Ras-stimulated Extracellular Signal-related Kinase 1 and RhoA Activities Coordinate Platelet-derived Growth Factor-induced G1 Progression through the Independent Regulation of Cyclin D1 and p27\(^{\text{KIP1}}\)*

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Platelet-derived growth factor (PDGF)-induced Ras activation is required for G1 progression in Chinese hamster embryo fibroblasts (IIC9 cells). Ras stimulates both extracellular signal-related kinase (ERK) activation and RhoA activation in response to PDGF stimulation. Inhibition of either of these Ras-stimulated pathways results in growth arrest. We have shown previously that Ras-stimulated ERK activation is essential for the induction and continued G1 expression of cyclin D1. In this study we examine the role of Ras-induced RhoA activity in G1 progression. Unstimulated IIC9 cells expressed high levels of the G1 cyclin-dependent kinase inhibitor p27\(^{\text{KIP1}}\). Stimulation with PDGF resulted in a dramatic decrease in p27\(^{\text{KIP1}}\) protein expression. This decrease was attributed to increased p27\(^{\text{KIP1}}\) protein degradation. Overexpression of dominant-negative forms of Ras or RhoA completely blocked PDGF-induced p27\(^{\text{KIP1}}\) degradation, but only dominant-negative Ras inhibited cyclin D1 protein expression. C3 transferase also inhibited PDGF-induced p27\(^{\text{KIP1}}\) degradation, thus further implicating RhoA in p27\(^{\text{KIP1}}\) regulation. Overexpression of dominant-negative ERK resulted in inhibition of PDGF-induced cyclin D1 expression but had no effect on PDGF-induced p27\(^{\text{KIP1}}\) degradation. These data suggest that Ras coordinates the independent regulation of cyclin D1 and p27\(^{\text{KIP1}}\) expression by the respective activation of ERK and RhoA and that these pathways converge to determine the activation state of complexes of cyclin D1 and cyclin-dependent kinase in response to mitogen.

Progression through the G1 phase of the mammalian cell cycle is mediated in part through the early induction of D-type cyclins by mitogenic stimulation (1–3). Cell cycle progression is orchestrated by distinct families of cyclin-dependent kinases (CDKs) whose activities depend upon cyclin binding, positive and negative phosphorylation, and association with inhibitory polypeptides (10). Progression through the G1 phase of the cell cycle is controlled by one of three D-type cyclins (D1, D2, or D3), which assemble with their catalytic partner CDK4 or CDK6, and cyclin E, which assembles with its catalytic partner CDK2 (1–9). D- and E-type CDKs are required for G1 progression, and both contribute to the phosphorylation and inactivation of the retinoblastoma (Rb) protein thus canceling its growth-inhibitory properties (1, 2, 5, 7, 10–17). The activation of CDK4/CDK6 following association with cyclin D is critical for G1 progression. Inhibition of cyclin D1 expression through antisense cDNA or microinjection of antibodies specific to cyclin D results in G1 growth arrest (18, 19). D-type cyclins have been referred to as G1 mitogenic sensors because their induction requires mitogen, and removal of mitogen in G1 results in their rapid degradation and subsequent growth arrest (1–3).

The Ras/ERK pathway (ERK) pathway has been implicated in transducing mitogenic signals from growth factor receptors to the cell cycle machinery. Inhibition of the Ras/ERK pathway blocks mitogen-induced expression of cyclin D1 in Chinese hamster fibroblasts, demonstrating the importance of this pathway in mediating the mitogenic signals responsible for cyclin D1 induction (20–22). We have shown recently that PDGF induces the sustained activation of ERK and that this sustained activation is required for the continued accumulation of cyclin D1, implicating ERK activation in the regulation of cyclin D1 expression (21).

