Characterization of a Rac1 Signaling Pathway to Cyclin D1 Expression in Airway Smooth Muscle Cells*

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Kristen Page‡, Jing Li‡, Joshua A. Hodge‡, Pai T. Liu‡, Terry L. Vanden Hoek§, Lance B. Becker§, Richard G. Pestell§, Marsha R. Rosner, and Marc B. Hershenson***

From the §Department of Pediatrics, ¶Department of Medicine, ‡Department of Pharmacological and Physiological Sciences and the Ben May Institute for Cancer Research, University of Chicago, Chicago, Illinois 60637 and the Albert Einstein Cancer Center, Department of Medicine and Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

We examined the importance of the Rho family GTPase Rac1 for cyclin D1 promoter transcriptional activation in bovine tracheal myocytes. Overexpression of active Rac1 induced transcription from the cyclin D1 promoter, whereas platelet-derived growth factor (PDGF)-induced transcription was inhibited by a dominant-negative allele of Rac1, suggesting that Rac1 functions as an upstream activator of cyclin D1 in this system. Rac1 forms part of the NADPH oxidase complex that generates reactive oxygen species, as measured by the fluorescence of dichlorofluorescein-loaded cells, and this was blocked by the glutathione peroxidase mimic ebselen. Pretreatment with ebselen, catalase, and the flavoprotein inhibitor diphenylene iodonium each attenuated PDGF- and Rac1-mediated cyclin D1 promoter activation, while having no effect on the induction of cyclin D1 by mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase-1 (MEK1), the upstream activator of ERKs. Antioxidant treatment also inhibited PDGF-induced cyclin D1 protein expression and DNA synthesis. Overexpression of an N-terminal fragment of p67phox, a component of NADPH oxidase which interacts with Rac1, attenuated PDGF-induced cyclin D1 promoter activity, whereas overexpression of the wild-type p67 did not. Finally, Rac1 was neither required nor sufficient for ERK activation. Taken together, these data suggest a model by which two distinct signaling pathways, the ERK and Rac1 pathways, positively regulate cyclin D1 and smooth muscle growth.

Excess airway smooth muscle cell proliferation is thought to contribute to airflow obstruction in patients with asthma (1). The signaling mechanisms underlying airway smooth muscle proliferation are not completely understood. We have investigated the role of extracellular signal regulated kinases (ERKs),\(^1\) cytosolic serine/threonine kinases of the mitogen-activated protein kinase superfamily, in bovine tracheal myocyte DNA synthesis. ERK activation is required for platelet-derived growth factor (PDGF)-induced DNA synthesis (2), and also regulates the transcriptional activation of cyclin D1 (3), a critical regulator of G1 progression in these cells (4).

Studies in immortalized cell lines (5, 6) and primary hepatocytes (7) have demonstrated a requirement for Rho family GTPases in G1 progression. The mechanisms underlying this requirement are not precisely known. Rac constitutes part of the phagocyte NADPH oxidase complex that generates reactive oxygen species such as H\(_2\)O\(_2\) (8, 9). This enzyme, by donating an electron, catalyzes the reaction: 2 O\(_2\) + NADPH → 2 O\(_2\) + NADP + H\(^+\). The superoxide produced is subsequently converted to H\(_2\)O\(_2\). The human NADPH oxidase consists of at least seven components: two membrane spanning polypeptides, p22phox and gp91phox (which comprise cytochrome b\(_{558}\)), three cytoplasmic polypeptides, p47phox, p67phox, and p40phox, Rap1A and Rac, the last of which is required for oxidase activation.

Increasing evidence suggests that reactive oxygen species may play a role in mitogen-activated cell signaling. Recent data suggest that the generation of H\(_2\)O\(_2\) by NADPH oxidase occurs in tissues other than phagocytes, including aortic adventitia, kidney, liver, vascular smooth muscle cells, and fibroblasts (10–13). Production of reactive oxygen species has been noted upon growth factor stimulation in arterial smooth muscle cells (14) and chondrocytes (15). Antioxidants have been shown to inhibit growth factor-induced ERK activation and DNA synthesis in arterial smooth muscle cells (14), growth factor-induced c-fos expression in chondrocytes (15), and phorbol ester-induced cyclin D1 expression and DNA synthesis in a subclone of NIH3T3 cells (16). Activation of Rac1 has been noted to increase intracellular reactive oxygen species in HeLa cells (17), NIH3T3 cells (18), and rabbit synovial fibroblasts (19). Finally, it has recently been demonstrated that a Rac1 effector site critical for the activation of NADPH oxidase is also required for the mitogenic effect of Rac1 in rat embryonic fibroblasts (20).

