Integrin-linked kinase regulates smooth muscle differentiation marker gene expression in airway tissue

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Wu Y, Huang Y, Herring BP, Gunst SJ. Integrin-linked kinase regulates smooth muscle differentiation marker gene expression in airway tissue. Am J Physiol Lung Cell Mol Physiol 295: L988–L997, 2008. First published September 19, 2008; doi:10.1152/ajplung.90202.2008.—Phenotypic changes in airway smooth muscle occur with airway inflammation and asthma. These changes may be induced by alterations in the extracellular matrix that initiate signaling pathways mediated by integrin receptors. We hypothesized that integrin-linked kinase (ILK), a multidomain protein kinase that binds to the cytoplasmic tail of β-integrins, may be an important mediator of signaling pathways that regulate the growth and differentiation state of airway smooth muscle. We disrupted signaling pathways mediated by ILK in intact differentiated tracheal muscle tissues by depleting ILK protein using ILK antisense. The depletion of ILK protein increased the expression of the smooth muscle differentiation marker genes myosin heavy chain (SmMHC), SM22α, and calponin and increased the expression of SmMHC protein. Conversely, the overexpression of ILK protein reduced the mRNA levels of SmMHC, SM22α, and calponin and SmMHC protein. Analysis by chromatin immunoprecipitation showed that the binding of the transcriptional regulator serum response factor (SRF) to the promoters of SmMHC, SM22α, and calponin genes was increased in ILK-depleted tissues and decreased in tissues overexpressing ILK. ILK depletion also increased the amount of SRF that localized within the nucleus. ILK depletion and overexpression, respectively, decreased and increased the activation of its downstream substrate protein kinase B (PKB/Akt). The pharmacological inhibition of Akt activity also increased SRF binding to the promoters of smooth muscle-specific genes and increased expression of smooth muscle proteins, suggesting that ILK may exert its effects by regulating the activity of Akt. We conclude that ILK is a critical regulator of airway smooth muscle differentiation. ILK may mediate signals from integrin receptors that control airway smooth muscle differentiation in response to alterations in the extracellular matrix.

smooth muscle myosin heavy chain; serum response factor

Chronic asthma is often characterized by structural remodeling of the airway wall and abnormalities in the extracellular matrix, which may induce changes in the airway smooth muscle phenotype (19, 32). The phenotypic properties of smooth muscle cells are sensitive to alterations in their extracellular environment (4). In cultured airway smooth muscle cells, changes in the composition of the extracellular matrix can modulate the expression of contractile marker proteins as well as their proliferative index (18, 19).

The principal receptors for extracellular matrix proteins are transmembrane heterodimeric integrin proteins, which are composed of α- and β-subunits. Integrin receptors have been implicated in the regulation of both smooth and skeletal muscle differentiation (4, 9, 22, 30, 36). However, the signaling pathways by which signals sensed by integrin receptors are transduced to regulate gene transcription and smooth muscle cell phenotype remain to be elucidated.

Integrin-linked kinase (ILK) is an integrin β1-cytoplasmic domain-binding protein that mediates structural connections between integrin proteins and the actin cytoskeleton through several of its binding partners (3, 10, 16, 26, 40). ILK is positioned to transduce integrin-mediated signals from the extracellular matrix to a variety of intracellular signaling cascades that regulate cytoskeletal organization and gene expression (3, 7, 16, 35, 38).

ILK associates with integrin receptors and regulates the organization of macromolecular complexes at the extracellular matrix/cytoskeletal junctions (26, 38, 40, 41). We previously demonstrated that ILK plays a critical role in the organization and localization of these macromolecular complexes to the integrin-associated adhesion sites during the contractile activation of airway smooth muscle and that these macromolecular adhesion complexes are critical for the regulation of cytoskeletal organization and contractility of airway smooth muscle tissues (38). However, a role for ILK in the regulation of smooth muscle differentiation has not been described. We hypothesized that ILK may also mediate signals to the nucleus that regulate airway smooth muscle phenotype and differentiation.

We evaluated the role of ILK in the regulation of airway smooth muscle phenotype by depleting or overexpressing ILK protein in airway smooth muscle tissues and then determining the subsequent effects on expression of smooth muscle differentiation marker genes and their transcriptional regulators. Because the extracellular matrix environment and cell morphology play a critical role in determining the effects of integrin-mediated signaling pathways, intact fully differenti-
ated tracheal smooth muscle tissues were used for these studies. Our results demonstrate that ILK plays a critical role in regulating the expression of smooth muscle differentiation marker genes in intact airway smooth muscle tissues.

