[Frontiers in Bioscience 5, d258-267, February 1, 2000]

# MITOGEN-ACTIVATED SIGNALING AND CELL CYCLE REGULATION IN AIRWAY SMOOTH MUSCLE

**Kristen Page and Marc B. Hershenson**

*Department of Pediatrics, University of Chicago Children's Hospital, 5841 S. Maryland Avenue, MC 4064, Chicago, IL 60637-1470*

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## 1. ABSTRACT

Increased airway smooth muscle mass has been demonstrated in patients with bronchopulmonary dysplasia and asthma. These data highlight the need for a precise understanding of the events involved in airway smooth muscle mitogenesis. To that end, investigators have developed cell culture systems adopting tracheal and bronchial myocytes from different species. A growing body of literature suggests that common signal transduction pathways regulate airway smooth muscle cell cycle entry across species lines. This review summarizes what is known about mitogen-activated signal transduction in airway smooth muscle cells. The extracellular signal regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI 3-kinase) pathways appear to be major positive regulators of airway smooth muscle proliferation. It is also conceivable that growth factor stimulation of airway smooth muscle simultaneously elicits signaling through negative regulatory pathways such as the p38 mitogen-activated protein (MAP) kinase pathway, perhaps as a safeguard against excessive growth.

## 2. INTRODUCTION

Growing airways are covered with a well-formed layer of smooth muscle cells by the end of the embryonic period of fetal lung development (1). With airway circumferential and axial growth, this layer increases in size, a result of both cellular hypertrophy and hyperplasia. It has been demonstrated that smooth muscle mass is abnormally increased in the airways of premature infants with bronchopulmonary dysplasia (2, 3), due in part to excess cell proliferation (4). Data describing the growth of airway smooth muscle during normal postnatal development are lacking, however.

Increased airway smooth muscle mass has also been demonstrated in patients with non-fatal (5) and fatal asthma (5-13). In the most convincing study to date, Ebina and colleagues (13) examined the airway thickness and smooth muscle cell number of patients with fatal asthma using state-of-the-art stereological techniques. Two subgroups of asthmatic airways were found, one in which smooth muscle mass was increased only in the central bronchi (Type I) and another in which smooth muscle thickness was increased throughout the airway tree (Type II). In Type I, smooth muscle hyperplasia was responsible for central airway smooth muscle thickening, whereas in Type II, cellular hypertrophy was present over the length of the airway tree.

Increased expression of epidermal growth factor (EGF), a mitogen for human airway smooth muscle (14), has been noted in the airways of asthmatic patients (15, 16). In addition, when added to cultured human airway smooth muscle cells, bronchoalveolar lavage fluid from asthmatic airways increased the ERK activation, cyclin $D_1$ protein abundance, [$^3$H]-thymidine incorporation and cell number, relative to that from control subjects (17). Finally, excess airway smooth muscle DNA synthesis has been demonstrated in two animal models of airways disease, hyperoxic exposure and allergen sensitization (18-20).

Taken together, the above data strongly suggest that excess smooth muscle proliferation is present in the airways of patients with bronchopulmonary dysplasia and asthma, and highlight the need for a precise understanding of the events involved in airway smooth muscle mitogenesis. To that end, investigators have developed cell culture systems adopting tracheal and bronchial myocytes

from different species. A growing body of literature suggests that common signal transduction pathways regulate airway smooth muscle cell cycle entry across species lines. A summary of mitogen-activated signal transduction in airway smooth muscle follows below.