In NIH3T3 cells, Rac1 activates transcription from the cyclin D1 promoter (21, 22), suggesting a mechanism by which Rac1 signaling may regulate G1 progression. However, the requirement of reactive oxygen species for transcriptional activation of cyclin D1 has not been tested.

In the present study, we examined the importance of the Rho

\(^1\) The abbreviations used are: ERK, extracellular signal-regulated kinase; BrdUrd, bromodeoxyuridine; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein; DMEM, Dulbecco’s minimum essential medium; DPI, diphenylene iodonium; FBS, fetal bovine serum; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1; PDGF, platelet-derived growth factor; MBP, myelin basic protein; FBS, fetal bovine serum.
family GTPase Rac1 for airway smooth muscle cyclin D1 expression. Overexpression of active Rac1 induced transcription from the cyclin D1 promoter. PDGF-induced transcription was also inhibited by a dominant-negative allele of Rac1, suggesting that Rac1 is a critical upstream activator of the cyclin D1 promoter in these cells. PDGF stimulated a substantial increase in the fluorescence of 2',7'-dichlorofluorescein diacetate (DCFH-DA)-loaded cells which was blocked by the glutathione peroxidase mimetic ebselen, implying that growth factor treatment increases the concentration of intracellular reactive oxygen species. Furthermore, antioxidants (ebselen, catalase) and inhibitors of NADPH oxidase (the flavoprotein inhibitor diphenylene iodonium, and the N-terminal fragment of p67phox) attenuated PDGF- and Rac1-mediated cyclin D1 promoter activation, but had no effect on the response induced by MEK1, the upstream activator of ERKs. Finally, Rac1 was neither required nor sufficient for ERK activation. Taken together, these data suggest that Rac1-induced cyclin D1 expression is mediated by the generation of intracellular reactive oxygen species. Furthermore, these data suggest a model by which two distinct signaling pathways, the ERK and Rac1 pathways, positively regulate smooth muscle growth.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-human α-smooth muscle actin, peroxidase-linked goat anti-rabbit IgG, protein A-Sepharose beads, myelin basic protein, ebselen (2-phenyl-1,2-benzisoxazol-3(2H)-one), catalase, diphenylene iodonium, α-nitrophenyl-β-d-galactosidase, and bromodeoxyuridine (BrduUr) were purchased from Sigma. PDGF was obtained from Upstate Biotechnology (Lake Placid, NY). PD98059 was obtained from New England Biolabs (Beverly, MA). Anti-γ-32P-ATP and an enhanced chemiluminescence kit were purchased from NEN Life Science Products. Antibodies against cyclin D1 and BrduUr were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Becton-Dickinson (San Jose, CA), respectively. A peroxidase-linked rat anti-mouse x light chain IgG was obtained from Zymed Laboratories Inc. (South San Francisco, CA). DCFH-DA was purchased from Molecular Probes (Eugene, OR). For in vitro phosphorylation assays, a monoclonal antibody against hemagglutinin (12CA5) was obtained from Babco (Berkeley, CA).

Plasmid DNA encoding a constitutively active (pEXV-Myo-V12Rac1) and dominant-negative forms of Rac1 (pEXV-N17Rac1) (23, 24) were gifts from Audrey Minden (Columbia University) and Simon Cook (Onyx Pharmaceuticals, Richmond, CA). Plasmid DNA encoding a constitutively active form of MEK1 (pCMV-MEK-2E) was provided by Dr. Dennis Templeton (Case Western Reserve University) (25). cDNAs encoding wild-type p67phox (pEXV-p67phox), and an N-terminal fragment (1–174) of p67phox (pEXV-p67phox1–174) were provided by Alan Hall (University College, London) (26). A hemagglutinin-tagged ERK2 (pCDNA3-HA-ERK2) was constructed by ligating a DNA fragment encoding the 7-amino acid influenza hemagglutinin epitope to the 5′ end of murine ERK2 (27). To construct the reporter −163CD1-Luc and −66CD1-Luc were also constructed, as described previously (28).

