# Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in $G_1$ phase

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In Chinese hamster embryo fibroblasts (IIC9 cells), platelet-derived growth factor (PDGF) stimulated mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAP kinase/ERK) activity, but not that of c-jun N-terminal kinase (JNK), and induced $G_1$ phase progression. ERK1 activation was biphasic and was sustained throughout the $G_1$ phase of the cell cycle. PDGF induced cyclin D1 protein and mRNA levels in a time-dependent manner. Inhibition of PDGF-induced ERK1 activity by the addition of a selective inhibitor of MEK1 (MAP kinase kinase/ERK kinase 1) activation, PD98059, or transfection with a dominant-negative ERK1 (dnERK$^-$) was correlated with growth arrest. In contrast, growth was unaffected by expression of dominant-negative JNK (dnJNK$^-$). Interestingly, addition of PD98059 or dnERK$^-$, but not dnJNK$^-$, resulted in a dramatic decrease in cyclin D1 protein and mRNA levels, concomitant with a decrease in cyclin D1–cyclin-dependent kinase activity. To investigate the importance of sustained ERK1 activation, ERK1 activity was blocked by the addition of PD98059 throughout $G_1$. Addition of PD98059 up to 4 h after PDGF treatment decreased ERK1 activity to the levels found in growth-arrested IIC9 cells. Loss of cyclin D1 mRNA and protein expression was observed within 1 h after inhibition of the second sustained phase of ERK1 activity. Disruption of sustained ERK1 activity also resulted in $G_1$ growth arrest. These data provide evidence for a role for sustained ERK activity in controlling $G_1$ progression through positive regulation of the continued expression of cyclin D1, a protein known to positively regulate $G_1$ progression.

## INTRODUCTION

Cyclin D1 is most closely linked to cell-cycle progression through $G_1$ phase and the commitment of cells to enter S phase. In mammalian cells, cyclin D1 binds to and activates its catalytic partner, cyclin-dependent kinase 4 (CDK4) [1–8], which helps to drive cells through $G_1$, presumably through the phosphorylation of the retinoblastoma gene product (Rb) [2–4,6,9–16]. Expression of cyclin D1 during $G_1$ phase is critical for this event, but is not required for continuation through the rest of the cell cycle [2,3,17]. Matsushime et al. [18] demonstrated the dependence of cyclin D1 expression on the presence of a growth factor, colony-stimulating factor-1. Recent studies have focused on the ability of cyclin D1 to mediate $G_1$ progression following mitogenic stimuli [3,19]. Overexpression of cyclin D1 shortens $G_1$ phase, suggesting that cyclin D1 levels have a role in progression through $G_1$ [2,3,11,20,21]. However, overexpression of cyclin D1 alone is not sufficient to transform cells, but instead may act in concert with the effects of other proto-oncogenes. The proto-oncogene *ras* has been identified with the transformation of murine fibroblasts, the resulting elevation of cyclin D1 levels and the subsequent acceleration of $G_1$ progression [22,23]. The mechanism(s) responsible for the induction of cyclin D1 in response to mitogenic stimuli or its role in cellular transformation remain unclear.

Efforts have recently shifted towards the identification of the signalling pathways involved in the regulation of expression of $G_1$ cell-cycle proteins. Overexpression of Ras and mitogen-activated protein kinase (MAP kinase)/extracellular-signal-regulated kinase (ERK) has been shown recently to stimulate cyclin D1 transcription in mink and human cell lines [24]. These studies provide evidence for a role for mitogen-induced intracellular signalling pathways in the regulation of cell-cycle proteins. A recent study by Lavoie et al. [19] demonstrated a positive regulation of cyclin D1 induction by thrombin-induced ERK activation. However, this regulation was limited to the initial activation of ERK. In the present study, we have focused on the role of platelet-derived growth factor (PDGF)-induced sustained ERK activity in the continued expression of cyclin D1 during $G_1$.

Vouret-Craviari et al. [25] demonstrated that addition of thrombin to quiescent CCL39 cells induced the biphasic activation of ERK. In contrast, a thrombin-mimicking peptide (TMP) stimulated a robust but transient increase in ERK activity [25]. While thrombin is a potent mitogen for CCL39 cells, TMP does not stimulate the growth of these cells. The observation that addition of TMP does not elicit the growth of CCL39 cells is striking, in view of the fact that TMP acts on the same receptor as thrombin and generates similar intracellular second messengers. Vouret-Craviari et al. [25] postulated that the sustained activation of ERK is essential for $G_1$ progression, but data linking sustained ERK activity to the machinery of the cell cycle has been lacking. Since cyclin D1 is thought to be a $G_1$ growth factor sensor [1], we hypothesized that PDGF-induced sustained ERK activity may in fact be an integral component in the continued expression of cyclin D1. We investigated the

Abbreviations used: CDK, cyclin-dependent kinase; ERK, extracellular-signal-regulated kinase; dnERK$^-$, dominant-negative ERK; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; IL-1, interleukin-1; JNK, c-jun N-terminal kinase; dnJNK$^-$, dominant-negative JNK; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase/ERK kinase; PDGF, platelet-derived growth factor; Rb, retinoblastoma gene product; TMP, thrombin-mimicking peptide.

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importance of sustained ERK activation for growth and for the expression of steady-state mRNA and protein levels of the $G_1$ cyclin, cyclin D1. In the present paper we demonstrate that PDGF induces a rapid transient peak of ERK activity, followed by sustained activation for more than 5 h. Inhibition of ERK activation by pretreatment with a dominant-negative ERK ($dnERK^-$) or an inhibitor of MAP kinase kinase/ERK kinase 1 (MEK1) activation, PD98059, results in the loss of ERK activity and the subsequent inhibition of cyclin D1 expression. Although c-jun N-terminal kinase (JNK) is thought to be important in the mitogen-induced expression of cyclin D1 through the phosphorylation of c-jun, no study has demonstrated a definitive role for JNK in cyclin D1 expression. In the present study we show that JNK is not activated by PDGF, and that the presence of dominant-negative JNK ($dnJNK^-$) does not affect cyclin D1–CDK-associated activities. This is the first study to demonstrate the requirement of sustained ERK activity in the regulation of the continued expression of cyclin D1, a protein known to regulate mitogen-induced $G_1$ progression, and therefore provides new insights into the mechanism(s) involved in growth-factor-induced $G_1$ progression.

