetracycline-operons and a KRAB silencing domain fused with a tetracycline-repressor protein (termed tetR) (Figure 1E and the Experimental section). Stable cell populations were generated with two shRNAs, shRNA PLCδ1#1 and shRNA PLCδ1#2. Figure 1(F) shows that after the addition of doxycycline, endogenous PLCδ1 levels were reduced by 99.0 ± 2.0 and 98.6 ± 2.2% respectively, compared with non-doxycycline-treated cells. In addition, no knockdown was observed in the absence of doxycycline compared with shRNA random cells, indicating that the system was not leaky. Furthermore, prior to induction with doxycycline, cells were expanded without loss of PLCδ1 knockdown or phenotypic effects upon induction.

In a similar manner to the unregulated cell lines, knockdown of PLCδ1 resulted in a phenotypic shape change (Figure 1G). However, in addition to the large round cells observed with PLCδ1 knockdown, knockdown with shRNA PLCδ1#2 also induced a subpopulation of elongated cells (Figure 1G). Interestingly, knockdown of PLCδ1 in NIH 3T3 cells did not exhibit a shape change (results not shown) suggesting a cell-specific or knockdown-dependent (PLCδ1 knockdown was greater in Rat-1 than NIH 3T3 cells) effect.

Regulated knockdown of PLCδ1 in Rat-1 cell lines also inhibited cellular proliferation. Figure 1(H) shows that cell accumulation measured by a WST-1 assay was significantly inhibited by greater than 50% at 2–3 days post-seeding in doxycycline-treated cells. In a similar manner, knockdown of PLCδ1 in NIH 3T3 cells inhibited cell accumulation by 43% (Figure 1I). The observed effects on proliferation were specific for PLCδ1 knockdown since doxycycline treatment had no effect on shRNA random control cells (Figure 1H) and knockdown of PLCε or PLCβ3 had no effect on cell viability (Figure 1I). In addition overexpression of human PLCδ1 with an shRNA PLCδ1#1-resistant construct, reconstituted the proliferation defect induced by this shRNA (Figure 1K). Thus PLCδ1 knockdown with two distinct shRNAs in two different species showed decreased viability consistent with a role for this isoform in the control of cellular proliferation. All subsequent experiments were performed with these tetracycline-regulated Rat-1 cells.

**Knockdown of endogenous PLCδ1 inhibits cell division but not apoptosis**

In order to understand the mechanism underlying the proliferation defect induced by PLCδ1 knockdown, mitogenesis was assessed with a [3H]thymidine incorporation assay performed at 1 and 2 days post-seeding. Figure 2(A) shows that PLCδ1 knockdown with shRNA PLCδ1#1 inhibited thymidine incorporation by 59% and 57% respectively, compared with random shRNA control cells (P < 0.04; n = 3). These results were specific to PLCδ1, as knockdown of PLCε and PLCβ3 did not produce changes in doubling time or differences in proliferation as seen by [3H]thymidine incorporation (results not shown). Consistent with the inhibition of thymidine incorporation, FACS analysis of BrdU-propidium-iodide-labelled cells revealed an accumulation of cells in G0/G1-phase. When compared with control cells, random shRNA cells treated with doxycycline or untreated shRNA PLCδ1 cells, PLCδ1 knockdown with shRNA PLCδ1#1 induced a 15–25% increase in total cell population in the G0/G1-phase (P < 0.001; n = 5), a concurrent decrease of 28–30% in S-phase (P < 0.001; n = 5), and a 34–38% increase in G2/M-phase (P < 0.05; n = 5) (Figure 2B). To demonstrate a direct correlation, a time course of PLCδ1 knockdown and cell-cycle distribution was determined. Figure 2(C) shows that the relative number of cells in G0/G1 compared with S-phase (cells in G0/G1 divided by cells in S-phase) increased only as levels of PLCδ1 decreased after addition of doxycycline, demonstrating the specificity of the PLCδ1-knockdown effect. Consistent with reversal of the proliferation defect with passage of the stable non-inducible clones (Figure 1D), marked knockdown of PLCδ1 is required before effects on proliferation are observed. Similar results were obtained for Rat-1 fibroblasts expressing shRNA PLCδ1#2 (results not shown).