**Cell Culture**—Bovine tracheal smooth muscle cells were isolated as described previously (30). Myocytes of passage number 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 Dulbecco’s minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS), 1% non-essential amino acids, penicillin (100 units/ml), and streptomycin (100 μg/ml).

**Determination of Cyclin D1 Promoter Transcriptional Activity**—Cells were seeded into 60-mm dishes at 50–80% confluence and incubated in 10% FBS/DMEM overnight. After rinsing, cells were incubated with a liposome solution consisting of serum- and antibiotic-free medium, plasmid DNA (total of 1.8 μg/plate) and LipofectAMINE (Life Technologies, Gaithersburg, MD; 12 μl/plate). Cells were transiently co-transfected with plasmids encoding the human cyclin D1 promoter subclone into a luciferase reporter vector (−1745CD1LUC) and either empty vector, constitutively active mutants of Rac1 (pEXV-Myo-V12Rac1) or MEK1 (pCMV-MEK2E), or a dominant-negative Rac1 (pEXV-N17Rac1). To control for transfection efficiency, cells were also co-transfected with pCMV-β-galactosidase. Data were calculated as luciferase/β-galactosidase normalized to the control vector (or for PDGF, untreated cells). Data shown represent mean ± S.E. for six independent experiments.

Finally, 8 h after PDGF treatment, cells were harvested for analysis of luciferase activity using lysis buffer provided with the Promega Luciferase Assay system (Madison, WI). Luciferase activity was measured at room temperature using a luminometer (Turner Designs, Sunnyvale, CA). Luciferase content was assessed by measuring the light emitted during the initial 30 s of the reaction and the values expressed in arbitrary light units. The background activity from cell extracts was typically less than 0.02 units, compared with signals on the order of 10^2-10^3 units.

Cyclin D1 promoter transcriptional activation was normalized for transfection efficiency by co-transfecting cells with a cDNA encoding β-galactosidase (30 ng/plate). β-Galactosidase activity was assessed by colorimetric assay using o-nitrophenyl-β-D-galactoside as a substrate (31).

The transfection of primary cells holds certain limitations that should be noted here. First, transfection efficiency, as assessed by β-galactosidase staining, was less than 50%. Second, co-transfection with viral promoter-driven expression vectors tended to suppress cyclin D1 promoter activity. Concentration-response curves were therefore generated for each expression vector to determine optimal concentration. In all cases, concentrations of 30–100 ng/plate were used. Also, due to unacceptably low cyclin D1 promoter activity levels, we were unable to co-transfect cells with more than two expression vectors (β-galactosidase plus one signaling intermediate of interest).

**Preparation of Cell Extracts for Immunoblotting**—Cells were cultured in 6-well plates and serum starved for 24 h prior to PDGF treatment (30 ng/ml for 16 h). Cells were washed in phosphate-buffered saline (150 mm NaCl, 0.1 μM 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 sodium fluoride, 200 μM Na3VO4, 20 μM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Lysates were centrifuged (13,000 rpm for 10 min at 4 °C) and the supernatant transferred to fresh microcentrifuge tubes.

**Western Analysis of Cyclin D1 Protein Levels**—Extracts (10 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose by semidy transfer (Hoefer, San Francisco, CA). After incubation with a polyclonal antibody against cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), signals were amplified and visualized using anti-rabbit IgG and enhanced chemiluminescence.