## MATERIALS AND METHODS

### Cells and cell culture

IIC9 cells, a subclone of Chinese hamster embryo fibroblasts [26], were grown and maintained in RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) containing 10 % (v/v) fetal calf serum and 2 mM L-glutamine (all chemicals from Sigma, St. Louis, MO, U.S.A., unless specified otherwise). Subconfluent (60–70 %) cultures were growth-arrested by washing twice with RPMI 1640 and incubating with RPMI 1640 containing 2 mM L-glutamine for 48 h. Human recombinant PDGF-BB (Calbiochem, La Jolla, CA, U.S.A.) was added to growth-arrested cells at 10 ng/ml in all experiments. Human recombinant interleukin-1$\beta$ (IL-1$\beta$) was added to growth-arrested cells at 2 ng/ml in all experiments. PD98059 (New England Biolabs, Beverly, MA, U.S.A.) was preincubated with growth-arrested cells for 30 min at 10 $\mu$M to allow for sufficient uptake before addition of mitogen. $dnERK^-$ (obtained from Dr. Jacques Pouyssegur, University of Nice, France) and $dnJNK^-$ (obtained from Dr. Roger Davis, University of Massachusetts, Worcester, MA, U.S.A.) were transfected (2 $\mu$g/ml) into IIC9 cells using LipofectAmine® Reagent (Gibco) as recommended by the manufacturer. Transient transfection of IIC9 cells resulted in $> 90$ % expression efficiency, as visualized by $\beta$-galactosidase staining.

### Western blots

PDGF (10 ng/ml) was added to growth-arrested IIC9 cells in the presence or absence of PD98059. Cells were then harvested at 0, 2, 4, 8 and 24 h after addition of PDGF by scraping in cold PBS. Harvested cells were pelleted at 10000 ***g*** for 5 min and then lysed and sonicated in 40 $\mu$l of solubilization buffer [25 mM Hepes, 300 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.1 % (v/v) Triton X-100, 20 mM $\beta$-glycerophosphate, 0.1 mM sodium vanadate, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin and 0.5 mM PMSF]. Protein concentrations of lysates were determined using a Bio-Rad (Hercules, CA, U.S.A.) Protein Assay, as recommended by manufacturer. Lysates/proteins (20 $\mu$g) were electrophoresed on SDS/12 %-polyacrylamide gels. Separated proteins were then transferred to PVDF membranes (Millipore, Boston, MA, U.S.A.). Membranes were probed with the respective primary antibodies against cyclin D1, CDK2 and CDK4 (Santa Cruz Biotechnology). Goat anti-rabbit IgG (heavy plus light chain)–horseradish peroxidase conjugate (Bio-Rad) was added as the secondary antibody, and specific protein bands were visualized using an ECL kit (Amersham, Arlington Heights, IL, U.S.A.), as recommended by the manufacturer.

### Northern blots

Total RNA was isolated from IIC9 cells cultured on 100 mm dishes with TRIZOL Reagent (Gibco) using the manufacturer's protocol. RNA (25 $\mu$g) was electrophoresed on 2 % (w/v) agarose/formaldehyde gels. Formaldehyde was removed by washing the gels in 0.5 M ammonium acetate. RNA was transferred on to Hybond $N^+$ nylon membranes (Amersham) using the Turboblotter® system (Schleicher & Schuell, Keene, NH, U.S.A.). RNA was cross-linked on to membranes using an Ultraviolet Crosslinker (Amersham) as recommended by the manufacturer. Transferred RNA was visualized using Methylene Blue/sodium acetate stain. Randomly [$\alpha$-$^{32}$P]dCTP-labelled cDNA probes (murine cDNAs for cyclin D1 and CDK4 were generously donated by Dr. Charles Sherr, St. Jude, Memphis, TN) were made using a Random Primed DNA Labeling Kit (Boehringer Mannheim). Blots were probed simultaneously with cyclin D1 or CDK4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for 1–2 h at 65 °C using Rapid-hyb buffer (Amersham), and washed once at room temperature with 5 × SSPE (20 mM EDTA, 1 M NaCl, 50 mM $NaH_2PO_4$,$H_2O$)/0.1 % (w/v) SDS and once at 65 °C with 1 × SSPE/0.1 % SDS. Membranes were then subjected to either autoradiography or direct quantification with a Phosphorimager® (Molecular Dynamics). More stringent washes were carried out at 65 °C with 0.1 % SDS when necessary.

### Thymidine incorporation

Growth-arrested IIC9 cells were stimulated with either serum (10 %, v/v) or PDGF (10 ng/ml) for approx. 20 h. Following the 20 h incubation, 1 $\mu$Ci/ml [$^3$H]thymidine (DuPont/NEN, Boston, MA, U.S.A.) was added and the cells were incubated for an additional 3 h. $^3$H-labelled cells were washed twice with cold PBS and the DNA was precipitated by incubating the cells for 30 min with cold 5 % (w/v) trichloroacetic acid. The trichloroacetic acid-precipitated DNA was washed twice with cold 5 % trichloroacetic acid and solubilized with 2 % (w/v) sodium bicarbonate/0.1 M NaOH. The solution was neutralized by the addition of 0.2 vol. of 5 % trichloroacetic acid, and the trichloroacetic acid-precipitated [$^3$H]DNA was quantified by scintillation counting.

### ERK assay

Growth-arrested IIC9 cells were stimulated with mitogen at 37 °C. In those experiments in which PD98059 was used, cells were preincubated for 30 min with PD98059 (10 $\mu$M) before the addition of mitogen. After 15 min the medium was removed and the cells were washed with PBS. After stimulation, the cells were lysed by scraping into 300 $\mu$l of solubilization buffer (20 mM Tris/HCl, pH 8, 1 mM sodium vanadate, 10 % glycerol, 1 mM PMSF, 2 mM EDTA, 0.1 % Triton X-100, 50 mM $\beta$-glycerophosphate, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin and 10 $\mu$g/ml pepstatin). The lysates were incubated at 4 °C for 30 min and then centrifuged for 5 min at 10000 ***g***. Anti-ERK1 antibody (2 $\mu$g) (Santa Cruz Biotechnology) was added to the lysate, followed by incubation with gentle rotation at 4 °C. After 5 h, Protein A–Sepharose was added, and the immune complexes were incubated overnight at 4 °C. ERK1 immune complexes were washed three times with fresh solubilization buffer,

three times with LiCl buffer (0.5 M LiCl, 100 mM Tris, pH 7.6), and finally with assay buffer [20 mM Tris/HCl, pH 7.6, 1.5 mM EGTA, 50 mM $\beta$-glycerophosphate, 1 mM dithiothreitol and 0.03% (w/v) Brij-35]. The ERK1 immune complexes were pelleted by centrifugation for 5 min at 10000 ***g***, and the pellets were resuspended in 30 $\mu$l of reaction buffer (20 mM Tris/HCl, pH 7.3, 10 mM $MgCl_2$, 50 $\mu$M ATP).

