Tension development during contractile stimulation of smooth muscle requires recruitment of paxillin and vinculin to the membrane

Anabelle Opazo Saez, Wenwu Zhang, Yidi Wu, Christopher E. Turner, Dale D. Tang, and Susan J. Gunst

Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202; and Department of Cell and Developmental Biology, State University of New York Upstate Medical University, Syracuse, New York 13210

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Saez, Anabelle Opazo, Wenwu Zhang, Yidi Wu, Christopher E. Turner, Dale D. Tang, and Susan J. Gunst. Tension development during contractile stimulation of smooth muscle requires recruitment of paxillin and vinculin to the membrane. Am J Physiol Cell Physiol 286: C433–C447, 2004. First published October 22, 2003; 10.1152/ajpcell.00030.2003.—Cytoskeletal reorganization of the smooth muscle cell in response to contractile stimulation may be an important fundamental process in regulation of tension development. We used confocal microscopy to analyze the effects of cholinergic stimulation on localization of the cytoskeletal proteins vinculin, paxillin, talin, and focal adhesion kinase (FAK) in freshly dissociated tracheal smooth muscle cells. All four proteins were localized at the membrane and throughout the cytoplasm of unstimulated cells, but their concentration at the membrane was greater in acetylcholine (ACh)-stimulated cells. Antisense oligonucleotides were introduced into tracheal smooth muscle tissue to deplete paxillin protein, which also inhibited contraction in response to ACh. In cells dissociated from paxillin-depleted muscle tissues, redistribution of vinculin to the membrane in response to ACh was prevented, but redistribution of FAK and talin was not inhibited. Muscle tissues were transfected with plasmids encoding a paxillin mutant containing a deletion of the LIM3 domain (paxillin LIM3 dl 444–494), the primary determinant for targeting paxillin to focal adhesions. Expression of paxillin LIM3 dl in muscle tissues also inhibited contractile force and prevented cellular redistribution of paxillin and vinculin to the membrane in response to ACh, but paxillin LIM3 dl did not inhibit increases in intracellular Ca2+ or myosin light chain phosphorylation. Our results demonstrate that recruitment of paxillin and vinculin to smooth muscle membrane is necessary for tension development and that recruitment of vinculin to the membrane is regulated by paxillin. Vinculin and paxillin may participate in regulating the formation of linkages between the cytoskeleton and integrin proteins that mediate tension transmission between the contractile apparatus and the extracellular matrix during smooth muscle contraction.

The cytoskeletal proteins vinculin, talin, and α-actinin have been implicated in the physical coupling of actin filaments to β-integrins (5, 6, 9, 28, 36, 38). Talin and α-actinin bind to the cytoplasmic domain of β-integrins as well as to actin filaments and thereby have the potential to provide direct mechanical coupling between integrin proteins and the actin cytoskeleton (6, 10, 36). Vinculin also binds to actin filaments, talin, and α-actinin, but there is no evidence that it can bind directly to β-integrins (10). Vinculin has been proposed to strengthen connections between integrins and actin filaments to support force transfer from the cytoskeleton to the extracellular matrix (7, 15, 34). There is evidence that the binding of vinculin to actin and talin can be actively regulated by phosphoinositides and inhibited by acidic phospholipids (20, 53), suggesting that the structural links between actin filaments and integrins may be regulated in some cell types.

We previously hypothesized (23–26, 31) that force development in smooth muscle in response to contractile stimulation involves cytoskeletal reorganization and the remodeling of structural linkages between actin filaments and integrin proteins at membrane-associated dense plaque sites. Contractile activation stimulates actin polymerization in tracheal smooth muscle and other smooth muscle tissues, and the polymerization of actin is required for tension generation in tracheal muscle (2, 8, 30, 31). In the present study, we evaluated the effects of stimulation with acetylcholine (ACh) on the localization of the cytoskeletal “linker” proteins vinculin and talin in freshly dissociated smooth muscle cells obtained from canine tracheal smooth muscle. Both proteins were found to redistribute to the membrane in response to stimulation with ACh, suggesting that contractile stimulation promotes the recruitment of these proteins to the smooth muscle cell membrane.

The proteins focal adhesion kinase (FAK) and paxillin, a substrate for FAK, localize to the focal adhesion sites of cultured cells and have been implicated in regulating focal adhesion assembly and stress fiber formation during cell adhesion (1, 5, 32, 37, 48). Both paxillin and FAK bind to peptides mimicking β-integrin cytoplasmic domains (35). In tracheal smooth muscle they undergo tyrosine phosphorylation in response to contractile stimulation, and this phosphorylation increases concurrently with force development (33, 40, 52).

Address for reprint requests and other correspondence: S. J. Gunst, Dept. of Cellular and Integrative Physiology, Indiana Univ. School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202 (E-mail: sgunst@iupui.edu).

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The depletion of paxillin from tracheal smooth muscle by antisense oligonucleotides inhibits force development and disrupts actin polymerization but does not affect myosin light chain (MLC) phosphorylation or myosin ATPase activity (43). Furthermore, the expression of nonphosphorylatable paxillin mutants in tracheal muscle that suppress ACh-induced paxillin tyrosine phosphorylation also inhibits tension development and actin polymerization, without affecting MLC phosphorylation (42). These findings suggest that paxillin plays an important role in regulating tension development in smooth muscle but that it is not involved in regulating contractile protein activation or cross-bridge cycling. Evidence from the present study indicates that FAK and paxillin also redistribute to the membrane in response to contractile stimulation. We therefore postulated that paxillin might be involved in regulating changes in the organization of the actin cytoskeleton or the linkage of actin filaments to integrin proteins.