Concomitant with increased G1 cyclin D expression, cyclin D-CDK-associated activity increases in G1 (1–9, 20–22). The increase in cyclin D-CDK activity is a result of both an increase in cyclin D and a decrease in G1 cyclin-dependent kinase inhibitor expression (1, 2, 7). Although several cyclin-dependent kinase inhibitors have been identified as potent inhibitors of cyclin-CDK complexes, p27\(^{\text{KIP1}}\) is the only cyclin-dependent kinase inhibitor whose protein expression decreases as mitogen-induced cells enter the cell cycle (7, 23–25). The decrease in p27\(^{\text{KIP1}}\) expression occurs through protein degradation via the ubiquitin-proteasome pathway (26). The retention of inhibitory levels of p27\(^{\text{KIP1}}\) appears to be involved in the growth-inhibitory properties of transforming growth factor-β, rapamycin, and cyclic AMP (27–29). In contrast, overexpression of p27\(^{\text{KIP1}}\) antisense cDNA results in mitogen-independent G1 progression, demonstrating the importance of p27\(^{\text{KIP1}}\) in maintaining cell quiescence (30, 31). The mitogenic signals responsible for extracellular signal-related kinase; PDGF, platelet-derived growth factor; dn, dominant-negative; PBS, phosphate-buffered saline; GST, glutathione S-transferase; MEK, MAP kinase/ERK kinase.
p27KIP1 degradation have not been defined clearly.

PDGF stimulation causes the rapid activation of Ras and the subsequent downstream activation of ERK (21, 22). In addition, Ras also stimulates the downstream activation of RhoA presumably to induce changes in cytoskeleton structure associated with growth (32–36). However, RhoA activation has not been linked directly to the regulation of the cell cycle. In this study we demonstrate that Ras coordinates G1 progression through two independent pathways: ERK regulation of cyclin D1 expression and RhoA regulation of p27KIP1 degradation to ensure the proper activation state of cyclin D1-CDK complexes following mitogenic stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—IIC9 cells, a subclone of Chinese hamster embryo fibroblasts (37), were grown and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal calf serum and 2 mM l-glutamine (Sigma). Subconfluent (60–70%) were growth arrested by washing once with fresh Dulbecco's modified Eagle's medium for 48 h. Human recombinant PDGF-BB (Calbiochem) was added to cultures at 10 ng/ml in all experiments. Growth-arrested IIC9 cells were covalently cross-linked with 10 μM PD98059 (New England BioLabs, MA) before the addition of PDGF. Dominant-negative ERK2 (dnERK+) was a generous gift from Dr. Jacques Pouyssegur (University of Nice, France). Dominant-negative Ras (dnRas+) and RhoA (dnRhoA+) and constitutively active RhoA (RhoA+) were constructed as described previously through site-directed mutagenesis of Thr to Asn at codon 17 and 19 or Gln to Leu at codon 63, respectively, with the TransformerTM site-directed mutagenesis kit (CLONTECH) (38). Transient transfection of IIC9 cells (50–60% confluence) using LipofectAMINE (Life Technologies, Inc.) as recommended by the manufacturer consistently resulted in >90% expression efficiency as visualized by β-galactosidase staining. Western Blots—PDGF was added to growth-arrested IIC9 cells in the presence (+) or absence (−) of PD98059 (10 μM), C3 transferase (40 μg/ml), or various dominant-negative plasmids. Cells were harvested 0–24 h after the addition of PDGF by scraping in cold 1× PBS. Harvested cells were lysed and sonicated in solubilization buffer (250 mM Hepes; 300 mM NaCl; 0.2 mM EDTA; 1.5 mM MgCl2; 0.1% Triton X-100; 20 mM β-glycerophosphate; 0.1 mM sodium vanadate; 10 μg/ml each aprotinin, leupeptin, and pepstatin; and 0.5 mM phenylmethylsulfonyl fluoride; and 2 mM EDTA; 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, and 20 μM ATP). Resuspended complexes were incubated with 2 μg of soluble GST-Rho fusion protein (generous gift from Dr. Mark Ewen) and 5 μCi of [γ-32P]ATP. Samples were subjected to SDS-polyacrylamide gel electrophoresis and developed on a PhosphorImager.