For the ERK assay, the immune complex was incubated for 45 min at 30 °C with 10 $\mu$g of myelin basic protein/sample and 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (Amersham). The reaction was stopped by the addition of 30 $\mu$l of 2 × Laemmli sample buffer. The samples were boiled for 5 min and subjected to SDS/PAGE. The gels were dried and subjected to autoradiography. It should be noted that all ERK assays were performed using either anti-ERK1 or anti-ERK2 antibodies for immunoprecipitation of ERK complexes. Results of assays using ERK1- or ERK2-immunoprecipitating antibodies were undistinguishable, and are therefore reported for ERK1 only.

### JNK assay

Growth-arrested IIC9 cells were washed twice with PBS and then lysed with ERK solubilization buffer (as described above). The lysates were incubated at 4 °C for 30 min, and insoluble material was pelleted by centrifugation at 10000 ***g*** for 10 min. The supernatant was collected and assayed for protein concentration (Bio-Rad Protein Assay), and then mixed with 10 $\mu$g of glutathione S-transferase (GST)–c-jun fusion protein complexed to glutathione–Sepharose beads and incubated overnight at 4 °C with gentle rotation. c-jun–JNK complexes were centrifuged at 10000 ***g*** for 5 min at 4 °C and the supernatant discarded. c-jun–JNK complexes were washed four times with HBIB buffer (20 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$ and 0.05% Triton X-100) and resuspended in 30 $\mu$l of kinase buffer (20 mM Hepes, 2 mM dithiothreitol, 20 mM $\beta$-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 20 mM $MgCl_2$, 0.1 mM sodium vanadate, 20 $\mu$M ATP and 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP/sample). Samples were incubated at 30 °C with agitation. After 20 min the complexes were pelleted by centrifugation at 10000 ***g***, the supernatants discarded and the pellets washed twice with HBIB buffer. Pellets were resuspended in 2 × Laemmli buffer, boiled for 3 min and centrifuged at 10000 ***g***. The resulting supernatants were electrophoresed on SDS/10%-polyacrylamide gels. The gels were dried and subjected to autoradiography or phosphorimaging for quantitative analysis.

### Cyclin D1–CDK assay

Cyclin D1–CDK activity was measured as described previously [1], with modifications. Briefly, growth-arrested IIC9 cells were washed twice with PBS and lysed in 50 $\mu$l of IP buffer [50 mM Hepes, 150 mM NaCl, 0.1 mM sodium vanadate, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 10 mM $\beta$-glycerophosphate, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, 1 mM PMSF, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin and 10 $\mu$g/ml pepstatin], and sonicated briefly. Insoluble material was pelleted by centrifugation at 10000 ***g*** for 10 min. Cyclin D1 or CDK4 complexes were immunoprecipitated and washed. Cyclin D1 or CDK4 immune complexes were then pelleted by centrifugation at 10000 ***g*** for 5 min and resuspended in 30 $\mu$l of reaction buffer (50 mM Hepes, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM $\beta$-glycerophosphate, 0.1 mM sodium vanadate and 20 $\mu$M ATP). The immune complexes were incubated with 2 $\mu$g/ml soluble GST–Rb fusion protein (Rb sequence encoding amino acids 379–928 inserted into the pGEX-2T plasmid was generously donated by Dr. Mark Ewen, Dana-Farber Cancer Institute, Boston, MA) and 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP. The reaction was stopped by the addition of 15 $\mu$l of 2 × Laemmli sample buffer. Samples were boiled for 5 min and subjected to SDS/PAGE. Gels were dried and subjected to autoradiography.

## RESULTS

### PDGF induces the sustained activation of ERK in IIC9 cells

Previous studies have shown that constitutively active MEK1 results in the transformation of several cell types. Other studies have demonstrated the activation of MEK1 and ERK1 by PDGF [27,28]. It has been postulated recently that both the ERK and JNK pathways play a key role in up-regulating initial cyclin D1 transcription [24]. To characterize the signalling pathways involved in the PDGF-stimulated growth of IIC9 cells, we measured the activities of ERK and JNK. Addition of PDGF to growth-arrested IIC9 cells stimulated a biphasic increase in ERK1 activity (Figure 1). ERK1 activation was maximal (10–16-fold) by 15–30 min after stimulation with PDGF (Table 1), and persisted at lower levels for approx. 15 h (Figure 1). However, PDGF failed to activate JNK (results not shown), indicating

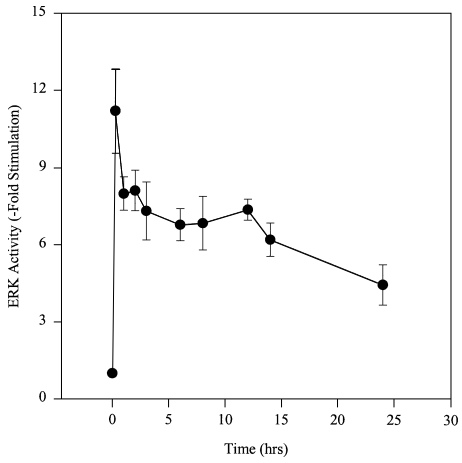


**Figure 1 PDGF induces sustained activation of ERK**

Growth-arrested IIC9 cells were harvested at 0, 0.25, 1, 2, 4, 6, 8, 12, 14 and 24 h after stimulation with PDGF (10 ng/ml) by scraping them into cold lysis buffer. ERK1 complexes were immunoprecipitated and assayed for their ability to phosphorylate myelin basic protein, as described in the Materials and methods section. Similar results were obtained in four separate experiments.