## 3. BRIEF OVERVIEW OF CELL CYCLE REGULATION

Regulation of mammalian cell proliferation by extracellular signals occurs in the transition from $G_0$ to $G_1$ of the cell cycle. A key event in this transition is phosphorylation of the 110 kD retinoblastoma protein (Rb) by the cyclin $D_1$/cyclin-dependent kinase-4 (cdk4) dimer. Cyclin $D_1$, cdk4, proliferating cell nuclear antigen (PCNA) and a cyclin-dependent kinase inhibitor, $p21^{Cip1}$, are induced as part of the delayed early response to mitogenic stimulation (21). The cyclin $D_1$/cdk4 dimer titrates $p27^{Kip1}$, another inhibitor of cdk activity (22), and also enters into complexes with PCNA and $p21^{Cip1}$ (23, 24). PCNA is a cofactor of DNA polymerase delta (25). $p21^{Cip1}$ may act as an assembly factor, promoting binding of cyclin $D_1$ with cdk4. As $G_1$ progresses, $p27^{Kip1}$ is degraded by the ubiquitin-proteasome pathway (26). Once enough cyclin $D_1$ and cdk4 are synthesized and enough $p27^{Kip1}$ degraded, steric inhibition by p27Kip1 is exceeded, leading to phosphorylation and activation of cdk4 by cdk4-activating kinase (CAK) (27, 28). Activation of cdk4, in turn, leads to hyperphosphorylation of Rb (29), a key regulator of S-phase traversal. Rb is phosphorylated near the G1/S restriction point and accumulates phosphate until cells exit from mitosis (30). Once phosphorylated, Rb releases the transcription factors E2F1-3, which activate genes required for DNA replication such as DNA polymerase alpha (31). Titration of p27 by cyclin $D_1$/cdk4 also frees cyclin E/cdk2 complexes, which contribute to Rb phosphorylation and the modification of preinitiation complexes which trigger DNA replication (32). Finally, in late $G_1$, cyclin A is induced and assembles with cdk2, marking the irreversible decision to enter S phase (G1 restriction point) (33).

Ectopic overexpression of either cyclin D or cyclin E accelerates progression through G and reduces the proliferative requirement for serum-derived growth factors (34-36). Conversely, abolition of cyclin D or cyclin E activity through the use of neutralizing antibodies or antisense oligonucleotides effectively blocks entry into S phase (34, 37, 38). The requirement of cyclin $D_1$ for S phase traversal has been confirmed in bovine tracheal myocytes (39).

## 4. GROWTH FACTOR STIMULATION OF AIRWAY SMOOTH MUSCLE CELLS

Many studies have focused on smooth muscle cell proliferation in response to growth factor stimulation. Airway smooth muscle cells proliferate in response to peptide growth factors ligating receptor tyrosine kinases (40-43) as well as to bronchoconstrictor substances associated with G protein-coupled seven transmembrane receptors (44-51). Sensitivity to growth factor activation appears to be species-specific. For instance, histamine is mitogenic for human airway smooth muscle (51, 52), but does not induce proliferation in bovine cells (41). Nevertheless, the signaling pathways and downstream targets initiated by growth factor stimulation appear to be remarkably constant across species lines (see below).

For the last five years, our laboratory has concentrated on the signaling pathways responsible for PDGF-induced DNA synthesis in bovine tracheal myocytes. We will therefore focus on this system as a model of airway smooth muscle proliferation. The PDGF receptor comprises two distinct chains (A and B) which are dimerized by a disulfide bond, and can exist in three possible isoforms, AA, AB, or BB. Typically, growth factor binding to a receptor tyrosine kinase activates the receptor's intracellular kinase domain, leading to the phosphorylation of specific tyrosine residues inside the kinase domain. Ligand binding of PDGF induces formation of a stable receptor dimer, which in turn causes one receptor molecule to phosphorylate the other in the dimer (53). EGF, a monomeric molecule, likely binds simultaneously to two receptor molecules (54). Phosphorylation of tyrosine residues inside the kinase domain further increases kinase activity, leading to phosphorylation of other sites in the receptor outside the kinase domain (55). These phosphotyrosine residues serve as binding sites for downstream signal transduction molecules containing Src-homology 2 (SH2) domains. For example, the PDGF receptor holds nine phosphotyrosine domains, one which is important for receptor tyrosine kinase activity and eight which interact with different signal transduction proteins. The latter include the growth factor receptor binding protein Grb2, phosphatidylinositol 3-kinase, phospholipase C-gamma, the GTPase-activating protein GAP, the Src tyrosine kinase, and protein tyrosine phosphatase 1D (56). We have characterized signaling pathways stemming from three of these PDGF receptor binding proteins (see below).