In the present study, we evaluated the role of paxillin in regulating the localization of cytoskeletal proteins that may mediate the formation of linkages between actin filaments and integrins. Antisense oligonucleotides (ODNs) were used to deplete tissues of paxillin, and the effects of paxillin depletion on the relocalization of FAK, talin, and vinculin in response to contractile stimulation were determined. Paxillin depletion by antisense depressed contractile force and inhibited the recruitment of vinculin, but not of FAK or talin, to the membrane in response to stimulation with ACh, suggesting that vinculin translocation to the membrane depends on paxillin. We further evaluated the role of paxillin in recruiting cytoskeletal proteins to the membrane by transfecting smooth muscle tissues with plasmids encoding mutant paxillin protein in which the focal adhesion targeting sequence was deleted (paxillin LIM3 dl) (4). Expression of the mutant paxillin LIM3 dl also inhibited the contraction of tracheal tissues and prevented the redistribution of paxillin and vinculin to the membrane in freshly isolated smooth muscle cells during stimulation.

Our results demonstrate that the contractile stimulation of smooth muscle causes the recruitment of vinculin, talin, FAK, and paxillin to the smooth muscle cell membrane. When the agonist-stimulated recruitment of paxillin and vinculin to the membrane is prevented, force development is inhibited, suggesting that this redistribution is required for tension development in smooth muscle. Furthermore, we find that the targeting of vinculin to the smooth muscle membrane in response to contractile stimulation depends on the relocalization of paxillin to the membrane.

METHODS

Preparation of smooth muscle tissues and measurement of force. Mongrel dogs (20–25 kg) were anesthetized with pentobarbital sodium (30 mg/kg iv) and quickly exsanguinated. Experiments were carried out in accordance with the guidelines of 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) 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). PSS was aerated with 95% O2-5% CO2 to maintain a pH of 7.4. Smooth muscle strips (1 mm wide × 0.5 mm thick × 10 mm long) were dissected free of connective tissue and epithelium. Muscle strips were 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, the optimal length for muscle contraction was determined by performing a complete length-tension curve. The muscle length was progressively increased, and the active isometric force elicited by ACh was measured at each length until the force of active contraction reached a maximum.

ODNs dissolved in Tris-EDTA buffer or plasmids encoding wild-type or mutant paxillin protein in which the focal adhesion targeting sequence was deleted (dl LIM3 444–494; Ref. 4) were introduced into muscle strips according to methods previously described (41–43). The strips were then transferred to organ baths containing PSS at 37°C and attached to Grass transducers for the measurement of isometric force. Tissue strips were then removed from the organ baths, and cells were dissociated from them for the analysis of intracellular protein localization as described in Dissociation of airway smooth muscle cells for evaluation of protein localization. In other experiments, after completion of force measurements, muscle strips were frozen with liquid N2-cooled tongs and pulverized under liquid N2 with a mortar and pestle for biochemical analysis.

Introduction of ODNs or plasmids into tracheal smooth muscle. Antisense and sense ODNs to paxillin were designed as previously described (43): paxillin antisense, 5′-GCCATTTAGGGCCTCACT-3′; paxillin sense, 5′-AGTGAGGGCTAAATGGC-3′. Phosphorothiolated ODNs were obtained from Life Technologies (Rockville, MD) or Integrated DNA Technologies (Coralville, IA). pcDNA3 vectors encoding full-length paxillin or a paxillin mutant (paxillin LIM3 dl) were described previously (3, 4). The paxillin LIM3 dl cDNA encodes a mutant paxillin protein in which the LIM3 domain (444–494) is deleted (4). Mutant and wild-type paxillin cDNA were subcloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). Escherichia coli (Bluescript) transformed with wild-type and mutant paxillin cDNA was grown in Luria-Bertani broth overnight. The bacterial cells were harvested by centrifugation at 4,000 rpm for 15 min at 4°C, and the plasmids were purified by standard methodology.

Paxillin antisense or sense ODNs or plasmids were introduced into tracheal smooth muscle strips by the method of reversible permeabilization, which we described previously (41, 43). After the optimal muscle length was determined, muscle strips were placed on metal hooks under tension to maintain them at optimal length. Strips were then 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 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES); solution 2 (at 4°C overnight) containing (in mM) 0.1 EGTA, 5 Na2ATP, 120 KCl, 2 MgCl2, and 20 TES, with 10 μM ODNs (antisense or sense ODNs) or 10 μg/ml plasmids (LIM3 dl mutant or wild type); solution 3 (at 4°C for 30 min) containing (in mM) 0.1 EGTA, 5 Na2ATP, 120 KCl, 10 MgCl2, and 20 TES; 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 aerated with 100% O2 to maintain a pH of 7.1, and solution 4 was aerated with 95% O2-5% CO2 to maintain a pH of 7.4. After 30 min in solution 4, CaCl2 was added gradually to reach a final concentration of 2.4 mM. The strips were then transferred to DMEM containing 5 mM Na2ATP, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml ODNs (antisense or sense) or 10 μg/ml plasmids (paxillin LIM3 dl mutant or wild-type paxillin) and incubated for 2 days at 37°C and 5% CO2. The media were changed every other day. The inclusion of ODNs or plasmids in the incubation medium was done to compensate for the degradation of DNA molecules by nucleases in cells (17, 50). ODNs and DNA molecules can be taken up by endocytosis, thus helping to maintain necessary levels in cells (39, 50). In preliminary experiments we found that the addition of plasmids or ODNs to the DMEM incubation medium enhanced their effectiveness, as evidenced by better expression of recombinant paxillin or enhanced downregulation of wild-type proteins.

The efficiency of tissue transfection was evaluated by immunostaining cells dissociated from plasmid-treated tracheal tissues with antibodies to chicken paxillin, which is specific for the recombinant
protein, and determining the percentage of positively stained cells. Consistent with observations in previous studies (42), we found that ~90% of cells dissociated from the transfected tissues expressed the recombinant proteins, indicating that the plasmids were incorporated into most of the cells throughout the tissue. (see Fig. 8).