**Measurement of ERK Activation**—Cells were transiently co-transfected with cDNAs encoding HA-tagged ERK2 and the expression vector of interest. Cells were seeded into 100-mm plates at a density of 5 x 10^5 cells/plate and incubated in 10% FBS/DMEM overnight. After rinsing, 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 h after transfection, cells were serum-starved in DMEM.
The next day, selected cultures were treated with PDGF (30 ng/ml for 10 min). Activation of ERK was then assessed by immunoprecipitation of the epitope tag, followed by an in vitro phosphorylation assay using MBP as a substrate, as described (27). Treated cells were washed twice with phosphate-buffered saline 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 sodium fluoride, 2 mM EDTA, 200 mM 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-hemagglutinin 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 (20 min at 30 °C) with 5 µCi of [α-32P]ATP and 0.25 mg/ml MBP. Reactions were terminated by adding Laemmli buffer and boiling. Samples were resolved on a 10% sodium dodecyl sulfate gel and the proteins transferred to a nitrocellulose membrane by semi-dry transfer. After Ponceau staining, the mem-
three independent experiments.

To confirm that apparent differences in ERK activity were not related to alterations in expression of the epitope-tagged ERK, nitrocellulose membranes were probed with the anti-hemagglutinin antibody 12CA5. Signals were amplified and visualized using peroxidase-linked rat anti-mouse 12 clone IgG and enhanced chemiluminescence.

Anti-phospho-ERK Immunoblots—In some experiments, the ERK activity of whole cell lysates was estimated by determining the level of phosphorylated ERKs. These experiments utilized a phosopho-specific antibody (32) which recognizes ERKs only when phosphorylated at Thr183 and Tyr185, which are required for full enzymatic activity (33). Cell extracts were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose, as described above. After incubation with antibody, signals were amplified and visualized using anti-rabbit IgG and enhanced chemiluminescence.

Measurement on Intracellular Hydrogen Peroxide Levels—Cells were grown on coverslips in 35-mm2 tissue culture-treated dishes. Coverslips were placed inside a perfusion chamber and imaged with an inverted phase/epifluorescence microscope. Fluorescence was measured with a cooled slow-scanning PC-controlled camera coupled to imaging software for quantification of changes in emission fluorescence. Cells were loaded with DCFH-DA (10 μM) and dichlorofluorescein (DCF) fluorescence was determined by imaging with an inverted phase/epifluorescence microscope, as described (34). After a 1-h stabilization period, selected cultures were treated with PDGF (30 ng/ml). In some instances, cells were pretreated with ebselen (30 μM).

Fractional Labeling with Bromodeoxyuridine—Subconfluent bovine tracheal myocytes were serum starved in DMEM for 24 h. Eight h following PDGF treatment, cells were incubated with bromodeoxyuridine (10 μM) and fluoroodeoxyuridine (1 μM). In some wells, catalase (100–1000 units/ml) was added 45 min prior to growth factor treatment. Sixteen h later, myocytes were fixed in periodate lysine paraformaldehyde buffer and the DNA precipitated with 2 M HCl. After acid neutralization with 0.1 M borate buffer, pH 9.0, cells were permeabilized with 0.2% Triton X-100. Cells were then immunostained with fluorescein isothiocyanate-labeled anti-BrdUrd and counterstained with propidium iodide.

RESULTS

Activation of the Cyclin D1 Promoter by Rac1—We examined the importance of the Rho family GTPase Rac1 for cyclin D1 promoter activation in bovine tracheal myocytes. Cells were co-transfected with cDNAs encoding the cyclin D1 promoter subcloned into luciferase and a constitutively active form of Rac1. Overexpression of active Rac1 induced transcription from the cyclin D1 promoter (Fig. 1). As reported previously (3), overexpression of a constitutively active MEK1 also increased

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cyclin D1 promoter activity. To determine the requirement of Rac1 for PDGF-induced cyclin D1 promoter activity, cells were transiently co-transfected with the dominant-negative Rac1 and the luciferase-tagged cyclin D1 promoter. N17Rac1 attenuated the PDGF-induced transcription from the cyclin D1 promoter (Fig. 1). Taken together, these experiments suggest that Rac1 is an important upstream activator of the cyclin D1 promoter in airway smooth muscle cells.