RESULTS

PDGF Induces the Loss of p27KIP1 Protein—Protein levels of p27KIP1 are increased in contact-inhibited or serum-deprived cells and decrease when cells are stimulated by mitogens to enter the cell cycle (7, 23–25). Various mitogens including epidermal growth factor, PDGF, and serum are capable of stimulating cell cycle entry and p27KIP1 degradation (23–25). However, the mechanism by which these mitogens stimulate p27KIP1 degradation remains unclear.

We have shown previously that PDGF is a potent mitogen for IIC9 cells, and addition of PDGF to quiescent IIC9 cells resulted in up-regulation of cyclin D1 protein expression and D-type cyclin-dependent kinase activity (21, 22). Stimulation with PDGF also resulted in the time-dependent degradation of p27KIP1 protein (Fig. 1). 2 h after PDGF stimulation, p27KIP1 protein levels decreased approximately 50%, and by 24 h they were nearly undetectable (Fig. 1). Levels of CDK4, which we have shown previously do not increase with PDGF stimulation (21), were measured to ensure equal protein loading (Fig. 1). Previous studies have shown that loss of p27KIP1 protein occurs via a ubiquitin-mediated degradation pathway (26). In agreement with these observations, incubation of IIC9 cells with a calpain 1 inhibitor resulted in the appearance of polyubiquitinated forms of p27KIP1 (data not shown).

Ras but Not ERK Activity Is Required for PDGF-induced Loss of p27KIP1—We have shown previously that PDGF-induced G1 progression requires the sustained activation of ERK in a MAP kinase/ERK kinase 1 (MEK1)-dependent manner (21). The sustained activation of ERK following PDGF stimulation was responsible for the continued accumulation of cyclin D1, and inhibition of this activity resulted in the loss of cyclin D1 protein expression (21). To determine whether PDGF-induced ERK activation also contributed to the degradation of p27KIP1, we overexpressed a dnERK+ in IIC9 cells. Although the dnERK+ inhibits PDGF-induced G1 progression (21), it did not inhibit the PDGF-induced loss of p27KIP1 (Fig. 2A). IIC9 cells preincubated with an inhibitor of MEK1 activation, PD98059, displayed normal PDGF-induced p27KIP1 protein degradation with p27KIP1 protein levels being reduced to 10% maximal levels by 16 h (Fig. 2B). These data suggest that downstream effectors of MEK1 and ERK are not responsible for the degradation of p27KIP1.
We next looked at Ras, an upstream activator of the MAP kinase pathway, which we have shown previously is activated rapidly by PDGF (22). The addition of PDGF to IIC9 cells overexpressing dnRas\(^{-}\) did not affect p27\(^{kip1}\) protein levels, demonstrating the requirement of Ras activation for PDGF-induced p27\(^{kip1}\) degradation. These data demonstrate clearly that mitogen-regulated destruction of p27\(^{kip1}\) is downstream of Ras.

**RhoA Regulates p27\(^{kip1}\) Degradation**—It has become apparent that both MAP kinase and Ras pathways are important in the control of cell proliferation (20–22, 32–36, 39, 40). Whereas the role of the MAP kinase cascade has been shown clearly to regulate cyclin D1 expression (20–22, 40, 42), the role of the Ras cascade in cell cycle progression is unknown. To investigate the importance of PDGF-induced RhoA activity, we transfected IIC9 cells with dnRhoA\(^{19}\) and examined the effect of dnRhoA\(^{19}\) expression on several proteins that control progression through G1. Overexpression of dnRhoA\(^{19}\) inhibited PDGF-induced reduction of p27\(^{kip1}\) protein levels in IIC9 cells (Fig. 3A) similar to that seen in dnRas\(^{-}\)-transfected cells (Fig. 2A), suggesting that RhoA is a downstream Ras-dependent signaling molecule required for PDGF-induced p27\(^{kip1}\) degradation. Incubation with C3 transferase, an inhibitor of RhoA activity, also resulted in the inhibition of PDGF-induced p27\(^{kip1}\) degradation, further implicating RhoA activation in p27\(^{kip1}\) destruction (Fig. 4A). Overexpression of a constitutively active RhoA mutant, RhoA\(^{63}\), resulted in the mitogen-independent decrease in p27\(^{kip1}\) protein expression (Fig. 5B) identical to that of PDGF-stimulated IIC9 cells. These data demonstrate that activated RhoA alone is sufficient for loss of p27\(^{kip1}\). The requirement of RhoA for PDGF-induced p27\(^{kip1}\) degradation and the ability of RhoA\(^{63}\) mutant to stimulate p27\(^{kip1}\) degradation independently show clearly that RhoA activation has an important role in G1 progression and provide further evidence of the separate and distinct properties of the Ras/ERK and Ras/RhoA pathways in cell cycle regulation.

