RhoA has been identified as an important regulator of cell proliferation. We recently showed that the Ras/RhoA pathway regulates the degradation of p27Kip and the progression of Chinese hamster embryo fibroblasts (IIC9 cells) through G1 into S phase (Weber, J. D., Hu, W., Jefcoat, S. C., Raben, D. M., and Baldassare, J. J. (1997) J. Biol. Chem. 272, 32966–32971). In this report, we have demonstrated that, in IIC9 cells, RhoA regulates cyclin E/CDK2 activity, which is required for p27Kip degradation. As previously shown in several fibroblast cell lines, expression of dominant-negative CDK2 in IIC9 cells blocked serum-induced cyclin E/CDK2 activity and p27Kip degradation. In the absence of serum, expression of constitutively active RhoA(63) resulted in significant stimulation of cyclin E/CDK2 activity and degradation of p27Kip. Cotransfection of dominant-negative CDK2 and RhoA(63) inhibited RhoA(63)-induced cyclin E/CDK2 activity and p27Kip degradation. In addition, expression of dominant-negative RhoA blocked serum-induced cyclin E/CDK2 and p27Kip degradation. Finally, expression of catalytically active cyclin E/CDK2 rescued the effect of expression of dominant-negative RhoA. Taken together, these data show that RhoA regulates p27Kip degradation through its regulation of cyclin E/CDK2 activity.

Cyclin-dependent kinases and their inhibitors are important for ordered progression through the cell cycle (1). Progression through G1 into S phase requires activation of both cyclins D/CDK4 and cyclin E/CDK2 activities (1). In addition to the cyclins, specific inhibitory proteins (CKI proteins) associate with distinct cyclin kinase complexes and regulate their activities. These CKI proteins are divided into two families on the basis of sequence homology: the Cip/Kip and Ink4 families. The Cip/Kip family, which includes p21Cip (2), p27Kip (3, 4), and p57Kip2 (5), inhibits CDK4, CDK6, and CDK2 activities. The Ink4 family, which includes p16, p15, p18, and p19, specifically inhibits CDK4 and CDK6 activities (6–8).

p27Kip is expressed in most cells and is thought to be an important negative regulator of mitogen-induced progression through G1 (9–11). Expression of p27Kip results in G1 arrest in a variety of cell types (12). The level of p27Kip increases when cells are growth-arrested by contact inhibition, mitogen withdrawal, or other anti-proliferative signals (9, 10, 14–18). Consistent with the notion that p27Kip is important in G1 progression, mitogens stimulate elimination of p27Kip during late G1 or as cells enter G/S. Interestingly, decreases in p27Kip protein levels are not a result of any changes in mRNA levels (18, 19), but mostly a result of decreased translation concomitant with increased ubiquitin-directed degradation (18–20). Recently, considerable effort has been directed at understanding the pathway important for ubiquitin-directed degradation and the upstream signaling pathway that regulates targeting ubiquitin-dependent proteolysis (21–23).

Mitogens stimulate p27Kip degradation by inducing cyclin E/CDK2 phosphorylation of p27Kip at Thr-187 (21–23). Expression of dominant-negative CDK2 in NIH-3T3 cells blocks serum-induced phosphorylation of p27Kip at Thr-187 and p27Kip degradation (21). Mutation of Thr-187 to alanine results in a stable form of p27Kip, which is resistant to phosphorylation by cyclin E/CDK2 and degradation (21, 22) and, when overexpressed, induces G1 arrest. Taken together, these data strongly indicate that inhibition of p27Kip degradation alone results in elevated levels of p27Kip and inhibition of G1 progression.

RhoA is a member of the Ras-like small G protein superfamily (24). In addition to its role in the formation of stress fibers and focal adhesion (25), RhoA also regulates Ras-dependent cell cycle progression and cell transformation (26, 27). Activation of RhoA is required for G1 cell cycle progression in Swiss 3T3 cells (26, 27) and, expression of geranylgeranylated RhoA facilitates the S phase entry in rat thyroid FRTL-5 cells (28). Moreover, Ras-mediated transformation in NIH-3T3 cells requires activation of both RhoA and Raf (26, 29–31). We previously demonstrated that overexpression of dominant-negative Ras or dominant-negative RhoA inhibits PDGF1-induced DNA synthesis of IIC9 cells (32).

Recent data suggest that RhoA regulates IIC9 cell cycle progression by stimulating p27Kip degradation. Expression of geranylgeranylated RhoA promotes the degradation of p27Kip in rat thyroid FRTL-5 cells (28). In IIC9 cells, we found that expression of dominant-negative RhoA inhibits p27Kip degradation (32). In the present study, we show that RhoA-induced activation of cyclin E/CDK2 precedes decreases in p27Kip protein amounts. This is the first study to demonstrate a requirement for RhoA in the activation of cyclin E/CDK2 activity and clearly identifies a role for RhoA in growth and transformation.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—IIC9 cells, a subculture of Chinese hamster embryo fibroblasts, were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum. Subconfluent cultures were growth-arrested by incubation for 2 days in serum-free DMEM. Serum-arrested cells were washed twice and equilibrated in basal medium for 30 min prior to the addition of serum. Dominant-negative (dnCDK2) and wild-type CDK2 were the generous gifts Dr. Ed Harlow. Cyclin E was a generous gift from the laboratory of

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1 The abbreviations used are: PDGF, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; dnCDK2 dominant-negative CDK2; dnRhoA, dominant-negative RhoA.
Dr. James Roberts. Dominant-negative RhoA (dnRhoA) and constitutively active RhoA (RhoA(63)) were constructed as described previously by site-directed mutagenesis of Thr to Asn at codon 19 or Glu to Leu at codon 63, respectively (32).

**Transient Transfection**—The cDNAs encoding wild-type CDK2, dnCDK2, wild-type cyclin E, dnRhoA, and RhoA(63) were transfected into subconfluent IIC9 cells using LipofectAMINE™ according to the manufacturer's protocol (Life Technologies, Inc.). Following expression of the cDNAs by incubation for 24 h in DMEM plus 10% serum, the cells were serum-arrested by incubation in DMEM for 48 h before agonist stimulation. Transient transfection using LipofectAMINE™ resulted in 80–90% expression efficiency as visualized by β-galactosidase staining.

**Cyclin E/CDK2 Kinase Assay**—Subconfluent (80%) growth-arrested IIC9 cells were stimulated with 10% fetal calf serum. Cells were harvested at the indicated times after serum addition by scraping into 150 µl of ice-cold lysis buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM NaF, 0.1 mM sodium vanadate, 50 mM β-glycerophosphate, 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 10 µg/ml leupeptin). The lysates were briefly sonicated, and insoluble material was pelleted by centrifugation at 14,000 × g for 5 min at 4 °C. Protein concentrations were determined using the Bio-Rad protein assay according to the manufacturer's specifications. Cyclin E-CDK2 immune complexes were isolated by incubation with anti-cyclin E antibody (Santa Cruz Biotechnology, Santa Cruz, CA) plus protein G. After several washes, the cyclin E-CDK2 complexes were incubated for 45 min at 30 °C with histone and 0.5 µCi of [γ-32P]ATP. The reactions were terminated by the addition of 5 µl of 4X Laemmli sample buffer. The samples were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis. The gels were dried, and cyclin E/CDK2 activities were quantified by PhosphorImager analysis (Molecular Dynamics, Inc.).

**Western Blot Analysis**—Serum-deprived cell cultures were incubated in the absence or presence of serum or PDGF. At the indicated times, the samples were scraped into 150 µl of cold phosphate-buffered saline, centrifuged at 4 °C for 5 min at 14,000 × g, and then lysed on ice in 30 µl of solubilization buffer (25 mM Hepes, 300 mM NaCl, 2 mM EDTA, 1.5 mM MgCl2, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% NaN3, 10 mM leupeptin, 10 µg/ml aprotinin, and 10 µg/ml pepstatin). The samples were subjected to brief sonication and centrifuged for 5 min at 4 °C. Protein concentrations of the supernatants were determined by the Bio-Rad protein assay as recommended by the manufacturer. Typically, 50 µg of lysate protein were resolved by 12% SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp., Bedford, MA). The membranes were probed with the appropriate antibody. Immunoreactive bands were visualized by chemiluminescence using the ECL Western blotting system (Amersham Pharmacia) as recommended by the manufacturer.

**RESULTS**

**Cyclin E/CDK2 Activation Precedes Degradation of p27Kip**—Recent studies have demonstrated that phosphorylation of p27Kip at Thr-187 by cyclin E/CDK2 is required for ubiquitin-dependent degradation of p27Kip (21–23). To determine whether cyclin E/CDK2 activity precedes serum-induced p27Kip degradation, we examined the time course of p27Kip degradation and cyclin E/CDK2 activity. Serum stimulated a rapid increase in cyclin E/CDK2 activity (Fig. 1A). Increased activity was detectable within 30 min, was maximal at 17 h, and remained elevated for 24 h (Fig. 1A). In contrast to cyclin E/CDK2 activity, a change in p27Kip protein was not detectable at 30 min (Fig. 1B). A significant decrease in p27Kip could be detected at 4 h, and p27Kip was barely detectable at 24 h (Fig. 1B). These data demonstrate that cyclin E/CDK2 activity precedes any detectable decrease in p27Kip.

The rapid increase in cyclin E/CDK2 activity was unexpected since, in most cell types, this activity does not increase until late in G1. Subsequent to activation of cyclin D/CDK4/CDK6. Previously, we showed that cyclin D/CDK4 activity is not detectable until 1–2 h after the addition of PDGF or serum (33), yet cyclin E/CDK4 activity increased within 30 min (Fig. 1A), indicating that, in IIC9 cells, cyclin E/CDK2 increases prior to a detectable increase in cyclin D/CDK4 activity. The timing of an increase in cyclin E/CDK2 in most cell types in late G1 is likely a result of the dependence of cyclin E expression on cyclin D/CDK4 activity (34). To understand the early activation of cyclin E/CDK2 observed with IIC9 cells, we examined whether expression of cyclin E in IIC9 cells was independent of cyclin D/CDK4 (Fig. 1C). Significant amounts of cyclin E were detectable in quiescent IIC9 cells (Fig. 1C). A modest increase in cyclin E protein was detectable within 4 h, was maximal (1.5–2-fold) at 17 h, and remained elevated for 24 h (Fig. 1C). Cyclin E is therefore detectable in quiescent IIC9 cells and remains elevated throughout G1 into S phase. The high level of cyclin E, which is expressed in quiescent IIC9 cells, is markedly different from the level of cyclin D1 protein (32, 33). Cyclin D1 protein is very low but detectable in quiescent IIC9 cells and increases at least 5-fold with PDGF (32, 33). Significant levels of cyclin E are present in quiescent cells, and serum stimulates only small increases. These data suggest that there are sufficient amounts of cyclin E in quiescent cells and indicate that the increases in this cyclin E/CDK2 activity occur independent of increased expression of cyclin E.

**RhoA Regulates the Activity of Cyclin E/CDK2 and the Degradation of p27Kip**—Data obtained from overexpression of dominant-negative mutants of both RhoA and CDK2 indicate that mitogen-induced degradation of p27Kip requires activation of RhoA (32) and cyclin E/CDK2 (21–23). To determine whether RhoA regulates cyclin E/CDK2 activity, we next examined the effect of blocking RhoA activation on cyclin E/CDK2 activity and p27Kip protein (Fig. 2). Expression of dnRhoA inhibited
RhoA Regulates Cyclin E/CDK2 Activation

**Fig. 2.** RhoA regulates cyclin E/CDK2 activity and p27Kip degradation. Subconfluent IIC9 cells were transfected with 1 µg of dnRhoA as described under “Experimental Procedures.” The transfected cells were grown for 36 h in DMEM plus 10% fetal calf serum and then growth-arrested for 48 h in DMEM. Cell lysates were isolated from growth-arrested cells and serum-stimulated cells. A, cyclin E immune complexes were isolated from 50 µg of cell lysate and assayed for their ability to phosphorylate histone protein (Histone-P) as described under “Experimental Procedures.” Similar results were obtained in four separate experiments. B, lysates containing 40 µg of protein were electrophoresed on 12% SDS-polyacrylamide gels and immunoblotted with anti-p27Kip1 antibody. Similar results were obtained in three separate experiments. WT, wild type.

**Fig. 3.** Expression of Rho(63) induces cyclin E/CDK2 activity and p27Kip degradation in quiescent IIC9 cells. Subconfluent IIC9 cells were transfected with 1 µg of RhoA(63) and growth-arrested as described for Fig. 2. A, cyclin E immune complexes were immunoprecipitated from lysates (50 µg of protein) of transfected cells. The immune complexes were assayed for their ability to phosphorylate histone protein (Histone-P) as described under “Experimental Procedures.” Similar results were obtained in four separate experiments. B, lysates from samples described in A containing 40 µg of protein were electrophoresed on 12% SDS-polyacrylamide gels and immunoblotted with anti-p27Kip1 antibody. Similar results were obtained in three separate experiments. WT, wild type.

Our data show that cyclin E levels were significantly elevated in quiescent IIC9 cells (Fig. 1C). Furthermore, expression of dnRhoA inhibited serum-induced cyclin E/CDK2 activity (Fig. 2A) and p27Kip degradation (Fig. 2B). To further demonstrate that RhoA regulates cyclin E/CDK2 activity, IIC9 cells were treated with botulinum C3 exoenzyme, which specifically ADP-ribosylates RhoA and blocks its function (35). This treatment significantly inhibited serum activation of cyclin E/CDK2 activity and the serum-induced decrease in p27Kip (data not shown).

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We therefore decided to determine whether expression of a constitutively active mutant of RhoA (RhoA(63)) stimulates cyclin E/CDK2 activity in the absence of agonist (Fig. 3A). Expression of RhoA(63) induced a significant increase in cyclin E/CDK2 activity in the absence of serum (Fig. 3A). This activity was ~80% of the maximal activity seen with serum-stimulated IIC9 cells. Although expression of RhoA(63) could induce cyclin E/CDK2 activity in quiescent IIC9 cells, serum stimulated a further increase (~1.4-fold) in the activity of cyclin E/CDK2 in IIC9 cells expressing RhoA(63) (Fig. 3A). This increase in activity is consistent with the ability of serum to induce a 2-fold increase in cyclin E (Fig. 1C). In addition to increasing cyclin E/CDK2 activity and consistent with the notion that cyclin E/CDK2 activity is necessary for p27Kip degradation, expression of RhoA(63) reduced p27Kip protein to levels comparable to those seen with serum stimulation (Fig. 3B). These data clearly demonstrate that expression of constitutively active RhoA alone can markedly increase cyclin E/CDK2 activity and induce p27Kip degradation. To further examine the ability of RhoA(63) to mediate the loss of p27Kip, we next determined whether coexpression of dnCDK2 with RhoA(63) blocks p27Kip breakdown. In contrast to expression of RhoA(63) alone, coexpression of dnCDK2 and RhoA(63) did not result in increased activity (data not shown) or a decrease in p27Kip protein in quiescent IIC9 cells (Fig. 4). These data are in agreement with the results demonstrating that serum-induced degradation of p27Kip is dependent on cyclin E/CDK2 activity and suggest that cyclin E/CDK2 activity is downstream of RhoA.

We next examined whether expression of cyclin E/CDK2 induces the degradation of p27Kip protein in cells expressing dnRhoA. Expression of cyclin E/CDK2 results in significant cyclin E/CDK2 activity in quiescent IIC9 cells (Fig. 5A) and loss of p27Kip (Fig. 5B). These changes occurred in the absence of cyclin D/CDK4 activity (data not shown). Therefore, up-regulation of cyclin E/CDK2 activity itself induced p27Kip degradation. To determine whether up-regulation of cyclin E/CDK2 activity rescues the effects of dnRhoA, we examined cyclin E/CDK2 activity and p27Kip levels in cells cotransfected with dnRhoA and cyclin E/CDK2 (Fig. 6A). Furthermore, p27Kip levels were reduced in these cells (Fig. 6B). Taken together, these data strongly suggest that
RhoA is upstream of cyclin E/CDK2.

Expression of Cyclin E/CDK2 Rescues dnRhoA-induced Growth Arrest—Inhibition of RhoA by expression of dnRhoA or treatment with botulinum C3 exoenzyme resulted in significant inhibition of growth (Fig. 7A). This is consistent with the observation that RhoA is important in mammalian cell growth and transformation (26, 27). Our data indicate that RhoA regulates cell cycle progression at least partially through its regulation of cyclin E/CDK2 activity and p27Kip levels. If cyclin E/CDK2 activity is the major cell cycle downstream effector, cotransfection of cyclin E/CDK2 should rescue the arrest observed with expression of dnRhoA. Indeed, serum stimulated significant growth in IIC9 cells expressing dnRhoA and cyclin E/CDK2 (Fig. 7A). In IIC9 cells coexpressing dnRhoA and cyclin E/CDK2, serum was less mitogenic than in wild-type IIC9 cells (Fig. 7A). However, growth under these conditions was virtually identical to growth in the presence of a potent mitogen, PDGF (Fig. 7B), indicating that cyclin E/CDK2 activation is a target for RhoA-dependent regulation of cell cycle progression through G1 into S phase. Whereas expression of dnRhoA could block PDGF-stimulated growth, overexpression of cyclin E/CDK2 rescued growth to near identical values seen in cells treated with PDGF alone (Fig. 7B). These data demonstrate that cyclin E/CDK2 activity is a major target of RhoA.

DISCUSSION

Observations from several independent studies suggest that oncogenic Ras may mediate its action through both Raf-dependent and RhoA-dependent pathways (29, 31). Expression of constitutively active Ras results in transformation in NIH-3T3 cells; expression of constitutively active Raf or RhoA alone only weakly transforms NIH-3T3 cells (29). Furthermore, cotransfection with either dominant-negative Rac or RhoA blocks Ras-induced transformation (29). Our previous studies have shown that the Ras/Raf/mitogen-dependent protein kinase pathway is important for the induction of cyclin D and that RhoA regulates p27Kip degradation (32, 33). In this study, we have demon-
strated for the first time that RhoA induces p27Kip degradation through regulation of cyclin E/CDK2 activity. Our data therefore indicate that the Ras/Erk pathway regulates cyclin D/CDK4/CDK6 activity and that the Ras/RhoA pathway regulates cyclin E/CDK2 activity. Both these activities are crucial for passage through G1 into S phase (Fig. 8).

Several recent reports (21–23) and the data in this paper clearly demonstrate that mitogen-induced p27Kip degradation is dependent on cyclin E/CDK2 activity. Previously, we found that, in IIC9 cells, PDGF stimulates p27Kip degradation in the absence of cyclin D/CDK4/CDK6 activity (32). This is likely a result of the high level of cyclin E expressed in quiescent IIC9 cells (Fig. 1C). Interestingly, progression through G1 in these cells still requires cyclin D expression, suggesting that, in cells containing normal levels of retinoblastoma protein,2 progression through G1 requires both cyclin D- and cyclin E-dependent kinases. These results are in agreement with data that indicate that retinoblastoma protein must be phosphorylated by cyclin D-dependent kinases prior to cyclin E/CDK2 (37).

Oddly, p27Kip is an inhibitor and substrate of cyclin E/CDK2. Phosphorylation of p27Kip by cyclin E/CDK2 is required for its degradation in G1 (21).

In vitro kinetic analysis of the association of p27Kip with cyclin E/CDK2 suggests that p27Kip binds to cyclin E/CDK2 with high and low affinities (21). Initially, it binds with low affinity, and then slowly, the binding shifts to high affinity (21). Furthermore, when it binds with low affinity, it is a substrate, and at high affinity, an inhibitor (21). At

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2 W. Hu, C. J. Bellone, and J. J. Baldassare, unpublished data.
equilibrium, p27Kip inhibits the activity. It is possible that, in vivo, certain conditions favor high or low affinity binding and thus determine whether it is a substrate or inhibitor. RhoA may be an important regulator of this association. The present data clearly demonstrate that RhoA regulates cyclin E/CDK2 activation and identify a role for RhoA in progression through G1 of the cell cycle.

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