As alterations in proliferation can also be due to cell death, apoptosis was assessed 72 h after doxycycline treatment by analysing nuclear fragmentation with DAPI staining (Figure 2D) and FACS analysis of BrdU/propidium-iodide-labelled cells (Figure 2E), and by measuring plasma membrane instability by FACS analysis of annexin-V-labelled cells (Figure 2F). These studies demonstrate that PLCδ1 knockdown had no effect on the number of apoptotic cells compared with controls (≤1%). In contrast, 50 μM staurosporine, a non-selective protein kinase inhibitor and potent inducer of apoptosis [21], markedly increased apoptosis to 14% of cells. These results suggest that the decreased cell number in the PLCδ1-knockdown cells is due to a cell-cycle defect and not to an increased rate of apoptosis.

**Kinetics of cell-cycle abnormality**

To elucidate the altered kinetics of the cell-cycle abnormality, cells were synchronized with serum starvation for 30 h (see Figure 3D for the protocol). Serum was then reintroduced and the progression through one cell cycle was examined from 0 to 23 h by FACS analysis. Figure 3(A) and Supplementary Figure S1(A) (at http://www.BiochemJ.org/bj415/bj4150439add.htm) shows the percentage of cells in G0/G1-, S- or G2/M-phase at the indicated time points post-synchronization, and Supplementary Figures S1(E) and S1(F) show original data for the 11 and 17 h time points. These results demonstrate that the rate of progression from quiescence through to G1-phase was not significantly different between knockdown and control cells. However, knockdown of PLCδ1 with shRNA PLCδ1#1 (Figure 3A and Supplementary Figure S1E) or shRNA PLCδ1#2 cells (Supplementary Figures S1A and S1F) delayed progression into S-phase, highlighted by a later G1-to-S-phase inflection point, and a gradual accumulation of cells in G0/G1 with a corresponding decrease in S-phase cells.

Since cell-cycle progression is dependent on CDK proteins and the temporal expression of cyclins which bind to and activate CDK enzymes, the levels of these proteins were measured. The identified cell-cycle abnormalities correlated with the levels of phase-specific cyclins and CDKs. Figure 3(B) and Supplementary Figure S1(B) show that, consistent with a normal progression through G1/G0, the amounts of G1-phase proteins, cyclin D2, cyclin D3 and CDK4, were unchanged between PLCδ1-knockdown and control cells. Cyclin D1 and CDK6 were not detected in these cells. Furthermore, CDK4-associated kinase activity was not affected (results not shown).

In contrast, lysates probed for G1-to-S-phase-associated proteins revealed unchanged levels of CDK2 but, surprisingly, increased levels of cyclin E. In control cells, cyclin E levels were elevated initially, began to decrease by 7 h and were undetectable at 13 h (Figure 3C and Supplementary Figure S1C). In contrast, cyclin E levels in PLCδ1-knockdown cells remained elevated and were further increased throughout the cell cycle (Figure 3C and Supplementary Figure S1C). On the other hand, the S-phase expression of cyclin A was delayed (Figure 3C and Supplementary Figure 1C), and levels of G2/M-phase proteins, cyclin B1 and CDK1, were decreased in PLCδ1-knockdown cell
E2 counted and scored for apoptosis based on the presence of pyknotic nuclear morphology. The histogram represents the percentage of cells undergoing apoptosis per visual field and represents an average of six fields per slide. Error bars represent the S.E.M. for a total of three slides. E2 Rat-1 cells expressing shRNA random or PLCδ1 were treated for 72 h, fixed and stained with the DNA-specific DAPI stain. Cells were harvested and double-stained with anti-BrdU antibodies and propidium iodide. DNA fragmentation by FACS analysis of hypodiploid DNA content was measured. The percentage of the cell population containing less than 2n of DNA in each condition is indicated. The histogram represents means ± S.D. of two independent studies, performed in triplicate.

Figure 2 Knockdown of endogenous PLCδ1 on proliferation and apoptosis rates in Rat-1 fibroblasts

(A) [3H]Thymidine proliferation assay. Cells were seeded at 10,000 cells per well in a 24-well plate, following 48 h pretreatment with 10 ng/ml doxycycline. A 6 h, thymidine incorporation was measured after 24 and 48 h of growth. Values are means ± S.D. of two independent studies, performed in triplicate. **P < 0.001, *P < 0.03. (B) The percentage of cells at each cell-cycle stage as determined by FACs analysis. Rat-1 fibroblasts expressing shRNA random or PLCδ1 were treated with 100 ng/ml doxycycline for 72 h. Cells were double-stained with anti-BrdU antibodies and propidium iodide. DNA fragmentation by FACs analysis of hypodiploid DNA content was measured. The percentage of the cell population containing less than 2n of DNA in each condition is indicated. The histogram represents means ± S.D. for two experiments. Doxy, doxycycline.

