Phosphatidylinositol 3-Kinase Activity Regulates α-Thrombin-Stimulated G1 Progression by Its Effect on Cyclin D1 Expression and CDK4 Activity

Polly J. Phillips-Mason†, Daniel M. Raben§ and Joseph J. Baldassare†¶*

From the †Departments of Cell and Molecular Biology and ¶Pharmacological and Physiological Sciences, St. Louis University School of Medicine, St. Louis, Missouri 63104 and the §Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Running Title: PI 3-kinase regulation of G1 progression

*To whom correspondence should be addressed: Department of Pharmacological and Physiological Sciences, St. Louis University School of Medicine, St. Louis, Missouri 63104, Tel.: 314-577-8543, E-mail: baldasjj@slu.edu.
This work was supported by United States Public Health Service Grant R01 DK46814 (JJB). The costs of publication of this article were defrayed in part by the payment of page charges. This article must, therefore, be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations are: CDK, cyclin-dependent kinase, ERK, extracellular-related kinase, GSK-3β, glycogen synthase kinase 3-β, PDGF, platelet-derived growth factor, PI 3-kinase, phosphatidylinositol 3-kinase, Rb, retinoblastoma.

2 Gardner, A.J. and Baldassare, J.J., unpublished results.

3 Phillips-Mason, P.J. and Baldassare, J.J., unpublished results.
Abstract

In this study we present evidence that PI 3-kinase is required for α-thrombin-stimulated DNA synthesis in Chinese hamster embryonic fibroblasts (IIC9 cells). Previous results from our laboratory demonstrate that the mitogen-activated protein kinase (ERK) pathway controls transit through G1 phase of the cell cycle by regulating the induction of cyclin D1 mRNA levels and CDK4/cyclin D1 activity. In IIC9 cells PI 3-kinase activation also is an important controller of the expression of cyclin D1 protein and CDK4/cyclin D1 activity. Pre-treatment of IIC9 cells with the selective PI 3-kinase inhibitor, LY294002 blocks the α-thrombin-stimulated increase in cyclin D1 protein and CDK4 activity. However, LY294002 does not affect α-thrombin-induced cyclin D1 steady state message levels, indicating PI 3-kinase acts independent of the ERK pathway. Interestingly, expression of a dominant-negative Ras significantly decreased both α-thrombin-stimulated ERK and PI 3-kinase activities. These data clearly demonstrate that the α-thrombin-induced Ras activation coordinately regulates ERK and PI 3-kinase activities, both of which are required for expression of cyclin D1 protein and progression through G1.
INTRODUCTION

Progression through the mammalian cell cycle requires the mitogen-stimulated induction of cyclin D1. In the presence of growth factor, cyclin D1 accumulates in the G1 phase of the cell cycle and assembles with its catalytic partner, CDK4 or 6 (1-4). The cyclin D1/CDK4 or CDK6 complex controls transit through the G1/S phase transition by phosphorylating and inactivating the growth suppressor, retinoblastoma protein (Rb) (5-9). In early G1 cyclin D1 levels increase and remain elevated. However, withdrawal of mitogen results in the rapid decline of cyclin D1 and growth arrest in G1 (3;10). The importance of cyclin D1 as a regulator of transit through the G1 phase is emphasized by its ability to accelerate passage through the G1 phase of the cell cycle when it is overexpressed (8;11;12). In addition, inhibition of cyclin D1 using antisense cDNA or microinjection of cyclin D1 specific antibodies, results in withdrawal from the cell cycle and G1 growth arrest (11;13).

It is well established that the Ras/ERK pathway is an important regulator of mitogen-stimulated expression of cyclin D1 (14-17). Inhibition of the Ras/ERK pathway blocks mitogen-induced upregulation of cyclin D1 in several cell types (14;15;18;19), including Chinese hamster fibroblasts (16;17), demonstrating the requirement of this pathway in the integration of extra-cellular signals responsible for cyclin D1 expression. We have shown previously that in IIC9 cells, platelet-derived growth factor (PDGF)-induced cyclin D1 accumulation is dependent on the sustained activation of ERK (16).

In addition to the Ras/MAPK pathway, recent data suggests a role for the phosphatidylinositol (PI) 3-kinase in cell growth (20-24). The PI 3-kinases comprise a family of lipid kinases that phosphorylate the 3-position of the inositol ring of phosphatidylinositol (PtdIns), PtdIns(4)P and PtdIns(4,5)P_2 to generate PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively. PI 3-kinase lipid products have been implicated as second messengers in several cellular processes including cell survival, mitogenesis, protein trafficking and metabolism (25-27). Activation of PI 3-kinase activity has been shown to be required for DNA synthesis in response to several mitogens (22). In addition, the intracellular levels of PI 3-kinase lipid products are elevated in response to mitogen stimulation or oncogenic transformation (25;28-30). The role of PI 3-kinase in growth likely
involves the serine/threonine kinase, Akt (PKB), a downstream effector of PI 3-kinase, thought to be important for cell proliferation and anti-apoptotic responses (31-36). Although the importance of the PI 3-kinase pathway in cell growth is well established, its role in the regulation of growth in not understood.

α-Thrombin is a potent mitogen in IIC9 cells. Addition of α-thrombin to growth-arrested IIC9 cells stimulates an increase in endogenous ERK1 activity, and this activity is required for growth (Gardner, A.J. and Baldassare, J.J., unpublished results). In this study, we show that PI 3-kinase is required for α-thrombin-stimulated growth in IIC9 cells. We provide evidence that PI 3-kinase is required for cyclin D1 accumulation independent of the ERK pathway. Furthermore, these pathways are regulated at the level of Ras. Our data indicate that both the PI 3-kinase and ERK pathways coordinately regulate cyclin D1 expression to promote cell cycle progression. This is the first study to examine the role of PI 3-kinase stimulated by a G-protein coupled receptor (GPCR) in the regulation of cyclin D1.
EXPERIMENTAL PROCEDURES

Cell Culture and Reagents
IIC9 cells, a subclone of Chinese hamster embryo fibroblasts were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) containing 4.5 G/L glucose and 2mM L-glutamine (BioWhittaker, Walkersville, MD) supplemented with 5% (v/v) fetal calf serum. Subconfluent IIC9 cells (80%) were growth-arrested by washing once with alpha-MEM (Life Technologies), containing 2mM L-glutamine (BioWhittaker) followed by a 48 hour incubation in the same media. Human α-thrombin isolated from plasma (Sigma, St. Louis, MO) was used at 1 U/ml in all experiments. PD98059 (New England Biolabs, Beverly, MA) was used at 15 µM. Wortmannin (Calbiochem, La Jolla, CA) was used at 100 nM. LY294002 (Calbiochem, La Jolla, CA) was used 10 µM. Calphostin C (Calbiochem) was used at 10 µM.