**Ras/ERK but Not Ras/Rho Pathway Regulates Cyclin D1 Expression**—We and others have demonstrated previously the importance of mitogen-stimulated Ras/ERK activation on cyclin D1 induction (20–22, 40, 42). The regulation of cyclin D1 induction and its continued G1 expression may be attributed to the ability of mitogens to stimulate the sustained activation of ERK (20, 21, 39). Overexpression of a dnRas mutant resulted in the inhibition of PDGF-stimulated cyclin D1 induction (Fig. 5A). In agreement with previous reports, overexpression of a dnERK mutant resulted in a similar inhibition in PDGF-stimulated cyclin D1 induction (Fig. 5B). However, it has not yet been determined whether other Ras-stimulated pathways are important for cyclin D1 induction. Overexpression of dnRhoA\(^{19}\), which resulted in an inhibition of PDGF-induced p27\(^{kip1}\) degradation, did not affect PDGF-stimulated ERK activation (Fig. 6). We hypothesized that the separation of these pathways would allow for their independent regulation of different G1 gene products: RhoA for p27\(^{kip1}\) degradation and ERK for cyclin D1 induction. In agreement with this hypothesis, overexpression of dnRhoA\(^{19}\) did not affect the induction and accumulation of cyclin D1 protein following PDGF stimulation (Fig. 5C), suggesting RhoA is not required for cyclin D1 protein expression.

**Constitutively Active Ras Requires ERK1 or RhoA Activity for the Downstream Regulation of Cyclin D1 or p27\(^{kip1}\)**—Ras has many downstream effectors of which two, ERK1 and RhoA, reside in separate and distinct growth-promoting pathways.

**FIG. 2.** Ras but not ERK activity is required for PDGF-induced p27\(^{kip1}\) degradation. Growth-arrested IIC9 cells (panel A) transfected with dnRas\(^{-}\) or dnERK\(^{-}\) or (panel B) preincubated with 10 μM PD98059 were harvested at 0, 8, and 16 h after the addition of PDGF (10 ng/ml) by scraping in cold 1× PBS and lysed. Lysates (15 μg) were electrophoresed on 12% SDS-polyacrylamide gels and immunoblotted with a polyclonal p27\(^{kip1}\) or CDK4 antibody.

**FIG. 3.** RhoA regulates the loss of p27\(^{kip1}\). Growth-arrested IIC9 cells (WT) transfected with (panel A) dnRhoA\(^{19}\) or (panel B) RhoA\(^{63}\) were harvested 0 and 24 h after the addition of PDGF (10 ng/ml) by scraping in cold 1× PBS and lysed. Lysates/proteins (15 μg) were electrophoresed on 12% SDS-polyacrylamide gels and immunoblotted with a polyclonal p27\(^{kip1}\) or CDK4 antibody.