**MATERIALS AND METHODS**

*Preparation of smooth muscle tissues.* All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Indiana University School of Medicine. Mongrel dogs were euthanized according to proscribed procedures, and the trachea was immediately removed and immersed in physiological saline solution (PSS) at 22°C (composition in mM: 110 NaCl, 3.4 KCl, 2.4 CaCl₂, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 glucose). The solution was aerated with 95% O₂-5% CO₂ to maintain a pH of 7.4. Rectangular strips of tracheal muscle 1 mm in diameter and 10 mm in length were dissected after removal of the epithelium and connective tissue layer. Each muscle strip was placed in PSS at 37°C in a 25-ml organ bath and attached to a Grass force transducer for the measurement of force. At the beginning of each experiment, muscle length was progressively increased until the force of active contraction in response to a contractile stimulus reached a maximum (optimal length).

The depletion or overexpression of ILK protein in the muscle tissues was induced by introducing oligodeoxynucleotides (ODNs) dissolved in Tris-EDTA buffer or plasmids encoding human ILK cDNA in muscle strips according to experimental procedures described below. Muscle tissues were then mounted on metal hooks at the optimal length and incubated for 1 or 2 days in DMEM with ODNs or plasmids in the medium. Tissues were then quickly frozen by 10.220.33.3 on June 20, 2017 http://ajplung.physiology.org/ Downloaded from using liquid N₂-cooled tongs and pulverized under liquid N₂ using a pestle and mortar. After homogenization, the tissue was then incubated for 1 day in DMEM containing 5 mM Na₂ATP, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 µM oligonucleotides at 37°C and 5% CO₂. Plasmid-treated tissues were incubated for 2 days in the same medium with 10 µg plasmids. Sham-treated tissues were studied in parallel in all protocols and were subjected to identical protocols except that plasmids and antisense were omitted from the solutions during the reversible permeabilization procedures.

*Quantitation of mRNA.* Total RNA was isolated from the pulverized muscle extracts using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. The eluted RNA was quantified using the Quant-iT RNA Assay Kit (Invitrogen). Total RNA from each muscle (2.5 µg) was converted to cDNA using SuperScript III reverse transcriptase (Invitrogen) with oligo(dT) as primer. The canine gene-specific primers were as follows: SmMH (5’-GAAGCCCTGCTGCTACACAAC-3’ and 5’-GCAGAAAGGGCCAGATCTGATATAT-3’), Sm22α primers (5’-AGCGGGGCTGAGGCTCT-3’ and 5’-CAACGTGTCAGACATGTCATT-3’), calponin (5’-GCGACCATTCCGGAAGAA-3’ and 5’-TGGGTGATGCCCCTTGATGAA-3’), and α-actin (5’-CTTCGCTCTGTGCTTGG-3’ and 5’-TAGATCAATCATAGCCTGCCC-3’) and were designed according to sequence-matching results obtained from The National Center for Biotechnology Information, these sequences are not homologous to sequences of any other contractile or cytoskeletal proteins.

Plasmids encoding full-length human ILK cDNA with a FLAG epitope at the amino terminus were cloned into the EcoRI/XhoI sites of the pFLAG-CMV-2 vector. Plasmids were kindly provided by Dr. Chuanyue Wu (Department of Pathology, University of Pittsburgh).