Transient Transfection
The cDNA encoding pcDNA3 (Invitrogen) or a HA-tagged dominant-negative Ras mutant, HA-RasN17 (a kind gift from Gary L. Johnson, University of Colorado) was transfected into subconfluent (60-80%) IIC9 cells using Liptofectamine™ (Gibco BRL, Gaithersburg, MD) following the manufacturer’s protocol. 2 µg of each plasmid was mixed with 10 µl Liptofectamine™ per 1 ml of media. Six hours post-transfection an equal volume DMEM supplemented with 0.2% (v/v) fetal calf serum was added to the transfection mix and the cells were incubated overnight. The following day, cells were growth-arrested by washing once with alpha-MEM followed by a 48 hour incubation in the same media prior to agonist stimulation. Transient transfection using Liptofectamine™ resulted in 80-90% expression efficiency as visualized by β-galactosidase staining.

Western Blot Analysis
Growth-arrested IIC9 cells were incubated in the absence or presence of 1 U/ml α-thrombin after pre-incubation in the absence or presence of 100 nM wortmannin, 10 µM LY294002 or 15 µM PD98059 for 30 minutes. At the indicated times, cells were washed twice in cold PBS and harvested by scraping into 150 µl cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, 1mM
phenylmethylsulfonylfluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin). The lysates were sonicated briefly and the insoluble material pelleted by centrifugation at 14,000 x g at 4°C for 5 minutes. Protein concentrations of the supernatants were determined using Coomassie® Plus (Pierce, Rockford, IL) as recommended by the manufacturer. Protein lysates (10-25 µg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Boston, MA). Membranes were probed with polyclonal antibodies to cyclin D1 (Santa Cruz Biotechnology), Akt (Santa Cruz Biotechnology), phopsho-Akt (New England Biolabs, Beverly, MA) or phospho-ERK1/2 (Santa Cruz Biotechnology). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL) as recommended by the manufacturer.

**Cyclin D1/CDK4 Assay**

Growth-arrested IIC9 cells were incubated in the absence or presence of 1 U/ml α-thrombin after pre-incubation in the absence or presence of 100 nM wortmannin, 10 µM LY294002 or 15 µM PD98059 for 30 minutes. Cells were harvested 10 hours after stimulation by washing twice in cold PBS and scraping into 100 µl cold retinoblastoma (Rb) assay buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM sodium vanadate, 1mM sodium flouride, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 50 mM β-glycerophosphate, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, 1 mM PMSF, 10µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin). Cell lysates were sonicated briefly and the insoluble material pelleted by centrifugation at 14,000 x g at 4°C for 5 minutes. Cyclin D1/CDK4 complexes were immunoprecipitated from supernatants containing equal amounts of protein by incubation with a monoclonal cyclin D1 antibody (Santa Cruz Biotechnology) for 3 hours at 4°C, followed by an incubation with protein G-agarose (Sigma) at 4°C overnight. Cyclin D1/CDK4 immune complexes were pelleted by centrifugation and washed twice with cold wash buffer (50 mM Hepes, pH 7.5, 1 mM DTT, 10 mM MgCl2). Cyclin D1/CDK4 immune complexes were then resuspended in 30 µl reaction buffer (50 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM DTT, 2.5 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM sodium flouride and 20 µM ATP) and incubated with 2 µg/ml soluble
GST-Rb fusion protein (Rb sequence encoding amino acids 379-928 inserted into pGEX-2T plasmid is a kind gift from Dr. Mark Ewen, Harvard University) and 5 μCi [γ-32P]ATP at 30°C for 30 minutes. Samples were subjected to SDS-polyacrylamide electrophoresis (9.75%) and developed using a Phosphoimager™ (Molecular Dynamics, Sunnyvale, CA).

**Northern Blot Analysis**
Growth-arrested IIC9 cells were incubated in the absence or presence of 1 U/ml α-thrombin after pre-incubation in the absence or presence of 100 nM wortmannin, 10 μM LY294002 or 15 μM PD98059 for 30 minutes. At the indicated times, total RNA was isolated using TRIZOL Reagent (Gibco BRL) according to the manufacturer’s protocol. RNA (20 μg) was electrophoresed in a 2% (w/v) agarose/formaldehyde gel. After electrophoresis, formaldehyde was removed from the gel washing gels in 0.5% ammonium acetate. RNA was then transferred to a Hybond N+ nylon membrane (Amersham) using the Turboblotter™ system (Schleicher & Schuell, Keene, NH) and cross-linked onto the membrane using an Ultraviolet Crosslinker (Amersham) as recommended by the manufacturer. Randomly-labeled [α-32P]dCTP cDNA probes were made using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany). Membranes were pre-incubated with Rapid-hybridization buffer (Amersham) for 1 hour at 65°C then probed simultaneously with cyclin D1 and GAPDH probes for 2 hours at 65°C. After hybridization, the membrane was washed twice with 5X SSPE (20 mM EDTA, 1 M NaCl, 50 mM NaH₂PO₄·H₂O), 0.1% (w/v) SDS at room temperature and once with 1X SSPE, 0.1% SDS at 65°C. The membrane was developed using a Phosphoimager™ (Molecular Dynamics).