CDK2 activity is inhibited in PLCδ1-knockdown cells

To determine the CDK2 and cyclin E-associated kinase activity, synchronized cells were examined at 7 h and 11 h post-serum-starvation, time points identified by FACs analysis as important for G1-to-S-phase transition and progression through S-phase respectively. Figures 4(A) and 4(B) show that, at these time points, CDK2 kinase activity in shRNA PLCδ1#1-expressing cells was significantly reduced by 73.9 ± 10.12 and 75.0 ± 10.3% respectively (P < 0.001; n = 3), despite similar levels of CDK2 and elevated cyclin E in the immunoprecipitation (Figure 4C) and lysates (Figure 4D). In a similar manner, cyclin E-associated kinase activity was inhibited 60.6 ± 12.2 and 49.5 ± 12.1% respectively (P < 0.01, n = 3) (Figures 4A and 4B). In addition, consistent with decreased CDK2 activity and delayed expression of cyclin A, cyclin A-associated kinase activity was inhibited 87.6 ± 1.4 and 76.1 ± 13.9% respectively (P < 0.003, n = 3) (results not shown). Similar results were observed in shRNA PLCδ1#2 cells (Supplementary Figures S2A–S2D at http://www.BiochemJ.org/bj/415/bj4150439add.htm).

Phosphorylation of inhibitory and activating sites of CDK2

CDK2 activity is dependent on the removal of inhibitory phosphorylations on Tyr15 and Thr14 by Cdc25 (cell-division cycle 25) phosphatases and the phosphorylation of Thr160 by Cdk1 (CDK-activating kinase) [1,2]. In lysates from unsynchronized and synchronized cells, PLCδ1 knockdown decreased phosphorylation of inhibitory residues, Tyr15 and Thr14 (Figure 5A and Supplementary Figure S3A at http://www.BiochemJ.org/bj/415/bj4150439add.htm), suggesting normal or overactive Cdc25 phosphatase activity. In a similar manner, stimulatory phosphorylation was not different between control and PLCδ1-knockdown cells as determined by equal amounts of the Thr160 phosphorylation-induced gel shift of CDK2 (Figure 5A and Supplementary Figure S3A).

Inhibitory regulators of cyclin E–CDK2

In addition to protein levels and regulation by post-translational phosphorylation, the cyclin E–CDK2 complex is regulated by binding of inhibitory proteins. Three important proteins that have been shown to regulate this complex are members of the Cip/Kip family, p21, p27 and p57 [3,4]. We therefore examined cells for the expression of these regulators in control and PLCδ1
PLCδ1 regulates cell-cycle progression

Figure 3 Effect of endogenous PLCδ1 knockdown on the cell cycle in synchronized Rat-1 fibroblasts

shRNA PLCδ1#1-expressing Rat-1 fibroblasts cells, ±100 ng/ml doxycycline, were synchronized and harvested at various time points from 0–23 h, following 1 h pulse with 10 μM BrdU. (A) FACS analysis of the DNA content of cells harvested over a 23 h period post-synchronization. The percentage of cells in a given phase is plotted against time after release from serum-starvation (n = 2). (B) Western blot analysis of G1-phase protein expression in cells harvested 0–7 h post-serum-starvation release. Hsp90 heat-shock protein 90 expression was used as a control. (C) Western blot analysis of cell-cycle protein expression in cells harvested 0–23 h post serum-starvation release. Hsp90 expression was used as a control. (D) Schematic diagram of the experimental procedure for cell synchronization as described in the Experimental section. Doxy, doxycycline.

Knocked-down cells. As previously shown [22], p21 or p57 were not detected in Rat-1 fibroblasts or induced by knockdown of PLCδ1 (results not shown). In addition, the DNA-damage response protein that induces p21, p53, was detected, but the levels did not differ between control and PLCδ1-knockdown cells (results not shown). Consistent with these findings [23], staining for SA-β-gal revealed no difference in the number of cells in senescence (results not shown).