Dissociation of airway smooth muscle cells for evaluation of protein localization. After completion of the force measurements, smooth muscle cells were enzymatically dissociated from tracheal muscle strips for the analysis of cellular protein distribution by confocal microscopy. Tracheal muscle strips were minced and homogenized in 5 ml of dissociation solution (composition in mM: 130 NaCl, 5 KCl, 1.0 CaCl2, 1.0 MgCl2, 10 HEPES, and 10 d-glucose, pH 7.4) and triturated with a pipette to liberate individual smooth muscle cells from the tissue. The solution containing the dissociated cells was poured over glass coverslips, and the cells were allowed to adhere to the coverslips for 2 h at room temperature. Cells on coverslips were incubated with a HEPES-buffered saline solution containing 20 mM 2,3-butanedione monoxime (BDM) to prevent shortening. In other experiments, cells were incubated in HEPES-buffered saline solution without BDM to verify that BDM treatment did not alter the cellular distribution of proteins of interest. Cells were stimulated with ACh (10−5 M) for 5 min at 37°C or left unstimulated and used as controls. Stimulated and unstimulated cells were fixed for 10 min in 4% paraformaldehyde (vol/vol) in phosphate-buffered saline (composition in mM: 137 NaCl, 4.3 Na2HPO4, 1.4 KH2PO4, and 2.7 KCl, pH 7.4).

Immunofluorescence staining. Stimulated and unstimulated smooth muscle cells on coverslips were washed three times in Tris-buffered saline (TBS) containing 50 mM Tris, 150 mM NaCl, and 0.1% NaN3 and permeabilized in 0.2% Triton X-100 dissolved in TBS for 2 min. Cells were washed again in TBS and placed in a blocking solution containing 2% goat serum and 1% bovine serum albumin for 1 h at room temperature. Cells were washed repeatedly and incubated with primary antibody against vinculin, talin, paxillin, or FAK for 1 h at 37°C. The primary antibodies used in this study were as follows: vinculin MAb clone Vin-11–5 and talin MAb clone 8D4 (Sigma), paxillin MAb clone 349 and FAK MAb clone 77 (Transduction Laboratories, Lexington, KY), chicken paxillin polyclonal antibody (3), and vinculin polyclonal antibody (43). The specificity of antibodies used for immunofluorescence analysis was documented by Western blot, except for that of the chicken paxillin antibody, which does not react well on Western blot (Fig. 1A). All enzymes were obtained from Sigma (St. Louis, MO). The strips were then washed three times in Tris-buffered saline solution (composition in mM: 130 NaCl, 1.0 CaCl2, 1.0 MgCl2, 10 HEPES, and 10 d-glucose, pH 7) with collagenase (type I, 400 U/ml), papain (type IV, 30 U/ml), bovine serum albumin (1 mg/ml), and dithiothreitol (DTT; 1 mM).

Confocal microscopy and image analysis. The cellular localization of fluorescently labeled vinculin, paxillin, talin, and FAK was assessed in the dissociated smooth muscle cells with a Zeiss LSM 510 laser scanning confocal microscope with an Apo ×63 oil-immersion objective (NA 1.4). Alexa 488-labeled (green) proteins were excited with a 488-nm argon laser light, and fluorescence emissions were collected at 500–550 nm. The fluorescence of Alexa 546-labeled (red) proteins was excited with a helium/neon laser at 543 nm, and emissions were collected at 565–615 nm. The optical pinhole was set to resolve optical sections of ~1 μm in cell thickness. The plane of focus was set midway between the bottom and top of the cell. Fluorescence intensity measurements were standardized among all cells compared within a single experiment by maintaining the same confocal settings for each fluoroprobe.

Images of smooth muscle cells were analyzed for regional differences in fluorescence intensity of stained proteins by quantifying the pixel intensity with a series of 6–10 cross-sectional line scans along the entire length of each cell (Fig. 1A). The area of the nucleus was excluded from the analysis. The ratio of pixel intensities between the

Fig. 1. A: method for the analysis of cytoskeletal protein distribution within each cell. Freshly dissociated smooth muscle cells were fixed, and cytoskeletal proteins were immunofluorescently labeled and visualized by confocal microscopy. Six to ten line scans were traced in each cell. Each line provided a fluorescence intensity profile that described protein distribution along a cross section of the cell (a–d). The ratio of pixel intensity between the cell periphery and the cell interior was calculated from the average of the peak pixel intensities to the lowest pixel intensity for each line. An average ratio for all lines was calculated for each cell. The ratio of pixel intensity between the cell periphery and the cell interior was used as an index of protein distribution. A ratio >1 indicated a higher protein concentration at the cell membrane than in the cell interior. B: immunoblots of extracts of whole cell homogenates were obtained with antibodies to paxillin (PAX), vinculin (VIN), focal adhesion kinase (FAK), and talin. The chicken paxillin antibody does not react well on Western blot; hence, the specificity of the antibody could not be confirmed.
Measurement of intracellular Ca\(^{2+}\) in freshly dissociated cells.

Freshly dissociated smooth muscle cells were allowed to settle on coverslips for 60 min and were washed with HEPES-buffered saline (acetone containing 10% (wt/vol) trichloroacetic acid and 10 mM DTT (acetic-TCA-DTT)), which was precooled with dry ice. Strips were thawed in acetic-TCA-DTT at room temperature and then washed four times with acetic-DTT. Proteins were extracted for 60 min in 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM DTT. MLCs were separated by gelatin-urea PAGE and transferred to nitrocellulose. The membranes were blocked with 5% bovine serum albumin and incubated with polyclonal affinity-purified rabbit MLC20 antibody. Unphosphorylated and phosphorylated bands of MLCs were detected by Western blotting and quantified by densitometry.