## 5. ROLE OF THE ERK SIGNALING PATHWAY IN CELL CYCLE PROGRESSION

Grb2 is found in a stable complex with the nucleotide exchange factor Son of sevenless (Sos). Binding of this complex to the receptor tyrosine kinase induces Sos to bind to Ras, a 21 kD membrane-bound GTPase. Once activated, Ras may activate a number of downstream signaling pathways, including the extracellular signal regulated kinase (ERK) pathway (figure 1). ERKs are members of the mitogen-activated protein (MAP) kinase superfamily of cytosolic serine/threonine kinases that participate in the transduction of growth and differentiation-promoting signals to the cell nucleus. ERK activation has been shown to be required for DNA synthesis in a wide variety of cell systems, including bovine, rat and human airway smooth muscle (45, 57-60). The classical route to ERK activation involves Ras, Raf-1, a 74 kD cytoplasmic serine/threonine kinase, and MAP kinase/ERK kinase kinase (MEK)-1, a 45 kD dual function kinase. Several reports confirm that these intermediates are involved in ERK activation in airway smooth muscle.

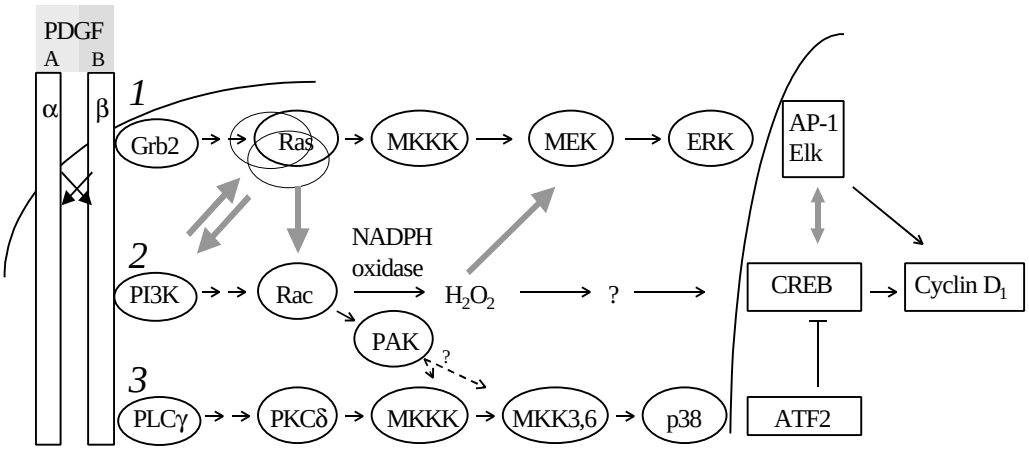


**Figure 1.** Model illustrating three signaling pathways regulating transcription from the cyclin $D_1$ promoter in airway smooth muscle. The ERK and Rac1/PI 3-kinase pathways positively regulate cyclin $D_1$ expression (pathways 1 and 2, respectively); a third pathway, the p38 pathway, negatively regulates transcription from the cyclin $D_1$ promoter. Potential pathways of crosstalk are shown by the broad grey arrows. MEKK refers to a specific MAP kinase kinase kinase called MAP kinase/ERK kinase kinase; MKKK refers to a generic MAP kinase kinase kinase.

Microinjection of the anti-pan Ras neutralizing antibody inhibited DNA synthesis in human airway smooth muscle cells (61), and overexpression of a dominant-negative form of H-Ras inhibited PDGF-mediated ERK activation in bovine tracheal myocytes (62). (Interestingly, Ras did not appear to be necessary for phorbol ester-induced ERK activation.) Overexpression of a kinase-dead mutant of Raf-1 inhibited endothelin-induced ERK activation in rat tracheal myocytes (44). Finally, chemical or dominant-negative inhibition of MEK-1 inhibits ERK activation and DNA synthesis in bovine, rat and human airway smooth muscle cells (45, 57-60).

One point at which bovine tracheal myocyte signaling may diverge from other cell types concerns the activation of MEK-1. Activation of MEK-1 occurred independently of Raf-1 in these cells (63) and instead involves a novel MEK-1 kinase (64).