The Rac1 Signaling Pathway to Cyclin D1 Transcriptional Activation Is Distinct from the ERK Pathway in Cultured Airway Smooth Muscle Cells—In HEK293 human kidney fibroblasts, co-expression of Raf-1 and Rac1 caused a synergistic increase in both MEK1 and ERK activation which was greater than the sum of their effects alone (35, 36). Since both active Raf1 and MEK1 induce cyclin D1 promoter activity (above), it is conceivable that activation of Rho family kinases enhances cyclin D1 transcriptional activation via activation of ERK. We therefore assessed the requirement and sufficiency of Rac1 for ERK activation. Cells were transiently co-transfected with hemagglutinin-tagged ERK2 and either V12Rac1 or N17Rac1. ERK activity was assessed by immunoprecipitation with an anti-hemagglutinin antibody followed by in vitro phosphorylation using MBP as the substrate. Rac1 was neither required nor sufficient for activation of ERK2 (Fig. 2, A and B). We also tested the effects of a synthetic MEK inhibitor, PD98059, on Rac1-induced responses. Pretreatment with PD98059 attenuated PDGF-induced cyclin D1 promoter activity, but did not decrease Rac1-activated transcription (Fig. 2C). These data strongly suggest that activation of ERK is not required for Rac1-induced transcription from the cyclin D1 promoter.

Since inhibition of either Rac1 or MEK1 attenuated PDGF-induced cyclin D1 promoter activity, we also sought to determine whether Rac1 might function downstream of MEK1 in this pathway. We reasoned that if this model were correct, then the downstream transcription factor targets of each intermediate would activate the same site on the cyclin D1 promoter. To test this, we examined the response of two 5′ cyclin D1 promoter fragments, −163CD1LUC and −66CD1LUC, to PDGF, Rac1, and MEK1 stimulation. Although cyclin D1 promoter basal activity was somewhat decreased in these 5′ deletion mutants (data not shown), PDGF and Rac1 responsiveness was maintained, whereas MEK1 responsiveness was lost upon deletion of base pairs −163 to −66 (Fig. 2D). These data imply that the downstream transcription factor targets of the MEK1 and Rac1 pathways act at different sites in the cyclin D1 promoter, and provide evidence that Rac1 and ERK function on distinct signaling pathways.

Growth Factor Treatment Increases the Generation of Reactive Oxygen Species—Production of reactive oxygen species has been noted upon growth factor stimulation of arterial smooth muscle cells (14) and chondrocytes (15). To test whether PDGF treatment induces the intracellular generation of reactive oxygen species in bovine tracheal myocytes, cells were loaded with DCFH-DA (10 μM). Upon loading, DCFH-DA enters the
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cell and the acetate group is cleaved by cellular esterases, trapping the non-fluorescent DCFH inside. Subsequent oxidation by reactive oxygen species, particularly \( \text{H}_2\text{O}_2 \) and hydroxyl radical, yields the fluorescent product DCF. PDGF (30 ng/ml) increased DCF fluorescence almost 2-fold over baseline (Fig. 3). This increase was similar in magnitude to that observed after hypoxic exposure of cardiac myocytes (34), and was blocked by ebselen (30 \( \mu \text{M} \)), a glutathione peroxidase mimetic (37).

Cyclin D\(_1\) Expression Is Sensitive to Antioxidants—To examine the potential role of reactive oxygen species in growth factor and Rac1-induced cyclin D\(_1\) promoter activity, we tested the effect of ebselen on PDGF-, Rac1-, and MEK1-mediated responses. PDGF- and V12Rac1-induced cyclin D\(_1\) promoter activities were attenuated by ebselen pretreatment, whereas MEK-2E-induced responses were not (Fig. 4A). Pretreatment with catalase also attenuated PDGF-induced cyclin D\(_1\) promoter activity (Fig. 4B). Finally, ebselen and catalase each decreased cyclin D\(_1\) protein abundance (Fig. 4C). Antioxidant pretreatment had no effect on PDGF-induced ERK phosphorylation, however (Fig. 4D). Taken together, these data suggest that there are antioxidant-sensitive (Rac1-mediated) and antioxidant-insensitive (mediated by MEK1/ERK) pathways to cyclin D\(_1\) expression in bovine tracheal myocytes, and provide further evidence that Rac1 and ERK function on distinct signaling pathways.

We have previously demonstrated in bovine tracheal myocytes that cyclin D\(_1\) is required for DNA synthesis (4). We therefore reasoned that antioxidant pretreatment would decrease DNA synthesis in these cells. Pretreatment of cells with catalase abolished PDGF-induced fractional BrdUrd labeling (Fig. 5).

