Integrin-linked Kinase Regulates N-WASp-mediated Actin Polymerization and Tension Development in Tracheal Smooth Muscle

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The contractile stimulation of smooth muscle tissues stimulates the recruitment of proteins to membrane adhesion complexes and the initiation of actin polymerization. We hypothesized that integrin-linked kinase (ILK), a β-integrin-binding scaffolding protein and serine/threonine kinase, and its binding proteins, PINCH, and α-parvin may be recruited to membrane adhesion sites during contractile stimulation of tracheal smooth muscle to mediate cytoskeletal processes required for tension development. Immunoprecipitation analysis indicated that ILK, PINCH, and α-parvin form a stable cytosolic complex and that the ILK-PINCH-α-parvin complex is recruited to integrin adhesion complexes in response to acetylcholine (ACh) stimulation where it associates with paxillin and vinculin. Green fluorescent protein (GFP)-ILK and GFP-PINCH were expressed in tracheal muscle tissues and both endogenous and recombinant ILK and PINCH were recruited to the membrane in response to ACh stimulation. The N-terminal LIM1 domain of PINCH binds to ILK and is required for the targeting of the ILK-PINCH complex to focal adhesion sites in fibroblasts during cell adhesion. We expressed the GFP-PINCH LIM1–2 fragment, consisting only of LIM1–2 domains, in tracheal smooth muscle tissues to competitively inhibit the interaction of ILK with PINCH. The PINCH LIM1–2 fragment inhibited the recruitment of endogenous ILK and PINCH to integrin adhesion sites and prevented their association of ILK with β-integrins, paxillin, and vinculin. The PINCH LIM1–2 fragment also inhibited tension development, actin polymerization, and activation of the actin nucleation initiator, N-WASp. We conclude that the recruitment of the ILK-PINCH-α-parvin complex to membrane adhesion complexes is required to initiate cytoskeletal processes required for tension development in smooth muscle.

Smooth muscle tissues from hollow organs are subjected to large changes in shape and volume under physiologic conditions in vivo. When subjected to external mechanical forces, the muscle must rapidly adapt its compliance and contractility to accommodate to changes in external mechanical forces. External forces that are imposed on smooth muscle tissues are transmitted to the interior of the cells via adhesion complexes, which link the cytoskeleton to the extracellular matrix. Transmembrane integrins, which are ligands for extracellular matrix proteins, can mediate the transduction of mechanical signals from the extracellular matrix to the cytoskeleton (1–3).

Recent data has suggested that the cytoskeletal organization of smooth muscle cells is dynamic and that it is regulated during contractile stimulation (4–10). Dynamic changes in cytoskeletal organization may enable smooth muscle cells to modulate their structure and contractility in response to changes in their external environment. The activation of smooth muscle contraction causes an increase in actin polymerization in airway and other smooth muscles (7, 9, 11–15). In tracheal smooth muscle tissues, contractile stimulation also initiates dynamic changes in the localization of cytoskeletal proteins and an increase in their association with integrin-associated protein complexes (10, 16, 17). Furthermore, the inhibition of actin polymerization or cytoskeletal reorganization can inhibit tension development in the absence of an effect on myosin light chain phosphorylation (7, 9, 18).

The actin nucleating protein, N-WASp, regulates the initiation of actin polymerization in tracheal smooth muscle during contractile activation (9). In tracheal smooth muscle, N-WASp activation can be regulated by phosphorylation of the adhesion complex protein, paxillin, which couples the SH2/SH3 adaptor protein CrkII to N-WASp to catalyze its activation by the small GTPase cdc42 (8, 9, 13). Paxillin undergoes tyrosine phosphorylation during the contractile activation of tracheal smooth muscle, and the tyrosine phosphorylation of paxillin is required for tension development and actin polymerization (18, 20). However, the mechanisms by which external signals mediated by integrin receptors are coupled to the cytoskeletal signaling pathways that regulate actin polymerization and cytoskeletal dynamics are not known.

Integrins are linked to the actin cytoskeleton via macromolecular protein complexes that interact with the cytoplasmic domains of integrin proteins and with actin filaments. These complexes are composed of adaptor and structural proteins as well as proteins with enzymatic activity. Integrin-linked kinase
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(ILK)² is a multidomain protein that binds directly to the cytoplasmic domain of β1 integrins and serves as a scaffolding protein for the organization of cytoskeletal signaling proteins at adhesion complexes (21). ILK forms a heterotrimeric complex with the adaptor protein PINCH, an adaptor protein that consists of a tandem array of 5 LIM domains, and α-parvin (also known as actopaxin and CH-ILKBP) (22–24). The N-terminal ankyrin domain of ILK binds to the N-terminal LIM domain of PINCH, and the C-terminal domain of ILK binds to α-parvin, which binds to actin filaments (25–27). The C-terminal domain of ILK also binds to paxillin (28, 29); thus ILK may transduce signals from integrin receptors to regulate actin polymerization through its interaction with paxillin (13, 18, 30). ILK has also been implicated in the regulation of N-WASp through its interactions with PINCH (31). ILK and its binding partners are thus positioned to coordinate signaling pathways that regulate cytoskeletal functions and the organization of structural links between integrin proteins and the actin cytoskeleton (22, 32).