In contrast, Figures 5(B), 5(C) and Supplementary Figure S3(B) show that p27 is present and that treatment with doxycycline significantly increased the amount of the inhibitory protein 2.51 ± 0.70-fold (P < 0.02; n = 6; two-tailed paired Student’s t test on band densities) and 2.24 ± 0.56-fold (P < 0.04; n = 5) in unsynchronized cells and synchronized cells at 11 h after release from serum starvation respectively. Furthermore, co-immunoprecipitation studies (Figure 5C) showed that CDK2-bound p27 was increased in doxycycline-treated cells, suggesting that elevated p27 inhibits cyclin E-CDK2 activity in PLCδ1-knockdown cells.

DISCUSSION

The present studies demonstrate that PLCδ1 plays an important role in cellular proliferation and specifically in cell-cycle control. We show that knockdown of PLCδ1 leads to decreased cell viability of Rat-1 and NIH 3T3 cells. To characterize this viability defect, studies in Rat-1 fibroblasts using an optimized doxycycline-regulated shRNA expression system were used to
and cyclin E immunoprecipitation. Error bars indicate the S.E.M; activity in doxycycline-treated cells with regards to non-doxycycline (control) cells for CDK2 activity following doxycycline treatment. The histogram indicates the level of residual kinase separated by SDS/PAGE and visualized by autoradiography. (respectively; n = 3. (B) Western blot analysis of CDK2 immunoprecipitation performed in 7 and 11 h lysates used in the immunoprecipitation and probed for the expression of cyclin E or CDK2.

Figure 4 Effect of endogenous PLCδ1 knockdown on kinase activity in 7 and 11 h synchronized Rat-1 fibroblasts

shRNA PLCδ1#1-expressing cells were synchronized and released from serum-starvation for 7 and 11 h prior to harvest, ± 100 ng/ml doxycycline (Dox). (A) Equal amounts of cell lysate (500 µg of protein) was immunoprecipitated (IP) with CDK2 or cyclin E antibody and the precipitate was incubated with [γ-32P]ATP and histone H1. 32P-Labelled histone H1 was separated by SDS/PAGE and visualized by autoradiography. (B) The change in associated kinase activity following doxycycline treatment. The histogram indicates the level of residual kinase activity in doxycycline-treated cells with regards to non-doxycycline (control) cells for CDK2 and cyclin E immunoprecipitation. Error bars indicate the S.E.M. *P < 0.001 and **P < 0.01 respectively; n = 3. (C) Western blot analysis of CDK2 immunoprecipitation performed in conjunction with the kinase assays. Blots were probed for either CDK2 or cyclin E expression. (D) Western blot analysis of 7 and 11 h lysates used in the immunoprecipitation and probed for the expression of cyclin E or CDK2.

demonstrate that knockdown of PLCδ1 induces a proliferation defect but has no effect on the rates of apoptosis or induction of SIPS (stress-induced premature senescence). Furthermore, we demonstrate that the decrease in proliferation is associated with an altered G1-to-S-phase transition and that this block is mediated by diminished cyclin E–CDK2 activity, which is critical for the initiation of DNA synthesis and transition through this cell-cycle phase [1,2].

This effect was specific for knockdown of PLCδ1 and not related to off-target effects because a similar effect on proliferation was observed in mouse and rat species, two shRNAs targeting distinct regions yielded similar results in all studies, and overexpression of an shRNA PLCδ1#1-resistant PLCδ1 rescued the proliferation defect induced by knockdown with the corresponding shRNA. Furthermore, effects on proliferation temporally correlated with knockdown of PLCδ1 and reversal of the proliferation defect in non-inducible clones with passage of cells was accompanied by an increased expression of PLCδ1. Similarly, no effect was observed in control cells expressing a random shRNA or in tetracycline-regulated cells not exposed to doxycycline. In addition, knockdown of PLCε or PLCβ3 had no effect on proliferation indicating that the observed cell-cycle-dependent effects are regulated by PLCδ1 and not PLCε or PLCβ3. Thus the present study suggests that endogenous PLCδ1 exerts a positive effect on the activity of the cyclin E–CDK2 complex and that removal of PLCδ1 leads to inhibition of this complex, decreased G1-to-S-phase transition and slowed cellular proliferation.