**Immune-Complex Kinase Assay for ERK1**
Growth IIC9 cells were incubated in the absence or presence of 1 U/ml α-thrombin after pre-incubation in the absence or presence of 100 nM wortmannin, 10 μM LY294002 or 15 μM PD98059 for 30 minutes. At the indicated times, cells were washed twice in cold PBS and harvested by scraping into 100 μl cold ERK lysis buffer and assayed as previously described (16).
**Thymidine Incorporation**

Growth-arrested IIC9 cells were incubated in the absence or presence of 1 U/ml α-thrombin for 17 hours after pre-incubation in the absence or presence of 100 nM wortamannin or 10 µM LY294002 for 30 minutes. Following the 17 hour incubation, 1 µCi/ml [3H]thymidine (Dupont/NEN, Boston, MA) was added to the cells for an additional 3 hour incubation. [3H]-labeled cells were washed twice with cold PBS and the DNA precipitated by incubating the cells in 5% (v/v) TCA for 30 minutes at 4°C. The TCA-precipitated DNA was washed twice with cold 5% TCA and solubilized with 500 µl of 2% (w/v) sodium bicarbonate/0.1 N NaOH. The solution was neutralized with 100 µl of 5% TCA and the TCA-precipitated [3H]DNA quantified by scintillation counting.

**Ras Activation Assay**

Growth-arrested IIC9 cells were labeled for 4 hours with [32P]Pi at 0.2 mCi/100 mm dish in phosphate-free DMEM (BioWhittaker). Cells were then washed twice with phosphate-free media and once with a saline buffer (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl). Cells were incubated in the presence or absence of 1U/ml α-thrombin for 5 minutes following pre-incubation in the absence or presence of 100 nM wortamannin or 10 µM LY294002 for 30 minutes. After stimulation, cells were washed two times with PBS and harvested by scraping into 500 µl IP buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 150 mM NaCl, 1% Triton X-100, 2 mM p-nitrophenylphosphate, 10 µg/ml pepstatin, 10 µg/ml aprotinin and 10 µg/ml leupeptin). Homogenates were incubated for 10 minutes on ice then centrifuged at 750 X g for 5 minutes. The supernatants were treated with 100 µl bovine serum albumin-coated charcoal for 5 minutes at 4°C and then centrifuged at 750 X g to remove charcoal. Ras immune complexes were immunoprecipitated by incubation with a monoclonal p21ras antibody (Oncogene) for 3 hours at 4°C, followed by an incubation with protein G plus agarose (Oncogene) overnight at 4°C. Ras complexes were washed twice with IP buffer and three times with wash buffer (Tris-HCl pH 7.5, 20 mM MgCl2, and 150 mM NaCl). Final pellets were drained and bound nucleotides
eluted in 20 µl of elution buffer (20 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2% SDS, 0.5 mM GDP, and 0.5 mM GTP). Eluants were heated at 65°C for 5 minutes and centrifuged. The supernatants were spotted onto a polyethyleneimine-cellulose thin layer plate (Merck) and developed with 0.75 M KH$_2$PO$_4$, pH 3.4. GDP and GTP $[^{32}p]$-labeled fractions were quantified using a Phosphoimager™ (Molecular Dynamics).

**Phosphatidylinositol 3-Kinase Assay**

Growth-arrested IIC9 cells were incubated in the absence or presence of 1 U/ml α-thrombin for 10 minutes after pre-incubation in the absence or presence of wortmannin. After stimulation, cells were washed twice with PBS and harvested by scraping into 400 µl cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl$_2$, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM EGTA, 100 mM sodium vanadate, 50 mM β-glycerolphosphate, 0.5 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin). The lysates were sonicated briefly and the insoluble material pelleted by centrifugation at 14,000 x g at 4°C for 5 minutes. p85 immune complexes were immunoprecipitated from lysates containing 300-400 µg of protein by incubation with polyclonal p85 antibody (Upstate Biotechnology, Lake Placid, NY) at 4°C for 3 hours, followed by an incubation with protein A-agarose (Sigma) at 4°C overnight. The p85 immune complexes were pelleted by centrifugation and washed three times with lysis buffer, three times with TNE (100 mM Tris-HCl, pH 7.4, 5 M LiCl and 100 mM sodium vanadate), and twice with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 100 mM sodium vanadate. After the last wash 50 µl of TNE, 10 µl phosphatidylinositol (suspended by sonication in 10 mM Tris-HCl, pH 7.4 and 1 mM EGTA at 2 µg/ml) and 10 µl of 100 mM MgCl$_2$ was added to the beads. The reaction was started by the addition of 5 µl of reaction buffer (0.88 mM ATP, 10 µCi [γ$^{32}$P]ATP and 20 mM MgCl$_2$). Samples were incubated for 10 minutes at 37°C and the reactions stopped by the addition of 20 µl 6 N HCl. Lipids were extracted by adding 160 µl of CHCl$_3$:MeOH [1:1] to the samples and vortexing briefly. Labeled lipids were resolved by spotting on a silicon TLC plate (J.T. Baker Inc., Phillipsburg, NJ) and developed with CHCl$_3$:MeOH:4N NH$_4$OH [9:7:2]. Phosphatidylinositol-3-phosphate production was quantitated using a
Phosphoimager™ (Molecular Dynamics). Phosphatidylinositol-4-phosphate isolated from bovine brain (Avanti Polar Lipids, Inc., Alabaster, AL) was included as a standard for TLC resolution of the lipids and visualized by iodine vapor.

**Phosphoinositide Hydrolysis Assay**

Subconfluent IIC9 cells were incubated for 24 hours in myoinositol-free basal media and then an additional 24 hours with 1 µCi [³H]myoinositol (Dupont/NEN) in the same media. LiCl was added to the cells 1 minute prior to incubation in the absence or presence of α-thrombin for 15 minutes. Cells were washed with cold PBS and total inositol phosphates extracted in 600 µl of cold 4% HClO₄ (v/v) for 30 minutes at 4°C. To each supernatant, 50 µl of phenol red/60 mM Hepes was added, supernatants were neutralized with Na₄OH, and then centrifuged. The supernatants were applied to a 0.5 ml column of Dowex AG1X8 (200-400 mesh size), formate form (Bio-Rad). Columns were washed three times with 3 ml water, and three times with 3 ml 0.05 M ammonium formate/0.005 M Borax. [³H]Inositol phosphates were eluted with 3 ml formic acid/1.8 M ammonium formate, and 1 ml quantified by scintillation counting.
RESULTS

PI 3-kinase is required for α-thrombin induced DNA synthesis.