Statistical analysis. Comparisons between groups were performed with paired \(t\)-tests. Values of \(n\) refer to the number of cells used to obtain mean values. \(P < 0.05\) was considered to be significant.

RESULTS

Paxillin, vinculin, talin, and FAK redistribute to cell periphery in response to ACh in freshly dissociated smooth muscle cells. Figure 2 illustrates freshly dissociated smooth muscle cells that were fluorescently labeled with antibodies for vinculin, paxillin, FAK, and talin. Each panel of Fig. 2 shows an unstimulated cell or a cell fixed 5 min after stimulation with ACh (10\(^{–4}\) M). In unstimulated cells vinculin, paxillin, FAK, and talin (Fig. 2, A–D, respectively) were distributed throughout the cell. The nucleus appeared as a dark area. In many cells, these proteins appeared to be distributed along longitudinal rows parallel to the long axis of the cell, suggesting that they are associated with cytoskeletal structures. In cells stimulated with ACh, the fluorescence intensity of vinculin, paxillin, FAK, and talin (Figs. 2, E–H) was greater at the cell periphery and lower in the cell interior.

The effect of stimulation with ACh on the cellular localization of each of the fluorescently stained proteins was assessed by quantifying the pixel intensity of fluorescence along multiple cross sections of each cell with line scans (Fig. 1A). The fluorescence intensity profile recorded from a single representative line scan is shown as an inset within each panel in Fig. 2. In unstimulated cells the fluorescence intensity profiles were relatively flat, indicating that cytoskeletal protein distribution was uniform throughout the cell. In contrast, in cells stimulated with ACh, fluorescence intensity was markedly higher at the periphery of each cell relative to the interior of the cell for all four proteins.

Ratios of fluorescence intensity between the cell periphery and the cell interior for vinculin, paxillin, FAK, and talin were calculated for unstimulated cells and for cells stimulated with ACh (Fig. 3). A total of 40 unstimulated and 40 stimulated smooth muscle cells obtained from 6 experiments were analyzed for each protein. Fluorescence intensity for all proteins was three to four times higher at the cell periphery after stimulation with ACh \((P < 0.05)\). These results indicate that stimulation with a contractile agonist promotes the recruitment of cytoskeletal proteins to the smooth muscle cell membrane.

Paxillin depletion by antisense depresses contractile force and inhibits cellular redistribution of vinculin, but not FAK or talin, that occurs with contractile stimulation. Vinculin binds to paxillin, and the localization of vinculin to focal adhesion sites requires a region within the COOH-terminal domain of vinculin that is near the binding site for paxillin (4, 54). We therefore questioned whether the localization of vinculin to the smooth muscle membrane would depend on its interaction with paxillin.
We evaluated the role of paxillin in vinculin localization to the smooth muscle cell membrane by depleting smooth muscle tissues of paxillin with antisense ODNs (43). In a previous study (43), we found that the depletion of paxillin from tracheal smooth muscle by treatment with antisense ODNs inhibited force development but did not prevent the increases in MLC phosphorylation, myosin ATPase activity, or intracellular Ca²⁺ that occur in response to contractile stimulation.

Fig. 2. Distribution of cytoskeletal proteins vinculin, paxillin, FAK, and talin in fixed freshly dissociated tracheal smooth muscle cells viewed by laser scanning confocal microscopy. A single line scan quantifying pixel intensity is indicated for each cell by the arrow. In unstimulated cells, vinculin (A), paxillin (B), FAK (C), and talin (D) were distributed throughout the cell. In cells stimulated with acetylcholine (ACH; E–H) there was a marked increase in fluorescence intensity at the cell periphery relative to the cell interior for all 4 proteins.
that the paxillin antisense selectively inhibited the expression of paxillin protein.

Paxillin antisense ODNs inhibited paxillin expression without affecting the expression of vinculin, MLC kinase (MLCK), or FAK (Fig. 4A). The ratios of expression of paxillin to vinculin, MLCK, and FAK were compared in antisense-treated and sense-treated tissues and expressed as a percentage of the ratios in untreated tissues. In sense-treated tissues, the ratios of paxillin to vinculin, MLCK, and FAK were 95.8 ± 5.6%, 95.3 ± 7.6%, and 99.5 ± 12.4%, respectively, and were not significantly different from those in untreated tissues (n = 4). In contrast, in the antisense-treated tissues, the ratios of expression of paxillin to vinculin, MLCK, and FAK were 24.6 ± 8.6%, 25.3 ± 7.6%, and 26.5 ± 13.2%, respectively, of the ratio in untreated tissues (n = 4). The ratios of FAK to vinculin were not significantly different in sense-treated and antisense-treated muscles from those in untreated tissues (97.6 ± 11.2% and 92.8 ± 9.8%, respectively).

In each experiment, the effect of paxillin depletion on tension development was evaluated in tracheal muscle strips; the muscle strips were then dissociated, and cytoskeletal protein localization was evaluated in the freshly dissociated smooth muscle cells. Isometric force development in response to 10−7 M ACh in muscle strips before and after treatment with paxillin antisense, paxillin sense, and no ODNs is shown in Fig. 4B. In strips incubated with paxillin sense or with no ODNs there were no differences in the force developed after 5-min stimulation with ACh and the force generated before incubation (sense: 96.4 ± 8.56%, untreated: 98.5 ± 7.45%; n = 11). In contrast, contractile force was markedly reduced in tissues treated with paxillin antisense to 12.4 ± 8.2% of the force before incubation (n = 11). These results verified the effectiveness of the paxillin depletion by the antisense treatment and also demonstrated that the contractile responses of the tissues were unaffected by the procedure for loading ODNs into the tissues and by the 2-day incubation period.