Although activated ERK has been demonstrated to induce phosphorylation or associate with numerous nuclear transcription factors, the precise downstream targets of ERK in airway smooth muscle cells are not known. However, as in other cell types (65-67), it has been demonstrated in bovine tracheal myocytes that ERK activation is an upstream activator of transcription from the cyclin $D_1$ promoter (68). Thus, the ERK pathway appears to constitute an important regulator of entry into the cell cycle and G1 progression in airway smooth muscle.

In NIH 3T3 cells, constitutive activation of MEK-1, while sufficient to increase ERK activation and expression of cyclin $D_1$, is insufficient for maximal phosphorylation of the retinoblastoma protein, degradation of the cyclin dependent kinase inhibitor p27, and cyclin A expression (69). Furthermore Ras, but not ERK was shown to be required for growth factor-induced degradation of p27 in IIC9 fibroblasts (70). Finally, ectopic overexpression of cyclin $D_1$ is insufficient for S-phase traversal (34, 36). Taken together, these data suggest that the ERK/cyclin $D_1$ pathway is insufficient for cell cycle progression, and that Ras coordinates cell cycle progression by regulating signaling through both ERK-dependent and ERK-independent signaling pathways.

## 6. ROLE OF PI 3-KINASE

PI3-kinase is a heterodimeric lipid kinase comprised of an 85 kD regulatory subunit and a 110 kD catalytic subunit which phosphorylates phosphatidylinositol at the D-3 hydroxyl of the inositol ring, forming the phosphatidylinositides phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-diphosphate and phosphatidylinositol 3,4,5-triphosphate. As noted above, ligation of the PDGF receptor may induce binding and activation of PI 3-kinase (figure 1). Ras has also been noted to interact with PI 3-kinase (71). Growth factors activate PI 3-kinase in human (43, 72) and bovine airway smooth muscle cells (73). Chemical inhibitors of PI 3-kinase, wortmannin and LY294002, inhibit airway smooth muscle cyclin $D_1$ expression (73) and DNA synthesis (73-75). Overexpression of the catalytic subdomain of PI 3-kinase in bovine tracheal myocytes induced transcription from the cyclin $D_1$ promoter but failed to activate ERK (73), suggesting that PI 3-kinase signaling occurs independently of ERK. Similarly, wortmannin and LY-294002 markedly inhibited EGF-induced PI 3-kinase activation in human airway smooth muscle cells but had no effect on ERK activation (75).

## 7. ROLE OF RAC1

Phosphoinositide products of PI 3-kinase may also influence the translocation of guanine nucleotide exchange factors, the upstream activators of GTPases (figure 1) (76). The Rho family GTPases (Rho A-C, Rac1 and 2, and Cdc42), through their regulation of the actin cytoskeleton and interactions with multiple target proteins, may influence cell cycle progression. In Swiss 3T3

fibroblasts, Rac1 is required for cell cycle progression (77, 78). In addition, Rac1 (79) and Cdc42, but not RhoA (C. Bauerfeld, K. Page, M. Hershenson, unpublished data) have been shown to function as upstream activators of cyclin $D_1$ expression in bovine tracheal myocytes.

Rac1 forms part of the NADPH oxidase complex that generates reactive oxygen species such as $H_2O_2$. Intracellular reactive oxygen intermediates are increased following growth factor treatment of both bovine (79) and rat tracheal myocytes (80). Accordingly, treatment with antioxidants attenuates both growth factor induced cyclin $D_1$ expression and DNA synthesis in these cells (79, 80). Overexpression of active Rac1 does not activate ERK in bovine tracheal myocytes, and Rac1-induced transcription from the cyclin $D_1$ promoter is insensitive to the chemical MEK inhibitor PD98059 (79), suggesting that Rac1-mediated cell cycle progression, like that following activation of PI 3-kinase, is independent of ERK activity.