**FIG. 4.** C3 transferase inhibits PDGF-induced p27\(^{kip1}\) degradation and cyclin D1-CDK activity. Growth-arrested IIC9 cells were preincubated for 2 h with C3 transferase (40 μg/ml) and harvested 0 and 24 h after the addition of PDGF (10 ng/ml) by scraping in cold 1× PBS and lysed. Panel A, lysates/proteins (15 μg) were electrophoresed on 12% SDS-polyacrylamide gels and immunoblotted with a polyclonal p27\(^{kip1}\) or CDK4 antibody. Panel B, conversely, lysates (100 μg) were incubated for 1–2 h at 4°C with a monoclonal cyclin D1 antibody. Cyclin D1 immune complexes were precipitated with protein G-Sepharose and assayed for their ability to phosphorylate soluble GST-Rb fusion protein in vitro as described under “Experimental Procedures.”
We and others have provided evidence previously which demonstrates the requirement of ERK1 for cyclin D1 up-regulation and active cyclin D1-CDK complexes following mitogenic stimulation (20, 21). We have also provided data in this study which strongly implicate RhoA activation in the regulation of p27KIP1 degradation. Constitutively active Ras mutants result in cellular transformation (35, 36), and in IIC9 cells a constitutively active Ras mutant (Ras12) resulted in ERK1 and RhoA activity independent of mitogen (data not shown) in agreement with several previous studies. We hypothesized that mitogen-independent regulation of cyclin D1 and p27KIP1 by Ras12 required ERK and RhoA activity, respectively. Ras12 stimulated cyclin D1 up-regulation as well as p27KIP1 degradation in the absence of mitogen (Fig. 7, A and B). In agreement with this hypothesis, IIC9 cells overexpressing Ras12 (IIC9-Ras12) required ERK1 activation by MEK1 to increase cyclin D1 expression in the absence of mitogen. IIC9-Ras12 cells incubated with PD98059 displayed reduced (6–8-fold) cyclin D1 protein expression levels (Fig. 7A), indicating a downstream requirement of ERK1 activity. Similarly, IIC9-Ras12 cells transfected with dnRhoA19 failed to induce the loss of p27KIP1 protein (Fig. 7B), demonstrating further the requirement of Ras-stimulated RhoA activity in p27KIP1 degradation. These data also provide evidence for the necessity of ERK and RhoA activities in the regulation of critical G1 events and suggest that other Ras-stimulated pathways are unable to compensate for the loss of either activity to regulate cyclin D1 and p27KIP1 protein expression.  

**PDGF-induced RhoA Activation Is Required for Active Cyclin D1-CDK Complexes and Subsequent G1 Progression—**Active cyclin D1-CDK complexes in concert with other G1 cyclin-CDKs are responsible for progression into S phase in part through their ability to phosphorylate and inactivate the Rb protein (1, 2, 10–17). Stimulation of growth-arrested IIC9 cells resulted in a 6–7-fold increase in cyclin D1-CDK activity (Figs. 4B and 8A). Although overexpression of dnRhoA19 did not affect cyclin D1 levels (Fig. 5C), overexpression of dnRhoA19 resulted in the complete inhibition of PDGF-induced cyclin D1-CDK activity (Fig. 8A). Incubation with C3 transferase, a specific inhibitor of RhoA activity, also resulted in the complete inhibition of PDGF-induced cyclin D1-CDK activity (Fig. 4B), further implicating RhoA in the downstream determination of the cyclin D1-CDK activation state. Concomitant with its ability to inhibit cyclin D1-CDK activity, dnRhoA19 inhibited PDGF-stimulated G1 progression (Fig. 8B), demonstrating further the importance of RhoA in mediating events important in G1 progression.

**DISCUSSION**

Ras/ERK are critical mediators of mitogen-dependent cyclin D1 expression (20–22, 40, 42). Inhibition of mitogen-induced MEK1 or ERK1 activation resulted in the inhibition of cyclin D1 induction (20, 21). Furthermore, the sustained activation of MEK1/ERK1 was required for the continued presence of cyclin D1, demonstrating the importance of ERK1 in regulating cyclin D1 expression positively (21). However, the ERK pathway does not appear to control p27KIP1 degradation. Expression of constitutively active ERK does not result in p27KIP1 degradation in the absence of mitogen, implicating an independent mitogenic pathway in the regulated destruction of p27KIP1 (31). We have focused our study on the Ras/RhoA mitogenic pathway in regulating p27KIP1 degradation. PDGF or serum stimulates a rapid induction of Ras activity followed by the sustained activation of ERK1 (20–22). We show for the first time that RhoA regulates mitogen-induced p27KIP1 degradation. Overexpression of dnRas inhibited the PDGF-induced loss of p27KIP1 (Fig. 2A). In contrast, inhibition of ERK activity or MEK1 activity did not affect the PDGF-induced degradation of p27KIP1 (Fig. 2, A and B).