Recently, much attention has focused on the role of PI 3-kinase in the regulation of cell growth. PI 3-kinase activity has been shown to be required for DNA synthesis in response to several mitogens (22-24). To determine whether PI 3-kinase is essential for α-thrombin-stimulated growth in IIC9 cells, we investigated the effect of a selective PI 3-kinase inhibitor, LY294002 on α-thrombin-induced DNA synthesis (Fig. 1). Pretreatment of growth-arrested IIC9 cells with LY294002 inhibits α-thrombin-induced DNA synthesis as measured by [3H]thymidine incorporation in a dose dependent manner (Fig. 1). Maximal inhibition of α-thrombin-induced DNA synthesis occurs at a concentration of 10µM LY294002 (Fig. 1). Wortmannin, a less selective inhibitor of PI3-kinase activity, also blocks α-thrombin-induced DNA synthesis in a dose dependent manner (data not shown).

We next examined whether α-thrombin-induces the activation of endogenous PI 3-kinase activity. α-Thrombin stimulates a 2-fold increase in PI 3-kinase activity over levels found in growth-arrested IIC9 cells (Fig. 2A). Although LY294002 is a potent selective inhibitor of PI 3-kinase activity, its effects are reversible (37), and subject to error when assayed in vitro by immunocomplex assays. To overcome this problem, we investigated whether LY294002 inhibits α-thrombin-induced Akt phosphorylation. This phosphorylation can be quantified by immunoblot analysis with a specific anti-phospho-Akt antibody. We reasoned that because the phosphorylation of Akt is dependent on PI 3-kinase activation (31;32;34), changes in its phosphorylation reflect changes in PI 3-kinase activity. α-Thrombin induces a rapid increase in Akt phosphorylation as detected by immunoblot analysis (Fig 2B). Increased activity is detectable within 10 minutes and remains elevated for at least 60 minutes. Pre-incubation of IIC9 cells with 10 µM LY294002 blocks Akt phosphorylation (Fig. 2C). Importantly, treatment with 10 µM LY294002 decreased Akt phosphorylation to values seen in growth-arrested IIC9 cells, in agreement with its effects on DNA synthesis. To confirm our results, we also determined the effect of wortmannin, a non-reversible but less selective inhibitor of PI 3-kinase activity on both PI 3-kinase (Fig 2A) and Akt phosphorylation (data not shown). When IIC9 cells are incubated
with 100 nM wortmannin 30 minutes prior to stimulation with α-thrombin, PI 3-kinase activity (Fig. 2A) and Akt phosphorylation (data not shown) are below levels found in growth-arrested IIC9 cells.

**PI 3-kinase is required for α-thrombin-induced CDK4 activity and accumulation of cyclin D1.**

Previous data from our laboratory (16) and others (15) have shown that inhibition of mitogen-induced signaling pathways affects CDK4 activity, and progression through the G1 phase of the cell cycle. We, therefore, decided to determine whether inhibition of PI 3-kinase activity affects cyclin D1/CDK4 activation by quantifying the effects of LY294002 on α-thrombin-stimulated CDK4 activity. Stimulation with α-thrombin produces a 4-fold induction of CDK4 activity that is blocked by pre-incubation of IIC9 cells with 10 µM LY294002 (Fig. 3). Interestingly, this concentration of LY294002 also reduces DNA synthesis and Akt phosphorylation to growth-arrested levels (Figs. 1 and 2C).

The effect of PI 3-kinase on CDK4 activity is similar to that seen with inhibitors of the ERK pathway in IIC9 cells (16). Previously, we (16) found that the ERK pathway controls PDGF-induced CDK4 activity by regulating expression of cyclin D1. We next determined the effect of inhibition of ERK activation on α-thrombin-induced cyclin D1 expression. Pretreatment of IIC9 cells with 15 µM PD98059, a selective MEK inhibitor, prior to treatment with α-thrombin blocks the α-thrombin-stimulated increase in cyclin D1 expression (Fig. 4B) and CDK4 activity (Fig. 3). These data suggested to us that PI 3-kinase also may regulate CDK4 activity by regulating cyclin D1 expression. Moreover, others have suggested that PI 3-kinase regulates mitogen-stimulated cyclin D1 expression (19;38-40). Western blot analysis shows that in IIC9 cells, α-thrombin stimulates a 3-5 fold induction in cyclin D1 by 4 hours and the increase in cyclin D1 protein is sustained for at least 10 hours (Fig. 4A). Indeed, pre-treatment of IIC9 cells with 10 µM LY294002 (Fig. 4B) or 100 nM wortmannin (data not shown), completely blocks the α-thrombin-induced upregulation of cyclin D1, suggesting PI 3-kinase regulates CDK4 activity through its effect on cyclin D1 expression.
**PI 3-kinase does not affect cyclin D1 steady state message levels.**

If PI 3-kinase prevents expression of cyclin D1 identical to ERK, we expect both pathways to block cyclin D1 protein in a similar manner. Previous data from our laboratory (16) and others (14;15;19) demonstrate that ERK controls cyclin D1 mRNA expression. α-Thrombin stimulates a 1.9-fold induction in cyclin D1 mRNA levels by four hours (Fig. 5), and reaches 2.7-fold by eight hours post-stimulation. Pre-incubation with LY294002 (Fig. 5) or wortmannin (data not shown) does not affect the α-thrombin-induced increase in cyclin D1 mRNA 6 hours post-stimulation, indicating PI 3-kinase is not regulating cyclin D1 steady state message levels. In contrast, PD98059 completely blocks the α-thrombin-induced increase in cyclin D1 mRNA.