Antibodies used in these experiments were as follows: mouse monoclonal anti-human ILK and rabbit polyclonal anti-ILK (Upstate Biotechnology); monoclonal anti-human smooth muscle myosin (clone HSM-V), monoclonal anti-human calponin (clone hCP), monoclonal anti-α-actin (clone EA-53), anti-FLAG (clone M2) (Sigma-Aldrich); polyclonal anti-human SM22α (custom made by Proteintech Group, Chicago, IL); rabbit polyclonal serum response factor (SRF; clone G-20, Santa Cruz Biotechnology); protein kinase B (Akt) anti-phospho Ser473 (Cell Signaling Technology) and anti-Akt (Abcam); and goat anti-rabbit conjugated to Alexa 488 (Molecular Probes). Propidium iodide (PI) was excited at 535 nm, and emissions were collected at 630 nm. PI was excited at 488 nm, and fluorescence emissions were collected at 500–530 nm. PI was excited at 535 nm, and emissions were collected at 630 nm. The optical pinhole was set to resolve optical sections of 1 µm in cell thickness. The plane of focus was determined using an oil-immersion lens objective (63 oil-immersion lens objective).
was set midway between the bottom of the nucleus and the top of the cell. Fluorescence intensity measurements were standardized among all cells compared within a single experiment by maintaining the same microscope settings. Images of smooth muscle cells were analyzed for regional differences in fluorescence intensity of labeled SRF by quantifying the pixel intensity using a series of cross-sectional line scans through the area of the nucleus (3 lines) and the cytoplasm (4 lines) (see Fig. 3, A and B). The ratio of nuclear to cytoplasmic SRF content (nuclear/cytoplasmic SRF) was determined for each cell by calculating the average pixel intensity of the lines across the nucleus relative to the lines across the cytoplasm.

Cell fractionation and extraction of nuclear and cytoplasmic protein. Nuclear and cytoplasmic protein extracts were prepared from sham-treated and ILK antisense-treated smooth muscle tissues using a commercially available kit (Active Motif) as follows: 300 mg muscle tissue from sham-treated or ILK-AS treated tissues were diced and homogenized. Homogenized tissues were centrifuged and the supernatant reserved as the cytoplasmic fraction. The pellet was resuspended in lysis buffer, centrifuged, and the supernatant reserved as the nuclear fraction. Proteins from nuclear and cytoplasmic extracts were analyzed by immunoblot. Glyceraldehyde-3-phosphate dehydrogenase was used as marker for the cytosolic fraction.

Chromatin immunoprecipitation assay. The binding of SRF to the promoters of smooth muscle-specific genes was performed according to the protocols described in a commercially available kit (Upstate Biotech) as follows: tissues were chopped into small pieces and treated with 1% formaldehyde for 15 min at room temperature to cross-link protein and DNA. After the tissues were homogenized using 1% SDS lysis buffer, 600–μl lystate of each group was sonicated using a Sonic Dismembranator (Fisher Scientific) and immunoprecipitated using SRF antibody or with IgG alone as a negative control. The immune complexes were recovered using agarose beads. DNA that was isolated from immunoprecipitated chromatin fragments (SRF and IgG) was subjected to quantitative RT-PCR using primers flanking the CCArGG box [CC (A/T-rich) GG] elements from SmMHC, SM22α, and calponin promoters. Primers for the α-actinin promoter were used as a negative control. Aliquots of sheared DNA from the tissue lysates that had not been immunoprecipitated were also included in the RT-PCR reaction (input) to adjust for differences in the amount of DNA in different tissue samples. Primer sequences used were as follows: SmMHC sense primer, 5′-CTGCCGGGGA CCATATTAGTCAGGGGAG-3′; SmMHC antisense primer, 5′-CTG GGGCGGAGCAACCC AAAAAGCCCA GG-3′ (24); SM22α sense primer, 5′-GCTCCATCTC CAAAGCAGG CAGG-3′; SM22α antisense primer, 5′-GACTCCACAGGCTC CATT TTG-3′; calponin sense primer, 5′-GGGC CCG GTTG CGTTTATAAA-3′; calponin antisense primer, 5′-CCCAGACAGGT TCGGAGACT-3′; α-actinin sense primer, 5′-TG TGGAGAGGCCGGCAGG-3′; and α-actinin antisense primer, 5′-GCTCGG TTCCATGCGG-3′. The amount of amplification of the target gene promoter for sample was quantitated from the difference in the cycle number (Ct) of the SRF immunoprecipitated DNA and the input DNA for that sample. The relative differences in the amount of SRF binding to the promoters of genes from ILK antisense-treated tissues and ILK wild-type-treated tissues, and for tissues treated or not treated with Akt inhibitor, were determined by calculating the difference in the values for the Ct for the treated and the sham-treated samples from each experiment as follows:

\[ RQ = 2^{-\Delta\Delta Ct} \Delta Ct = (Ct_{SRF} - Ct_{input}) \text{treated tissue} \]
\[ - (Ct_{SRF} - Ct_{input}) \text{sham-treated tissue} \]

Statistical analysis. Data are expressed as means ± SE. N represents the number of experiments. Differences between treatment groups were determined using paired or unpaired two-tailed Student’s t-test or ANOVA. Differences were considered statistically significant when P < 0.05.