We hypothesized that ILK may play a pivotal role in the regulation of actin polymerization and cytoskeletal dynamics during contractile activation and tension generation in tracheal smooth muscle. To evaluate the role of ILK in these processes, we inhibited the recruitment of ILK to integrin complexes by expressing a truncated N-terminal fragment of PINCH (PINCH LIM1–2) in tracheal smooth muscle tissues. PINCH LIM1–2 contains only the first 2 LIM domains and competes with endogenous PINCH for binding to ILK, thereby disrupting the recruitment of ILK to integrin adhesion sites (33). Our results demonstrate that the contractile activation of tracheal smooth muscle stimulates the recruitment of the ILK–PINCH complex to membrane adhesion complexes and increases its interaction with β1 integrins and other adhesion complex proteins. We find that the assembly of the ILK protein complex at membrane adhesion sites in tracheal smooth muscle tissues is critical for the regulation of N-WASp-mediated actin polymerization and tension development during contractile activation. Our findings suggest that integrin-linked kinase is an important mediator of signals from integrin receptors to downstream pathways that regulate actin polymerization and cytoskeletal organization in smooth muscle during contractile stimulation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies used in these studies were obtained from the following sources: mouse β1 integrin (clone 18), mouse PINCH-FL (clone 49, reacts at LIM3 domain of PINCH), polyclonal and monoclonal (IL-8) GFP and paxillin (clone 349), BD Biosciences; ILK (polyclonal antibody), Upstate; PINCH-N (monoclonal antibody, clone N173, reacts at amino acids 14–31 of human PINCH-1) and actin (monoclonal antibody, clone AC40), Sigma; polyclonal vinculin and polyclonal myosin light chain antibodies were custom made by BABCO, Richmond, CA; polyclonal N-WASp (H-100) and polyclonal Arp2 (H-84), Santa Cruz Biochemicals; polyclonal paxillin tyrosine phosphorylation antibodies Y118 and Y31, BIOSOURCE. Monoclonal anti-α-parvin used in immunoprecipitation has been previously described (25). Polyclonal anti-α-parvin, from Sigma was used in immunoblot analysis. Secondary antibodies used were: Alexa Fluor 488 and Alexa Fluor 546, obtained from Molecular Probes Co. All other reagents were purchased from Sigma. EGFP-C2 vector encoding human full-length ILK (residues 1–452), EGFP-C2 vectors encoding FLAG-tagged human full-length PINCH (residues 1–315), and the PINCH LIM1–2 domains (residues 1–130) (GFP-PINCH LIM1–2) were used in these studies (34).

Preparation of Smooth Muscle Tissues and Measurement of Force—Mongrel dogs (20–25 kg) were anesthetized with pentobarbital sodium (30 mg/kg, intravenously) and quickly exsanguinated in accordance with procedures approved by the Institutional Animal Care and Use Committee, Indiana University School of Medicine. A segment of the trachea was immediately removed and immersed in physiological saline solution (PSS) (composition in mM: 110 NaCl, 3.4 KCl, 2.4 CaCl₂, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 glucose). Smooth muscle strips (1.0 × 0.2–0.5 × 15 mm) were dissected free of connective and epithelial tissues. For the measurement of contractile force, muscle tissues were attached to force transducers and maintained within a tissue bath in PSS at 37 °C. Transfection of Smooth Muscle Tissues with Plasmids—Plasmids were introduced into tracheal smooth muscle strips by the method of reversible permeabilization (9, 10, 13, 16). After determination of the length of maximal isometric force (Lo), muscle strips were attached to metal mounts at Lo. The strips were incubated successively in each of the following solutions: Solution 1 (at 4 °C for 120 min) containing (in mM): 10 EGTA, 5 Na₂ATP, 120 KCl, 2 MgCl₂, and 20 TES; Solution 2 (at 4 °C overnight) containing (in mM): 0.1 EGTA, 5 Na₂ATP, 120 KCl, 2 MgCl₂, 20 TES, and 10 µg/ml plasmids. Solution 3 (at 4 °C for 30 min) containing (in mM): 0.1 EGTA, 5 Na₂ATP, 120 KCl, 10 MgCl₂, 20 TES; and Solution 4 (at 22 °C for 60 min) containing (in mM): 110 NaCl, 3.4 KCl, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 dextrose. Solutions 1–3 were maintained at pH 7.1 and aerated with 100% O₂. Solution 4 was maintained at pH 7.4 and aerated with 95% O₂, 5% CO₂. After 30 min in Solution 4, CaCl₂ was added gradually to reach a final concentration of 2.4 mM. The strips were then incubated in a CO₂ incubator at 37 °C for 2 days in serum-free Dulbecco's modified Eagle's medium containing 5 mM Na₂ATP, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml plasmids.

Cell Dissociation and Live Cell Imaging—Smooth muscle cells were enzymatically dissociated from tracheal muscle strips as previously described (9, 10). Muscle strips were minced and transferred to 5 ml of dissociation solution (in mM: 130 NaCl, 5 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 10 HEPES, 0.25 EDTA, 10 d-glucose, and 10 tauroine, pH 7) with collagenase (type I, 400 units/ml), papain (type IV, 30 units/ml), bovine serum albumin (1 mg/ml), and dithiothreitol (1 mM). All enzymes were obtained from Sigma. The strips were then placed in a 37 °C shaking water bath at 80 oscillations/min for 20–30 min, followed by washing three times with a HEPES-buffered saline solution (in mM: 130 NaCl, 5 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 20 HEPES, and 10 d-glucose,
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pH 7.4) and trituration with a pipette to liberate individual smooth muscle cells from the tissue. Live freshly dissociated cells were plated onto glass coverslips and allowed to adhere for 30–120 min before visualization. The localization of GFP-labeled ILK and PINCH was monitored in live cells by scanning them once/s for 60 s using a Zeiss LSM 510 laser scanning confocal microscope with an Apo ×40 (NA 1.2) water immersion objective. EGFP was excited with a 488-nm argon laser light, and fluorescence emissions were collected at 500–530 nm. The optical pinhole was set to resolve optical sections of ~1 μm in cell thickness. Contraction was stimulated by adding acetylcholine (ACh) to the PSS bathing the cell on the coverslip to achieve a concentration of 100 μM.

Analysis of Protein Localization by Immunofluorescence—The effects of ACh stimulation on the localization of endogenous and recombinant cytoskeletal proteins was evaluated in freshly dissociated smooth muscle cells (9, 10, 16). The dissociated smooth muscle cells were stimulated with 10−4 M ACh or left unstimulated, fixed, and reacted with primary antibodies specific for the proteins of interest (ILK, PINCH, paxillin, and N-WASP) and with secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546). The cellular localization of fluorescently labeled proteins was determined using a Zeiss LSM 510 laser scanning confocal microscope.