**PI 3-kinase is not required for α-thrombin-induced ERK1 activation.**

Several recent reports indicate a role for PI 3-kinase in the regulation of ERK in response to activation by G-protein coupled receptors. In Cos-7 cells, treatment with wortmannin, LY294002 or expression of a dominant-negative mutant of PI 3-kinase, block ERK activation by lysophosphatidic acid (41). Similar results are seen with carbachol in Cos-7 cells expressing the m2 muscarinic receptor (42). Our data, however, indicate differential regulation of cyclin D1 expression by ERK and PI 3-kinase, suggesting these pathways are parallel and not dependent on each other. To confirm that PI 3-kinase is not required for α-thrombin-stimulated ERK activity in IIC9 cells, we examined the effect of LY294002 and wortmannin on endogenous ERK1 activity. α-Thrombin stimulates a biphasic activation of ERK, with a rapid initial phase within the first few minutes of activation and a late sustained phase lasting at least 4 hours (Fig. 6A). Pre-treatment of IIC9 cells with 10 µM LY294002 (Fig. 6A) or 100 nM wortmannin (data not shown) does not affect early or sustained ERK1 activity, although these concentrations completely block PI 3-kinase activation (Fig. 2C and data not shown) as determined by the phosphorylation of Akt. In addition, pretreatment with PD98059 does not affect Akt phosphorylation (data not shown). These data clearly demonstrate that PI 3-kinase does not regulate the ERK pathway and both differentially contribute to cyclin D1 upregulation and cell cycle progression. As expected, pre-treatment of IIC9 cells with PD98059 (Fig. 6A) blocks α-thrombin-stimulated ERK1 activity. In agreement with these data, pre-treatment of IIC9 cells with LY294002 (Fig. 6B) or wortmannin (data not shown) had no effect on α-thrombin-
stimulated Ras activity, which is required for α-thrombin-stimulated ERK1 activation in IIC9 cells (see below).

Ras coordinately regulates PI 3-kinase and ERK1 Activities.

Previous data from our laboratory (16) clearly demonstrate that activation of Ras results in cyclin D1 expression and transformation of IIC9 cells. We reasoned that coordinate regulation of the ERK and PI 3-kinase pathway could account for the ability of Ras to transform IIC9 cells. To determine whether PI 3-kinase and ERK are coordinately controlled by Ras, we quantified the effect of expression of HA-tagged dominant negative Ras (HA-RasN17) on endogenous Ras, ERK and PI 3-kinase activities (Fig. 7). Because of variation in the transfection efficiency between experiments, the endogenous activities were quantified from the same lysates. In IIC9 cells α-thrombin induces a 4-fold increase (GTP/[GTP +GDP] increased from 9% to 35%) in GTP-loading of Ras (Fig. 7A). Expression of HA-RasN17 (Fig. 7A) blocks the increase of GTP-loading of Ras by approximately 75% (GTP/[GTP +GDP] increases from 9% to 14%). Transient expression of HA-RasN17 inhibits PI 3-kinase activity by approximately 70% as measured by Akt phosphorylation (Fig. 7B). Taken together these data strongly indicate that α-thrombin-induced PI 3-kinase activity is mediated solely through Ras.

We next determined the effect of expression of HA-RasN17 on ERK activity (Fig. 7C). As shown for other cell types (41), whereas sustained ERK activity (30 minutes to several hours) is solely dependent on Ras, multiple signaling pathways regulate the initial phase (between 5 and 20 minutes) of ERK activation in IIC9 cells. Approximately 50% of the initial phase of α-thrombin-induced ERK activity is Ras-dependent. In agreement with others (41), the Ras-
independent activation is mediated through the Gq family by activation of phospholipase Cβ1 and protein kinase C (PKC) \(^2\). In serum arrested IIC9 cells ERK1 activity is approximately 11% of the maximal activity (100%) found in IIC9 cells stimulated with α-thrombin for 5 minutes (Fig. 7C). ERK1 activity in cells transient transfected with HA-RasN17 increases to 53% of the maximal activity when stimulated with α-thrombin, indicating that expression of HA-RasN17 inhibits the initial phase by approximately 40-45% (Fig. 7C). Furthermore, in the presence of the PKC inhibitor, calphostin C, ERK1 activity increases to 23% of maximal (Fig. 7C).

Therefore, treatment with both the calphostin C and HA-RasN17 ERK1 activity blocks ERK1 activation by greater than 80%. Taken together with the data showing that expression of HA-RasN17 blocks Ras GTP-loading by 90%, these data strongly indicate that Ras mediates a significant portion of the initial phase of α-thrombin ERK1 activation. Moreover, treatment with calphostin C does not further block Ras activation (GTP/[GTP + GDP] increased from 9% to 15%) in the presence of HA-RasN17 (Fig. 7A), indicating PKC activity is not involved in Ras activation.

Because expression of RasN17 inhibits growth, we next examined whether the inhibition of the ERK and PI 3-kinase activities are specific or a consequence of the inability of these cells to grow. In IIC9 cells α-thrombin-induced inositol phosphate release is mediated through Gq, and is independent of Ras \(^2\). We next examined the effect of expression of HA-RasN17 on PI hydrolysis (Fig. 8). Treatment of IIC9 cells with α-thrombin increases inositol phosphate release approximately 5-fold. Ectopic expression of HA-RasN17 does not affect inositol phosphate release (Fig. 8), indicating the effect of HA-RasN17 is specific.
DISCUSSION

Progression from the G1 to S phase of the cell cycle requires activation of CDK4, and CDK4 activation is controlled, in part, by complex formation with its catalytic partner, cyclin D1 (1-4). Cyclin D1 levels are low in serum-arrested cells and increase in the G1 phase of the cell cycle. Several laboratories have demonstrated that the Ras/ERK pathway is an essential regulator of mitogen-stimulated expression of cyclin D1 and its assembly with its catalytic partner, CDK4 or 6 (14-17). Although the importance of PI 3-kinase activity as a controller of cell growth is recognized (20)(21;2); little is known of how PI 3-kinase affects growth. Recent data with several cell types suggest that PI 3-kinase contributes to the upregulation of cyclin D1 (18;19;38-40). Here we examine the effect of PI 3-kinase in α-thrombin-stimulated cyclin D1 expression and CDK4 activity. Our results clearly demonstrate that PI 3-kinase activity is required for the upregulation of cyclin D1 protein expression. Treatment of IIC9 cells with LY294002, a selective inhibitor of PI 3-kinase, results in the significant reduction in α-thrombin-induced cyclin D1 protein expression (Fig. 4B), CDK4 activity (Fig. 3) and DNA synthesis (Fig.1). In agreement with previous data from our laboratory (16) and others (15;19), the inhibition of ERK activation also markedly suppresses mitogen-induced cyclin D1 expression. As previously reported for PDGF-induced cyclin D1 in IIC9 cells (16), inhibition of ERK affects cyclin D1 mRNA expression stimulated by α-thrombin in IIC9 cells. In contrast, no detectable change in cyclin D1 mRNA is observed when PI 3-kinase is inhibited to levels found in growth-arrested IIC9 cells (Fig. 5). These data indicate that ERK and PI3-kinase activities regulate α-thrombin-induced cyclin D1 protein expression by different mechanisms.