Role of NADPH Oxidase Complex in Rac1-mediated Cyclin D\(_1\) Promoter Activation—Recent data suggest that the generation of \( \text{H}_2\text{O}_2 \) by NADPH oxidase occurs in tissues other than phagocytes, including vascular smooth muscle cells (10–13). The human NADPH oxidase consists of at least seven components including two membrane spanning polypeptides, p22\(^{\text{phox}}\) and gp91\(^{\text{phox}}\) (which comprise the flavoprotein cytochrome \( b_{558} \)), p67\(^{\text{phox}}\), and Rac, the last of which is required for oxidative activation. We tested the effects of a flavoprotein inhibitor, diphenylene iodonium (DPI), on transcription from the cyclin D\(_1\) promoter. PDGF- and V12Rac1-stimulated cyclin D\(_1\) promoter activity were attenuated by DPI, whereas MEK2E-induced responses were unaffected (Fig. 6A), consistent with the notion that Rac1 and ERK function on distinct pathways to cyclin D\(_1\) promoter activation. DPI also decreased PDGF-induced cyclin D\(_1\) protein abundance (Fig. 6B). Pretreatment of cells with DPI did not alter ERK phosphorylation (Fig. 6C).

The further examine the role of the NADPH oxidase complex in growth factor-induced cyclin D\(_1\) promoter activity, we transiently co-transfected cells with cDNAs encoding the full-length cyclin D\(_1\) promoter and either wild-type p67\(^{\text{phox}}\) or an N-terminal fragment of this protein, p67\(^{\text{phox}\text{N}}\) (1-199). Since Rac1 interacts directly with p67\(^{\text{phox}}\), previous studies have shown the N-terminal fragment to function as a specific inhibitor of the Rac1 signaling pathway (26). PDGF stimulation increased cyclin D\(_1\) promoter activity in control and wild-type p67\(^{\text{phox}}\)-transfected cells, whereas those transfected with the N-terminal fragment no longer responded to PDGF (Fig. 6D). Taken together, these data suggest that NADPH oxidase function is required for PDGF- and Rac1-induced transcription from the cyclin D\(_1\) promoter in bovine tracheal myocytes.

Discussion

Studies in NIH 3T3 cells (5, 6) and rat hepatocytes (7) have demonstrated a requirement for Rac1 in \( G_1 \) progression. The mechanisms underlying this requirement are not precisely known. Activation of Rac1, a constitutent of the NADPH oxidase complex, has been noted to increase intracellular reactive oxygen species (17–19). It has also been demonstrated that a Rac1 effector site critical for the activation of NADPH oxidase is required for the mitogenic effect of Rac1 in rat embryonic fibroblasts (20). In NIH3T3 cells, Rac1 activates transcription from the cyclin D\(_1\) promoter (21, 22), suggesting a mechanism by which Rac1 signaling may regulate \( G_1 \) progression. However, the requirement of reactive oxygen species for transcriptional activation of cyclin D\(_1\) has not been tested.

In this study, we found in primary bovine tracheal myocytes that (i) overexpression of dominant-negative Rac1 inhibits PDGF-induced promoter activity, whereas active Rac1 induces transcription from the cyclin D\(_1\) promoter; (ii) growth factor treatment increases the intracellular generation of reactive oxygen species; (iii) antioxidant pretreatment attenuates PDGF- and Rac1-induced cyclin D\(_1\) promoter activity, as well as PDGF-induced cyclin D\(_1\) protein abundance and DNA synthesis; and (iv) inhibitors of NADPH oxidase decrease PDGF- and Rac1-induced responses. Taken together, these data strongly suggest that Rac1 is an important upstream activator of the cyclin D\(_1\) promoter in airway smooth muscle cells. Furthermore, these findings suggest that Rac1-induced transcription from the cyclin D\(_1\) promoter is dependent on the generation of reactive oxygen species by NADPH oxidase.

