Cdc42, but not RhoA, regulates cyclin D1 expression in bovine tracheal myocytes

CHRISTIAN P. BAUERFELD, MARC B. HERSHENSON, AND KRISTEN PAGE

Department of Pediatrics, University of Chicago, Chicago, Illinois 60637-1470

Received 2 August 2000; accepted in final form 27 November 2000

Bauerfeld, Christian P., Marc B. Hershenson, and Kristen Page. Cdc42, but not RhoA, regulates cyclin D1 expression in bovine tracheal myocytes. Am J Physiol Lung Cell Mol Physiol 280: L974–L982, 2001.—We previously demonstrated that Rac1 increased cyclin D1 promoter activity in an extracellular signal-regulated kinase (ERK)-independent, antioxidant-sensitive manner. Here, we examined the regulation of cyclin D1 expression by Cdc42 and RhoA. Overexpression of active Cdc42, but not of RhoA, induced transcription from the cyclin D1 promoter. Furthermore, dominant negative Cdc42, but not RhoA, attenuated platelet-derived growth factor-mediated activation of the cyclin D1 promoter. Overexpression of active Cdc42 increased cyclin D1 protein abundance in COS cells. Cdc42-induced cyclin D1 promoter activation was independent of ERK as evidenced by insensitivity to PD-98059, an inhibitor of mitogen-activated protein kinase/ERK kinase (MEK). Furthermore, Cdc42 was neither sufficient nor required for activation of ERK. Similar to Rac1-induced cyclin D1 expression, pretreatment with the antioxidants catalase and ebselen inhibited Cdc42-mediated transcription from the cyclin D1 promoter. Finally, like Rac1, active Cdc42 induced transactivation of the cyclin D1 promoter cAMP response element binding protein/activating transcription factor-2 binding site. Together, these data suggest that in airway smooth muscle cells, Cdc42 and Rac1 share a common signaling pathway to cyclin D1 promoter activation.

activating transcription factor-2; antioxidant; adenosine 5′-cyclic monophosphate response element binding protein; extracellular signal-regulated kinase; guanosine 5′-triphosphatase; platelet-derived growth factor; Rac1

INCREASED AIRWAY SMOOTH MUSCLE proliferation is thought to contribute to airflow obstruction in patients with asthma (8). The signaling mechanisms underlying airway smooth muscle proliferation are not completely understood. Our laboratory and others have shown that extracellular signal-regulated protein kinases (ERKs) increase cyclin D1 expression (30) and DNA synthesis (16, 24) in bovine tracheal myocytes. However, in NIH/3T3 cells, activation of the ERK pathway does not induce all the key events required for transition from the G1 to the S phase of the cell cycle (23), suggesting that an additional signaling pathway is required for cell proliferation. Our laboratory (26) recently demonstrated that Rac1 activates transcription from the cyclin D1 promoter in an ERK-independent, antioxidant-sensitive manner. We now investigated the roles of two additional Rho GTPases, Cdc42 and RhoA, in the regulation of cyclin D1 expression.

The Rho family of GTPases (RhoA, -B, and -C, Rac1 and -2, and Cdc42) control the assembly and organization of the actin cytoskeleton (13). Rho activation leads to stress fiber formation, and Rac induces the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles, whereas Cdc42 induces surface protrusions called filopodia. Rac1 and Cdc42 have also been reported to activate the stress-activated mitogen-activated protein (MAP) kinases, c-Jun amino-terminal kinase (JNK), and p38 (5–7, 9, 10, 20, 33, 38). In addition, there may be significant cross talk between the members of the Rho GTPase family. For example, Cdc42 can activate Rac1, whereas Rac1 can activate RhoA (13, 22, 31).