Recent data indicate that Ras activates two independent pathways that are important for Ras-transforming ability (35, 36, 38). Whereas overexpression of constitutively active Ras in NIH 3T3 cells resulted in transformation, expression of constitutively active ERK or RhoA alone was ineffective (38). However, expression of both constitutively active ERK and RhoA was as effective as overexpression of constitutively active Ras. Our findings are consistent with these results and demonstrate for the first time that RhoA signaling regulates p27KIP1, a protein important in the regulation of G1 progression. Expression of dnRhoA19 inhibited the PDGF-induced degradation of p27KIP1 (Fig. 3A), and RhoA63 stimulated a loss of p27KIP1 in the absence of mitogen, demonstrating the requirement of RhoA activity for Ras-dependent p27KIP1 degradation. C3 transferase also inhibited PDGF-induced p27KIP1 degradation (Fig. 4A), further implicating RhoA activation in the stimulation of this process. In contrast, cyclin D1 induction was not affected by dnRhoA19 expression after PDGF stimulation (Fig. 5C), suggesting separate pathways for Ras-dependent cyclin D1 and p27KIP1 regulation. PDGF-stimulated induction of cyclin D1 protein was not sufficient for progression through G1 because C3 transferase or dnRhoA19 blocked PDGF-induced cyclin D1-CDK activity and subsequent G1 progression (Figs. 4B and 8A). These data demonstrate the coordinated signaling between ERK and RhoA required for G1 progression. We cannot rule out the possibility that overexpression of dnRhoA19 may affect or inhibit other G1 events necessary for cell growth. However, it is clear that RhoA activity is required for PDGF-induced cyclin D1-CDK activity and that the phosphorylation of Rb by these activated complexes is an integral component of G1 progression.

Anti-mitogens (transforming growth factor-β) promote growth arrest through their ability to maintain high levels of p27KIP1 (27–29). High levels of p27KIP1 stoichiometrically inhibit cyclin D-CDK complexes (7, 29), and a loss of p27KIP1 through mitogenic stimulation or antisense cDNA expression promotes G/S transit (7, 23–25, 30, 31). We were able to disrupt the normal PDGF-induced degradation of p27KIP1 in cycling cells by overexpressing dnRhoA19 (Fig. 3A) or inhibiting RhoA activity with C3 transferase (Fig. 4A). The imbalance of p27KIP1 protein levels was sufficient to arrest PDGF-stimu-
The ability of p27 KIP1 to inhibit the activation of cyclin D is an important aspect of G1 progression (21). It is not clear whether the mitogen-induced degradation requires sustained growth factor signaling, but evidence showing p27 KIP1 up-regulation following mitogen removal suggests that such regulation may exist. We have shown previously the requirement of sustained ERK activation for continued cyclin D1 mRNA and protein expression following PDGF stimulation, demonstrating the importance of continued growth factor signaling in G1 progression (21). It is not clear whether the mitogen-induced degradation requires sustained growth factor signaling, but evidence showing p27 KIP1 up-regulation following mitogen removal suggests that such regulation may exist. We have shown that Ras/RhoA activities are required for PDGF-stimulated p27 KIP1 degradation (Figs. 2-4) and that constitutively active RhoA stimulates a loss of p27 KIP1 independent of mitogen. It is unclear, however, whether active RhoA acts directly or indirectly on p27 KIP1 to target p27 KIP1 for ubiquitin-mediated degradation.

p27 KIP1 is primarily a nuclear protein, which raises the possibility that active RhoA or downstream effectors of RhoA may translocate to the nucleus where they promote the degradation of p27 KIP1. Although few proteins have been identified as downstream nuclear effectors involved in p27 KIP1 destruction, it seems warranted. However, the identification of RhoA as a necessary mediator of p27 KIP1 degradation clearly implicates RhoA as a signaling pathway for gene products important for G1 progression.