RESULTS

Depletion of ILK protein in airway smooth muscle tissues increases the expression of smooth muscle-specific genes. We evaluated the role of ILK in regulating the phenotype of intact tracheal smooth muscle tissues by introducing ILK antisense into the muscle tissues to deplete ILK protein using the reversible permeabilization procedure. Tissues were then incubated in DMEM at 37°C without serum for 1 day, after which gene and protein expression was evaluated. Sham-treated tissues were subjected to identical procedures and incubation protocols except that antisense was omitted.

Treatment of the tissues with ILK antisense resulted in significant depletion of ILK protein to 48 ± 9% (n = 6) of that of sham-treated tissues, whereas treatment with ILK sense ODNs had no significant effect on the expression of ILK (Fig. 1, A and B). ILK depletion also significantly decreased the contractile response of the tissues to ACh to 40.9 ± 7.2% (n = 6) of force in sham-treated tissues (data not shown). Myosin light chain phosphorylation was reduced from 49.2 ± 2.2% in sham-treated muscles to 42.5 ± 2.2% in ILK antisense-treated tissues (n = 4, P < 0.05). Neither ILK sense nor sham-treatment significantly affected the contractile response to ACh.

Real-time quantitative RT-PCR was performed to evaluate the mRNA levels for SmMHC, SM22α, calponin h1, and α-actinin in extracts from smooth muscle tissues treated with ILK antisense. ILK depletion significantly increased the levels of mRNA for SmMHC to 2.41 ± 0.23 (n = 4), for SM22α to 2.08 ± 0.08 (n = 6), and for calponin to 1.47 ± 0.15 (n = 5).

Fig. 1. Treatment with integrin-linked kinase (ILK) antisense depresses ILK protein expression in airway smooth muscle tissues. A: immunoblot illustrating the effects of ILK antisense treatment on ILK protein expression. ILK protein was depleted by treating smooth muscle tissues with ILK antisense (ILK-AS) for 24 h. Treatment with ILK sense (ILK-S) had no effect on the expression of ILK protein. Sham-treated muscles were subjected to identical procedures in the absence of oligonucleotides. B: mean expression levels of ILK protein in tissues treated with ILK antisense or ILK-S, normalized to protein levels in sham-treated tissues. ILK protein was significantly decreased in tissues treated with ILK antisense but was unaffected by treatment with ILK sense. Means ± SE (n = 6). *Significantly different from sham treated, P < 0.05.
times that of sham-treated tissues. Depletion of ILK did not significantly affect the levels of mRNA for α-actinin (n = 3) (Fig. 2A).

The effect of depleting ILK protein on the expression of SmMHC, SM22α, calponin h1, and α-actinin protein was evaluated in tracheal smooth muscle tissues by immunoblot (Fig. 2, B and C). Expression levels of SmMHC, SM22α, and calponin h1 were normalized to that of α-actinin. ILK depletion significantly increased the expression of SmMHC by 2.31 ± 0.58 (n = 6) times that of sham-treated tissues, but it did not significantly affect the expression of SM22α (n = 6) or calponin (n = 6).

The depletion of ILK protein increases the amount of SRF in the nucleus of freshly dissociated smooth muscle cells (Fig. 3). Smooth muscle cells were enzymatically dissociated from sham-treated or ILK antisense-treated tracheal smooth muscle tissues and immunofluorescently stained with SRF antibody. SRF localization in the cells was analyzed by confocal microscopy. More SRF protein was observed in the nucleus of cells dissociated from tissues depleted of ILK compared with cells dissociated from sham-treated tissues (Fig. 3A). Differences in the ratio of SRF in the nucleus vs. the cytoplasm were quantitated in individual cells by comparing the ratios of average pixel intensity of cross-sectional line scans through the nucleus and the cytoplasm (Fig. 3, A–C). The ratio of nuclear to cytoplasmic SRF was significantly elevated in both sham-treated cells (1.3 ± 0.1, 47 cells from 12 muscle tissues) and cells from ILK antisense-treated tissues (2.7 ± 0.2, 43 cells from 8 muscle tissues); however, the ratio of nuclear to cytoplasmic SRF was significantly higher in ILK antisense-treated tissues than in sham-treated tissues (Fig. 3C). We also used immunoblots to compare the amount of SRF in extracts from nuclear and cytoplasmic fractions prepared from sham-treated smooth muscle tissues and tissues treated with ILK antisense (Fig. 3, D and E). Significantly more SRF was found in the nuclear fractions obtained from ILK antisense-treated tissues compared with those from sham-treated tissues (n = 3, P < 0.05). Taken together, these results indicate that the depletion of ILK from smooth muscle tissues leads to an increase in the amount of SRF in the nucleus.