## 8. INHIBITION OF AIRWAY SMOOTH MUSCLE CELL PROLIFERATION

Substances which induce a sustained increase in the intracellular concentration of cyclic AMP (cAMP) have been long known to inhibit airway smooth muscle growth (52, 81-87). However, the precise mechanism by which this occurs is unknown. In bovine tracheal myocytes, pre-treatment with forskolin decreases cyclin $D_1$ protein abundance and promoter activity while inducing the phosphorylation and DNA binding of cAMP response element binding protein (CREB)-1. Taken together, these data suggest that cAMP suppresses cyclin $D_1$ gene expression via phosphorylation and transactivation of CREB. However, it remains unclear whether this is the primary mechanism of cAMP-induced growth inhibition, or whether the inhibition of upstream signaling pathways is involved. cAMP does not inhibit growth factor-induced activation of ERKs in bovine (63) or rat (58) airway smooth muscle, suggesting that the effect of cAMP on growth does not involve the ERK signaling pathway. Glucocorticoids also inhibit human airway smooth muscle cyclin $D_1$ expression and DNA synthesis in an ERK-independent manner (88). On the other hand, PI 3-kinase (74) has been shown to be cAMP-sensitive.

Growth factor treatment of airway smooth muscle cells also induces activation of the two stress-activated MAP kinases, p38 and Jun amino-terminal kinase (JNK) (47, 62, 89), consistent with the notion that these intermediates, like ERKs, play a role in the growth regulation (figure 1). However, based on the other types of signals which activate p38 and JNK (cellular stress and proinflammatory cytokines), it is possible that these pathways are involved in growth inhibition, rather than mitogenesis (see below). The p38 MAP kinase family now consists of four isoforms. p38alpha was originally identified in lipopolysaccharide-stimulated mouse macrophages and was found to have substantial homology to the yeast high osmolarity glycerol kinase (90-93). Since then, three additional isoforms, beta, gamma and delta have been cloned (94-97). p38alpha, beta and delta are somewhat ubiquitously expressed, whereas p38gamma is primarily restricted to skeletal muscle (96). The p38 MAP kinases are phosphorylated and activated by MAP kinase kinase (MKK)-3, MKK-4, and MKK-6. MKK-6 appears to strongly activate all p38 isoforms, whereas MKK-3 preferentially activates p38alpha and beta (98-102). MKK-4 appears to phosphorylate and activate both JNK1 and p38alpha (92, 93). A number of distinct MAP kinase kinase kinases have been found to activate MKK-3 and MKK-6, including MAP kinase/ERK kinase kinase (MEKK)-1 (103, 104), mixed lineage kinase (MLK)-2 (104) and MLK-3 (105), MAP three kinase (MTK)-1 (106), apoptosis signal-regulating kinase (ASK) (107) and TAK-1, a potential mediator of TGF-beta signaling (108).

We have recently obtained data suggesting that selective activation of p38 inhibits airway smooth muscle cell cycle progression (109), as it does in CCL39 hamster lung fibroblasts (67). Chemical inhibition of p38 by SB202190 and SB203580 increased transcription from the cyclin $D_1$ promoter and cyclin $D_1$ protein abundance. Furthermore, transient transfection of bovine myocytes with dominant-negative forms of MKK3 or MKK6 increased transcription from the cyclin $D_1$ promoter. On the other hand, overexpression of constitutively active mutants of MKK3 or MKK6 decreased both basal and PDGF-mediated cyclin $D_1$ promoter activity. Since cyclin $D_1$ is a critical regulator of cell cycle progression (39), these data suggest that p38 is an important negative regulator of cell cycle progression in airway smooth muscle cells.

Paradoxically, p38 may be stimulated by Ras and Rac1 (62, 110-112), suggesting that GTPases may simultaneously activate positive and negative growth regulatory pathways, perhaps as a safeguard against excessive growth.