After the 2-day incubation period and subsequent measurement of contractile force, smooth muscle cells were enzymatically dissociated from tissues treated with paxillin antisense ODNs, paxillin sense ODNs, or with no ODNs. Cells dissociated from smooth muscle strips treated with paxillin antisense oligonucleotides (ODNs), sense ODNs, or no ODNs for 2 days, and the contractile responses and protein expression were determined. A: representative immunoblot illustrating paxillin expression in antisense-treated, sense-treated, and no-ODN-treated muscle strips from a single experiment. Paxillin expression in the antisense-treated smooth muscle tissue after 2-day incubation was dramatically lower than that in sense-treated or no-ODN-treated muscle strips. The expression of vinculin, myosin light chain (MLC) kinase (MLCK), and FAK was similar in no-ODN-treated, sense-treated, and antisense-treated smooth muscle tissues after 2-day incubation. B: representative isometric force responses of 3 muscle strips from 1 experiment obtained before and after 2-day incubation with paxillin sense ODNs, paxillin antisense ODNs, or no ODNs. Contractile force in response to ACh was dramatically inhibited in the tissue treated with paxillin antisense. The contractile responses of sense-treated or no-ODN-treated muscle strips were similar before and after 2-day incubation.

In the present study, smooth muscle strips were treated with paxillin antisense ODNs, sense ODNs, or no ODNs and incubated for 2 days to allow for the inhibition of protein expression. Protein extracts from muscle strips were then analyzed by Western blot to confirm the suppression of paxillin antisense selectively inhibited the expression of paxillin protein.

mean change in the distribution of all 4 proteins at the membrane relative to the cell interior. Forty cells were studied for each protein. *Significant difference between the fluorescence intensity ratio for stimulated and unstimulated cells (P < 0.05).
exhibited low paxillin immunofluorescence compared with untreated strips or strips treated with paxillin sense (Fig. 5), further confirming that paxillin expression was suppressed by the treatment with paxillin antisense. There was no detectable effect of paxillin depletion by antisense on the distribution of vinculin in unstimulated smooth muscle cells.

The effect of paxillin depletion on the localization of vinculin, talin, and FAK was compared in cells dissociated from tissue strips treated with paxillin antisense ODNs, paxillin sense ODNs, or no ODNs (Fig. 6). Paxillin depletion inhibited the redistribution of vinculin to the cell periphery in response to stimulation with ACh. In contrast, paxillin depletion did not alter the redistribution of talin or FAK in response to stimulation with ACh. In cells treated with paxillin sense and untreated cells (data not shown), vinculin, FAK, and talin all redistributed to the membrane in response to ACh stimulation.

Figure 7 shows mean data for vinculin (A), talin (B), and FAK (C), in which the ratio of fluorescence intensity between the cell periphery and the cell interior was quantified for 20 cells from each treatment group from a total of 4 experiments. In paxillin antisense-treated cells, there was no significant difference between unstimulated and stimulated cells in the ratios of vinculin fluorescence intensity between the cell periphery and cell interior. In contrast, the distribution of talin and FAK at the membrane increased significantly relative to the cell interior in paxillin-depleted cells in response to cholinergic stimulation ($P < 0.05$). The increase in the ratio of talin, vinculin, and FAK at the membrane relative to the cell periphery in response to ACh was similar in untreated cells and in paxillin sense-treated cells. These results demonstrate that paxillin depletion inhibits the cellular translocation of vinculin to the membrane in response to contractile stimulation and that it does not affect the translocation of talin or FAK. Thus the presence of paxillin is required for the translocation of vinculin to the cell periphery in response to cholinergic stimulation.

Expression of mutant paxillin with deletion of its LIM3 domain inhibits force but not MLC phosphorylation in tracheal muscle strips. The LIM3 domain of paxillin near its COOH terminus (residues 444–494) is required for the localization of paxillin to focal adhesions in cultured fibroblasts during cell adhesion (4), whereas the vinculin binding site on paxillin has been localized to a contiguous stretch of 21 amino acids spanning residues 143–164 near the NH2 terminus of paxillin (49). We attempted to determine whether the recruitment of paxillin to the membrane is required for tension development during contractile stimulation, whether the LIM3 domain of paxillin is required for paxillin translocation to the membrane, and whether the translocation of vinculin during cholinergic stimulation depends on the translocation of paxillin.

We expressed a chicken paxillin mutant with a deletion of the LIM3 domain (paxillin LIM3 dl; Ref. 4) in tracheal muscle strips by introducing an expression vector encoding the mutant paxillin protein into the tissues. Plasmids encoding wild-type chicken paxillin were introduced as controls. After transfection, tracheal muscle strips were incubated for 2 days to allow for expression of the recombinant proteins.

![Fig. 5. Immunofluorescence of vinculin (top) and paxillin (bottom) in cells freshly dissociated from smooth muscle strips after treatment for 2 days with no ODNs (A), paxillin sense ODNs (B), or paxillin antisense ODNs (C). Cells were double-stained for vinculin and paxillin. Cells from paxillin-depleted tissues showed a markedly lower level of paxillin immunofluorescence, whereas vinculin fluorescence was similar in cells from strips treated with paxillin antisense ODNs, paxillin sense ODNs, or no ODNs. IF, protein labeled by immunofluorescence.](https://example.com/fig5.png)
The efficiency of tissue transfection was evaluated by immunostaining cells dissociated from plasmid-treated and untreated muscle tissues with antibody to chicken paxillin, which is specific for the recombinant protein, and determining the percentage of positively stained cells. As we previously reported (42), ~90% of the cells dissociated from the transfected tissues stained positively for the recombinant proteins whereas no cells dissociated from untreated tissues stained positively (Fig. 8A).

Expression of recombinant chicken paxillin proteins was also confirmed by immunoprecipitation with a paxillin antibody specific for chicken paxillin, followed by an antibody recognizing both species (Ref. 3; Fig. 8B). The mutant and wild-type chicken recombinant proteins were expressed in the tracheal muscle strips transfected with the plasmids. A faint band was also observed in muscle strips that were not treated with plasmids, which probably resulted from some cross-reactivity of the chicken paxillin antibody with the endogenous paxillin.