ILK protein depletion increases SRF binding to the CArG box sequences of the SmMHC, SM22α, and calponin promoters in smooth muscle tissues (Fig. 4). Chromatin immunoprecipitation was used to evaluate the effect of ILK depletion on the binding of SRF to CArG box sequences within the promoter regions of SmMHC, SM22α, and calponin genes. DNA fragments bound to SRF were immunoprecipitated using SRF antibody from extracts of smooth muscle tissues treated with ILK antisense and from sham-treated tissues. The amounts of SmMHC, SM22α, and calponin promoters in the immunoprecipitates were quantitated by real-time PCR. Binding of SRF to the α-actinin promoter was also evaluated as a negative control. The amounts of promoter DNA bound to SRF in chromatin extracted from ILK antisense-treated tissues was significantly higher than that in sham-treated tissues for SmMHC (2.24 ± 0.42, n = 4), SM22α (2.10 ± 0.40, n = 4), and calponin h1 (1.54 ± 0.26, n = 4) (Fig. 4). There was no detectible binding of SRF to the α-actinin promoter.

Overexpression of ILK protein expression regulates smooth muscle-specific gene and protein expression in airway smooth muscle tissues. ILK depletion may prevent the assembly of macromolecular protein complexes at adhesion sites (38, 40) and thus might affect gene and protein expression by disrupting signaling pathways mediated by other protein constituents of these adhesion complexes. We therefore evaluated whether an increase in ILK expression also modulates the expression of smooth muscle-specific proteins and genes, since an increase in...
ILK expression would not disrupt the assembly of macromolecular protein complexes at adhesion sites.

ILK was overexpressed by introducing plasmids encoding recombinant human ILK-FLAG in intact tissues strips by reversible permeabilization. The overexpression of ILK in the tissues was confirmed 2 days after the introduction of ILK plasmids by immunoblotting with an ILK antibody (Fig. 5, A and B) and further confirmed by immunoprecipitation using a FLAG antibody (Fig. 5 C). Tissues treated with plasmids encoding recombinant ILK-FLAG wild type showed a 44% increase in total ILK protein expression relative to sham-treated tissues (1.44 ± 0.052, n = 5), for SM22α to 0.62 ± 0.11 (n = 8), and for calponin to 0.72 ± 0.04 (n = 4) relative to sham-treated tissues. The level of mRNA for α-actinin was not significantly different from that in sham-treated muscles (n = 4).

We also used chromatin immunoprecipitation to evaluate the effect of ILK overexpression on the binding of SRF to CArG box sequences within the promoter regions of SmMHC, SM22α, and calponin genes (Fig. 6B). The amounts of SmMHC (0.58 ± 0.06, n = 4), α-SM22α (0.65 ± 0.07, n = 4), and calponin (0.79 ± 0.05, n = 4) promoters in SRF immunoprecipitates were significantly decreased in tissues overexpressing ILK relative to sham-treated tissues (P < 0.05).

The effect of overexpressing ILK protein on the expression of SmMHC, SM22α, calponin h1, and α-actinin protein was evaluated in tracheal smooth muscle tissues by immunoblotting with an ILK antibody. The overexpression of ILK did not significantly affect contractile force in response to ACh (102 ± 1.5% of force in sham-treated tissues, n = 5).

Real-time quantitative RT-PCR was performed to evaluate the mRNA levels for SmMHC, SM22α, calponin h1, and α-actinin in extracts from smooth muscle tissues treated with ILK wild-type plasmids (Fig. 6A). ILK overexpression resulted in a significant decrease in the level of mRNA for SmMHC to 0.44 ± 0.052 (n = 5), for SM22α to 0.62 ± 0.11 (n = 8), and for calponin to 0.72 ± 0.04 (n = 4) relative to sham-treated tissues. The level of mRNA for α-actinin was not significantly different from that in sham-treated muscles (n = 4).