The PI 3-kinase G1 target, cyclin D1

In IIC9 cells, PI 3-kinase is required for the regulation of cyclin D1 protein expression (Fig. 4B). This data is consistent with several reports indicating a role for PI 3-kinase in mitogen-stimulated cyclin D1 upregulation (18;19;38-40). However, in IIC9 cells, LY294002 had no effect α-thrombin-induced steady state message levels (Fig. 5) which is inconsistent with results in NIH3T3 cells stimulated with serum (18;19). We do not think the effect of PI 3-kinase on cyclin D1 is cell type specific. In support of this view, Diehl and coworkers (38), also working in NIH3T3 cells, found PI 3-kinase to be important in cyclin D1 protein stabilization. They (38) found that glycogen
Synthase kinase-3β (GSK-3β) phosphorylates and targets cyclin D1 for ubiquitin-mediated degradation. PI 3-kinase activates Akt, which phosphorylates and inhibits glycogen synthase kinase-3β (38). Furthermore, treatment of α-thrombin-stimulated IIC9 cells with the ubiquitin-dependent proteosome inhibitor, MG132, blocks the effects of LY294002 and results in levels of cyclin D1 protein seen with α-thrombin-stimulated IIC9 cells.

**Role of Ras**

Previous studies in IIC9 cells show that activation of Ras is sufficient for transformation (17;43). Because PI 3-kinase is essential for G1 transit, and Ras activation results in growth, we reasoned that PI 3-kinase must be downstream of Ras. Consistent with this model and the ability of Ras to transform IIC9 cells, expression of RasN17 inhibits both ERK and PI 3-kinase activities (Fig. 7). Several reports have suggested Ras as key regulator of several divergence signaling pathways (19;38;44;45). In agreement with Ras being a divergence point for several activities, we (45) have shown in IIC9 cells that Ras stimulates both ERK and RhoA, which control cyclin D1 upregulation and p27KIP1 degradation, respectively. Proper transit through the cell cycle depends on the timely synthesis and degradation of cyclin D1. The regulation of cyclin D1 by both synthesis and degradation allows for the rapid changes necessary to secure the timely appearance and disappearance of cyclin D1. We propose that, in α-thrombin-stimulated cells, Ras coordinates the activation of both PI 3-kinase and ERK leading to the rapid changes in cyclin D1 expression. This model is in agreement with Diehl and coworkers (38) who show PI 3-kinase and ERK act independently to contribute to cyclin D1 accumulation.
REFERENCES

1. Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.-Y., Hanks, S.K., Roussel, M.F., and Sherr, C.J. (1992) *Cell* **71**, 323-334

2. Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J., and Kato, J. (1994) *Mol. Cell. Biol.* **14**, 2066-2076

3. Sherr, C.J. (1994) *Cell* **79**, 551-555

4. Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C., and Peters, G. (1994) *Oncogene* **9**, 71-79

5. Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and Sherr, C.J. (1993) *Genes Dev.* **7**, 331-342

6. Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J., and Livingston, D.M. (1993) *Cell* **73**, 487-497

7. Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A., and Weinberg, R.A. (1993) *Cell* **73**, 499-511

8. Resnitzky, D.M., Gossen, M., Bujard, H., and Reed, S.I. (1994) *Mol. Cell. Biol.* **14**, 1669-1679

9. Weinberg, R.A. (1995) *Cell* **81**, 323-330

10. Matsushime, H., Roussel, M.F., Ashmun, R.A., and Sherr, C.J. (1991) *Cell* **65**, 701-713

11. Quelle, D.E., Ashmun, R.A., Shurtleff, S.A., Kato, J., Bar-Sagi, D., Roussel, M.F., and Sherr, C.J. (1993) *Genes Dev.* **7**, 1559-1571

12. Liu, J., Chao, J., Jiang, M., Ng, S., Yen, J.J., and Yang-Yen, H. (1995) *Mol.Cell. Biol.* **15**, 3654-3663

13. Baldin, V., Lukas, J., Marcote, M.J., Pagano, M., and Draetta, G. (1993) *Genes Dev.* **7**, 812-821

14. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R.G. (1995) *J. Biol. Chem.* **270**, 23589-23597

15. Lavoie, J.N., L’Alleman, G., Brunet, A., Müller, R., and Pouysségur, J. (1996) *J. Biol. Chem.* **271**, 20608-20616

16. Weber, J.D., Raben, D.M., Phillips, P.J., and Baldassare, J.J. (1997) *Biochem. J.* **326**, 61-68
17. Weber, J.D., Cheng, J., Raben, D.M., Gardner, A., and Baldassare, J.J. (1997) J. Biol. Chem. 272, 17320-17326

18. Takuwa, N., Fukui, Y., and Takuwa, Y. (1999) Mol.Cell.Biol. 19, 1346-1358

19. Gille, H. and Downward, J. (1999) J. Biol. Chem. 274, 22033-22040

20. Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., and Cantley, L.C. (1989) Cell 57, 167-175

21. Coughlin, S.R., Escobedo, J.A., and Willimas, L.T. (1989) Science 243, 1191-1194

22. Roche, S., Koegl, M., and Courtneidge, S.A. (1994) Proc. Natl. Acad. Sci.U.S.A. 91, 9185-9189

23. Cheatham, B., Vlahos, C.J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C.R. (1994) Mol. Cell. Biol. 14, 4902-4911