Our findings that dominant-negative and constitutively active forms of Rac1 regulate cyclin D\(_1\) promoter activity in cultured bovine tracheal myocytes imply that Rac1 is required and sufficient for transcription from the cyclin D\(_1\) promoter in airway smooth muscle. However, because these experiments depend on the transient overexpression of mutant alleles of Rac1 in the cell, their results should be interpreted with caution. First, dominant-negative proteins may bind upstream signaling intermediates, thereby blocking the activity of other proteins that bind to the same site. An alternative approach for confirming the requirement of Rac1 for growth factor-induced cyclin D\(_1\) promoter activity would be to use other inhibitors. However, reagents such as \textit{Clostridium botulinum} exoenzyme C3 transferase, \textit{Clostridium sordellii} lethal toxin, \textit{Clostridium difficile} toxin B, and lovastatin are each poorly specific for Rac1 (38–41). Second, it is possible that overexpression of a constitutively active protein could induce supraphysiologic outcomes. Thus, activation of Rac1 may be insufficient for transcription from the cyclin D\(_1")promoter under normal physiologic conditions. Nevertheless, when evaluated in the context of additional experiments employing inhibitors of NADPH oxidase, the function of which depends on Rac1, we believe that our results strongly suggest that Rac1 is an upstream activator of cyclin D\(_1\) promoter activity in airway smooth muscle cells. Whether it is sufficient to do so under physiologic conditions, or requires the activation of other pathways (such as the ERK pathway, see below) will require further investigation.

Transcription from the cyclin D\(_1\) promoter has been demonstrated to be a consequence of ERK activation in several cell types, including bovine tracheal myocytes (3, 28, 29, 42). In HEK293 human kidney fibroblasts, co-expression of the serine threonine kinase Raf-1 and Rac1, Rac2, Cdc42, RhoA, or RhoB caused a synergistic increase in both MEK1 and ERK activation which was greater than the sum of their effects alone (35, 36), suggesting that Rac1 activation of cyclin D\(_1\) promoter activity might be mediated by the activation of the ERK pathway. The enhancement of ERK activity by Rac1 was mediated by p21 (Cdc42/Rac)-activated kinase-1, or PAK1, which phosphorylates MEK1 on a site important for Raf-1-MEK1 interaction. Using a panel of Rac1 mutants, Westwick and colleagues (21) demonstrated that PAK1 binding was required for Rac1-
induced transcription from the cyclin D1 promoter, consistent with this model. We therefore examined potential interactions between the Rac1 and ERK pathways in our system. Rac1 was neither required nor sufficient for ERK activation. Furthermore, inhibition of ERK activation by pretreatment with the chemical inhibitor PD98059 did not attenuate Rac1-induced responses. These data suggest that, in cultured airway smooth muscle cells, ERK activation is not the major mechanism of Rac1-induced cyclin D1 transcription. We also found that Rac1-but not MEK1-induced transcription was sensitive to pretreatment with antioxidants or inhibitors of NADPH oxidase, and that Rac1 and MEK1 target different response elements in the cyclin D1 promoter. Taken together, these data strongly suggest that Rac1 and MEK1 stimulate transcription from the cyclin D1 promoter through distinct signaling pathways.

Once generated, reactive oxygen species may influence a number of signaling processes, including activation of NF-κB (43–45) and p38 mitogen-activated protein kinase (46). Treatment with antioxidants has been demonstrated to attenuate Rac1-induced activation of NF-κB in HeLa cells (17), and PDGF treatment induces NF-κB activity in mouse fibroblasts (47), consistent with the notion that NF-κB may mediate the Rac1/reactive oxygen species signal. The smallest 5’ promoter fragment we studied (−66CD1LUC) holds consensus sequences for both NF-κB and cAMP response element/activation transcription factor family proteins, the downstream phosphorylation targets of p38 (48, 49). Since this deletion fragment remained both PDGF and Rac1 responsive, it is possible that one or both of these transcription factors is involved in growth factor-induced cyclin D1 promoter activation.

We and others have previously shown in cultured airway smooth muscle cells that activation of the ERK pathway is required for cyclin D1 expression (3) and DNA synthesis (2, 50). In the present study, we demonstrate the importance of the Rho family GTPase Rac1 for airway smooth muscle cyclin D1 expression. Taken together, these data suggest a model whereby two distinct signaling pathways, the ERK pathway and Rac1 pathway, regulate airway smooth muscle G1 progression.

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