Paxillin immunoprecipitates were also immunoblotted for vinculin. Vinculin and metavinculin were detected in immunoprecipitates obtained from untreated fresh muscle tissues (Fig. 8B) and immunoprecipitates of both the recombinant LIM3 dl paxillin and wild-type recombinant paxillin, confirming that vinculin was associated with the mutant LIM3 dl paxillin and wild-type recombinant paxillin (4). Neither PKC-α nor myosin heavy chain (MHC) are detected in paxillin immunoprecipitates, even though both proteins are present in homogenates of tracheal smooth muscle strips (42). Thus the immunoprecipitation procedure with paxillin antibody
specifically concentrates paxillin and paxillin-associated proteins.

The total amount of paxillin protein expressed was compared in extracts of whole cell homogenates from fresh muscle tissues and muscle tissues incubated for 2 days with plasmids encoding wild-type paxillin, mutant LIM3 dl paxillin, or no plasmids by immunoblotting with a monoclonal paxillin antibody that reacts with both recombinant and endogenous species (Fig. 8C). The level of paxillin expression in muscle strips that had been treated with plasmids was significantly higher than that in fresh or untreated muscle strips; there were no differences in paxillin expression between fresh tissues and tissues that were incubated without plasmids (untreated). The ratios of expression of paxillin in transfected tissues to that in untransfected tissues were 2.1 ± 0.2 for LIM3 dl paxillin and 2.2 ± 0.1 for wild-type paxillin (n = 4). These results are consistent with our previous observations (42) that the expression of recombinant mutant paxillin does not inhibit the expression of endogenous paxillin.

We evaluated contractile responses to 10⁻⁵ M ACh in tissues treated with plasmids encoding the mutant recombinant LIM3 dl paxillin, wild-type paxillin, or no plasmids (Fig. 9A). The contractile force generated after stimulation with ACh for 5 min was markedly inhibited in tissues expressing the LIM3 dl paxillin mutant (7.4 ± 3% of preincubation force; n = 6). In muscle strips incubated with plasmids encoding the wild-type paxillin or with no plasmids, there was no significant inhibition of tension development in response to stimulation with ACh. The contractile responses were 100 ± 4% of the preincubation force for tissues not treated with plasmids and 94 ± 5% of the preincubation force for tissues expressing wild-type paxillin. There were no significant differences in force measured before incubation among the three groups.

MLC phosphorylation was also evaluated in muscle strips incubated after treatment with plasmids encoding recombinant wild-type paxillin, LIM3 dl mutant paxillin, or no plasmids by freezing muscle strips after the measurement of contractile tension. Whereas the tension development was markedly inhibited, there were no significant differences in MLC phosphorylation in the three groups of muscles in response to stimulation with ACh (Fig. 9B).

Membrane localization of vinculin during contractile stimulation depends on paxillin localization to membrane mediated by LIM3 domain of paxillin. We evaluated whether the expression of the paxillin mutant LIM3 dl affected the redistribution of paxillin and vinculin to the membrane in response to stimulation with ACh. Tissues expressing mutant LIM3 dl paxillin protein, wild-type paxillin protein, or no recombinant protein were enzymatically dissociated. Freshly dissociated cells were double-stained for both paxillin and vinculin. Deletion of the LIM3 domain of paxillin prevented the redistribution of paxillin to the membrane in response to stimulation with ACh (Fig. 10). Vinculin redistribution to the membrane in response to ACh was also inhibited in cells expressing the paxillin LIM3 dl mutant. Redistribution of both paxillin and vinculin to the membrane in response to ACh was observed in cells expressing the wild-type recombinant paxillin, as well as in untreated cells (data not shown).

The ratios of protein distribution for vinculin and paxillin are shown in Fig. 10B (n = 12; P < 0.05). In cells expressing wild-type recombinant paxillin, the ratio of distribution of vinculin and paxillin between the cell periphery and the cell interior was approximately four times higher than the ratio in unstimulated cells. In contrast, in cells expressing LIM3 dl paxillin, the ratio of distribution of both paxillin and vinculin remained close to 1 in both unstimulated and stimulated cells. These results indicate that the LIM3 domain of paxillin is necessary for it to localize to the membrane in smooth muscle cells in response to stimulation with ACh and that the localization of vinculin to the membrane in response to ACh stimulation depends on the membrane localization of paxillin.