24. Hu, S.-W., Shi, X.-Y., Lin, R.Z., and Hoffman, B.B. (1996) J. Biol. Chem. 271, 8977-8982

25. Carpenter, C.L. and Cantley, L.C. (1996) Curr. Opin. Cell Biol. 8, 153-158

26. Vanhaesebroeck, B., Leevers, S.J., Panayotou, G., and Waterfield, M.D. (1997) Trends Biochem. Sci. 22, 267-272

27. Leevers, S.J., Vanhaesebroeck, B., and Waterfield, M.D. (1999) Curr. Opin. Cell Biol 11, 219-225

28. Toker, A. and Cantley, L.C. (1997) Nature 387, 673-676

29. Franke, T.F., Kaplan, D.R., and Cantley, L.C. (1997) Cell 88, 435-437

30. Rameh, L.E. and Cantley, L.C. (1999) J. Biol. Chem. 274, 8347-8350

31. Franke, T.F., Yang, S.-I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., and Tsichlis, P.N. (1995) Cell 81, 727-736

32. Burgering, B.M.Th. and Coffer, P.J. (1995) Nature 376, 599-602

33. Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordán, J., Bellacosa, A., Tsichlis, P.N., and Hay, N. (1997) Genes Dev. 11, 701-713

34. Franke, T.F., Kaplan, D.R., Cantley, L.C., and Toker, A. (1997) Science 275, 665-668

35. Coffer, P.J., Jin, J., and Woodgett, J.R. (1998) Biochem. J. 335, 1-13

36. Downward, J. (1998) Curr. Opin. Cell Biol 10, 262-267
37. Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994) *J. Biol. Chem.* **269**, 5241-5248

38. Diehl, J.A., Cheng, M., Roussel, M.F., and Sherr, C.J. (1998) *Genes Dev.* **12**, 3499-3511

39. Muise-Helmericks, R.C., Grimes, H.L., Bellacosa, A., Malstrom, S.E., Tsichlis, P.N., and Rosen, N. (1998) *J. Biol. Chem.* **273**, 29864-29874

40. Treinies, I., Paterson, H.F., Hooper, S., Wilson, R., and Marshall, C.J. (1999) *Mol. Cell. Biol.* **19**, 321-329

41. Hawes, B.E., van Biesen, T., Koch, W.J., Luttrell, L.M., and Lefkowitz, R.J. (1995) *J. Biol. Chem.* **270**, 17148-17153

42. Lopez-Ilasaca, M., Crespo, P., Pellici, P.G., Gutkind, J.S., and Wetzker, R. (1997) *Science* **275**, 394-397

43. Cheng, J., Weber, J.D., Baldassare, J.J., and Raben, D.M. (1997) *J. Biol. Chem.* **272**, 17312-17319

44. Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D., and Downward, J. (1994) *Nature* **370**, 527-532

45. Weber, J.D., Hu, W., Jefcoat, S.J., Jr., Raben, D.M., and Baldassare, J.J. (1997) *J. Biol. Chem.* **272**, 32966-32971
Figure 1. **PI 3-kinase is required for α-thrombin-stimulated DNA synthesis.** Growth-arrested IIC9 cells were pre-incubated in the absence or presence 0.1-20 µM LY294002, 30 minutes prior to incubation in the absence or presence of 1U/ml α-thrombin for 17 hours. Cells were then incubated for an additional 3 hours with 1µCi [3H]thymidine. Cells were washed, the DNA precipitated as described in “Experimental Procedures”. [3H]DNA was quantified by scintillation counting. The data indicate the mean ± SD of two independent experiments done in triplicate.

Figure 2. **α-Thrombin stimulated PI 3-kinase activity is sensitive to inhibition by wortmannin and LY294002.** A). Growth-arrested IIC9 cells were pre-incubated in the absence or presence of wortmannin at 1, 10, 50 and 100 nM, 30 minutes prior to incubation in the absence or presence of 1U/ml α-thrombin for 10 minutes. Cells were harvested by scraping into cold PI 3-kinase lysis buffer (see “Experimental Procedures”). PI 3-kinase complexes were immunoprecipitated from lysates containing equal protein using an anti-p85 polyclonal antibody and assayed for their ability to phosphorylate PI in vitro as described in “Experimental Procedures”. Total lipids were extracted and resolved by TLC. [32P]PI3P was quantitated using a Molecular Dynamics Phosphoimager™. B). Growth-arrested IIC9 cells were incubated in the absence or presence of 1U/ml α-thrombin for 0, 10, 20, 30, 45, or 60 minutes. Cells lysates were prepared in 1 X Laemmli buffer, separated by SDS-PAGE (9.75%) and immunoblotted with an anti-phospho-Akt or anti-Akt polyclonal antibody. C). Growth-arrested IIC9 cells were incubated in the absence or presence of 10 µM LY294002, 30 minutes prior to incubation in the absence or presence of 1U/ml α-thrombin for 30 minutes. Cell lysates were prepared and immunoblots performed as described above. Data are representative of at least two independent experiments.

Figure 3. **PI 3-kinase is required for α-thrombin-stimulated Cdk4 Activity.** Growth-arrested IIC9 cells were pre-incubated in the absence or presence of 10 µM LY294002, or 15 µM PD98059, 30 minutes prior to incubation in the absence or presence of 1U/ml α-thrombin for 10
hours. Cells were harvested by scraping into cold Rb lysis buffer (see “Experimental Procedures”). Cyclin D1/Cdk4 complexes were immunoprecipitated from lysates containing equal protein with a monoclonal cyclin D1 antibody and assayed for their ability to phosphorylate soluble GST-Rb fusion protein in vitro as described under “Experimental Procedures”. The data indicate the mean ± SD of three independent experiments.

Figure 4. **PI 3-kinase is required for α-thrombin-induced accumulation of cyclin D1.**  
A.) Growth-arrested IIC9 cells were incubated in the absence or presence of 1U/ml α-thrombin for 2, 4, 6, 8 and 10 hours. At the indicated times cells were harvested by scraping into cold lysis buffer. Lysates/proteins (10-15 µg) were separated by SDS-PAGE (12.75%) and immunoblotted with a polyclonal cyclin D1 antibody. B). Growth-arrested IIC9 cells were pre-incubated in the absence or presence of 10 µM LY294002, or 15 µM PD98059, 30 minutes prior to incubation in the absence or presence of 1U/ml α-thrombin for 4 and 6 hours. At the indicated times cells were harvested by scraping into cold lysis buffer. Lysates were analyzed for cyclin D1 as described above. Data are representative of three independent experiments.