By disrupting the expression of newly formed cyclin D1-CDK complexes and p27 KIP1 by altering protein levels of cyclin D1 and p27 KIP1, we were able to inhibit the mitogenic properties of PDGF. Overexpression of dnRhoA19 was able to inhibit PDGF-induced cyclin D1-CDK complexes (Fig. 8A) overriding PDGF-stimulated Ras/ERK signals. Inhibition of ERK activation blocked cyclin D1 induction but had no effect on PDGF-induced p27 KIP1 degradation, and inhibition of RhoA activity blocked p27 KIP1 degradation but had no effect on PDGF-induced cyclin D1 expression. These data provide evidence of the separate actions of these Ras-stimulated pathways. Although these pathways appear to determine the fate of distinct cell cycle transitions of the RhoA signaling for G1 progression.

The ERK cascade is largely responsible for the immediate-early induction and/or activation of mitogen-induced transcription factors including, but not limited to, c-myc, Elk-1, c-fos, and c-jun (41). Recent studies have focused on the role of these transcription factors in promoting cyclin D1 transcription (20, 40, 42). We have shown previously the requirement of sustained ERK activation for continued cyclin D1 mRNA and protein expression following PDGF stimulation, demonstrating the importance of continued growth factor signaling in G1 progression. Ras/RhoA activities are required for PDGF-stimulated p27 KIP1 degradation (Figs. 2-4) and that constitutively active RhoA stimulates a loss of p27 KIP1 independent of mitogen. It is unclear, however, whether active RhoA acts directly or indirectly on p27 KIP1 to target p27 KIP1 for ubiquitin-mediated degradation.

**FIG. 6**. Overexpression of dnRhoA19 does not affect PDGF-induced ERK1 activation. Growth-arrested IIC9 cells (WT) transfected with dnRhoA19 were stimulated with PDGF (10 ng/ml) for 15 min. Stimulated cells were harvested by scraping in cold 1× PBS and lysed. ERK1 immune complexes were immunoprecipitated and assayed for their ability to phosphorylate myelin basic protein in vitro as described under “Experimental Procedures.” Results reported are the mean ± S.D. (n = 3).

**FIG. 7**. Ras12 requires ERK1 or RhoA activity for the downstream regulation of cyclin D1 and p27 KIP1. Panel A, IIC9-Ras12 cells treated with PD98059 (10 μM) for 24 h were harvested by scraping in cold 1× PBS and lysed. Panel B, conversely, IIC9-Ras12 cells transfected with dnRhoA19 were harvested by scraping in cold 1× PBS and lysed. Lysates/proteins (15 μg) were electrophoresed on 12% SDS-polyacrylamide and immunoblotted with (panel A) cyclin D1 or (panel B) p27 KIP1 polyclonal antibodies.

A

- PDGF
- PD98059
- Ras12
- p27 KIP1

B

- PDGF
- PD98059
- dnRhoA19
- Ras12
- p27 KIP1

Our data provide further evidence that p27 KIP1 acts downstream of cyclin D1 induction. Kato et al. (29) demonstrated the ability of p27 KIP1 to inhibit the activation of cyclin D-CDK4 complexes. Overexpression of dnRhoA19 inhibited PDGF-induced cyclin D1-CDK activity (Fig. 8A) likely because of the maintenance of high levels of p27 KIP1 and thus inhibited G1/S transit (Fig. 8B). These results clearly demonstrate the importance of continued growth factor signaling in G1 progression.

2 J. D. Weber and J. J. Baldassare, unpublished observations.
proteins independently, this is the first study to show that they converge downstream to determine the activation state of cyclin D1 CDK complexes and subsequently coordinate mitogen-induced G1 progression.

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