Rho GTPases have also been demonstrated to play an essential role in cell cycle progression through G1 in NIH/3T3 cells (4, 18, 23). Rac1 has been shown to activate transcription from the cyclin D1 promoter in NIH/3T3 cells (36) and, as noted above, primary bovine tracheal smooth muscle cells (26). Rac1 constitutes part of the NADPH oxidase complex that generates reactive oxygen species (1, 2). In airway smooth muscle, NADPH oxidase activity is required for platelet-derived growth factor (PDGF)- and Rac1-mediated transcription from the cyclin D1 promoter, and growth factor-induced promoter activity is antioxidant sensitive, suggesting that Rac1 activates transcription from the cyclin D1 promoter via the generation of reactive oxygen species (26).

It has also been demonstrated that active Cdc42 (12) and RhoA (36, 39) are each sufficient to induce cyclin D1 protein accumulation in NIH/3T3 cells. However, the precise mechanism that underlies this regulation has not been studied. In this report, we investigated the role of Cdc42 and RhoA in the regulation of cyclin D1 expression in airway smooth muscle cells. We found that Cdc42, but not RhoA, activates transcription from the cyclin D1 promoter in an ERK-independent, antioxidant-sensitive manner similar to that of Rac1. Fur-
thermore, both Rac1 and Cdc42 induce transactivation of the cyclin D1 promoter cAMP response element binding protein (CREB/activating transcription factor (ATF)-2 binding site. These data suggest that in airway smooth muscle cells, Cdc42 and Rac1 share a common signaling pathway to cyclin D1 promoter activation.

EXPERIMENTAL PROCEDURES

Materials. Peroxidase-linked goat anti-rabbit IgG, protein A Sepharose beads, myelin basic protein (MBP), catalase, ebselen, and o-nitrophenyl-β-d-galactoside were purchased from Sigma (St. Louis, MO). PDGF and an anti-Myc tag antibody (clone 9E10) were obtained from Upstate Biotechnology (Lake Placid, NY). PD-98059 was obtained from New England Biolabs (Beverly, MA). [γ-32P]ATP and an enhanced chemiluminescence kit were purchased from DuPont/NEN Research Products (Wilmington, DE). A polyclonal antibody to the light chain IgG was obtained from Zymed Laboratories (South San Francisco, CA). For in vitro phosphorylation assays, a monoclonal antibody against hemagglutinin (HA; 12CA5) was obtained from Babco (Berkeley, CA). The MAC-Select Kk-transfected cell selection kit was purchased from Miltenyi Biotec (Auburn, CA).

Plasmid DNAs encoding constitutively active Cdc42 (pCMV-Myc-V12Cdc42), dominant negative Cdc42 (pCMV-Myc-N17Cdc42), constitutively active RhoA (pEXV-Myc-V14RhoA), and dominant negative RhoA (pEXV-Myc-N19RhoA) were gifts from Richard Pestell (Albert Einstein College of Medicine, Bronx, NY). Plasmid DNA encoding a constitutively active Rac1 (pEXV-Myc-V12Rac1) was a gift from Audrey Minden (Columbia University, New York, NY) (31). HA-tagged ERK2 (pCDNA3-HA-ERK2) was constructed by ligating a DNA fragment that encoded the seven-amino acid influenza HA epitope to the 5′-end of murine ERK2 (14). Plasmid DNA encoding HA-tagged JNK1 was provided by Marsha Rosner (University of Chicago, Chicago, IL) (21). Plasmid DNA encoding recombinant c-Jun was provided by James Posada (University of Vermont, Burlington, VT) (32). Luciferase reporter plasmids encoding the full-length human cyclin D1 promoter (~1745CD1LUC) and the promoter sequences –66 to –40 bp from the transcriptional start site, under the control of a minimal thymidine kinase promoter (CRE-TK81LUC), were gifts from Richard Pestell (3, 34). The PathDetect cis-reporting pSRF-Luc plasmid was purchased from Stratagene (La Jolla, CA). A cDNA-encoding green lantern protein was purchased from GIBCO BRL (Life Technologies, Rockville, MD).