## 9. POTENTIAL ROLE OF PROTEIN KINASE C (PKC) ISOFORMS

PKC is a complex family including three types of isoenzymes. The conventional isoforms (alpha, $beta_1$, $beta_2$ and gamma) are activated by calcium, phorbol esters and phosphatidylserine, whereas the novel isoforms (delta, epsilon, eta, theta and mu) are calcium-insensitive and activated by phorbol esters and phosphatidylserine. The atypical isoforms (zeta, tau/lambda) are calcium and phorbol ester-insensitive and activated by phosphatidylserine. PKC alpha, $beta_1$, $beta_2$, delta, epsilon, and zeta, but not gamma or eta, are expressed in bovine tracheal myocytes (113), whereas PKC alpha, $beta_1$, $beta_2$, delta, epsilon, theta, eta, zeta, tau and mu have each been identified in human tracheal myocytes (114). Conventional and novel PKCs may be activated in vivo by diacylglycerol that is formed from phospholipids upon receptor-mediated activation of phospholipases. As noted above, ligand binding of the PDGF receptor leads to the phosphorylation of tyrosine residues which then serve as binding sites for signal transduction molecules containing Src-homology 2 (SH2) domains, including phospholipase C-gamma. Accordingly, growth factor stimulation has been noted to activate PKC delta in vascular smooth muscle (115), NIH 3T3 cells and monocytes (116).

Different PKC isoforms may have distinct roles in the regulation of cell proliferation. For example, PKCzeta activity increases in proliferating human airway smooth muscle (114). In NIH3T3 cells, PKCepsilon is a powerful growth stimulus, whereas PKCalpha and delta inhibit growth (117). PKCdelta also inhibits cell cycle progression in vascular smooth muscle cells (118), capillary endothelial cells (119) and rat colonic epithelial cells (120). Finally, in preliminary studies, we have found that overexpression of active PKCdelta attenuates growth factor-induced transcription from the cyclin $D_1$ promoter, perhaps via the aforementioned p38 inhibitory pathway (K. Page, M. Hershenson, unpublished data).

## 10. PERSPECTIVE

In recent years, the signaling pathways regulating airway smooth muscle growth have been elucidated. Although the substances mitogenic for airway smooth muscle may vary across species lines, the signal transduction mechanisms linking receptor ligation with DNA synthesis appear to be highly conserved. For example, the ERK and PI 3-kinase signaling pathways appear to constitute the major paths required for cell proliferation in both human (60, 72, 121) and bovine airway smooth muscle cells (57, 73, 74).

The signaling pathways regulating cell proliferation appear to be comparable in vascular and airway smooth muscle. Activation of ERK and PI 3-kinase has been shown to be required for vascular smooth muscle proliferation (122-124), and inhibition of proliferation by cAMP occurs via suppression of cyclin $D_1$ expression (125, 126), as it does in airway smooth muscle (87). Finally, reactive oxygen species may be critical for vascular smooth muscle cell mitogenic signaling (127, 128), as they are in airway myocytes (79). Perhaps these species and tissue similarities are to be expected, as many aspects of MAP kinase cascades, GTPase signaling pathways and cell cycle regulation are highly conserved in eukaryotic species, including mammals, Drosophila, nematodes and yeast (129-135).

Data are lacking regarding the roles of cyclins and their inhibitors in lung diseases. As might be expected, expression of cyclin $D_1$ antisense RNA retards lung cancer growth (136, 137). In rodents exposed to hyperoxia, whole lung p21Cip1 accumulates during hyperoxic exposure (138), whereas after exposure, alveolar epithelial cell cyclin $D_1$ expression increases, coincident with proliferative repair (139). Finally, as noted above, bronchoalveolar lavage fluid from asthmatic airways increases cultured human airway smooth muscle cell cyclin $D_1$ protein abundance relative to that from control subjects (17). Elucidation of the signal transduction and cell cycle mechanisms regulating airway smooth muscle growth may provide insight into similar mechanisms that occur in the airways of patients with bronchopulmonary dysplasia and chronic severe asthma, and lead to therapeutic interventions.

## 11. ACKNOWLEDGEMENTS

These studies were supported by National Institutes of Health Grants HL54685, HL56399, HL63314 and a grant from the Blowitz-Ridgeway Foundation.

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**Key words:** Asthma, Cyclin $D_1$, Extracellular Signal Regulated Kinase, P38 Mitogen-Activated Protein Kinase, Phosphatidylinositol 3-Kinase, Platelet-Derived Growth Factor, Rac, Ras, Signal Transduction, Review

**Send correspondence to:** Marc B. Hershenson, M.D., University of Chicago Children's Hospital, 5841 S. Maryland Ave., MC4064, Chicago, IL 60637-1470, Tel:773-702-9659, Fax: 773-702-4041, E-mail: mhershen@midway.uchicago.edu

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