The effect of overexpressing ILK protein on the expression of SmMHC, SM22α, calponin h1, and α-actinin protein was evaluated in tracheal smooth muscle tissues by immunoblotting with a FLAG antibody. The overexpression of ILK did not significantly affect contractile force in response to ACh (102 ± 1.5% of force in sham-treated tissues, n = 5).

We also used chromatin immunoprecipitation to evaluate the effect of ILK overexpression on the binding of SRF to CArG box sequences within the promoter regions of SmMHC, SM22α, and calponin genes (Fig. 6B). The amounts of SmMHC (0.58 ± 0.06, n = 4), α-SM22α (0.65 ± 0.07, n = 4), and calponin (0.79 ± 0.05, n = 4) promoters in SRF immunoprecipitates were significantly decreased in tissues overexpressing ILK relative to sham-treated tissues (P < 0.05).
ILK REGULATES AIRWAY SMOOTH MUSCLE GENE EXPRESSION

The results of our study demonstrate that the adhesion complex protein ILK regulates the expression of smooth muscle-specific genes in intact differentiated tracheal smooth muscle tissues. This is the first demonstration that ILK mediates signaling pathways that regulate the phenotype of smooth muscle. We found that ILK modulates its effects on airway smooth muscle gene transcription by activating the phosphoinositide-dependent kinase Akt/PKB, a downstream effector and substrate of ILK (1, 8, 29, 33), and that the activation of Akt suppresses gene transcription by inhibiting activity of the

sequences within the promoter regions of SmMHC, SM22α, and calponin genes. The amounts of promoter DNA bound to SRF in chromatin extracted from tissues treated with the Akt inhibitor were significantly higher than those in untreated tissues for SmMHC (1.6 ± 0.25, n = 3) and SM22α (1.4 ± 0.15, n = 3). The increase in SRF binding to calponin was not significantly different from that of untreated muscles (1.2 ± 0.12, n = 3) (Fig. 9C). There was no detectible binding of SRF to the α-actinin promoter.

DISCUSSION

The overexpression of ILK protein resulted in a significant decrease in the expression of SmMHC expression to 0.67 ± 0.07 (n = 9) of the level in sham-treated muscles, whereas the expression of SM22α (n = 4) and calponin (n = 4) was not significantly affected (Fig. 7B).

ILK may regulate smooth muscle-specific gene expression by regulating Akt/PKB activity. ILK has been implicated in the activation of PKB/Akt (8, 29, 33), which can regulate the expression of smooth muscle differentiation marker genes and proteins (6, 13, 17, 20). We evaluated the effects of ILK depletion and ILK overexpression on the activation of PKB/Akt in tracheal smooth muscle tissues by measuring the phosphorylation of Akt at Ser473. Akt phosphorylation at Ser473 was significantly increased in ILK-depleted tissues to 58.9 ± 0.8% of sham-treated tissues (n = 3), and Akt phosphorylation at Ser473 was significantly increased in ILK-overexpressing tissues to 1.76 ± 0.33 of sham-treated muscles (n = 4) (Fig. 8, A and B).

Inhibition of Akt increases smooth muscle-specific gene expression and SRF binding to CArG box sequences of the SmMHC and SM22α genes. We then evaluated the effect of inhibiting PKB/Akt activity on the expression of SmMHC, SM22α, and calponin h1 in tracheal smooth muscle tissues. Smooth muscle tissues were treated for 6–16 h with 50 μM Akt inhibitor VIII (21) (Fig. 9, A and B). Akt phosphorylation at Ser473 was depressed after this treatment, confirming the effectiveness of the Akt inhibitor at inactivating Akt. Treatment of muscle tissues for 6–16 h with the Akt inhibitor resulted in a 1.9 ± 0.30-fold increase in SmMHC expression and a 1.28 ± 0.05-fold increase in SM22α but did not significantly affect expression of calponin. The increase in SmMHC expression was evident 6 h after the inhibitor was administered and persisted for at least 20 h.