Cell culture. Bovine tracheal smooth muscle cells were isolated as described previously (17). Myocytes of passage 5 or less were studied. Confluent cultures exhibited the typical “hill and valley” appearance and showed specific immunostaining for α-smooth muscle actin. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. In some studies, COS cells (American Type Culture Collection, Manassas, VA) were used and cultured as described above.

Determination of cyclin D1 promoter transcriptional activity. Cells were seeded in 60-mm dishes at 50–80% confluence and incubated in 10% FBS-DMEM overnight. After being rinsed, the cells were incubated with a liposome solution consisting of serum- and antibiotic-free medium, plasmid DNA (total of 1.8 μg/plate), and LipofectAMINE (12 μl/plate; Life Technologies, Gaithersburg, MD). The transfection efficiency in primary cultures of bovine tracheal myocytes was on the order of 10–20%. Cotransfection with viral promoter-driven expression vectors tends to suppress cyclin D1 promoter activity. Therefore, concentration-response curves were generated for each expression vector to determine optimal concentration. cDNA concentrations of 30–50 ng/plate were used. Cells were transiently cotransfected with plasmids that encode the human cyclin D1 promoter subcloned into a luciferase reporter and either the relevant expression vector or the control vector. After 5 h, the liposome solution was replaced with 10% FBS-DMEM. The next day, cells were serum starved for 8 h before stimulation with PDGF (30 ng/ml). Finally, 16 h after PDGF treatment, cells were harvested for analysis of luciferase activity with the lysis buffer provided with the Promega (Madison, WI) luciferase assay system. Luciferase activity was measured at room temperature with a luminometer (Turner Designs, Sunnyvale, CA). Luciferase content was assessed by measuring the light emitted during the initial 30 s of the transcription and the values are expressed in arbitrary light units. The background activity from the cell extracts was typically <0.02 U compared with signals on the order of 102 to 103 U.

Cyclin D1 promoter transcriptional activation was normalized for transfection efficiency by cotransfecting cells with a cDNA that encoded β-galactosidase (30 ng/plate). β-Galactosidase activity was assessed by colorimetric assay with o-nitrophenyl-β-d-galactoside as a substrate (29).

Serum response factor reporter assay. Cells were transiently transfected with the serum response factor (SRF)-luciferase reporter plasmid from Stratagene and either the constitutively active or dominant negative RhoA mutants or the vector control as described in Determination of cyclin D1 promoter transcriptional activity. Selected cells were treated with 10% serum for 16 h, extracted, and assayed for luciferase activity.

Magnetic cell separation. To verify the expression of relevant plasmids in bovine tracheal myocytes, we used a magnetic cell selection system to increase the proportion of transfected cells in whole cell extracts, thereby facilitating immunostaining for the Myc epitope tag (11). Cells were transiently cotransfected as described in Determination of cyclin D1 promoter transcriptional activity with pMACS Kk (2.5 μg/plate) and either constitutively active forms of Cdc42, RhoA or Rac1, dominant negative forms of Cdc42 or RhoA, or their respective control vectors (7.5 μg/plate). After 48 h, cells were rinsed with PBS, trypsinized, pooled, and centrifuged (1,000 rpm for 3 min at room temperature). The resulting pellet was resuspended in 320 μl of PBS supplemented with 0.5% BSA and 5 mM EDTA (PBE buffer) and incubated with 80 μl of magnetic beads for 15 min at room temperature. After incubation, the volume of the respective samples was adjusted to 2 ml with PBE buffer and loaded onto the magnetized separation column in 500-μl aliquots. After collection of the flow-through (nonselected cells), the column was washed four times with 2 ml of PBE. After being washed, the column was detached from the magnet, 1 ml of PBE was added, and the selected cells were flushed out with a plunger. The collected cells were centrifuged (1,000 rpm for 3 min at room temperature), the supernatant was discarded, and the pellet was resuspended in lysis buffer.