Fig. 7. Mean ratios of pixel intensity for vinculin (A), talin (B), and FAK (C) in unstimulated and stimulated smooth muscle cells after treatment with paxillin sense, paxillin antisense, or no ODNs (untreated). Protein distribution is expressed as the ratio of fluorescence intensity of the cell periphery to the cell interior. In both stimulated and unstimulated cells, the fluorescence intensity ratios were >1, indicating that there was a higher protein concentration at the cell periphery than in the cell interior. After stimulation with ACh, there was a marked increase in the localization of all 3 proteins at the membrane relative to the cell interior in both the untreated and paxillin sense-treated cells. In the paxillin antisense-treated cells, there were no significant differences in vinculin distribution between unstimulated and stimulated (ACh) cells, indicating that paxillin depletion inhibited the redistribution of vinculin to the cell periphery in response to contractile stimulation. The redistribution of talin and FAK to the membrane in response to ACh was not affected by paxillin depletion and increased relative to the cell interior to the same extent in paxillin antisense-treated, sense-treated, and untreated cells. Each mean value was obtained from the average of 6–10 line scans in each of 20 cells. *Significant difference between fluorescence intensity ratio of stimulated and unstimulated cells (P < 0.05); NS, not significant.
Fig. 8. A: cells freshly dissociated from muscle strips that were not treated with plasmids or were treated with plasmids encoding chicken paxillin mutant LIM3 dl were immunostained with anti-chicken paxillin antibody followed by secondary antibody conjugated with Alexa 488 fluoroprobe. Cells were examined at low power under a confocal microscope to detect expression of recombinant chicken paxillin. Immunofluorescence images are shown on left and phase-contrast images of the same fields of cells on right. The arrow points to a cell not expressing recombinant chicken paxillin. B: representative immunoblots of paxillin immunoprecipitates from extracts of unstimulated smooth muscle strips after no incubation, 2-day incubation with plasmids encoding wild-type paxillin or LIM3 dl paxillin, or 2-day incubation without plasmid treatment. Paxillin was immunoprecipitated from extracts of fresh muscle strips with monoclonal paxillin antibody and from extracts of plasmid-treated and parallel untreated muscle strips with anti-chicken paxillin antibody, which reacts selectively with the recombinant chicken protein. Both endogenous and paxillin immunoprecipitates were detected with a monoclonal paxillin antibody and then stripped and reprobed with vinculin antibody. Recombinant wild-type or mutant paxillin was immunoprecipitated from extracts of muscle strips treated with plasmids, indicating that the recombinant proteins were expressed in the tissue strips. Vinculin and metavinculin coprecipitated with both mutant and wild-type recombinant paxillin, indicating that the mutant paxillin retains its ability to bind vinculin. Paxillin immunoprecipitates were also probed with antibodies to myosin heavy chain (MHC) to evaluate nonspecific protein associations with the immunocomplexes. Although MHC was present in whole muscle extracts (not shown), no MHC was found in the paxillin immunoprecipitates. C: protein stain of paxillin immunoprecipitate (Gelcode blue; Pierce). A distinct band is visible at the molecular mass of paxillin. This stain is not sensitive enough to detect other proteins in the immunoprecipitate. D: representative immunoblots of extracts of whole tissue homogenates obtained with a paxillin MAb that reacts with both endogenous and chicken paxillin. More total paxillin is expressed in tissues transfected with plasmids encoding recombinant mutant or wild-type paxillin. The amount of paxillin expressed in tissues incubated without plasmids is similar to that in fresh tissue. Similar results were obtained in immunoblots of homogenates from muscle strips studied in 3 experiments.
Expression of the paxillin LIM3 dl mutant did not inhibit the rise in intracellular Ca\(^{2+}\) in freshly dissociated cells in response to stimulation with ACh. Cells were freshly dissociated from tissues that had been transfected with mutant LIM3 dl paxillin or that had been incubated for 2 days without transfection and loaded with the Ca\(^{2+}\) indicator fluo 3. Changes in intracellular Ca\(^{2+}\) in response to ACh administered to the live cells were evaluated by monitoring the tissues continuously for 1–1 min after stimulation with ACh by using a laser scanning confocal microscope. Twenty-six cells from tissues that had not been transfected and nineteen cells from transfected tracheal muscle strips from three different dogs were studied. An increase in Ca\(^{2+}\) was observed in 23 of 26 cells from the untransfected tissues and in 17 of 19 cells from tissues transfected with plasmids encoding the LIM3 dl mutant paxillin (Fig. 11).

**DISCUSSION**

**Role of cytoskeletal protein recruitment in smooth muscle contraction.** The widely accepted paradigm for active tension development in smooth muscle presumes that connections between the cytoskeleton and cell matrix remain constant during contractile activation and tension generation. Within this paradigm, tension generation during muscle contraction is attributed to the activation of acto-myosin cross bridges and the interaction of actin and myosin filaments whereas the membrane dense plaques and cytosolic dense bodies are presumed to act as stable anchor points for the contractile system (12, 13, 16). Talin and vinculin are well-documented constituents of the membrane-associated dense plaques in smooth muscle and are believed to be important in linking actin filaments to integrin proteins to connect the cytoskeleton to the extracellular matrix (14, 19, 38). In the present study we provide evidence that vinculin and talin, as well as paxillin and FAK, are rapidly recruited to the membrane of freshly dissociated smooth muscle cells in response to stimulation with the contractile agonist ACh. This suggests that the structural organization of the cytoskeleton-cell matrix junctions of smooth muscle is far more dynamic than previously supposed. These observations, combined with evidence that contractile activation stimulates actin polymerization in smooth muscle tissues (2, 8, 31, 42, 43), suggest that regions of the cytoskeleton of smooth muscle cells may undergo rapid remodeling in response to a physiological stimulus for contraction. Analysis of the physiological properties of smooth muscle has led to the proposal that smooth muscle cells undergo cytoskeletal reorganization in response to contraction under different mechanical conditions (23–26, 31). Reorganization of the connections between the actin cytoskeleton and the extracellular matrix may enable smooth muscle cells to adapt their structure to the mechanical conditions present in their external environment at the time of activation.

The formation of focal adhesion plaques during cell adhesion and migration has been proposed to strengthen cytoskeleton-extracellular matrix connections to support the force applied by the cytoskeleton to the extracellular matrix through integrin molecules (18). Vinculin and talin have been identified as constituents of membrane plaques in smooth muscle cells and tissues (14, 19, 38). The recruitment of cytoskeletal proteins to the adhesion sites of smooth muscle during contractile stimulation may serve an analogous function. Our observations that talin and vinculin are recruited to the smooth muscle membrane during active contraction, and our previous observations (33) that talin undergoes serine-threonine phosphorylation during the contractile activation of tracheal smooth muscle, provide evidence that the function of these proteins is actively regulated during smooth muscle contraction.