**Table 1 Effects of PD98059 and dnERK$^-$ on ERK1 activity**

Growth-arrested IIC9 cells were incubated in the absence or presence of 10 $\mu$M PD98059 or transfected with dnERK$^-$ and then stimulated for 15 min with PDGF (10 ng/ml). Cells were solubilized and lysates (150 $\mu$g) were assayed for ERK1 activity, based on their ability to phosphorylate soluble myelin basic protein, as described in the Materials and methods section. Results are means ± S.D. from four independent experiments. Significance of differences from control PDGF response: $^*P < 0.05$.

| | Increase in ERK1 activity (fold) | | |
|---|---|---|---|
| Stimulus | Control | + PD98059 | + dnERK$^-$ |
| None | 1.0 | 1.7 ± 0.5 | 2.2 ± 0.8 |
| PDGF | 16.4 ± 3.3 | 0.9 ± 0.7* | 1.2 ± 0.7* |

**Table 2 PDGF-induced growth requires ERK1 activity in IIC9 cells**

Growth-arrested IIC9 cells were incubated in the absence or presence of PD98059 (10 $\mu$M) or transfected with dnERK$^-$ and then stimulated with 10% (v/v) serum or 10 ng/ml PDGF for 20 h. Cells were then incubated for an additional 3 h with 1 $\mu$Ci of [$^3$H]thymidine. $^3$H-labelled cells were washed with PBS, and DNA was precipitated and washed as described in the Materials and methods section. [$^3$H]DNA was quantified in triplicate samples by scintillation counting, and values are reported as means $\pm$ S.D. from five independent experiments. Significance of differences from control PDGF response: $^*P < 0.01$. nd, not determined.

| | [$^3$H]Thymidine incorporation (c.p.m.) | | |
|---|---|---|---|
| Stimulus | Control | + PD98059 | + dnERK$^-$ |
| None | 3105 $\pm$ 619 | nd | nd |
| Serum | 33651 $\pm$ 3092 | 18155 $\pm$ 4688* | nd |
| PDGF | 21806 $\pm$ 4116 | 3090 $\pm$ 650* | 5320 $\pm$ 1108* |

that, in IIC9 cells, PDGF, which is a potent mitogen (Table 2), does not activate the JNK pathway. In contrast with PDGF, both serum and IL-1 stimulated significant increases in JNK activity (results not shown), demonstrating that known activators of the JNK pathway can stimulate JNK activity in IIC9 cells.

### PD98059 and dnERK$^-$ inhibit initial ERK1 activity

Alessi et al. [29] have recently characterized a novel selective inhibitor of MEK1 activation, PD98059, that significantly inhibits PDGF-induced MEK1 and ERK1 activity, but not JNK or cyclin–CDK activity, in Swiss 3T3 cells [29]. Pretreatment of IIC9 cells with 10 $\mu$M PD98059 decreased PDGF-stimulated ERK1 activity to below the levels in resting cells (Table 1). IIC9 cells transfected with dnERK$^-$ also displayed significantly reduced PDGF-stimulated ERK1 (Table 1) and ERK2 (results not shown) activity. As expected, PD98059 did not affect IL-1- or serum-stimulated JNK activity (results not shown).

### PDGF-induced growth requires initial ERK activation

PDGF is a potent growth factor in several cell types, and is capable of stimulating cell-cycle progression in quiescent cells. To establish that PDGF is a potent mitogen in IIC9 cells, we compared the mitogenic potency of PDGF with that of serum. Addition of serum to quiescent IIC9 cells resulted in a 10-fold increase in [$^3$H]thymidine incorporation compared with control cells (Table 2). Maximal levels of [$^3$H]thymidine incorporation with PDGF were 60–70 % of the levels seen with serum (Table 2). Epidermal growth factor and thrombin also stimulated DNA synthesis (results not shown), with maximal levels of approx. 60–70 % of that observed with PDGF. These data demonstrate that PDGF is a potent mitogen for IIC9 cells. To demonstrate the effects of PD98059 and dnERK$^-$ on IIC9 cell growth in response to PDGF, PDGF-induced [$^3$H]thymidine incorporation and cell number were examined. Pretreatment of IIC9 cells with PD98059 or transfection with dnERK$^-$ markedly inhibited PDGF-induced DNA synthesis (Table 2) and proliferation (results not shown). However, PD98059 inhibited serum-induced DNA synthesis by only 45–50 % (Table 2). These data indicate that inhibition of PDGF-stimulated ERK1 activation blocks the PDGF-dependent re-entry of IIC9 cells into the cell cycle.