Preparation of cell extracts for immunoblotting. Cells were cultured in six-well plates and serum starved for 24 h before PDGF treatment (30 ng/ml for 16 h). The cells were washed in PBS (150 mM NaCl and 0.1 M phosphate, pH 7.5) and extracted in a lysis buffer containing 50 mM Tris, pH 7.5, 40
mM β-glycerophosphate, 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 200 μM NaN₃, 200 μM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Insoluble materials were removed by centrifugation (13,000 rpm for 10 min at 4°C), and the supernatant was transferred to fresh microcentrifuge tubes.

Immunoblotting. Cell extracts (10 μg for cyclin D1, 50 μg for Myc) were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose by semidry transfer (Hoefer, San Francisco, CA). After incubation with the relevant antibody, signals were amplified and visualized with anti-rabbit or anti-mouse IgG and enhanced chemiluminescence.

Measurement of ERK and JNK activation. Cells were transiently cotransfected with cDNAs that encoded HA-tagged ERK2 or HA-JNK1 and the expression vector of interest. Cells were seeded in 100-mm plates at a density of 5 × 10⁵ cells/plate and incubated in 10% FBS-DMEM overnight. After being rinsed, the cells were incubated in a solution consisting of serum- and antibiotic-free medium, plasmid DNA (10 μg/plate), and LipofectAMINE (40 μl/plate). After 5 h, the solution was replaced with 10% FBS-DMEM. Forty-eight hours after transfection, the cells were serum-starved in DMEM. The next day, selected cultures were treated with PDGF (30 ng/ml for 10 min). Activation of ERK and JNK was then assessed by immunoprecipitation of the epitope tag followed by an in vitro phosphorylation assay with MBP or recombinant c-Jun as substrates, respectively (16, 26).

Fig. 1. Cdc42 and RhoA mutants are expressed and functional in primary bovine myocytes. A: cells were transiently cotransfected with plasmids encoding MACS K⁺ and either constitutively active forms of Cdc42 (pCMV-Myc-V12Cdc42), RhoA (pEXV-Myc-V14RhoA), or Rac1 (pEXV-Myc-V12Rac1); dominant negative forms of Cdc42 (pCMV-Myc-N17Cdc42) or RhoA (pEXV-Myc-N19RhoA); or their vector controls. 1, Presence; 2, absence. Cells selected by immunomagnetic separation were centrifuged and extracted. Cellular proteins were resolved by SDS-PAGE and probed for the Myc epitope tag. B: cells were transiently cotransfected with hemagglutinin (HA)-tagged c-Jun amino-terminal kinase (JNK) 1 and either constitutively active (V12Cdc42), dominant negative (N17Cdc42) Cdc42, or empty vector. Selected cultures were treated with anisomycin (50 ng/ml for 30 min). JNK activity was assessed by immunoprecipitating cell extracts with an anti-HA antibody (12CA5) and measuring in vitro phosphorylation with c-Jun as the substrate (top). The level of HA-JNK1 expression was determined by immunoblotting (bottom). Data are representative of 2 separate experiments. C: cells were transiently cotransfected with plasmids encoding serum response factor (SRF)-LUC along with the constitutively active (V14RhoA) or dominant negative (N19RhoA) RhoA mutants or vector control. To control for transfection efficiency, cells were also cotransfected with pCMV-β-galactosidase. Data are means ± SE; n = 4 independent experiments. D: cells were cotransfected with active Cdc42 or active RhoA and green lantern protein (GLP). Two days after transfection, cells were visualized with a fluorescence microscope. Transfected cells were distinguished by the green fluorescence. Cdc42- and RhoA-transfected cells demonstrated substantial cytoplasmic spreading relative to empty vector-transfected cells, consistent with Rho GTPase-induced cytoskeletal changes.
Treated cells were washed twice with PBS and incubated in a lysis buffer consisting of 50 mM Tris·HCl, pH 7.5, 1% Triton X-100, 40 mM β-glycerophosphate, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 200 μM Na$_3$VO$_4$, and 0.2 mM phenylmethylsulfonyl fluoride (30 min at 4°C). Insoluble materials were removed by centrifugation (13,000 rpm for 10 min at 4°C). Cell lysates were then incubated for 3 h with 30 μl of protein A Sepharose beads precoupled with the 12CA5 anti-HA antibody. Immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl$_2$, 1 mM dithiothreitol, 200 μM Na$_3$VO$_4$, and 10 mM p-nitrophenyl phosphate. Immune complexes were resuspended in a final volume of 30 μl of kinase buffer and incubated for 20 min at 30°C with 5 μCi of [α-32P]ATP and 0.25 mg/ml of MBP. Reactions were terminated by adding Laemmli buffer and boiling. Samples were resolved on a 10% sodium dodecyl sulfate gel, and the proteins were transferred to a nitrocellulose membrane by semidry transfer. After being studied with Ponceau stain, the membrane was exposed to film, and substrate phosphorylation was assessed by optical scanning (Jandel Scientific, San Rafael, CA).