Image Analysis—Images of smooth muscle cells were analyzed for regional differences in fluorescence intensity of labeled proteins by quantifying the pixel intensity with a series of six cross-sectional line scans along the entire length of each cell as previously described (9, 10, 16). The area of the nucleus was excluded from the analysis. The ratio of pixel intensity between the cell periphery and the cell interior was computed for each line scan by calculating the ratio of the average maximum pixel intensity at the cell periphery to the average minimum pixel intensity in the cell interior. The ratios of pixel intensities between the cell periphery and the cell interior for all of the six line scans performed on a given cell were averaged to obtain a single value for the ratio for each cell. The ratio of fluorescence intensity at the cell periphery to that at the cell interior was compared in cells at rest and in cells stimulated with ACh (10−4 M).

Immunoprecipitation of Proteins—Pulverized muscle tissues were mixed with extraction buffer containing 1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 0.3% NaCl, 10% glycerol, 2 mM EDTA, phosphatase inhibitors (in mM: 2 sodium orthovanadate, 2 molybdate, and 2 sodium pyrophosphate), and protease inhibitors (in mM: 2 benzamidine, 0.5 aprotinin, and 1 phenylmethylsulfonyl fluoride). Each sample was centrifuged (14,000 × g) for the collection of supernatant. Muscle extracts containing equal amounts of protein were precleared for 30 min with 50 μl of 10% protein A-Sepharose and then incubated overnight with primary antibodies. Samples were then incubated for 2 h with 125 μl of a 10% suspension of protein A-Sepharose beads. Immunocomplexes were washed three times in a buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100. All procedures of immunoprecipitation were performed at 4 °C.

Measurement of Intracellular Ca2+ Concentration—Tracheal smooth muscle strips were pinned in a dish at a slightly stretched length (1.2 times slack length) and incubated in PSS containing 20 μM fura-2-AM, which was dissolved in 0.5% Me2SO premixed with 0.01% Pluronic 127 for 3.5 h at room temperature. They were then washed in PSS for 30 min to remove extracellular fura-2-AM and allowed time for the hydrolytic conversion of intracellular fura-2-AM to fura-2. Tissues were mounted in a cuvette and attached to a force transducer for the simultaneous measurement of force and fura-2 fluorescence using a ratio fluorescence spectrophotometer (system model C-14, Photon Technology International). The muscle was illuminated alternately at excitation wavelengths of 340 and 380 nm at a frequency of 2 Hz. Emitted light was collected through a single long-pass filter (510 nm) and detected with a photomultiplier tube. The ratio of fluorescence at 340 nm to fluorescence at 380 nm was continuously computed by a dedicated computer.

Measurement of Myosin Light Chain (MLC) Phosphorylation—Frozen muscle strips were immersed in dry ice precooled acetone containing 10% (w/v) trichloroacetic acid and 10 mM dithiothreitol. Proteins were extracted in 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM dithiothreitol. Phosphorylated and unphosphorylated myosin light chains were separated by gel-cylinder.polyacrylamide gel electrophoresis, transferred to nitrocellulose, then immunoblotted for proteins of MLC (9, 10). The ratio of phosphorylated to unphosphorylated MLCs was determined by scanning densitometry.

Analysis of F-actin and G-actin—The relative proportions of F-actin and G-actin in smooth muscle tissues were analyzed using an assay kit from Cytoskeleton (Denver, CO) as previously described (9, 10). Briefly, each of the tracheal smooth muscle strips was homogenized in 200 μl of F-actin stabilization buffer (50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% antifoam, 1 mM ATP, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 10 μg/ml benzamidine, and 500 μg/ml tosyl arginine methyl ester). Supernatants of the protein extracts were collected after centrifugation at 150,000 × g for 60 min at 37 °C. The pellets were resuspended in 200 μl of ice-cold distilled water containing 10 μM cytochalasin D and then incubated on ice for 1 h to depolymerize F-actin. The resuspended pellets were gently mixed every 15 min. Four microliters of supernatant (G-actin) and pellet (F-actin) fractions were subjected to immunoblot analysis using anti-actin antibody (clone AC-40; Sigma). The ratios of F-actin to G-actin were determined using densitometry.

RESULTS

Endogenous ILK and PINCH Are Recruited to the Cell Membrane during Contractile Stimulation of Smooth Muscle with ACh—The effect of stimulation with acetylcholine (ACh) on the cellular localization of endogenous ILK and PINCH was evaluated in smooth muscle cells freshly dissociated from intact tissues. Freshly dissociated cells were plated onto glass coverslips, stimulated with 10−4 M ACh or left unstimulated, then fixed and stained for immunofluorescence analysis of the distribution of ILK and PINCH (Fig. 1). Fig. 1 shows 4 optical sections from an unstimulated smooth muscle cell and from a stimulated smooth muscle cell, each double stained for ILK and...
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The cellular distribution of ILK and PINCH was quantified using line scans across a mid-level optical section of each cell as previously described (9, 10, 16). Examples of a single profile of pixel intensity from one line scan on each cell are shown in Fig. 1A. In unstimulated cells (n = 32), the ratio of the fluorescence intensity between the cell periphery to the cell interior for both ILK and PINCH was ~1.3, indicating a higher fluorescence of both proteins at the membrane of the cell relative to the interior. In cells stimulated with ACh (n = 32), the fluorescence intensity ratio rose to about 3 for both ILK and PINCH, suggesting that contractile stimulation with ACh leads to the recruitment of endogenous ILK and PINCH to the membrane of smooth muscle cells.

The Stimulation of Freshly Dissociated Tracheal Smooth Muscle Cells with ACh Causes the Rapid Recruitment of GFP-ILK and GFP-PINCH to the Cell Periphery—GFP-ILK and GFP-PINCH were expressed in tracheal smooth muscle tissues to monitor the localization of ILK and PINCH during contractile stimulation. Cells were then enzymatically dissociated from the tissues and evaluated by confocal fluorescence microscopy. As observed previously for other proteins, GFP-ILK or GFP-PINCH fluorescence was observed in ~90% of the dissociated cells (9, 10, 16, 18).

Freshly dissociated smooth muscle cells expressing the recombinant GFP-ILK or GFP-PINCH were visualized live during stimulation with 10^{-4} M ACh. Cells were scanned once per second for 5 s before stimulation and for 30–60 s after stimulation with 10^{-4} M ACh (Fig. 2 and Fig. 2 video (supplemental file)). Within seconds after stimulation with ACh, the intensity of GFP fluorescence for ILK and PINCH increased at the cell periphery and decreased in the cell interior. The heightened localization of ILK and PINCH at the cell periphery was maintained for the duration of the stimulation period. Similar results were observed in 16 cells dissociated from 6 different experiments (4–6 cells each experiment).

ILK, PINCH, and α-Parvin Form a Stable Complex That Associates with β1 Integrin, Vinculin, and Paxillin in Response to Stimulation of Tracheal Muscle Tissues with ACh—In cultured mammalian cell lines such as Chinese hamster ovary K1...
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with adhesion site proteins in response to contractile stimulation.

We evaluated the interaction of ILK with PINCH and \( \alpha \)-parvin during the formation of focal adhesions (26, 27). We confirmed the expression of GFP-PINCH and GFP LIM1–2 by immunoblotting extracts from muscle tissues that had been treated with plasmids for either PINCH or PINCH LIM1–2, or were not treated with plasmids. The PINCH N-terminal antibody reacts at the LIM1 domain of PINCH and can detect both the full-length PINCH and the PINCH LIM1–2 peptide (Fig. 4A).

We then evaluated the effect of expression of the PINCH LIM1–2 peptide on the localization of endogenous ILK and PINCH and on their recruitment to the cell periphery in response to ACh stimulation. Cells were dissociated from the PINCH wild type-treated, PINCH LIM1–2-treated, or untreated tracheal smooth muscle tissues and analyzed by immunofluorescence to determine the cellular localization of endogenous ILK and PINCH. Cells were fixed either unstimulated or after ACh stimulation, and double stained for ILK and PINCH using an antibody against the LIM3 domain of PINCH (PINCH-C49 antibody), which does not react with the N-terminal PINCH LIM1–2 fragment. This PINCH-LIM3 antibody was used to selectively stain for endogenous PINCH in PINCH LIM1–2-treated tissues. However, as it does react with the full-length recombinant GFP-PINCH, it therefore does not distinguish endogenous PINCH from full-length recombinant GFP-PINCH (Fig. 4B).

In smooth muscle cells dissociated from tissues expressing the GFP-PINCH LIM1–2 peptide, the distribution of both ILK and endogenous PINCH was similar in both unstimulated cells and stimulated cells. The fluorescence intensity ratios for ILK and PINCH were both 1.3 in unstimulated cells (\( n = 30–33 \)) and only increased to 1.5 in stimulated cells (\( n = 30–33 \)) (Fig. 3A).
Expression of the PINCH LIM1–2 protein fragment inhibits the recruitment of endogenous PINCH and ILK to the cell periphery and inhibits their association with adhesion site proteins. A, immunoblots (IB) show that EGFP wild-type PINCH and EGFP-PINCH LIM1–2 domain peptides were expressed in smooth muscle strips transfected with plasmids. PINCH-N antibody recognizes the N-terminal of PINCH and therefore reacts with both full-length PINCH and GFP-PINCH LIM1–2 proteins. B, optical longitudinal sections were taken at the midsection of cells double-immunostained for ILK (green) and PINCH-FL (full-length PINCH) (red). Smooth muscle cells were freshly dissociated from tissue strips expressing PINCH LIM1–2 fragments or expressing PINCH WT. The PINCH-FL antibody reacts with the endogenous PINCH but does not react with the PINCH LIM1–2 peptide. Expression of PINCH wild type does not inhibit the recruitment of endogenous ILK and PINCH to the membrane in response to ACh stimulation. C, expression of the PINCH LIM1–2 fragment inhibits the recruitment of ILK and endogenous PINCH to the membrane in response to ACh stimulation. * indicates the difference in ratio between ACh (n = 30–33 cells) and unstimulated (US) (n = 30) cells (p < 0.05). Error bars are S.E. D and E, optical longitudinal sections were taken at the midsection of cells stained for PINCH-FL (full-length PINCH) (red). The localization of GFP PINCH LIM1–2 in the PINCH LIM1–2-treated cells was determined by evaluating GFP fluorescence (green). Neither full-length endogenous PINCH nor the GFP PINCH LIM1–2 fragment localized at the cell membrane in ACh-stimulated cells (n = 15–18 cells, p > 0.05).
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4C). This suggests that expression of the PINCH LIM1–2 peptide inhibited the recruitment of ILK and endogenous PINCH to the membrane in response to ACh stimulation. In contrast, treatment with GFP-PINCH did not inhibit the recruitment of endogenous ILK or PINCH to the membrane in response to ACh, indicating that overexpression of full-length PINCH protein did not affect the localization of PINCH or ILK.

GFP fluorescence was also evaluated in unstimulated and ACh-stimulated cells from tissues transfected with PINCH LIM1–2 plasmids to determine the localization of the expressed PINCH LIM1–2 peptide. Our results confirmed that the PINCH LIM1–2 peptide does not redistribute to the cell membrane in response to ACh stimulation (Fig. 4, D and E; n = 16–18).

Expression of the GFP-PINCH LIM1–2 Peptide Inhibits the Association of ILK and PINCH with β1 Integrin, Paxillin, and Vinculin in Response to Stimulation with ACh—We evaluated homogenates from muscle tissues to determine the effect of expressing the PINCH LIM1–2 fragment on the interaction of ILK with PINCH. Full-length PINCH was distinguished from the PINCH LIM1–2 peptide using the PINCH-C49 antibody that reacts only with full-length PINCH. The effects of expression of wild-type PINCH were also evaluated to control for any possible effects of overexpression of the PINCH protein. The recombinant PINCH LIM1–2 peptides were detected in both unstimulated and ACh-stimulated tissues treated with the PINCH LIM1–2 plasmids.

In extracts from muscle tissues expressing the PINCH LIM1–2 peptide, the amount of endogenous PINCH that co-immunoprecipitated with ILK was significantly decreased in both stimulated and unstimulated tissues compared with the amount of endogenous PINCH that co-precipitated with ILK in untreated tissues (Fig. 5A). These results suggest that the PINCH LIM1–2 peptide binds to ILK and disrupts the interaction of ILK with endogenous PINCH.

The effect of PINCH LIM1–2 on the ACh-stimulated increase in the association of β1 integrin, paxillin, and vinculin with ILK was determined. In muscle tissues expressing the PINCH LIM1–2 peptide, the amount of β1 integrin and paxillin that co-precipitated with ILK was similar in tissues stimulated with ACh compared with unstimulated muscles. The expression of wild-type PINCH did not alter the effects of ACh stimulation on the coprecipitation of PINCH, β1 integrin, or paxillin with ILK (Fig. 5). These observations provide further evidence that expression of the PINCH LIM1–2 peptide in the muscle tissues disrupts the interaction of ILK with endogenous PINCH and thereby inhibits the recruitment of ILK and endogenous PINCH to integrin complexes during stimulation with ACh.

Expression of the PINCH LIM1–2 Peptide Inhibits Tension Development in Smooth Muscle Tissues—We evaluated the effect of expression of the PINCH LIM1–2 peptide on tension development in response to stimulation with 10^{-5} M ACh in intact smooth muscle tissue strips (Fig. 6). In smooth muscle tissues expressing the PINCH LIM1–2 peptide, contractile force in response to 5 min stimulation with ACh was significantly reduced to 36.4 ± 8.2% of that in untreated smooth muscle tissues (n = 30, p < 0.05). In contrast, in tissues expressing wild-type PINCH and in untreated tissues, isometric force in response to stimulation with ACh was not significantly different from the preincubation force. There were no significant differences in tension among the 3 groups of tissues before incubation. To determine whether the recruitment of the ILK-PINCH complex depended on receptor-coupled signaling pathways, we also evaluated the effect of expression of the PINCH LIM1–2 peptide on contractile responses to stimulation with KCl. Expression of the PINCH LIM1–2 peptide caused similar inhibition of contractions induced by 60 mM KCl (Fig. 6B). These observations suggest that the recruitment of ILK and PINCH to cell membrane integrin adhesion sites is necessary for tension development in smooth muscle tissues, and that this recruitment does not depend on receptor-mediated mechanisms.

Expression of the PINCH LIM1–2 Peptide Slightly Reduces Myosin Light Chain Phosphorylation in Smooth Muscle Tissues—The effects of stimulation with 10^{-5} M ACh on myosin light chain phosphorylation were compared in muscle tissues transfected with the PINCH LIM1–2 plasmids, plasmids encoding wild-type PINCH (PINCH WT), and tissues incubated without plasmids (Fig. 7). There were no significant differences in MLC phosphorylation in unstimulated muscles expressing the PINCH LIM1–2 peptide, PINCH WT, or untreated muscles.

[Image 318x505 to 558x733]
force in response to ACh stimulation was significantly lower in tissues transfected with the PINCH LIM 1–2 plasmids (n = 4, p < 0.05).

Expression of the PINCH LIM1–2 Peptide Inhibits Actin Polymerization in Smooth Muscle Tissues—A cell fractionation assay (Cytoskeleton, Inc.) was used to analyze the proportions of G-actin and F-actin in extracts from unstimulated and stimulated muscle tissues expressing the PINCH LIM1–2 peptide (8–10, 13) (Fig. 9A). Expression of the PINCH LIM1–2 peptide markedly inhibited the increase in the ratio of F- to G-actin in response to stimulation with ACh. Changes in the ratio of F-ac-
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expression of PINCH LIM1–2 in tracheal muscle tissues does not affect ACh-induced intracellular Ca2+ signals. A, typical traces of fura-2 fluorescence and force in response to 10^{-4} M ACh stimulation are shown for muscle strips that have not been treated with plasmids, or have been treated with PINCH wild type plasmids (PINCH WT) or PINCH LIM1–2 plasmids. Fura-2 fluorescence is quantified as an increase in the ratio of fluorescence at excitation wavelengths of 340 and 380 over baseline. B, the intracellular calcium signals in response to ACh were similar in all groups (n = 4, p > 0.05). Force in the PINCH LIM1–2 plasmid-treated strips was significantly depressed relative to strips treated with no plasmid and PINCH WT plasmid.

FIGURE 9. Expression of PINCH LIM1–2 in tracheal muscle tissues inhibits ACh-induced actin polymerization. A, representative immunoblots of actin from soluble (G-actin) and insoluble (F-actin) fractions obtained from one experiment on 6 muscle strips. Tracheal smooth muscle strips incubated with plasmids encoding PINCH wild type (WT), PINCH LIM1–2 domain, or incubated without plasmids for 2 days were stimulated with 10^{-4} M ACh for 5 min. The amount G-actin was lower and the amount of F-actin was higher in tissues stimulated with ACh in the no plasmid and PINCH WT tissues. In PINCH LIM1–2–treated tissues, the amounts of G- and F-actin were similar in untreated and in ACh-treated muscle strips. B, mean ratios of F-actin/G-actin in strips stimulated with ACh (black bars) or unstimulated (US, open bars). Expression of the PINCH LIM1–2 peptide inhibited the increase in F-actin/G-actin ratio in response to ACh stimulation. *, significant difference between ACh-stimulated tissue and unstimulated tissues (p < 0.05). Values are mean ± S.E. (n = 4).

Expression of the PINCH LIM1–2 Domain Peptide Inhibits N-WASp Activation in Response to Stimulation with ACh—We previously demonstrated that the activation of N-WASp mediates actin polymerization in tracheal smooth muscle tissues (9). We evaluated the role of the ILK-PINCH complex in the regulation of N-WASp activation as a possible mechanism by which it might regulate actin polymerization. N-WASp activation was evaluated by measuring changes in the association of N-WASp with the Arp2/3 complex, by measuring the phosphorylation of N-WASp on tyrosine 256, and by analyzing changes in the cellular localization of N-WASp. N-WASp is recruited to the cell membrane in activated tracheal muscle cells, and activated N-WASp associates with the Arp2/3 complex to mediate actin polymerization (9). In studies of NIH3T3 cells, N-WASp has also been found to undergo phosphorylation on tyrosine 256 by FAK (focal adhesion kinase), and this phosphorylation regulates its cellular localization and increases when N-WASp undergoes activation (36).

Immunoprecipitation was used to evaluate the association of N-WASp and the Arp2/3 complex in stimulated and unstimulated smooth muscle tissues (Fig. 10, A and B). In untreated tissues and tissues treated with PINCH WT, ACh stimulation increased the amount of Arp2 associated with N-WASp more than 2-fold. The increase in the amount of Arp2 in N-WASp immunoprecipitates in response to ACh stimulation was inhibited by the expression of the PINCH LIM1–2 fragments.

ACh stimulation increased N-WASp Tyr-256 phosphorylation in tracheal muscle tissues without plasmid treatment and in tissues treated with PINCH WT. The expression of PINCH LIM1–2 fragments inhibited the increase in N-WASp Tyr-256 phosphorylation in response to ACh (Fig. 10, C and D).

The recruitment of N-WASp to the cell membrane in response to ACh in tracheal smooth muscle was evaluated by immunofluorescence in tracheal smooth muscle cells freshly dissociated from tissues expressing PINCH LIM1–2. The expression of PINCH LIM1–2 fragments in smooth muscle tissue strips inhibited the recruitment of N-WASp to the cell membrane in freshly dissociated smooth muscle cells (Fig. 10E). In cells dissociated from PINCH WT–treated tissues, stimulation with ACh elicited the recruitment of N-WASp to the cell membrane similarly to that observed in cells from untreated muscle tissues (9). All of these observations suggest that the inhibition of the recruitment of the ILK-PINCH complex to the membrane by the PINCH LIM1–2 fragment inhibits...
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actin polymerization by inhibiting activation of the actin nucleating protein, N-WASP.

Expression of the PINCH LIM1–2 Domain in Smooth Muscle Inhibits the Tyrosine Phosphorylation of Paxillin—In tracheal smooth muscle, the activation of N-WASP and the Arp2/3 complex is regulated by tyrosine phosphorylation of paxillin at tyrosine 31 and tyrosine 118 (13). Paxillin undergoes tyrosine phosphorylation in response to contractile stimulation (13, 18). ILK binds to paxillin (28), and we observed that the association of paxillin with the ILK-PINCH complex is inhibited in muscles expressing the PINCH LIM1–2 fragment (Fig. 5). We therefore considered that PINCH LIM1–2 might prevent paxillin phosphorylation and thereby inhibit N-WASP activation.

Both Tyr-31 and Tyr-118 phosphorylation of paxillin were suppressed in tracheal smooth muscle tissues expressing the PINCH LIM1–2 domains (Fig. 11); whereas expression of the PINCH WT had no effect on the tyrosine phosphorylation of paxillin in response to ACh stimulation. The results suggest that the recruitment of the ILK-PINCH complex to the membrane and the binding of paxillin to ILK is required for paxillin phosphorylation.

DISCUSSION

Our results suggest that the activation of tracheal smooth muscle by a contractile stimulus initiates the assembly of a macromolecular protein complex at adhesion junctions between the cell-matrix and the cytoplasm, and that the assembly of this protein complex is necessary for the transmission of signals that initiate actin polymerization, cytoskeletal organization, and tension development in differentiated smooth muscle. We find that contractile stimulation of tracheal smooth muscle with acetylcholine catalyzes the recruitment of the ILK-PINCH-α-parvin complex to the periphery of the smooth muscle cell where it binds to transmembrane integrin proteins. Paxillin is also recruited to the cell periphery (16), where it associates with the ILK-PINCH-α-parvin complex and undergoes tyrosine phosphorylation. The phosphorylation of paxillin leads to its coupling with the actin nucleation initiating protein, N-WASP, resulting in actin polymerization and tension development. These observations suggest a mechanism by which external stimuli that modulate integrin activation, such as mechanical forces, can directly regulate cytoskeletal organization and modulate smooth muscle contractility.

ILK has been shown to form a heterotrimeric complex with the LIM-domain containing protein PINCH and the calponin homology protein, α-parvin (also called CH-ILKBP or actopaxin), which has been referred to as the IPP complex (37). In cultured mammalian cells, such as Chinese hamster ovary K1 cells and mouse C2C12 cells, the assembly of this trimeric phosphorylation in ACh-stimulated tissues and unstimulated tissues (p < 0.05). Values are mean ± S.E. (n = 4), *, significant difference between ACh-stimulated tissue (ACh) and unstimulated tissues (US) (p < 0.05). C, immunoblots of N-WASP immunoprecipitates from muscle tissues treated with PINCH WT or PINCH LIM1–2 or no plasmid-treated tissues. Expression of PINCH LIM1–2 inhibited the 10^{-5} M ACh-induced increase in the co-precipitation of Arp2 with N-WASP. D, mean ratios of Arp2/N-WASP in ACh-stimulated and unstimulated muscle tissues. Values are mean ± S.E. (n = 4), *, significant difference between ACh-stimulated tissue (ACh) and unstimulated tissues (US) (p < 0.05). C, immunoblots of N-WASP immunoprecipitates from muscle tissues treated with PINCH WT or PINCH LIM1–2 or no plasmid-treated tissues. Expression of PINCH LIM1–2 inhibited the 10^{-5} M ACh-induced increase in the co-precipitation of Arp2 with N-WASP. E, optical longitudinal sections were taken at the midsection of cells immunostained for N-WASP (red). Smooth muscle cells were freshly dissociated from tissue strips expressing PINCH LIM1–2 fragments or expressing PINCH wild type (WT). Expression of PINCH wild type does not inhibit the recruitment of N-WASP to the membrane in response to 5 min 10^{-5} M ACh stimulation. Expression of the PINCH LIM1–2 fragment inhibits the recruitment of N-WASP to the membrane in response to 5 min 10^{-5} M ACh stimulation. Similar results were obtained in four experiments for 30 cells.

Figure 10: Expression of PINCH LIM1–2 inhibits the activation of N-WASP in tracheal muscle tissues. A, immunoblots of N-WASP immunoprecipitates from muscle tissues transfected with PINCH WT or PINCH LIM1–2 or no plasmid-treated tissues. Expression of PINCH LIM1–2 inhibited the 10^{-5} M ACh-induced increase in the co-precipitation of Arp2 with N-WASP. B, mean ratios of Arp2/N-WASP in ACh-stimulated and unstimulated muscle tissues. Values are mean ± S.E. (n = 4), *, significant difference between ACh-stimulated tissue (ACh) and unstimulated tissues (US) (p < 0.05). C, immunoblots of N-WASP immunoprecipitates from muscle tissues treated with PINCH WT or PINCH LIM1–2 or no plasmid-treated tissues. Expression of PINCH LIM1–2 inhibited the 10^{-5} M ACh-induced increase in the co-precipitation of Arp2 with N-WASP. D, mean ratios of Arp2/N-WASP in ACh-stimulated and unstimulated muscle tissues. Values are mean ± S.E. (n = 4), *, significant difference between ACh-stimulated tissue (ACh) and unstimulated tissues (US) (p < 0.05). C, immunoblots of N-WASP immunoprecipitates from muscle tissues treated with PINCH WT or PINCH LIM1–2 or no plasmid-treated tissues. Expression of PINCH LIM1–2 inhibited the 10^{-5} M ACh-induced increase in the co-precipitation of Arp2 with N-WASP. E, optical longitudinal sections were taken at the midsection of cells immunostained for N-WASP (red). Smooth muscle cells were freshly dissociated from tissue strips expressing PINCH LIM1–2 fragments or expressing PINCH wild type (WT). Expression of PINCH wild type does not inhibit the recruitment of N-WASP to the membrane in response to 5 min 10^{-5} M ACh stimulation. Expression of the PINCH LIM1–2 fragment inhibits the recruitment of N-WASP to the membrane in response to 5 min 10^{-5} M ACh stimulation. Similar results were obtained in four experiments for 30 cells.
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**FIGURE 11.** Expression of PINCH LIM1–2 inhibits paxillin phosphorylation in tracheal muscle tissues. A, immunoblots of paxillin tyrosine 31 phosphorylation in extracts of muscle tissues transfected with PINCH WT or PINCH LIM1–2 or tissues not treated with plasmids. Five minutes ACh stimulation (10−5 M) increases paxillin Tyr-31 phosphorylation in tracheal smooth muscle tissues transfected with PINCH wild type plasmid (PINCH WT) and tissues without plasmid transfection (No Plasmid). Expression of PINCH LIM1–2 peptide inhibits ACh-induced paxillin Tyr-31 phosphorylation in tracheal smooth muscle tissues. *, significant difference between ACh-stimulated tissue (ACh) and unstimulated tissues (US) (p < 0.05). Values are mean ± S.E. (n = 5). B, immunoblots of paxillin tyrosine 118 phosphorylation from muscle tissues transfected with PINCH WT or PINCH LIM1–2 or tissues not treated with plasmids. Similar results were obtained as for paxillin tyrosine 31 (Y31) phosphorylation. Expression of PINCH LIM1–2 peptide inhibits ACh-induced paxillin Tyr-118 (Y118) phosphorylation in tracheal smooth muscle tissues. *, significant difference between ACh-stimulated tissue and unstimulated tissues (p < 0.05). Values are mean ± S.E. (n = 5).

complex occurs in the cytosol, independently of adhesion signals (27). We found that the amount of PINCH and α-parvin co-precipitate with ILK is similar in both stimulated and unstimulated smooth muscle tissues, suggesting that these proteins also form a stable complex in smooth muscle cells. Our evidence suggests that this complex is recruited to integrin-cell matrix contact points in response to a contractile stimulus (Fig. 3).

We present several lines of evidence to demonstrate that the ILK-PINCH-α-parvin complex is recruited to integrin cell-matrix junctions during the contractile activation of tracheal muscle tissues. First, we find more β1 integrin and paxillin proteins are associated with ILK immuno-complexes precipitated from extracts of stimulated muscle tissues than from unstimulated muscle tissues (Fig. 3). Second, we observe that recombinant GFP-ILK or GFP-PINCH that is expressed in smooth muscle tissues is recruited to the periphery of freshly dissociated smooth muscle cells within seconds of exposure to ACh (Fig. 2). Third, immunofluorescence analysis of the localization of endogenous ILK and PINCH in unstimulated and ACh-stimulated freshly dissociated smooth muscle cells demonstrates higher levels of ILK and PINCH fluorescence at the membrane of stimulated cells than in unstimulated cells (Fig. 1). These observations support the concept that the organization of a macromolecular protein complex at integrin-cell-matrix contact points in smooth muscle tissues is labile and that its assembly is dynamically regulated during contraction.

Wu and colleagues (26, 33, 35) have shown that in C2C12 mouse myoblast cells, the interaction of ILK with PINCH is critical for the efficient localization of both ILK and PINCH to cell-matrix contact sites. In these cells, a PINCH fragment consisting only of its first two N-terminal LIM domains, which contain the ILK binding site, can act as a dominant-negative inhibitor of the PINCH-ILK interaction and can partially inhibit the localization of ILK to cell-matrix contact sites (27).

We expressed the PINCH LIM1–2 peptide fragment in tracheal muscle tissues to inhibit the binding of endogenous PINCH to ILK with the goal of suppressing the recruitment of ILK to cell-matrix adhesion sites. Co-immunoprecipitation analysis of extracts of tracheal tissues confirmed that expression of PINCH LIM1–2 inhibited the association between ILK and endogenous PINCH in both unstimulated and ACh-stimulated tissues (Fig. 5A). Immunofluorescence analysis showed that expression of the PINCH LIM1–2 peptide inhibits the recruitment of ILK and endogenous PINCH to the membrane in response to stimulation with acetylcholine (Fig. 4). Co-precipitation analysis of extracts from intact muscle tissues showed that the expression of the PINCH LIM1–2 peptide inhibited the increase in the association of ILK with β1 integrin (Fig. 5B). Thus, expression of the recombinant PINCH LIM1–2 domain peptide in tracheal muscle tissues can inhibit the recruitment of the ILK-PINCH-α-parvin complex to cell-matrix adhesion junctions in response to contractile stimulation. Furthermore, preventing the recruitment of the ILK-PINCH-α-parvin complex to cell membrane adhesion sites markedly inhibited the development of tension in response to ACh stimulation (Fig. 6). The expression of PINCH LIM1–2 caused a similar depression of the contractile response to KCl, indicating that the effect is not specific to receptor-coupled activation mechanisms. Thus, the recruitment of the ILK-PINCH-α-parvin complex to cell-matrix contact points is a critical step in the development of active tension.

Previous studies of smooth muscle cells and tissues have shown that stimulation with contractile agonists initiates the polymerization of actin, and that this actin polymerization is required for tension development (6, 7, 9, 12, 16). Our studies suggest that this pool of actin is relatively small in differentiated muscle tracheal muscle tissues, probably less than 15% of the
total pool of cellular actin (9, 38). We have proposed that this pool of actin may be submembranous cortical actin, and that it may function to provide support and rigidity to the membrane during muscle contraction to support the transmission of tension and enable the muscle cell to adapt its shape to accommodate to external mechanical forces (10, 38).

Actin polymerization in tracheal smooth muscle is initiated by the activation of the actin-nucleating protein N-WASp, which binds to and activates the Arp2/3 complex, catalyzing the formation of new actin filaments (9, 39–42). The activation of N-WASp and the Arp2/3 complex is regulated by paxillin, which undergoes tyrosine phosphorylation in response to contractile stimulation (18, 43, 44). Paxillin is a scaffolding protein that associates with integrin adhesion complexes and can bind to ILK (28). In tracheal smooth muscle, the phosphorylation of paxillin at tyrosine 31 and 118 enables it to couple to the SH2/SH3 adapter protein CrkII, and catalyze the activation of cdc42, which activates N-WASp (13). We considered that the recruitment of the ILK-PINCHα-parvin complex to cell-matrix-integrin adhesion junctions might be necessary for the tyrosine phosphorylation of paxillin, N-WASp activation, and the initiation of ACh-induced actin polymerization.

We found that inhibiting the recruitment of the ILK-PINCHα-parvin complex to the membrane in tracheal muscle tissues in response to stimulation with ACh almost completely inhibited actin polymerization (Fig. 9). We also found that it inhibited the increase in the association of N-WASp with the Arp2/3 complex and its recruitment to the cell periphery (Fig. 10). The phosphorylation of N-WASp at tyrosine 256, an indicator of N-WASp activation (36), was also suppressed. These findings indicate that recruitment of the ILK-PINCHα-parvin complex to the membrane is necessary for N-WASp activation.

Preventing the recruitment of ILK to cell membrane also suppressed the increase in tyrosine phosphorylation of paxillin in response to ACh (Fig. 11). This suggests that association of the ILK-PINCH complex with β-integrins regulates actin polymerization by promoting the tyrosine phosphorylation of paxillin at integrin-cell matrix contact points, which may then initiate the activation of N-WASp-mediated actin polymerization through its coupling to CrkII. Paxillin phosphorylation is sensitive to mechanical stimuli in tracheal smooth muscle (44); thus, ILK-paxillin interaction may regulate the mechanosensitivity of pathways downstream of paxillin phosphorylation.

ILK has been implicated as a regulator of smooth muscle MLC phosphorylation in vascular smooth muscle (45–48). Walsh and colleagues (45, 46, 48) have proposed that ILK may function as a Ca2+-independent regulator of myosin light chain phosphorylation and tension development in these tissues. We therefore evaluated the possibility that the recruitment of ILK to integrin complexes is necessary for the activation of signaling pathways that regulate myosin light chain phosphorylation. We observed that blocking the recruitment of ILK to cell membrane by expressing the PINCH LIM1–2 domain peptide caused a small but statistically significant inhibition of myosin light chain phosphorylation (Fig. 7), whereas tension development was depressed by ~60% compared with untreated tissues. Expression of the PINCH LIM-2 domain did not have a detectable effect on intracellular Ca2+, as measured by fura-2 in intact tissues (Fig. 8), indicating that PINCH LIM1–2 expression does not disrupt receptor-coupling mechanisms that regulate intracellular Ca2+. The small inhibition of myosin light chain phosphorylation appears inadequate to account for the dramatic inhibition of tension development observed in muscles expressing the PINCH LIM1–2 domain.

The M3 muscarinic receptor is the predominant receptor activated by ACh in tracheal smooth muscle (49). The mechanisms for coupling muscarinic receptor activation to the activation of ILK-PINCHα-parvin complex are unclear; however, collaboration between integrin-mediated signaling pathways and pathways activated by heterotrimeric G-proteins have been documented in a variety of cultured cell lines (50). In HEK cells stably transfected with M3 muscarinic receptors, muscarinic receptor activation can induce the tyrosine phosphorylation of paxillin and focal adhesion kinase as well as focal adhesion assembly through a protein kinase C-dependent mechanism (51). The effects of muscarinic activation are dependent on integrin engagement with the extracellular matrix (19).

In migrating cells, new adhesive complexes form at the leading edge of the cell to provide the traction necessary to move the cell body forward. Integrins are first activated and then associate with the ILK-PINCHα-parvin macromolecular complex. This complex acts as a platform to recruit paxillin, vinculin, and other adhesion proteins to membrane integrin sites to regulate processes that control adhesion and motility (31). Our results showed that ILK localization at adhesion sites and the formation of an ILK-PINCHα-parvin macromolecular complex that associates with integrin proteins is necessary for the activation and regulation of signaling pathways that are important for tension generation in smooth muscle. These observations provide further evidence that tension generation in smooth muscle is a complex event involving dynamic cytoskeletal processes that occur concurrently with the activation of cross-bridge cycling. The role of ILK in this process may enable the cell to modulate actin polymerization and tension development in response to external forces on the cell that are sensed by integrin receptors.

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