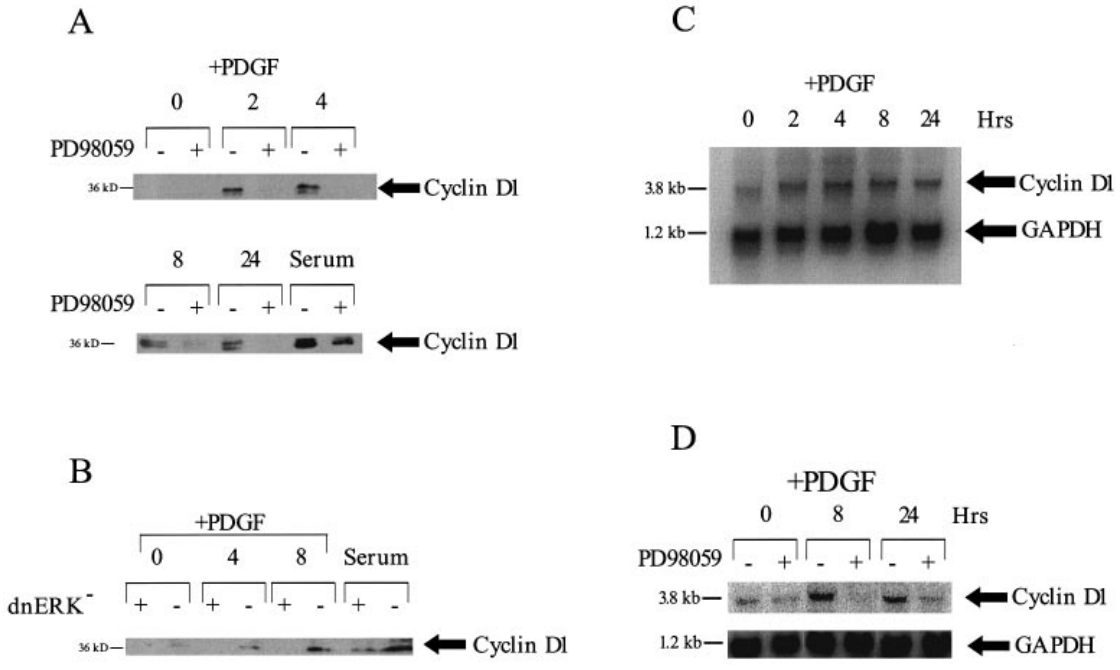


**Figure 2 PDGF-induced cyclin D1 expression requires ERK activation**

Growth-arrested IIC9 cells preincubated for 30 min in the absence (−) or presence (+) of 10 $\mu$M PD98059 (**A**), or transfected (+) or not (−) with dnERK$^-$ (**B**), were harvested at 0, 2, 4, 8 and 24 h (**A**) or 0, 4 and 8 h (**B**) after the addition of PDGF (10 ng/ml), or 24 h after the addition of serum (10%, v/v) by scraping them into cold PBS, and lysed. Lysates/proteins (15 $\mu$g) were electrophoresed on SDS/10%-polyacrylamide gels and immunoblotted with a polyclonal antibody against cyclin D1. In another experiment, growth-arrested IIC9 cells were isolated and lysed at 0, 2, 4, 8 and 24 h after the addition of PDGF (**C**), or in the absence (−) or presence (+) of 10 $\mu$M PD98059 (**D**) using Trizol Reagent. RNA (25 $\mu$g) was electrophoresed on 2% agarose/formaldehyde gels and transferred to Hybond N$^+$ nylon membranes. Membranes were probed simultaneously with randomly [$\alpha$-$^{32}$P]dCTP-labelled murine cyclin D1 cDNA and murine GAPDH (to ensure equal RNA loading). Similar results were obtained in three (**B**, **D**) or four (**A**, **C**) separate experiments. Abbreviation: kD, kDa.

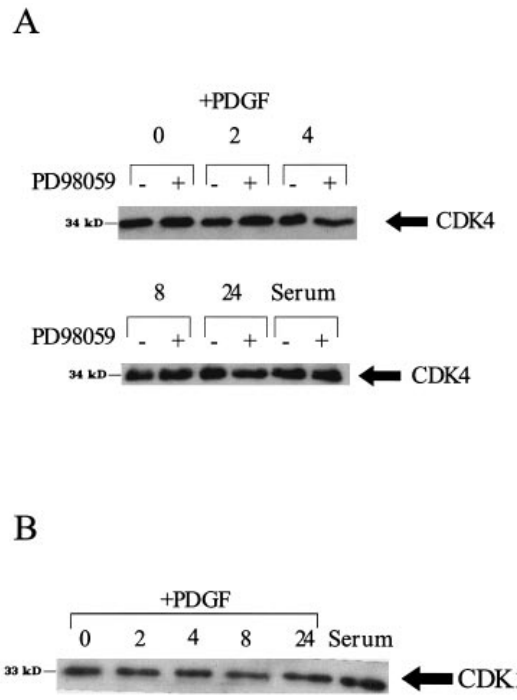


**Figure 3 PDGF and PD98059 do not induce changes in cyclin D1-associated CDKs**

Growth-arrested IIC9 cells preincubated in the absence (−) or presence (+) of 10 $\mu$M PD98059 were harvested at 0, 2, 4, 8 and 24 h after the addition of PDGF (10 ng/ml), or 24 h after the addition of serum, by scraping them into cold PBS, and then lysed. Lysates/proteins (15 $\mu$g) were electrophoresed on SDS/10%-polyacrylamide gels and immunoblotted with a polyclonal anti-CDK4 (**A**) or anti-CDK2 (**B**) antibody. Similar results were obtained in three separate experiments. Abbreviation: kD, kDa.

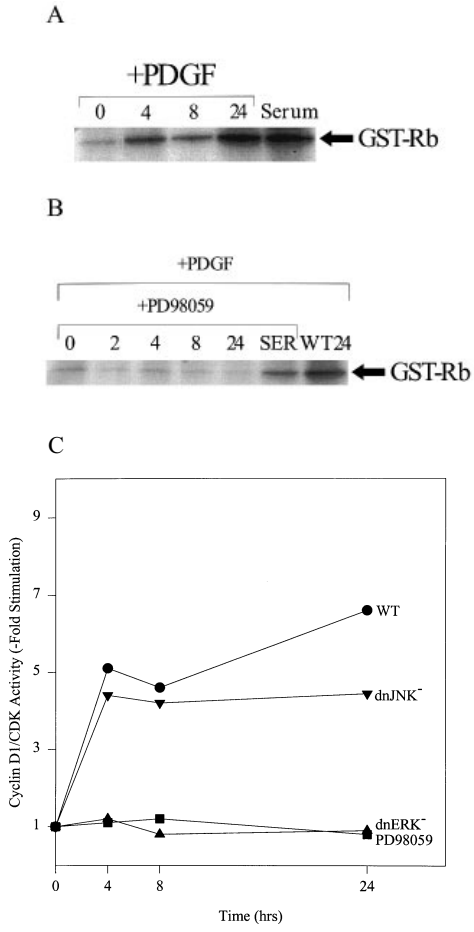


**Figure 4 ERK1, but not JNK, activity is required for PDGF-induced cyclin D1–CDK activity**

Growth-arrested IIC9 cells preincubated in the absence (**A**) or presence (**B**) of 10 $\mu$M PD98059, or transfected with dnERK⁻ or dnJNK⁻ (**C**), were harvested at 0, 4, 8 and 24 h after the addition of PDGF (10 ng/ml), or 24 h after the addition of serum, by scraping them into cold PBS, and then lysed in 50 $\mu$l of IP buffer (see theMaterials and methods section). Cyclin D1 complexes were immunoprecipitated and their activity was assayed based on their ability to phosphorylate GST–Rb, as described in the Materials and methods section, In (**B**), SER indicates the presence of serum and WT24 indicates IIC9 cells stimulated with PDGF in the absence of PD98059 for 24 h. Similar results were obtained in three separate experiments. In (**C**), WT is wild-type (non-transfected).

### Initial up-regulation of cyclin D1 expression by PDGF requires ERK activation

To correlate PDGF-stimulated growth with cell-cycle progression, we measured the levels of several cell-cycle proteins. Cyclin D1 has been shown to be a necessary component for progression through $G_1$ phase and for the commitment of cells to replicate their DNA [2,3]. Its expression is induced early in $G_1$ in response to growth factor stimulation [2,3,19], and its over-expression is responsible for the shortening of $G_1$ phase [11,18,20,21]. These findings made cyclin D1 an ideal candidate for a PDGF-induced gene in IIC9 cells.