The activity of CDK2 is temporally regulated by the expression of cyclin E, which rises in late G1-phase and is rapidly degraded in early S-phase [1]. In our PLCδ1-knockdown cells, however, CDK2 levels were unchanged and total cellular and CDK2-complexed cyclin E levels were paradoxically elevated compared with control PLCδ1-expressing cells. As the degradation of cyclin E is dependent on the active cyclin E–CDK2 complex, which targets cyclin E for ubiquitination by SCF (Skp1–cullin–F-box protein complex) ubiquitin ligase [24], an elevated level of this protein is consistent with decreased cyclin E–CDK2 activity. Since levels and activity of cyclin D–CDK4 were unaffected, these studies indicate that cyclin E–CDK2 is a primary site regulated by PLCδ1.

In addition to temporal changes in cyclin E, cyclin E–CDK2 activity is also regulated by post-translational inhibitory and stimulatory phosphorylations and the Cip/Kip family of CKIs, p21, p27 and p57 [1,3,4]. Phosphorylation of CDK2 Thr187/Tyr189 residues has been shown to inhibit cyclin E–CDK2 activity and removal of these modifications by Cdc25 family phosphatases is necessary for activation of this complex [1]. Likewise, CAK phosphorylation of Thr160 on CDK2 allows for complete activation of the cyclin–CDK complex [1]. Interestingly, in PLCδ1-knockdown cells, we showed that inhibitory phosphorylation of Thr187/Tyr189 was decreased and that stimulatory phosphorylation was intact, indicating that altered Cdc25 or CAK activity do not mediate the inhibitory effects of PLCδ1 knockdown on cyclin E-associated CDK2 activity.

On the other hand, the level of the CKI p27 and its association with cyclin E–CDK2 were elevated in PLCδ1-knockdown cells. p27 binds to cyclin E–CDK2 and cyclin A–CDK2 and inhibits the activity of these complexes by obstructing the ATP-binding site [4,25]. Thus elevated levels of this inhibitor could explain the decreased cyclin E–CDK2 activity in PLCδ1-knockdown cells. Normally, during the cell cycle, p27 levels are initially elevated but decrease with progression, releasing the inhibition of cyclin E–CDK2 and allowing cells to transition from G1- into S-phase [4]. Several mechanisms regulate the effective levels of p27 including localization and sequestration by cyclin D-dependent kinases, but the amount of p27 is predominantly regulated by SCF ubiquitination-mediated proteolytic degradation that is stimulated by cyclin E–CDK2-mediated phosphorylation of Thr187 on p27 [26–28]. Recent evidence, however, suggests that p27 degradation is regulated by phosphorylation at multiple other sites by other kinases [25,29]. Further studies are required to elucidate the underlying mechanism by which PLCδ1 regulates the level of p27.

A role for PLCδ1 regulating cellular proliferation is also consistent with previous studies examining localization of PLCδ1. PLCδ1 shuttles between the cytoplasm and the nucleus and...
accumulates in the nucleus at the G1/S-phase boundary and during the G1-phase of the cell cycle [14–16,18,30], consistent with a G1-to-S-phase transition regulatory role as shown in the present studies. Similarly, PLCδ1 is primarily expressed in the nucleus, initially in the G1-to-S-phase transition, and knockout of this isoform delays the onset of DNA synthesis [11]. Furthermore, the simultaneous disruption of PLCδ1 and PLCδ3 is embryonic lethal between E11.5 and E13.5 (where E is embryonic day) due to abnormal placental development from decreased proliferation and aberrant apoptosis of cells [31]. Taken together, these studies suggest a possible commonality in cell-cycle regulation between PLCδ isoforms. In contrast, while PLCβ1 overexpression also regulates mitogenesis, the underlying mechanism is distinct from PLCδ1 and involves increasing the expression of the cyclin D3–CDK4 complex [5,6]. Similarly, PLCγ1 overexpression stimulates DNA synthesis but in a phosphodiesterase-independent SH2 domain-dependent manner [32]. Thus distinct families of PLC regulate proliferation by diverse mechanisms.