To confirm that apparent differences in ERK or JNK activity were not related to alterations in expression of the epitope-tagged ERK, nitrocellulose membranes were probed with the anti-HA antibody 12CA5. Signals were amplified.

---

**Fig. 2.** Cdc42, but not RhoA, is required and sufficient for cyclin D1 expression in bovine tracheal myocytes. **A:** cells were transiently cotransfected with cDNAs encoding the full-length cyclin D1 promoter subcloned into a luciferase reporter gene (−1745CD1LUC) and either empty vector, active Cdc42, or dominant negative Cdc42 (pCMV-Myc-N17Cdc42). Selected cultures were treated with platelet-derived growth factor (PDGF; 30 ng/ml for 16 h). To control for transfection efficiency, cells were also cotransfected with pCMV-β-galactosidase. Data are means ± SE; n = 5–8 independent experiments. Overexpression of active Cdc42 significantly increased cyclin D1 promoter activity, whereas overexpression of dominant negative Cdc42 significantly reduced PDGF-mediated activation of the promoter (P < 0.05 by ANOVA and Student-Newman-Keuls multiple range test). **B:** cells were transiently cotransfected with cDNAs encoding the full-length cyclin D1 promoter and either empty vector, active RhoA, or dominant negative RhoA (pEXV-Myc-N19RhoA). RhoA did not significantly alter cyclin D1 promoter activity. Data are means ± SE for 3–5 independent experiments. **C:** COS cells were transiently transfected with active Cdc42. Cellular proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal antibody against cyclin D1 (1:1,000 dilution). These results are typical of 2 separate experiments.
and visualized with peroxidase-linked rat anti-mouse κ light chain IgG and enhanced chemiluminescence.

Statistical analysis. When applicable, significance was assessed by one-way analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by the Student-Newman-Keuls multiple range test.

RESULTS

Verification of plasmid expression and function in bovine tracheal myocytes. To verify the expression of Myc-tagged Cdc42, RhoA, and Rac1 in bovine tracheal myocytes, we enriched the population of transfected cells using immunomagnetic separation and then performed immunoblots to detect protein expression. Anti-Myc immunoblots of transfected cells demonstrated expression of the Myc-tagged Cdc42, RhoA, and Rac1 proteins (Fig. 1A).

To confirm that the Cdc42 mutants we employed were functional, we assessed the effects of active and dominant negative Cdc42 on the activation of JNK, a known downstream target of Cdc42 (5, 7, 20). Overexpression of active Cdc42 increased JNK activity, whereas overexpression of dominant negative Cdc42 attenuated anisomycin-induced activity (Fig. 1B). Our laboratory has previously shown (25) that overexpression of active Rac1 induces JNK1 activation.