Chromatin immunoprecipitation was used to evaluate the effect of Akt inhibition on the binding of SRF to CArG box

Fig. 4. Depletion of ILK protein expression increased the binding of SRF to the promoters of SmMHC, SM22α, and calponin in tracheal smooth muscle tissues. SRF was immunoprecipitated from chromatin extracts from ILK-depleted smooth muscle tissues. SRF immunoprecipitates were subjected to real-time PCR to amplify SmMHC, SM22α, and calponin promoter DNA. α-Actinin promoter DNA was used as a negative control for SRF binding. Depletion of ILK protein significantly increased the amount of SRF bound to the SmMHC promoter relative to sham-treated tissues (2.24 ± 0.42). ILK depletion also significantly increased the amount of SRF bound to the SM22α (2.10 ± 0.40) and calponin (1.54 ± 0.26) promoters compared with sham-treated tissues. Binding of SRF to the α-actinin promoter was negligible in extracts from both ILK-AS-treated and sham-treated tissues. Results represent means from 4 separate experiments. Values are means ± SE (n = 4).

*Significantly different from sham-treated tissues (P < 0.05).

Fig. 5. Treatment of airway smooth muscle tissues with ILK plasmids caused an increase in ILK protein expression. A: immunoblot indicating the effects of transfection with plasmids encoding WT-ILK on ILK protein expression. Treatment of smooth muscle tissues with ILK-WT plasmids followed by 2 days incubation caused upregulation of ILK protein expression (ILK Overexp). B: mean expression level of ILK protein in tissues treated with ILK plasmids relative to sham-treated tissues. ILK protein was significantly increased in tissues transfected with ILK plasmids (n = 14). Values are means ± SE. *Significantly different from 1 (Sham treated), P < 0.05. C: expression of recombinant FLAG-ILK protein was confirmed by immunoprecipitation of recombinant ILK from muscle extracts using anti-Flag antibody (Ab) followed by immunoblot using ILK Ab.

Fig. 6. Western analysis showing that ILK expression is induced in tracheal smooth muscle tissues transfected with ILK plasmids. ILK expression increased in tissues transfected with ILK plasmids (n = 14). Values are means ± SE. *Significantly different from 1 (Sham treated), P < 0.05.
transcriptional regulator SRF. ILK binds to the cytoplasmic tails of \( \beta \)-integrin proteins where it regulates the organization of macromolecular signaling complexes at extracellular matrix/cytoskeletal junctions in tracheal smooth muscle tissues (38). Thus our results suggest that ILK may regulate nuclear signaling pathways that control the differentiation and phenotype of airway smooth muscle in response to signals from integrin receptors. This pathway may represent a physiologically important mechanism for regulating the phenotype of airway smooth muscle in response to physiological or pathophysiological conditions that alter the extracellular matrix.

Extensive previous studies have shown that the binding of SRF to CArG box elements within genes encoding SmMHC, calponin h1, and SM22\( \alpha \) is essential for their transcription (24, 25). Our analysis by chromatin immunoprecipitation detected a pronounced increase in the amount of SRF bound to the promoter regions of genes encoding SmMHC, calponin h1, and SM22\( \alpha \) in nuclear extracts from ILK-depleted tissues and a reduction in SRF bound to the promoter regions of these genes in tissues overexpressing ILK. We also observed a marked increase in nuclear SRF in cells dissociated from tracheal tissues depleted of ILK and more SRF in the nuclear fractions of extracts from the ILK-depleted tissues. Together, these data suggest that ILK regulates the activation of SRF to control the transcription of genes of smooth muscle-specific contractile proteins.

Our results suggest that ILK regulates SRF activity in tracheal smooth muscle tissues through its effects on the activity of the protein kinase Akt/PKB, a substrate of ILK (1, 8, 29, 33). Akt has been implicated in the control of airway and vascular smooth muscle phenotypic modulation by both transcriptional and posttranscriptional mechanisms (2, 6, 13, 20). We observed that Akt activation, as indicated by its phosphorylation on Ser\textsuperscript{473}, was significantly lower in ILK-depleted tracheal smooth muscle tissues and significantly higher in tissues overexpressing ILK. We also found that the pharmacological inhibition of Akt activity increased the binding of SRF to SmMHC promoter and increased the expression of SmMHC in tissues. Previous studies have also implicated Akt in the regulation of SRF activity (11). Kaplan-Alburquerque et al. (20) reported that, in cultured rat aortic vascular smooth muscle cells, the platelet-derived growth factor-mediated activation of Akt suppressed the expression of smooth muscle \( \alpha \)-actin and SM22\( \alpha \) proteins by decreasing SRF-mediated activation of Akt.