Low levels of cyclin D1 were observed in growth-arrested IIC9 cells (Figure 2A). Upon addition of PDGF, cyclin D1 protein levels increased 5-fold within 4 h (Figure 2A). The induction of cyclin D1 was maximal (6.5-fold) by 8 h and remained elevated for 24 h. Treatment of IIC9 cells with serum increased cyclin D1 levels 10-fold by 24 h (Figure 2A), but IL-1 was unable to stimulate an increase in cyclin D1 protein expression (results not shown). Northern blot analysis showed a time-dependent increase in the steady-state level of cyclin D1 mRNA that correlated well with the time course for cyclin D1 protein up-regulation. Cyclin D1 mRNA reached a maximal 5-fold increase by 4 h, and remained at approx. 3–4-fold above quiescent levels for 24 h (Figure 2C). In contrast with cyclin D1, the protein levels of CDK4 (Figure 3A) and CDK2 (Figure 3B), which associate independently with D cyclins to form active cyclin–CDK complexes [1–6], were not affected by the addition of PDGF.

Although recent data have suggested the presence of an ERK-responsive region on the cyclin D1 promoter, there has been a lack of evidence linking mitogen-induced ERK activity to the control of cell-cycle protein expression. Our results demonstrate a requirement for ERK1 activity for the up-regulation of cyclin D1 protein and mRNA levels following PDGF stimulation by two methods: the use of a selective inhibitor of MEK1 activation and of dnERK⁻. PD98059 and dnERK⁻ did not significantly inhibit basal cyclin D1 protein (Figures 2A and 2B) or mRNA (Figure 2D) expression in growth-arrested IIC9 cells, as expected. Treatment of IIC9 cells with PD98059 or dnERK⁻ markedly reduced and completely blocked cyclin D1 protein levels by 2 h (Figure 2A) and 4–24 h (Figures 2A and 2B) respectively after PDGF addition. PDGF-induced increases in steady-state cyclin D1 mRNA levels were inhibited similarly by pretreatment of IIC9 cells with PD98059 (Figure 2D). The inhibition of PDGF-

**Table 3 PD98059 inhibits sustained ERK1 activity**

Growth-arrested IIC9 cells were stimulated with 10 ng/ml PDGF and treated with PD98059 (10 $\mu$M) at 0.5, 1, 2 and 3 h after stimulation. Cells were harvested 30 min after treatment with PD98059 by scraping them into cold lysis buffer. ERK1 complexes were immunoprecipitated and assayed for their activity, as described in the Materials and methods section. Values represent means $\pm$ S.D. from three independent experiments. Significance of differences from control PDGF response: $^{*}P < 0.05$; $^{**}P < 0.01$.

| | Increase in ERK1 activity (fold) | |
|---|---|---|
| Time (h) | PDGF | PDGF + PD98059 |
| 0.5 | 13.6 $\pm$ 1.4 | 1.6 $\pm$ 0.7** |
| 1 | 8.1 $\pm$ 1.1 | 2.2 $\pm$ 1.6* |
| 2 | 7.8 $\pm$ 1.9 | 1.7 $\pm$ 0.9* |
| 3 | 7.4 $\pm$ 0.2 | 1.0 $\pm$ 0.5** |

**Table 4 Inhibition of sustained ERK1 activity results in $G_1$ growth arrest**

Growth-arrested IIC9 cells were stimulated with PDGF (10 ng/ml) and treated with PD98059 (10 $\mu$M) at 0, 0.5, 1, 4, 16 and 20 h after stimulation. Following a 21 h incubation, the cells were incubated for an additional 3 h with 1 $\mu$Ci of [$^3$H]thymidine. $^3$H-labelled cells were washed with PBS, and DNA was precipitated and washed as described in the Materials and methods section. [$^3$H]DNA was quantified in triplicate samples by scintillation counting, and values are means $\pm$ S.D. from four independent experiments. Maximal [$^3$H]thymidine incorporation was achieved in the presence of 10 ng/ml PDGF. Significance of differences from PDGF response (100%): $^{*}P < 0.01$.

| Time (h) | [$^3$H]Thymidine incorporation (% of maximal) |
|---|---|
| 0 | 20 $\pm$ 2.3* |
| 0.5 | 21 $\pm$ 1.1* |
| 1 | 27 $\pm$ 1.8* |
| 4 | 24 $\pm$ 2.5* |
| 16 | 91 $\pm$ 3.8 |
| 20 | 98 $\pm$ 3.3 |

induced mRNA expression ranged from 75 to 85%. In contrast with cyclin D1, CDK4 protein levels (Figure 3A) and mRNA levels (results not shown) were unchanged in the presence of PD98059. These results suggest that, in IIC9 cells, initial ERK1 activity is required for the increased expression of cyclin D1, a protein known to regulate $G_1$ progression, but not by the CDKs associated with cyclin D1.

### ERK1, but not JNK, activity is required for PDGF-induced cyclin D1–CDK activity

To further demonstrate the correlation between ERK1 activity, cyclin D1 regulation and $G_1$ progression, we examined the activity of cyclin D1–CDK complexes in the presence or absence of ERK1 activity. Growth-arrested IIC9 cells displayed minimal cyclin D1-immunoprecipitatable cyclin D1–CDK activity, whereas addition of PDGF induced a 5-fold increase in activity by 4 h (Figures 4A and 4C). Cyclin D1–CDK activity increased to a maximum level (6.7-fold) by 24 h after addition of PDGF or serum (Figures 4A and 4C). Pretreatment of IIC9 cells with PD98059 completely inhibited PDGF-induced cyclin D1–CDK activity by 2 h and sustained basal activity for 24 h (Figures 4B and 4C). PDGF-induced cyclin D1–CDK activity was also completely inhibited by dnERK1$^-$ for 24 h (Figure 4C). Serum-induced cyclin D1–CDK activity was inhibited 60–70% by PD98059 (Figure 4B). However, PDGF-induced cyclin D1–CDK activity was not inhibited by dnJNK$^-$ (Figure 4C). Cyclin D1–CDK activity was increased 4-fold by 4 h after addition of PDGF, and remained at maximal levels for 24 h (Figure 4C). Similar results were obtained using CDK4-immunoprecipitated cyclin–CDK4 activities (results not shown).

### Sustained activation of ERK is required for cell growth and the continued $G_1$ expression of cyclin D1

PD98059 was added at various times (0.5, 1, 2 and 4 h after PDGF addition) after the initial PDGF-induced peak increase in ERK activity. These times were chosen because we have found that IIC9 cells require the continued presence of mitogen for at least 6 h to progress into S phase (results not shown). Addition of PD98059 to PDGF-treated IIC9 cells resulted in a significant decrease in PDGF-induced ERK activity within 30 min (Table 3). Treatment of PDGF-stimulated IIC9 cells with PD98059 (0.5–6 h after PDGF), therefore, did not block the initial burst of ERK activity, only its sustained activation.