The Rho family GTPases regulate transcriptional activation by SRF (15). We therefore verified the function of the RhoA mutants by examining their effects on the expression of an SRF-sensitive reporter plasmid. Overexpression of active RhoA was sufficient to induce transcription from the SRF-LUC promoter, whereas overexpression of the dominant negative RhoA attenuated serum-induced transcription (Fig. 1C).

Finally, we examined the morphology of cells cotransfected with green lantern protein and cDNAs encoding active Cdc42 or RhoA (Fig. 1D). Cdc42 and RhoA each induced cytoplasmic spreading. The observed changes in cytoskeletal organization confirmed the ability of these reagents to affect downstream biological pathways in cultured bovine tracheal myocytes.

Cdc42, but not RhoA, induces transcription from the cyclin D1 promoter. Bovine tracheal smooth muscle cells were cotransfected with cDNAs encoding the human full-length cyclin D1 promoter subcloned into a luciferase reporter gene and empty vector or a constitutively active Cdc42. Selected cultures were pretreated with PD-98059 (30 μM for 1 h) before stimulation with PDGF (30 ng/ml for 15 h). Data are means ± SE; n = 4 independent experiments. Treatment with PD-98059 significantly inhibited PDGF- but not Cdc42-induced activation of the cyclin D1 promoter (P < 0.05 by ANOVA and Student-Newman-Keuls multiple range test).

The Rho family GTPases regulate transcriptional activation by SRF (15). We therefore verified the function of the RhoA mutants by examining their effects on the expression of an SRF-sensitive reporter plasmid. Overexpression of active RhoA was sufficient to induce transcription from the SRF-LUC promoter, whereas overexpression of the dominant negative RhoA attenuated serum-induced transcription (Fig. 1C).

Finally, we examined the morphology of cells cotransfected with green lantern protein and cDNAs encoding active Cdc42 or RhoA (Fig. 1D). Cdc42 and RhoA each induced cytoplasmic spreading. The observed changes in cytoskeletal organization confirmed the ability of these reagents to affect downstream biological pathways in cultured bovine tracheal myocytes.

Cdc42, but not RhoA, induces transcription from the cyclin D1 promoter. Bovine tracheal smooth muscle cells were cotransfected with cDNAs encoding the human full-length cyclin D1 promoter subcloned into a luciferase reporter and mutant forms of Cdc42 or RhoA. Overexpression of active Cdc42 (V12Cdc42) increased transcription from the cyclin D1 promoter, whereas overexpression of the dominant negative RhoA attenuated serum-induced transcription (Fig. 1C).

Finally, we examined the morphology of cells cotransfected with green lantern protein and cDNAs encoding active Cdc42 or RhoA (Fig. 1D). Cdc42 and RhoA each induced cytoplasmic spreading. The observed changes in cytoskeletal organization confirmed the ability of these reagents to affect downstream biological pathways in cultured bovine tracheal myocytes.

Cdc42, but not RhoA, induces transcription from the cyclin D1 promoter. Bovine tracheal smooth muscle cells were cotransfected with cDNAs encoding the human full-length cyclin D1 promoter subcloned into a luciferase reporter and mutant forms of Cdc42 or RhoA. Overexpression of active Cdc42 (V12Cdc42) increased transcription from the cyclin D1 promoter, whereas overexpression of the dominant negative RhoA attenuated serum-induced transcription (Fig. 1C).
active Cdc42 in the presence of PDGF stimulation increased cyclin D1 transcription in an additive fashion. In contrast, overexpression of active RhoA (V14RhoA) did not increase basal or PDGF-mediated cyclin D1 transcription. Furthermore, PDGF-mediated activation of the cyclin D1 promoter was not attenuated by overexpression of dominant negative RhoA (N19RhoA) (Fig. 2B). These data suggest that Cdc42, but not RhoA, regulates cyclin D1 expression.