Recently, evidence has been presented [33] that suggests that PLCδ1 functions as a novel tumour-suppressor in ESCC (oesophageal squamous cell carcinoma). This study found that a high percentage (~50%) of ESCC cell lines and primary tumours lack PLCδ1 expression and that overexpression of PLCδ1 in KYSE30 cells was associated with decreased cell growth rate and cell-cycle arrest at the G1-to-S-phase checkpoint through increased expression of p21. It is unclear why KYSE30 cells respond differently to PLCδ1 than Rat-1 cells; however, KYSE30 cells are derived from ESCC and other tumour suppressors deleted in the 3p region may have a role. Also, it is important to note that the effect of the cell-cycle arrest in KYSE30 cells is mediated by the increased expression of p21, and that only p27 is expressed in Rat-1 cells. Although these Cip/Kip proteins have overlapping functions, they are regulated differently and have independent cell-cycle regulatory roles [4,25,26]. Further studies are needed to address these differences, but they hint at the complexity of the role of PLCδ1 to regulate the cell cycle. Given the significance of the cyclin E–CDK2 complex and CKI proteins in cell-cycle control and malignancy [4,25,26], it is important to elucidate these pathways.

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SUPPLEMENTARY ONLINE DATA

Phospholipase Cδ1 regulates cell proliferation and cell-cycle progression from G1- to S-phase by control of cyclin E–CDK2 activity

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Figure S1  Effect of endogenous PLCδ1 knockdown on the cell cycle in synchronized Rat-1 fibroblasts

shRNA PLCδ1#2-expressing Rat-1 fibroblasts cells, ± 100 ng/ml doxycycline (Dox), were synchronized and harvested at various time points 0–23 h, following a 1 h pulse with 10 μM BrdU. (A) FACS analysis of DNA content of cells harvested over a 23 h period post-synchronization. The percentage of cells in a given phase is plotted against time after release from serum-starvation (n = 2). (B) Western blot analysis of G1-phase protein expression in cells harvested 0–7 h post serum-starvation release. Hsp90 expression was used as a control. (C) Western blot analysis of cell-cycle protein expression in cells harvested 0–23 h post serum-starvation release. Hsp90 expression was used as a control. (D) Schematic diagram of the experimental procedure for cell synchronization as described in the Experimental section of the main text. (E and F) Representative images of FACS analysis of DNA content of shRNA PLCδ1#1 (E) and shRNA PLCδ1#2 (F), shown in (A), at 11 and 17 h time points and ± doxycycline (100 ng/ml), as shown.
Figure S2  Effect of endogenous PLCδ1 knockdown on kinase activity in 7 and 11 h synchronized Rat-1 fibroblasts

shRNA PLCδ1#2-expressing cells were synchronized and released from serum-starvation for 7 and 11 h prior to harvest, ± 100 ng/ml doxycycline. (A) Equal amounts of cell lysate (500 μg of protein) was immunoprecipitated (IP) with CDK2 or cyclin E antibody and the precipitate was incubated with [γ-32P]ATP and histone H1. 32P-labelled histone H1 was separated by SDS/PAGE and visualized by autoradiography. Doxy, doxycycline. (B) Change in associated kinase activity following doxycycline treatment. The histogram indicates the level of residual kinase activity in doxycycline-treated cells compared with non-doxycycline (control) treated cells for CDK2 and cyclin E immunoprecipitation. Values are means ± S.E.M. **P < 0.002, *P < 0.02, #P < 0.02, n = 3. (C) Western blot analysis of CDK2 immunoprecipitation (IP) performed in conjunction with the kinase assays. Blots were probed for either CDK2 or cyclin E expression. (D) Western blot analysis of 7 and 11 h lysates used in the immunoprecipitation and probed for the expression of cyclin E or CDK2.

Figure S3  Regulation of the cyclin E–CDK2 complex

Western blot analysis of shRNA PLCδ1#2-expressing cells treated for 72 h ± 100 ng/ml doxycycline, synchronized for 7 or 11 h or unsynchronized. (A) Western blots of whole cell lysates were probed for post-translational phosphorylations of CDK2 with: a p-CDK2 (Thr14/Tyr15) antibody to identify inhibitory phosphorylations, a CDK2 antibody to identify the stimulatory CAK phosphorylation on Thr160 (lower band represents CAK-phosphorylated CDK2 with higher electrophoretic mobility compared to unphosphorylated), and a Hsp90 antibody for normalization. (B) Western blots probed for the induction of p27. Western blots of synchronized whole cell lysates collected 7 and 11 h after release from serum starvation were stained with p27 and β-actin. The blot is representative of two similar experiments. IB, immunoblot.