The inhibition of sustained ERK activation occurring after stimulation with PDGF also resulted in an inhibition of growth. Growth-arrested IIC9 cells stimulated with PDGF entered S phase within 20 h. When sustained ERK activity was inhibited by addition of PD98059 (0.5, 1 or 4 h after PDGF), cells arrested (Table 4). However, ERK activity was not required for PDGF-induced growth once cells had neared or entered S phase (16–20 h) (Table 4).

Since we and others have shown that inhibition of initial ERK activation blocks cyclin D1 expression, we hypothesized that the sustained activation of ERK1 in $G_1$ phase may be critical for the continued expression of cyclin D1. We next examined whether inhibition of sustained activation blocked the continued expression of cyclin D1 mRNA and protein. Addition of PD98059 (1, 2, 4 or 6 h after PDGF) resulted in a significant decrease (5–7-fold) in cyclin D1 protein (Figure 5A) and mRNA (Figure 5B) expression by 10 h after the initial stimulation with PDGF. The continued expression of cyclin D1 protein and mRNA consistently was decreased 5–7-fold after the inhibition of sustained ERK activity when compared with uninhibited cells, demonstrating a requirement for sustained ERK1 activation throughout $G_1$ phase for the positive regulation of the $G_1$ cyclin, cyclin D1.

## DISCUSSION

Until recently, it was unclear how growth factors might influence the progression of cells through the cell cycle. Recent observations have provided evidence for a requirement of the continued presence of growth factors for the entry of quiescent cells into $G_1$ phase and their accelerated progression through the $G_1$/S boundary [2,3,18]. The focus has now shifted to the proteins thought to be responsible for $G_1$ progression, mainly the CDKs and the D and E cyclins that regulate their activity. Several human tumour cell lines express high levels of cyclin D1, leading to a correlation between transformation and deregulation of cyclin D1 expression. Understanding the mechanisms that control the expression and activity of this $G_1$-associated protein and its catalytic partners is an important element in our understanding of the overall mechanism of mitogen-induced progression through $G_1$.

Here we demonstrate that levels of CDK2 and CDK4 are unaffected by the PDGF-induced entry of quiescent IIC9 cells

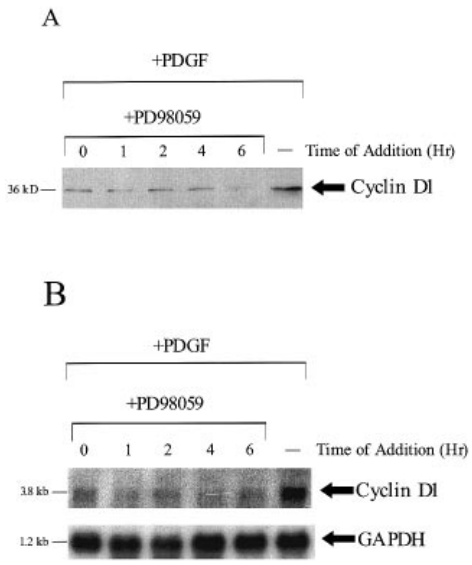


**Figure 5 Continued expression of cyclin D1 requires sustained ERK activation**

Growth-arrested IIC9 cells were stimulated with PDGF (10 ng/ml) and treated with 10 $\mu$M PD98059 at 0, 1, 2, 4 and 6 h after stimulation. (**A**) Cells were harvested 10 h after stimulation with PDGF by scraping them into cold lysis buffer. Lysates/proteins (15 $\mu$g) were electrophoresed on SDS/10%-polyacrylamide gels and immunoblotted with a polyclonal antibody against cyclin D1. Abbreviation: kD, kDa. (**B**) Cells were harvested using Trizol Reagent. RNA (20 $\mu$g) was electrophoresed on 2% agarose/formaldehyde gels and transferred to Hybond $N^+$ nylon membranes. Membranes were probed with randomly [$\alpha$-$^{32}$P]dCTP-labelled murine cyclin D1 cDNA. Membranes were then stripped and re-probed with randomly [$\alpha$-$^{32}$P]dCTP-labelled murine GAPDH to ensure equal RNA loading. Similar results were seen in three separate experiments.

into $G_1$ and their progression into S phase (Figures 3A and 3B). In contrast, addition of PDGF to quiescent IIC9 cells resulted in significant increases in cyclin D1 protein and mRNA expression (Figures 2A and 2C). While some reports have suggested that fluctuations in the levels of $G_1$ CDKs may be responsible for the control of $G_1$ progression [4,9], IIC9 cells did not display such regulation. Fluctuations in the levels of these proteins, therefore, seem to be cell-type- and stimulus-dependent. Our results indicate that, in IIC9 cells, PDGF induces increased levels of cyclin D1, and suggest that changes in the expression of cyclin D1 regulate entry into the cell cycle and progression to S phase through the time-dependent phosphorylation of Rb by active cyclin D1–CDK complexes (Figures 4A and 4C).

There has been a lack of understanding as to how growth factors and their signalling cascades regulate the machinery of the cell cycle. Here we show that PDGF stimulates the activation of ERK1 (Figure 1, Table 1), but not of JNK (results not shown). Addition of a MEK1 inhibitor, PD98059, which blocked the PDGF-induced increases in ERK1 activity at concentrations similar to those used by Alessi et al. [29], inhibited the progression of IIC9 cells into S phase, as determined by [$^3$H]thymidine incorporation (Table 2). It is clear from these data that ERK1 is essential for the mitogenic action of PDGF. Inhibition of initial ERK1 activity by PD98059 or dnERK$^-$ resulted in the elimination of cyclin D1 expression at the protein level (Figures 2A and 2B), and a dramatic down-regulation of mRNA levels (Figure 2D). Concomitant with the inhibition of ERK1 activity and cyclin D1 expression, cyclin D1–CDK activity was completely inhibited (Figures 4B and 4C). However, inhibition of JNK activity by dnJNK$^-$ did not affect PDGF-induced cyclin D1–CDK activity (Figure 4C), suggesting that the JNK pathway does not play a role in PDGF-induced cyclin D1–CDK activity in IIC9 cells. The mechanism of the decrease in cyclin D1 mRNA, however, is unclear. Our data show that the levels of $G_1$ CDKs are not affected by PDGF stimulation, and that these CDKs do not require the presence of ERK activity for their expression throughout the cell cycle (Figures 3A and 3B).