To determine whether induction of Cdc42 increases cyclin D1 protein abundance as well as promoter activity, we transiently transfected COS cells with active Cdc42 and analyzed the amount of cyclin D1 protein. COS cells were used because of their increased transfection efficiency relative to primary bovine tracheal myocytes. Overexpression of active Cdc42 was sufficient to increase cyclin D1 protein accumulation (Fig. 2C).

**Regulation of cyclin D1 by Cdc42 is ERK independent.** We asked whether Cdc42 induces transcription from the cyclin D1 promoter via activation of ERK. Cells were transiently transfected with cDNAs encoding HA-tagged ERK2 and either constitutively active or dominant negative Cdc42. ERK activity was assessed by immunoprecipitation of the HA tag followed by an in vitro phosphorylation assay with MBP as a substrate. Overexpression of dominant negative Cdc42 did not attenuate PDGF-mediated ERK activation, nor did overexpression of active Cdc42 increase ERK2 activity (Fig. 3A). To further examine the requirement of ERK for Cdc42 regulation of cyclin D1 transcription, we used the MAP kinase/ERK kinase (MEK) chemical inhibitor PD-98059. Pretreatment of cells with PD-98059 had no effect on Cdc42-mediated activation of the cyclin D1 promoter (Fig. 3B). Together, these data suggest that Cdc42-induced activation of cyclin D1 is independent of ERK.
Regulation of cyclin D1 by Cdc42 is antioxidant sensitive. Previously, our laboratory (26) has shown that Rac1-mediated transcription from the cyclin D1 promoter is dependent on the generation of reactive oxygen intermediates by NADPH oxidase. We therefore investigated the effect of antioxidants on Cdc42-induced activation of cyclin D1 promoter activity. Treatment with catalase attenuated Cdc42-mediated transcription from the cyclin D1 promoter in a concentration-dependent manner (Fig. 4A). In addition, treatment with ebselen, a glutathione peroxidase mimic, also attenuated Cdc42-mediated activation (Fig. 4B). These data suggest that activation of the cyclin D1 promoter by Cdc42 is sensitive to antioxidants.

Rac1 and Cdc42 each activate the cyclin D1 promoter at the CREB/ATF-2 binding site. We have identified the cyclin D1 promoter CREB/ATF-2 response element located −58 to −52 bp 5′ from the transcription start site to be a Rac1 response element (28). To determine whether Cdc42 and Rac1 activate the cyclin D1 promoter by a similar mechanism, cells were transiently cotransfected with a luciferase reporter plasmid that encoded the cyclin D1 promoter sequences from −66 to −40 bp under the control of a minimal thymidine kinase promoter (CRE-TK81LUC) (34), and active alleles of Rac1, Cdc42, or RhoA. Rac1 and Cdc42, but not RhoA, were sufficient to activate this reporter plasmid (Fig. 5).

DISCUSSION

The Rho family of GTPases has recently been implicated in the regulation of cell cycle progression. In both NIH/3T3 cells (36) and primary bovine myocytes (26), Rac1 regulated transcription from the cyclin D1 promoter. However, the other GTPase family members have not been thoroughly studied. Reports of cross talk between the small GTPases led us to investigate the role of Cdc42 and RhoA in transcriptional regulation of cyclin D1.

In this study, we found that in primary bovine tracheal myocytes 1) overexpression of active Cdc42 increased transcription from the cyclin D1 promoter, whereas overexpression of dominant negative Cdc42 attenuated PDGF-induced activation; 2) overexpression of constitutively active or dominant negative RhoA had no effect on cyclin D1 promoter activity; 3) Cdc42 activated the cyclin D1 promoter in an ERK-independent, antioxidant-sensitive manner, similar to that of Rac1 (26); and 4) both Rac1 and Cdc42 induced transcription of the cyclin D1 promoter at a common CREB/ATF-2 binding site. Taken together, these data suggest that in airway smooth muscle cells, Cdc42 and Rac1 share a common signaling pathway to cyclin D1 promoter activation.