We observed that the depletion of ILK protein in airway muscle tissues increased the levels of mRNA transcripts for the
smooth muscle differentiation marker genes SmMHC, calponin h1, and SM22α, and it also increased the binding of SRF to the promoter regions of their genes. In contrast, ILK overexpression decreased the levels of mRNA transcripts for these proteins and decreased binding of SRF to their promoters. In each of our experiments, changes in the expression of SmMHC were greater than the changes in calponin h1 or SM22α. Our observations are consistent with observations of the effects of serum deprivation on cultured canine tracheal smooth muscle cells, in which serum deprivation was found to increase the expression of SmMHC proportionally more than that of other smooth muscle phenotypic marker proteins (12). At the protein level, we were able to detect parallel changes in the levels of SmMHC but not of calponin h1 or SM22α. Our inability to detect changes in expression of Sm22α and calponin proteins that correspond to the changes in their mRNA levels may simply reflect the more quantitative nature of our mRNA analysis compared with our Western blot analysis of protein expression. Differences in the sensitivity of the expression of smooth muscle phenotypic marker proteins to stimuli that regulate differentiation might result from a number of factors: differences in the rate of protein turnover or degradation, the amount of protein synthesis relative to basal protein expres-
sion, the activity of transcriptional cofactors that interact with SRF to regulate promoter activity (23), or posttranscriptional mechanisms that regulate the stability of mRNA or protein expression (2, 13). However, our results demonstrate that the activity of all of these SRF-dependent genes is regulated by ILK.

Akt can regulate protein expression in airway smooth muscle cells by modulating ribosomal translation through its downstream targets mammalian target of rapamycin (mTOR) and p70 ribosomal S6 kinase (p70S6K) (2, 13). The stimulation of serum-deprived cultured canine airway smooth muscle cells with insulin activates a signaling pathway mediated by phosphatidylinositol 3-kinase, Akt, and ribosomal protein regulator p70S6K that leads to smooth muscle cell hypertrophy and the accumulation of SM22α and SmMHC without affecting levels of mRNA for these proteins (13). Our studies do not exclude the possibility that ILK regulates protein translation in addition to gene transcription. However, we observed that ILK depletion was associated with a decrease in Akt phosphorylation and an increase in expression of SRF-dependent gene transcripts and that the inhibition of Akt activation also increased SmMHC mRNA. These observations are inconsistent with ILK/Akt-mediated stimulation of ribosomal translation of SmMHC through the mTOR p70S6K pathway. The differences between the results of our study and those of previous investigators may reflect differences in effects of Akt activity in cultured tracheal smooth muscle cells and intact tracheal tissues, or they may have resulted from the presence of insulin and other growth factors in the serum of the tracheal cells in culture that were not present in the incubation medium of the tracheal muscle tissues in our study (13).

We previously demonstrated that ILK provides a scaffold for the formation of a macromolecular protein complex that associates with the cytoplasmic tail of β-integrins in airway smooth muscle (38). Contractile stimulation causes the recruitment of structural and signaling proteins to this complex, and this complex then regulates changes in cytoskeletal structure and organization that occur during contractile activation (35, 40). When we inhibited the formation of this ILK-associated macromolecular complex in tracheal muscle tissues, actin polymerization and the development of active tension were inhibited, and myosin light chain phosphorylation was somewhat reduced. Consistent with our previous results, the depletion of ILK from tracheal muscle tissues by antisense also depressed contractile force and somewhat reduced myosin light chain phosphorylation. It is likely that this effect dominated any possible effects on contractile force that might have resulted from the enhanced expression of SmMHC. The overexpression of ILK, which reduced the expression of SmMHC, did not have a detectable effect on force; however, given the variability in force development between different strips of muscle, subtle effects on tension would be difficult to detect experimentally.

In conclusion, our results demonstrate that ILK is a critical mediator of a pathway that regulates gene transcription and smooth muscle phenotype expression in intact airway smooth muscle tissues. Phenotypic changes in airway smooth muscle are associated with pathophysiological conditions such as asthma that cause inflammation of the smooth muscle tissues and alter the extracellular matrix of the smooth muscle cells. ILK may be an important intermediary in a pathway by which signals from transmembrane integrin receptors regulate the differentiation state of airway smooth muscle cells in intact tissues in vivo in response to physiological and pathophysiological conditions that lead to alterations in the extracellular matrix.

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