We and others have shown that mitogen-induced ERK activity is required for the initial expression of cyclin D1 in response to mitogen, and that some mitogens (thrombin and PDGF) stimulate sustained ERK activity (Figure 1) [19,25]. Until now, a precise role for the sustained activation of ERK has remained unknown. Here we have shown that sustained ERK activity is required for the continued expression of cyclin D1 in $G_1$. Inhibition of PDGF-stimulated ERK activity throughout $G_1$ by the addition of PD98059 resulted in a significant decrease in cyclin D1 protein and mRNA expression (Figures 5A and 5B) and subsequent growth arrest (Table 4). Although the mechanism behind the loss of cyclin D1 protein and mRNA expression is unclear, it is consistent with other studies showing the rapid degradation of cyclin D1 protein upon removal of mitogen [18]. The inhibition of sustained activity may result in the loss of cyclin D1 transcription and the consequential rapid degradation of accumulated cyclin D1 protein. The influence of sustained ERK activity on growth was not seen after cells had neared or entered S phase (Table 4), suggesting that the role of such sustained activity may be limited to the regulation of events in $G_1$.

We have provided evidence for the novel regulation of PDGF-induced $G_1$ progression through the control of cyclin D1 expression. We have demonstrated a need for ERK1 (but not JNK) activity in the initial up-regulation of cyclin D1 and subsequent cell growth. We have shown that ERK1 (but not JNK) activity is required for PDGF-induced cyclin D1–CDK activity, providing a necessary link between mitogen-induced signalling events and cell-cycle events leading to mitogen-induced progression through $G_1$. How ERK1 exerts its control over cyclin D1 is unclear as yet, although preliminary results from our laboratory indicate that the activation, but not induction, of immediate-early genes by ERK1 is important for cyclin D1 up-regulation (J. D. Weber and J. J. Baldassare, unpublished work).

This is the first study to suggest a role for the sustained activation of ERK. Our data show that the continued $G_1$ expression of cyclin D1 in the presence of mitogen requires the sustained activation of ERK1. Although we have shown that the sustained activation of ERK1 is required for $G_1$ progression, preliminary results from our laboratory indicate that it is not sufficient and that $G_1$-mediated events (i.e. $p27^{KIP1}$ degradation) are regulated by the combined activities of ras-dependent kinases (J. D. Weber and J. J. Baldassare, unpublished work). Therefore other upstream signalling molecules remain to be linked to the control of the cell cycle. Our data demonstrate a correlation between the sustained activation of ERK1, which is required for growth, and the continued expression of cyclin D1, a mitogen-induced $G_1$ protein known to be an integral component in the regulation of $G_1$ progression. This provides a physiological link between sustained activation of the ERK signalling pathway and the regulation of essential components of cell-cycle progression through $G_1$.

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## REFERENCES


1 Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J. and Kato, J.-Y. (1994) Mol. Cell. Biol. **14**, 2066–2076
2 Sherr, C. J. (1994) Cell **79**, 551–555
3 Sherr, C. J. (1995) Trends Biochem. Sci. **20**, 187–190
4 Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C. and Peters, G. (1994) Oncogene **9**, 71–79
5 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
6 Sherr, C. J. and Roberts, J. M. (1995) Genes Dev. **9**, 1149–1163
7 Xiong, Y., Shang, H. and Beach, D. (1992) Cell **71**, 505–514
8 Lukas, J., Bartkova, J., Welcker, M., Peterson, O. W., Peters, G., Strauss, M. and Bartek, J. (1995) Oncogene **10**, 2125–2134
9 Grana, X. and Reddy, E. P. (1995) Oncogene **11**, 211–219
10 Dowdy, S. F., Hinds, P. W., Louie, K., Reed, S. I., Arnold, A. and Weinberg, R. A. (1993) Cell **73**, 499–511
11 Resnitzky, D., Gossen, M., Bujard, H. and Reed, S. I. (1994) Mol. Cell. Biol. **14**, 1669–1679
12 Kato, J.-Y., Matsushime, H., Hiebert, S. W., Ewen, M. E. and Sherr, C. J. (1993) Genes Dev. **7**, 331–342
13 Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J.-Y. and Livingston, D. M. (1993) Cell **73**, 487–497
14 Hatakeyama, M., Brill, J. A., Fink, G. R. and Weinberg, R. A. (1994) Genes Dev. **8**, 1759–1771
15 Resnitzky, D. and Reed, S. I. (1995) Mol. Cell. Biol. **15**, 3463–3469
16 Horton, L. E., Qian, Y. and Templeton, D. J. (1995) Cell Growth Differ. **6**, 395–407
17 Baldin, V., Likas, J., Marcote, M. J., Pagano, M., Bartek, J. and Draetta, G. (1993) Genes Dev. **7**, 812–821
18 Matsushime, H., Roussel, M. F., Ashmun, R. A. and Sherr, C. J. (1991) Cell **65**, 701–713
19 Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R. and Pouyssegur, J. (1996) J. Biol. Chem. **271**, 20608–20616
20 Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J.-Y., Bar-Sagi, D., Roussel, M. F. and Sherr, C. J. (1993) Genes Dev. **7**, 1559–1571
21 Jiang, W., Kahn, S. M., Zhou, P., Zhang, Y., Cacace, A. M., Infante, A. S., Doi, S., Santella, R. M. and Weinstein, I. B. (1993) Oncogene **8**, 3447–3457
22 Liu, J., Chao, J., Jiang, M., Yung, S., Yen, J. J. and Yang-Yen, H. (1995) Mol. Cell. Biol. **15**, 3654–3663
23 Winston, J. T., Coats, S. R., Wang, Y.-Z. and Pledger, W. J. (1996) Oncogene **12**, 127–134
24 Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. and Pestell, R. G. (1995) J. Biol. Chem. **270**, 23589–23597
25 Vouret-Craviari, V., Van Obberghen-Schilling, E., Scimeca, J. C., Van Obberghen, E. and Pouyssegur, J. (1993) Biochem. J. **289**, 209–214
26 Low, D. A., Scott, R. W., Baker, J. B. and Cunningham, D. D. (1982) Nature (London) **298**, 476–478
27 Reuter, C. W. M., Catling, A. D., Jelinik, T. and Weber, M. J. (1995) J. Biol. Chem. **270**, 7644–7655
28 Graves, L. M., Bornfeld, K. E., Raines, E. W., Potts, B. C., MacDonald, S. G., Ross, R. and Krebs, E. G. (1993) Proc. Natl. Acad. Sci. U.S.A. **91**, 6030–6034
29 Alessi, D., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) J. Biol. Chem. **270**, 27489–27494


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