Overexpression of active Cdc42 increased cyclin promoter activity in bovine tracheal myocytes two- to threefold. Although such increases may not be as great as those generated in cell lines (3), they are nearly so. Stimulation by PDGF gave similar results. Finally, observed changes in promoter activity correlated with changes in protein abundance. Taken together, these data suggest that the observed increases are meaningful.

It has previously been demonstrated (12) that Cdc42 is sufficient to induce cyclin D1 protein accumulation in NIH/3T3 cells. In addition, overexpression of Dbl family members, which serve as guanine nucleotide exchange factors for Cdc42 and RhoA, has been shown to induce transcription from the cyclin D1 promoter in NIH/3T3 cells (37), consistent with the notion that Cdc42 regulates cyclin D1 expression. In the present study, we confirm that Cdc42 is a positive regulator of cyclin D1 expression and show that this effect is mediated at the transcriptional level.

Previous reports examining the contribution of RhoA to cyclin D1 expression have been less conclusive. In NIH/3T3 cells, overexpression of active RhoA was sufficient to induce transcription from the cyclin D1 promoter (36, 39). In IIC9 cells, a subclone of Chinese hamster embryo fibroblasts, overexpression of a dominant negative RhoA did not decrease PDGF-mediated cyclin D1 protein abundance (35), suggesting that RhoA is not required for transcription from the cyclin D1 promoter. In the present study, we found that although RhoA was sufficient to induce cytoskeletal...
reorganization, it was neither required nor sufficient for cyclin D1 transcription. Discrepancies between these studies may be due to the use of different cyclin D1 reporter constructs (we used the full-length promoter) or, perhaps, cell type-specific differences in RhoA signaling.

Significant cross talk between Rho GTPase family members exists. In Swiss 3T3 cells, Cdc42 activates Rac1, whereas Rac1 can activate RhoA (13, 22, 31). Because Cdc42 and Rac1 regulate cyclin D1 transcription in a similar manner, we speculate that Cdc42 acts upstream of Rac1 in this signaling pathway. However, due to the suppressive effects of multiple expression vectors on cyclin D1 promoter activity, we were unable to test this hypothesis directly (e.g., by coexpressing active Cdc42 and dominant negative Rac). On the other hand, because RhoA and Rac1/Cdc42 have different effects on bovine tracheal smooth muscle cyclin D1 promoter activity, it appears that in the context of cyclin D1 expression, Cdc42 and Rac1 were not upstream activators of RhoA in our system. This model is consistent with a study (15) in NIH/3T3 cells that showed that activated forms of Cdc42 or Rac1 signal to the serum response element in a Rho-independent manner, suggesting that Rac1 and/or Cdc42 and RhoA do not necessarily function in a linked cascade.

Our laboratory (26) previously showed that Rac1-induced transcription from the cyclin D1 promoter is dependent on the generation of reactive oxygen species by NADPH oxidase. However, Rac1 and Cdc42 interact with multiple target proteins, including the JNK and p38 MAP kinases (5–7, 9, 10, 20, 33, 38). We have confirmed that Rac and Cdc42 activate both stress-activated MAP kinases in bovine tracheal myocytes (Page and Hershenson, unpublished data). Because p38 negatively regulates transcription from the cyclin D1 promoter in NIH/3T3 cells (19) and bovine tracheal myocytes (27), it is conceivable that Rac1 and Cdc42 activate both stimulatory and inhibitory pathways to cyclin D1 expression. The relative activities of these opposing pathways, in conjunction with other signaling pathways, may determine the final outcome of Rho GTPase signaling. This model may explain the divergent results described above, including differences in Rho GTPase signaling between cell types.

REFERENCES


24. Orsini MJ, Krymskaya VP, Esztheras AD, Benovic JL, Panettieri RA, and Penn RB. MAPK superfamily activation...