Figure 5. **α-Thrombin-stimulated PI 3-kinase does not regulate cyclin D1 steady state message levels.** Growth-arrested IIC9 cells were pre-incubated in the absence or presence of 15 µM PD98059 or 10 µM LY294002, 30 minutes prior to incubation in the absence or presence of 1U/ml α-thrombin for 0, 4, 6 and 8 hours. At the indicated times, total RNA was isolated with Trizol Reagent (Gibco-BRL) according to the manufacturer’s protocol. RNA (15µg) was separated on a 2% agarose/formaldehyde gel and transferred to Hybond N+ nylon membrane. The membrane was probed simultaneously with randomly [γ-32P]dCTP-labeled cyclin D1 and GAPDH and washed as described in “Experimental Procedures”. Cyclin D1 and GAPDH transcripts were visualized using a Molecular Dynamics Phosphoimager™. Data are representative of two independent experiments.

Figure 6. **PI 3-kinase acts independent of the MAPK pathway.** A). Growth-arrested IIC9 cells were pre-incubated in the absence (I) or presence of 10 µM LY294002 ( ) or 15 µM PD98059 ( ).
prior to incubation in the absence or presence of 1U/ml α-thrombin for 5, 30, 120, 240 and 360 minutes. Cells were harvested by scraping into cold ERK lysis buffer (see “Experimental Procedures”). ERK1 complexes were immunoprecipitated from lysates containing equal protein and assayed for their ability to phosphorylate myelin basic protein in vitro as described under “Experimental Procedures”. ERK1 activity was quantitated using a Molecular Dynamics Phosphoimager™. Data are representative of three independent experiments. B). 32P-labeled IIC9 cells were growth-arrested and pre-incubated in the absence or presence of 10 µM LY294002, 30 minutes prior to incubation in the absence or presence of 1U/ml α-thrombin for 5 minutes. Cells were harvested by scraping into cold lysis buffer (see “Experimental Procedures”). Ras immune complexes were immunoprecipitated with a monoclonal Ras antibody and analyzed for 32P-labeled guanine nucleotides by TLC as described in “Experimental Procedures”. Resolved nucleotides were quantitated using a Molecular Dynamics Phosphoimager™ and Ras activity reported as the ratio of GTP/(GTP + GDP). The results are the mean ± SD of two independent experiments.

Figure 7. **PI 3-kinase and ERK activities are mediated by Ras.** IIC9 cells were transiently transfected with HA-RasN17 using Lipofectamine™ as described in “Experimental Procedures”. Transfected IIC9 cells were 32P-labeled and growth-arrested. In those samples treated with calphostin C, cells were pre-incubated with 10 µM calphostin C for 1 hour prior to incubation with 1U/ml α-thrombin for 5 minutes. A). HA-RasN17 was removed by immunoprecipitation with anti-HA antibody for 1 hour. Immediately after treatment with antibodies directed against HA, endogenous Ras immune complexes were immunoprecipitated by treatment with a monoclonal Ras antibody and analyzed for 32P-labeled guanine nucleotides by TLC as described in “Experimental Procedures”. Resolved nucleotides were quantitated using a Molecular Dynamics Phosphoimager™ and Ras activity reported as the ratio of GTP/(GTP + GDP). B). After removal of unreacted 32P by treatment with albumin-coated charcoal, the lysates containing equal protein were prepared in 1 X Laemmli buffer, separated by SDS-PAGE (9.75%) and immunoblotted with an anti-phospho-Akt antibody. C). ERK1 complexes were immunoprecipitated from lysates containing equal protein and assayed for their
ability to phosphorylate myelin basic protein in vitro as described under “Experimental Procedures”. ERK1 activity was quantitated using a Molecular Dynamics Phosphoimager™. Data are representative of three independent experiments.

Figure 8. **Expression of dominant negative Ras does not affect α-thrombin-induced PI hydrolysis.** IIC9 cells were transiently transfected with RasN17, growth-arrested and labeled with myo-[3H]inositol (1µCi/ml) in the presence of 20 mM LiCl. Cells were stimulated in the absence or presence of 1 U/ml α-thrombin for 15 minutes. IP3 levels were quantified as described in “Experimental Procedures”. The results are the mean ± SD of two independent experiments.
Figure 1
Figure 2B and 2C

B.

| Time (min) | P-Akt | Akt |
|-----------|-------|-----|
| 0         |       |     |
| 10        |       |     |
| 20        |       |     |
| 30        |       |     |
| 45        |       |     |
| 60        |       |     |

C.

| Condition   | P-Akt | Akt |
|-------------|-------|-----|
| α-Thrombin  | -     | +   |
| +           |       |     |
| LY294002    | -     | -   |
|             |       | +   |
Figure 5

![Image of a diagram showing a gel blot with bands labeled Cyclin D1 and GAPDH. The image also includes a table with conditions for various treatments: α-Thrombin, PD98059, and LY294002 at different time points (0, 4, 6, 8 hours). The table indicates the presence (+) or absence (-) of each condition.]
Figure 6B

GTP/(GTP + GDP) on p21 Ras (%)

| Variable       | -  | +  | +  |
|----------------|----|----|----|
| α-Thrombin     |    | 30 | +  |
| LY294002       |  10|    | +  |
Figure 7C

Erk Activity (% of Maximal)

|                | - | + | + | + |
|----------------|---|---|---|---|
| $\alpha$-Thrombin |   |   |   |   |
| RasN17         |   |   | + | + |
| Calphostin C   |   |   |   | + |
Figure 8

[\text{[^3H]inositolphosphates (cpm)}]

|          | $\alpha$-Thrombin | RasN17 |
|----------|-------------------|--------|
| $\text{--}$ | -                 | -      |
| $\text{--}$ | -                 | +      |
| $\text{+}$  | +                 | -      |
| $\text{+}$  | +                 | +      |
Phosphatidylinositol 3-Kinase Activity regulates α-Thrombin-stimulated G1 Progression by its Effect on Cyclin D1 Expression and CDK4 Activity
Polly J. Phillips-Mason, Daniel M. Raben and Joseph J. Baldassare

J. Biol. Chem. published online April 3, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909194199

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts