Cdc42 Promotes G₁ Progression through p70 S6 Kinase-mediated Induction of Cyclin E Expression*

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The Rho family GTPase Cdc42 is recognized for its role in cellular proliferation and transformation. However, the mechanism by which it promotes cell cycle progression has remained undefined. Using an inducible expression system, we show that constitutively active Cdc42 (Cdc42V12) is sufficient by itself to induce anchorage-independent but not mitogen-independent growth in NIH3T3 cells. However, Cdc42V12 markedly accelerates activation of cyclin E-Cdk2 in response to mitogen. These effects were highly specific, as the kinetics of cyclin D-Cdk4 activation was unaltered. Cdc42V12 promotes Cdk2 activation by selectively inducing cyclin E expression without affecting other regulatory proteins such as the p27 Cdk inhibitor or Cdc25A. Furthermore, Cdc42V12 was able to activate a reporter gene driven by the cyclin E promoter in the absence of exogenous mitogen or adhesion. Cyclin E induction was sensitive to rapamycin but not inhibitors of mitogen-activated protein kinases, implicating p70 S6 kinase (p70S6k) as the relevant mediator. Consistent with this notion, wild type and constitutively active alleles of p70S6k were sufficient to activate the cyclin E promoter. In sum, these studies provide novel insights into the mechanism by which Cdc42 promotes G₁ progression.

Rho family GTPases play a central role in controlling cellular proliferation and transformation. Work from many laboratories has demonstrated that members of this family can induce various aspects of the transformed phenotype including mitogen- and anchorage-independent growth and invasiveness in multiple cell types (for reviews, see Refs. 1–5). These effects are elicited through the ability of the GTPases to regulate diverse functions, such as cell cycle progression, cytoskeletal remodeling, survival, adhesion, and vesicular trafficking. Furthermore, many effectors for Rho GTPases have been identified in recent years, and in some cases specific effectors have been linked to distinct responses, such as actin reorganization and cell cycle progression (for reviews, see Refs. 1–5).

Cdc42, a member of the Rho GTPase subfamily, has been the focus of intense study in recent years (for review, see Ref. 6). Multiple studies demonstrate a role for Cdc42 in promoting cell cycle progression. In certain cell types, activated Cdc42 is sufficient to induce S phase entry in the absence of exogenous mitogen (7–10). In others, although it is not sufficient by itself, it nevertheless promotes proliferation and G₁ progression (10, 11). In addition, activated Cdc42 induces anchorage-independent growth (8, 11). Despite these studies, little is known about the mechanism by which Cdc42 functions. Many effectors for Cdc42 have been identified, including the PAK family kinases (12–14), the mitogen-activated protein kinases (15–20), WASP family proteins (21, 22), and p70 S6 kinase (p70S6k) (23). Several studies have identified proximal effectors of Cdc42 that are dispensable for their ability to induce S phase entry, but none has identified those that are required (7, 24).

Another point that remains unresolved is the component of the cell cycle machinery that is targeted by Cdc42. One report identified the retinoblastoma (Rb) tumor suppressor protein as a distal target of Cdc42 (10). Rb plays a central role in inhibiting the activity of the E2F family transcription factors, which are activated during G₁ phase and drive expression of genes required for S phase, such as cyclin A (for review, see Ref. 25). Transient overexpression of activated Cdc42 induced hyperphosphorylation of Rb, resulting in the release of E2F. Accordingly, Cdc42 activated a synthetic reporter construct driven by E2F-responsive elements (10). An important unanswered question is the identity of the kinase that mediates Cdc42-induced Rb hyperphosphorylation. Both cyclin D-Cdk4 and cyclin E-Cdk2 phosphorylate Rb on multiple sites that regulate its ability to bind E2F (26–29). The study above suggested that transient overexpression of activated Cdc42 can induce a modest increase in cyclin D protein (10); however, cyclin D-associated kinase activity was not examined. Another report showed that activated Cdc42 more potently stimulates expression of cyclin A (24). However, cyclin A-Cdk2 activation occurs after Rb phosphorylation in vivo, excluding it as the relevant kinase. Thus, the primary cell cycle target(s) of Cdc42 remain incompletely defined.

In the current study we define the effects of Cdc42 on the cell cycle machinery and identify the signaling pathway utilized to promote G₁ progression. Using stable NIH3T3 cell lines that inducibly express activated Cdc42 (Cdc42 G12V; Cdc42V12), we show that Cdc42V12 is sufficient by itself to induce anchorage-independent but not mitogen-independent growth. However, Cdc42V12 specifically accelerates cyclin E-Cdk2 activation in response to growth factor. Strikingly, the kinetics of cyclin D-Cdk4 activation was not affected by Cdc42V12. We further demonstrate that Cdc42V12 functions by specifically

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activating the cyclin E promoter without affecting other regulators of Cdk2, such as p27 or Cdc25A. Induction of the cyclin E promoter by Cdc42V12 is sensitive to rapamycin but not to pharmacological inhibitors of mitogen-activated protein kinases, implicating p70S6k as an important mediator.Consistent with this interpretation, the cyclin E promoter was also activated by co-expression of wild type or constitutively active alleles of p70S6k. Together, these results define a signaling pathway through which Cdc42 contributes to G1 progression.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—NIH3T3 cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% calf serum, Glutamax, and penicillin/streptomycin (5% calf serum/DMEM). The Cdc42V12-expressing cells were derived from LAP3/NIH3T3 cells (denoted LAP3 in the text), which express the Lac repressor/VP16 activator (a generous give from Drs. Lester Lau and Dmitri Peskov) (30). LAP3 clones expressing HA-tagged Cdc42V12 were isolated by co-transfection of HA-Cdc42V12/pX11 with a hygromycin resistance plasmid. Clonal cell lines were isolated and maintained in 5% calf serum/DMEM. Expression of HA-Cdc42V12 was induced by the addition of 1 mg isopropyl-1-thio-β-D-galactopyranoside (IPTG). Rapamycin, SB203580, and PD98059 were purchased from Calbiochem. For plating of cells in the absence of adhesion, cells were trypsinized then replated in tissue culture dishes coated with 1% agarose or in the presence of 1 µg cytochalasin D, as previously described (24, 31–34).

Constructs and Plasmids—The HA-tagged p70S6k constructs have been described previously (23). The murine cyclin E promoter-luciferase construct, kindly provided by Dr. Fedler Jansen-Durr, encodes nucleotide positions 543 to +263 relative to the transcriptional start site in pXP1 (35).

Colony Formation Assays—Cdc42V12 (CV12)/LAP3 cells were resuspended in 5% calf serum/DMEM containing 0.4% agarose, then overlaid in 5% calf serum/DMEM containing 0.4% agarose in the absence or presence of IPTG. The next morning, cells were trypsinized, then replated in starvation medium (0.5% FBS/DMEM) in the absence or presence of IPTG. Cells were grown for 5–7 days, supplemented with 1 mM IPTG every second day. Constructs and Plasmids—The HA-tagged p70S6k constructs have been described previously (23). The murine cyclin E promoter-luciferase construct, kindly provided by Dr. Fedler Jansen-Durr, encodes nucleotide positions 543 to +263 relative to the transcriptional start site in pXP1 (35).

Cyclin Kinase Assays—LAP3 or CV12/LAP3 cells were treated with 1 mM IPTG overnight where indicated, then starved for 24 h in 0.5% FBS/DMEM. PGDF (20 ng/ml) was added to induce cell cycle entry, and cells were harvested at various times. For cyclin E kinase assays, cells were lysed in Cdk2 lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.5% Nonidet P-40, 0.1% Brij-35, 5 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol). Clarified supernatants were immunoprecipitated with anti-cyclin D antibody and protein G-Sepharose for 2–3 h. Immunocomplexes were washed 3 times in Cdk2 lysis buffer, once in ST buffer (100 mM Tris, pH 7.2, 150 mM NaCl, 1 mM dithiothreitol), then once in 1.5× kinase buffer (30 mM HEPES, pH 7.4, 15 mM MgCl2, 150 µg/ml bovine serum albumin, 1 mM dithiothreitol). Pellets were resuspended in 20 µl of 1.5× kinase buffer, and kinase reactions were initiated by the addition of 10 µl of Cdk2 kinase mixture containing 10 µCi of [γ-32P]ATP (PerkinElmer Life Sciences), 50 µM ATP, and 4 µg of histone HI Sigma. Reactions were performed at 30 °C for 30 min. Reactions were fractionated on SDS-polyacrylamide gels followed by autoradiography to assess histone phosphorylation. For cyclin D kinase assays, cells were lysed in Cdk4 lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 10% glycerol). Clarified supernatants were immunoprecipitated with anti-cyclin D antibody and protein G-Sepharose for 2–3 h. Immunocomplexes were washed 4 times in Cdk4 lysis buffer, once in 50 mM HEPES, pH 7.2, 1 mM dithiothreitol), then once in Cdk4 kinase buffer (75 mM HEPES, pH 7.2, 15 mM MgCl2, 7.5 mM β-glycerophosphate, 1.5 mM sodium vanadate). Samples were resuspended in 20 µl of Cdk4 kinase buffer, and kinase reactions were initiated by the addition of 10 µl of Cdk4 kinase mixture containing 10 µCi of [γ-32P]ATP, 20 µM ATP, and 1 µg of glutathione S-transferase-Rb (kindly provided by Dr. Peter Adams, Fox Chase Cancer Center). Reactions were performed at 30 °C for 30 min. Reactions were fractionated on SDS-polyacrylamide gels followed by autoradiography.

Cyclin E Promoter Activation Assays—LAP3 or CV12/LAP3 cells were seeded at 2.4–2.7 × 105 cells/10-cm dish the day before transfection. The following day, the cyclin E-luciferase reporter plasmid was transfected using LipofectAMINE. After a 4–5 h incubation, cells were allowed to recover overnight in 5% calf serum/DMEM in the presence or absence of IPTG. The next morning, cells were trypsinized, then replated in starvation medium (0.5% FBS/DMEM) in the absence or presence of IPTG. Cells were harvested the following day according to manufacturer instructions (Promega Corp.). Lysates were also immunoblotted in parallel with p70S6k or mitogen-activated protein kinase antibodies to confirm that equal amounts of protein were present in each sample (data not shown). For co-transfections of cyclin E-luciferase with p70S6k, CV12/LAP3 cells were seeded at 3.0–3.4 × 105 cells per 35-mm dish. Cyclin E-luciferase and p70S6k DNA transfections were transfected at a 1:1 ratio, and cells were treated as described above.

Antibodies—Anti-HA, cyclin E, cyclin D, Cdc25A, Cdc42, and p27 antibodies were purchased from Santa Cruz Biotechnologies, Inc. Cyclin E antibody was also purchased from Upstate Biotechnology, Inc. Anti-glutathione S-transferase was affinity-purified from rabbits immunized with glutathione S-transferase peptide. Anti-Erk and p70S6k antisera were kindly provided by Dr. John Blenis. Anti-p70S6k antisera against the C terminus of the rat enzyme was used for immunoblotting; antisera against the N terminus (anti-p70S6k-N2) was used for immunoprecipitation.

Cdc42V12 induction of cyclin E mediated by p70S6k and Cdc42V12/CV12/LAP3 cells were treated with IPTG as indicated, then lysed and immunoprecipitated as previously described (23). Anti-p70S6k-N2 and pre-immune sera were covalently cross-linked to protein A-Sepharose beads. Immunoprecipitates were fractionated on 12% Laemmli gels; the top portion was immunoblotted with anti-p70S6k, and the bottom portion was immunoblotted with anti-HA.

RESULTS

Cdc42V12 Induces Anchorage-independent but Not Mitogen-independent Growth—To elucidate the mechanism by which Cdc42 stimulates progression through G1 phase of the cell cycle, we generated NIH3T3 cells stably expressing Cdc42V12 in an inducible manner (CV12/LAP3). Expression of Cdc42V12 was controlled by a chimeric Lac repressor-VP16 protein, which drives expression of target genes in the presence of IPTG. As seen in Fig. 1A, expression of Cdc42V12 was strongly induced in the presence of IPTG. Anti-Cdc42 immunoblotting revealed that the transgene was expressed at a level below that of endogenous Cdc42. Strikingly, however, this level was sufficient to induce anchorage-independent growth, as judged by the ability of Cdc42V12 to form colonies in soft agar strictly in the presence of IPTG (Fig. 1B). Approximately 10% of CV12/LAP3 cells seeded in the presence of IPTG gave rise to colonies containing at least four cells; ~5% gave rise to macroscopically

![Image](35242.png)
visible colonies as typified in Fig. 1B. In contrast, LAP3 or CV12/LAP3 in the absence of IPTG remained as single cells in suspension.

To determine whether Cdc42V12 could also induce mitogen-independent growth, S phase entry was monitored using tritiated thymidine incorporation of adherent cells. No difference in thymidine incorporation was observed between CV12/LAP3 or parental LAP3 cells under conditions of serum starvation or mitogen addition (data not shown). These results were confirmed using bromodeoxyuridine incorporation (data not shown). Thus, Cdc42V12 is sufficient to induce only a subset of hallmarks of the transformed phenotype, namely anchorage-independent but not mitogen-independent growth, in NIH3T3 cells.

Cdc42V12 Accelerates PDGF-dependent Activation of Cyclin E—but Not Cyclin-D-associated Kinase—The inability of Cdc42 to induce mitogen-independent growth is consistent with a previous report using Rat1 fibroblasts stably and inducibly expressing Cdc42V12 (11). However, other studies suggest that Cdc42 can promote Rb phosphorylation when transiently over-expressed (10), suggesting that it can induce specific molecular events that contribute to G1 progression. We, therefore, undertook a detailed analysis of the cell cycle machinery, examining cyclin D- and cyclin E-associated kinase activity, CV12/LAP3 or parental LAP3 cells were grown either in the absence or presence of IPTG, starved of serum for 24 h, then stimulated for various times with platelet-derived growth factor (PDGF). Anti-cyclin E or anti-cyclin D immunoprecipitates were prepared followed by in vitro kinase assays using histone H1 or Rb as substrate, respectively. Under starved conditions, no induction of cyclin D- or cyclin E-associated kinase activity was seen in CV12/LAP3 cells treated with IPTG, consistent with the notion that Cdc42V12 is insufficient by itself to drive cell cycle entry (the modest increase in cyclin E-Cdk2 activity in CV12/LAP3 treated with IPTG was not consistently observed) (Fig. 2A).

However, a marked acceleration of cyclin E-Cdk2 activation was observed in response to PDGF in induced CV12/LAP3 cells. As seen in Fig. 2A, in parental cells (LAP3) treated with IPTG or CV12/LAP3 in the absence of IPTG, cyclin E activity began to increase at 9–12 h and peaked 15 h after the addition of PDGF. In contrast, in CV12/LAP3 cells treated with IPTG, cyclin E-Cdk2 activation was accelerated, first appearing at 6 h and peaking at 9–12 h. Thus, Cdc42V12 accelerates mitogen-induced activation of cyclin E-Cdk2.

Given that activation of cyclin D normally precedes cyclin E, we analyzed whether cyclin D-Cdk4 activity was similarly accelerated in CV12/LAP3 cells. Surprisingly, the kinetics of cyclin D activation were identical in parental LAP3 cells and CV12/LAP3 cells regardless of the presence of IPTG (Fig. 2B). These results suggest that signals generated by Cdc42 feed into the cell cycle machinery downstream of cyclin D activation and specifically promote activation of cyclin E-Cdk2 complexes.

Cdc42V12 Activates the Cyclin E Promoter in a Mitogen- and Adhesion-independent Manner—The activation of cyclin E-Cdk2 is a multi-step process that requires the coordinated induction of cyclin E mRNA and dephosphorylation of inhibitory sites on Cdk2 as well as degradation of the p27 Cdk inhibitor. Any of these events is a potential target for Cdc42 in modulating cyclin E-Cdk2 activation. We first examined expression of cyclin E by immunoblotting. In LAP3 and uninduced CV12/LAP3, cyclin E levels were low under serum-starved conditions and increased upon PDGF addition (Fig. 3A). Strikingly, induction of Cdc42V12 expression in the absence of mitogen was sufficient to drive cyclin E expression to levels induced by PDGF.

To confirm that the above effects reflected a transcriptional response, we examined the ability of Cdc42V12 to activate a luciferase reporter construct driven by the murine cyclin E promoter. Cells were grown in the presence or absence of IPTG, starved, then assayed for luciferase activity. As shown in Fig. 3B, luciferase activity was strongly increased in the presence of IPTG in CV12/LAP3 cells. The effect of Cdc42V12 on the cyclin E promoter was specific, as no activation of the cyclin D promoter was observed (data not shown). Together, these data reveal that Cdc42V12 is sufficient to induce cyclin E expression in the absence of mitogen.

Because Cdc42V12 induces anchorage-independent growth (Fig. 1), we wished to test whether this correlated with its ability to induce cyclin E expression in the absence of adhesion. Adhesion-mediated proliferative signals are conveyed through integrin-dependent regulation of the actin cytoskeleton (for review, see Ref. 32). A commonly used method for testing whether a signaling event can occur in the absence of adhesion is to test its sensitivity to cytochalasin D (cytoD), which disrupts the actin cytoskeleton (33, 36). We found that Cdc42V12 was able to fully activate the cyclin E promoter in cells treated with cytoD; in fact, activity was consistently enhanced (Fig. 3B). Thus, Cdc42V12 induces activation of the cyclin E promoter in an adhesion- and mitogen-independent manner.

We next examined whether Cdc42V12 might regulate other events associated with cyclin E-Cdk2 activation. Previous reports suggest that Rho family GTPases contribute to the deg-
radiation of the Cdk2 inhibitor p27 (37, 38). However, induction of Cdc42V12 expression had no effect on the accumulation of p27 in serum-starved cells (Fig. 3C). We also examined expression of Cdc25A, the phosphatase that mediates de-phosphorylation of inhibitory sites on Cdk2 (39, 40). Cdc25A expression is induced in response to mitogenic stimulation (39, 40). As seen in Fig. 3D, Cdc42V12 had no effect on basal or mitogen-induced expression of Cdc25A. Together, these results indicate that the effects of Cdc42V12 on cyclin E-Cdk2 are specifically mediated through induction of cyclin E expression.

**Fig. 3.** Cdc42V12 specifically activates the cyclin E promoter in a mitogen- and adhesion-independent manner. A, CV12/LAP3 or LAP3 cells were starved for 24 h in the absence or presence of IPTG. Cells were stimulated with PDGF (20 ng/ml) for 15 h, lysed, then subjected to immunoblotting using anti-cyclin E antibody. B, CV12/ LAP3 or LAP3 cells were transfected with a luciferase reporter construct driven by the murine cyclin E promoter. After transfection, cells were treated with IPTG or not overnight and then starved for 24 h in the presence of IPTG as indicated. Cell extracts were prepared, and luciferase activity was measured. Data are represented as fold activation over LAP3 cells grown in the absence of IPTG. C, CV12/LAP3 or LAP3 cells were grown in the presence or absence of IPTG and then starved for 24 h, again with or without IPTG. After PDGF stimulation for 15 h, cell extracts were prepared and subjected to immunoblotting using anti-p27 antibody. D, cells were grown as in B, except PDGF stimulation was for 8 h. Cell extracts were subjected to immunoblotting using anti-Cdc25A antibody.

**Fig. 4.** Cdc42V12 activation of the cyclin E promoter is mediated by p70 S6 kinase. A, CV12/LAP3 cells were transfected with the cyclin E promoter-luciferase construct. After transfection, cells were treated with IPTG or not overnight and then starved for 24 h in the presence of the indicated pharmacological inhibitor and IPTG. Cell extracts were prepared, and luciferase activity was measured. Data are represented as fold activation over CV12/LAP3 cells grown in the absence of IPTG. Rapamycin (Rap) was used at a final concentration of 20 nM, PD98059 (PD) was at 20 μM, and SB203580 (SB) was at 10 μM. B, CV12/LAP3 cells were co-transfected with the cyclin E promoter-luciferase construct and the indicated vector. p70S6k, WT p70S6k; p70ED4, constitutively active p70S6k.

Cdc42V12 Activates the Cyclin E Promoter through a Rapamycin-sensitive Pathway—Multiple downstream effectors for Cdc42 have been identified, including p70S6k and the mitogen-activated protein kinases Erk and p38. To determine whether any of these molecules mediate Cdc42V12 induction of the cyclin E promoter, pharmacological agents that inhibit the activation of these molecules were used. CV12/LAP3 cells were transfected with the cyclin E-luciferase reporter construct, starved, then treated with various pharmacological inhibitors. Rapamycin, which inhibits the activation of p70S6k but not the mitogen-activated protein kinases Erk and p38, was used at a final concentration of 20 nM. PD98059 (PD) was at 20 μM, and SB203580 (SB) was at 10 μM. B, CV12/LAP3 cells were co-transfected with the cyclin E promoter-luciferase construct and the indicated vector. p70S6k, WT p70S6k; p70ED4, constitutively active p70S6k.

**p70S6k Activates the Cyclin E Promoter—**Rapamycin is an indirect inhibitor of p70S6k activation. Its direct target is mTOR, a protein kinase that phosphorylates and activates at least one substrate, elf4E-BP1, in addition to p70S6k (41, 42). Therefore, to confirm that p70S6k is the relevant mediator of Cdc42V12-dependent activation of the cyclin E promoter, p70S6k constructs were tested for their ability to activate the luciferase reporter. Co-expression of wild type (WT) p70S6k stimulated the cyclin E promoter ~2.5-fold (Fig. 4B). We also examined an allele of p70S6k, p70ED4, that has elevated basal...
activity. In this mutant, five regulatory phosphorylation sites are substituted with acidic amino acids, resulting in elevated kinase activity under serum-starved conditions (43). This mutant activated the cyclin E promoter even more strongly than did wild type p70S6k. Together, these results implicate p70S6k as a key mediator of Cdc42 in activation of the cyclin E promoter and define a novel pathway leading from Cdc42 to the cell cycle machinery.

FIG. 5. Basal p70S6k activity is elevated in Cdc42V12/LAP3 cells. A, CV12/LAP3 or LAP3 cells were starved for 24 h in the absence or presence of IPTG. Cell extracts were immunoblotted with anti-p70S6k or anti-Erk antibodies. Migrations of Erk1 and Erk2 are indicated. B, CV12/LAP3 cells were starved for 24 h in the absence or presence of IPTG and then stimulated where indicated with PDGF (20 ng/ml) for 10 min. Cell extracts were immunoprecipitated with anti-p70S6k or pre-immune serum and then immunoblotted with anti-HA to detect associated Cdc42V12 (top panel) or anti-p70S6k (bottom panel).

c70S6k Activity Is Elevated in CV12/LAP3 Cells and Occurs in the Absence of Adhesion—Our results above indicate that cyclin E promoter activation is induced by Cdc42V12 under starved conditions and is resistant to cytoD. We therefore wished to confirm the activation state of p70S6k under these conditions. We have previously reported that Cdc42V12 co-immunoprecipitates with p70S6k and promotes its hyperphosphorylation and activation (23). p70S6k appears as a quadruplet of bands, with each representing a distinctly phosphorylated species. Activation of p70S6k can be discerned from the relative abundance of the four bands, with the slowest migrating form being catalytically active. In parental LAP3 cells or CV12/LAP3 in the absence of IPTG, the predominant species was the fastest migrating, inactive form of p70S6k (Fig. 5A). The addition of IPTG to CV12/LAP3 cells led to an increase in the proportion of the hyperphosphorylated species of p70S6k (Fig. 5A). Although the effects were modest (likely due to the low level of HA-Cdc42V12 expression), they were nonetheless specific since activation of Erk was unaffected (as indicated by lack of a shift in its electrophoretic mobility) (Fig. 5A). Furthermore, immunoprecipitation of endogenous p70S6k revealed its association with Cdc42V12 in a mitogen-independent manner (Fig. 5B). Thus, induction of the cyclin E promoter by Cdc42V12 correlates with its ability to activate p70S6k in the absence of additional mitogen.

Cdc42V12 is able to activate the cyclin E promoter in the absence of adhesion, as indicated by its resistance to cytoD (Fig. 5B). Immunoblotting of lysates from these cells confirmed that p70S6k was not inhibited by cytoD (Fig. 6). As an alternative method for examining signaling in the absence of adhesion, cells were plated on 1% agarose (suspension) or 5% calf serum and then seeded on tissue culture plates (adherent). Alternatively, cells were allowed to adhere normally to tissue culture plates (adherent (A)) or plated in suspension on 1% agarose (suspension (S)) overnight (right panels). Cell extracts were immunoblotted with anti-p70S6k, anti-phospho-S6, or anti-Erk antibodies as indicated. The active, mobility shifted forms of Erk1 and Erk2 are indicated.

FIG. 6. Activation of p70S6k by Cdc42V12 occurs in the absence of adhesion. CV12/LAP3 cells were resuspended in medium containing 5% calf serum and then seeded on tissue culture plates either in the absence or presence of cytoD (1 μM). Alternatively, cells were allowed to adhere normally to tissue culture plates (adherent (A)) or plated in suspension on 1% agarose (suspension (S)) overnight (right panels). Cell extracts were immunoblotted with anti-p70S6k, anti-phospho-S6, or anti-Erk antibodies as indicated. The active, mobility shifted forms of Erk1 and Erk2 are indicated.

DISCUSSION

The mechanism by which Cdc42 contributes to G1 to S phase progression has remained poorly defined. In the current study, we identify both a proximal effector pathway and a cell cycle target for this Rho GTPase in NIH3T3 cells. Our data reveal that Cdc42V12 stimulates expression of cyclin E through p70S6k. Because additional events (such as Cdk2 de-phosphorylation and p27 degradation) still require the addition of growth factor, Cdc42V12 by itself cannot induce mitogen-independent activation of cyclin E-Cdk2 complexes. However, upon the addition of PDGF these additional events proceed, and cyclin E-Cdk2 activation is accelerated by ∼3–4 h relative to control cells. These results suggest that accumulation of cyclin E is the rate-limiting step in this process in NIH3T3 cells. Strikingly, the kinetics and degree of acceleration induced by Cdc42V12 is comparable with that induced by constitutive expression of cyclin E itself (44–46). In human fibroblasts constitutively expressing cyclin E, cyclin E-associated kinase activity first appears 6–8 h after mitogen addition, 4 h earlier than in control cells.

The activation of cyclin D, E, and A complexes that is required for passage through the restriction point is normally dependent on cell adhesion (for reviews, see Refs. 47–49). Adhesion has been shown to promote cyclin E-Cdk2 activation by different mechanisms in different cell types. At least three mechanisms have been described, including induction of cyclin E expression, down-regulation of p27, and down-regulation of p21 (50–52). Our current studies suggest that in NIH3T3 cells, Cdc42V12 may circumvent the requirement for adhesion in part by inducing cyclin E expression. In addition, others have
shown that activated Cdc42 can induce cyclin D and cyclin A expression in various cell types (10, 24). For cyclin A, induction occurred in an adhesion-independent manner (24); in the case of cyclin D, adhesion dependence was not examined (10). Thus, Cdc42 may induce anchorage-independent growth by promoting activation of both cyclin A and cyclin E complexes. The ability of Cdc42 to induce expression of the various G1 cyclins may be distinct in different cell types. Indeed, we failed to observe activation of the cyclin E promoter by Cdc42V12 in HeLa or Rat1 cells. A model incorporating our current findings with previous studies is shown in Fig. 7. As a final note, our results highlight the complementary role of Rho GTPases in cell cycle machinery, perhaps in a cell type-specific manner.

Because TOR has at least one substrate other than p70S6k that regulates the proliferative status of the cell, eIF4E-BP (41, 42, 64, 65), it was imperative to demonstrate that p70S6k, like Cdc42V12, could also activate the cyclin E promoter. We show that under conditions where cells are mitogenically quiescent, p70S6k is competent to induce the cyclin E promoter. Our results identifying the cyclin E promoter as a target of p70S6k are consistent with two genetic studies linking the TOR/p70S6k/S6 pathway to cyclin E. First, in Drosophila disruption of TOR causes a cell cycle arrest phenotype that can be rescued by cyclin E expression (66). Even more striking are studies of G1 progression in mice with a conditional disruption of S6 (31). In liver cells from these mice, cyclin D-Cdk4 activation proceeded normally, but expression of cyclin E was specifically blocked. In summary, our studies demonstrate that the TOR/p70S6k/S6 pathway plays a central role in Cdc42-mediated G1 progression through induction of cyclin E expression.

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