Current evidence suggests that both talin and vinculin are primary constituents of integrin-cytoskeletal connections in both smooth muscle and nonmuscle cells. Talin can bind directly to the cytoplasmic domains of β-integrins and to actin filaments and can thereby form a direct link between integrin proteins and actin filaments (6, 27). Talin assembles into the initial sites of adhesion formed between integrin receptors and
the extracellular matrix at the leading edge of migratory cells (11, 29). Vinculin binds to talin and to actin filaments, as well as to α-actinin and paxillin (9, 10). In cultured fibroblasts, mechanical force acts as a stimulus to recruit vinculin into integrin-extracellular matrix connections (18). The addition of vinculin to vinculin-null cells from an embryonic carcinoma cell line increases the stiffness of integrin-cytoskeleton linkages, suggesting that vinculin reinforces extracellular matrix-cytoskeleton linkages to support the transfer of mechanical stresses from the cytoskeleton across the cell surface (15).

Fig. 10. Paxillin and vinculin immunofluorescence in smooth muscle cells freshly dissociated from muscle strips treated with plasmids encoding wild-type or mutant paxillin (LIM3 deletion) and stimulated with ACh. Cells were fixed, either stimulated with ACh or left unstimulated, and double-stained for paxillin and vinculin. A: paxillin and vinculin were concentrated at the membrane of ACh-stimulated cells dissociated from smooth muscle strips treated with wild-type (WT) paxillin. In the cells expressing mutant LIM3 dl paxillin, paxillin and vinculin were distributed throughout the cytoplasm. B: mean ratios of pixel intensity for vinculin and paxillin in unstimulated and stimulated smooth muscle cells after treatment with plasmids encoding wild-type paxillin or mutant paxillin LIM3 dl. Protein and vinculin distribution throughout the cell is expressed as the ratio of fluorescence intensity of the cell periphery to the cell interior. In cells expressing the wild-type recombinant paxillin, the distribution of both proteins at the membrane relative to the cell interior was significantly greater in ACh-stimulated cells. In cells expressing the recombinant mutant paxillin LIM3 dl, there was no significant difference in paxillin or vinculin distribution between unstimulated and stimulated (ACh) cells. Each mean value was obtained from an average of 6–10 line scans in each of 12 cells from 3 muscle tissues. *Significant difference in fluorescence intensity ratio between stimulated and unstimulated cells (P < 0.05).

Fig. 11. Live cells freshly dissociated from tissue strips treated with plasmids for LIM3 dl paxillin or left untreated respond to stimulation with ACh. Changes in intracellular Ca²⁺ were visualized by confocal fluorescence microscopy in living smooth muscle cells loaded with the Ca²⁺ indicator fluo 3. Muscle strips were incubated for 2 days after treatment with plasmids encoding paxillin LIM3 dl or no plasmids and cells dissociated from them; freshly dissociated cells were monitored for 60 s during stimulation with ACh. Each row of panels shows a sequence of images of the same cell at different time points before and after stimulation with ACh. Intracellular Ca²⁺ increased in response to ACh in both untreated cells and cells expressing paxillin LIM3 dl paxillin. Increases in Ca²⁺ were observed in 23 of 26 untreated cells and 17 of 19 paxillin LIM3 dl-treated cells.
Our data are consistent with previous studies demonstrating the presence of vinculin and talin in the adhesion junctions of smooth muscle cells and tissues (14, 19, 38). In our study, these proteins were more concentrated at membrane sites in unstimulated smooth muscle cells. However, we also demonstrate a process of active recruitment of these proteins to the membrane caused by contractile stimulation: the concentration of these proteins at the membrane increased significantly in response to contractile stimulation. The recruitment of these proteins during active contraction may strengthen existing connections or contribute to the formation of new connections between the cytoskeleton and transmembrane proteins. This may provide additional support for the transmission of force generated by the contractile apparatus to the extracellular matrix.

Role of paxillin in recruitment of cytoskeletal proteins to adhesion plaques. Paxillin functions as a scaffolding protein and has been demonstrated to play an important role in regulating cytoskeletal organization in a variety of cell types (46). The depletion of paxillin from tracheal smooth muscles by treatment with antisense ODNs dramatically inhibits active force development but does not affect MLC phosphorylation, myosin ATPase activity, or intracellular Ca^{2+} (43). Furthermore, the expression of nonphosphorylatable paxillin proteins in smooth muscle inhibits tension development, also without affecting MLC phosphorylation (42). These findings indicate that the role of paxillin in tension development in smooth muscle is independent of contractile protein activation or cross bridge cycling. Because paxillin has been implicated in regulating the formation of adhesion plaques in cultured cells (4), we speculated that paxillin might be important in regulating cytoskeletal linkages between actin filaments and the extracellular matrix during smooth muscle contraction. Furthermore, vinculin and FAK have been shown to bind directly to paxillin and talin binds to vinculin (21, 45, 47, 54). We evaluated the role of paxillin in the recruitment of vinculin, talin, and FAK during contractile stimulation by introducing antisense ODNs into smooth muscle tissues to selectively suppress the expression of paxillin protein. We then determined the effects of contractile stimulation on the localization of cytoskeletal proteins in cells dissociated from those strips. We found that the recruitment of vinculin to the smooth muscle membrane during contractile stimulation was inhibited by depletion of paxillin protein, whereas depletion of paxillin by antisense did not prevent the translocation of FAK or talin to the membrane in response to contractile stimulation (Fig. 7). This suggests a critical role for paxillin in regulating the organization of cytoskeletal linkages during contractile stimulation.

We used a mutant paxillin protein to evaluate whether the translocation of paxillin to the smooth muscle membrane is required for tension development. Four LIM domains have been mapped on the COOH terminus of paxillin; one of these, LIM3, is the primary determinant for the targeting of paxillin to focal adhesions in cultured fibroblasts (4). We transfected smooth muscle tissues with plasmids encoding paxillin LIM3 dl and expressed the mutant paxillin in the muscle tissues. Immunofluorescence analysis of cells freshly dissociated from these tissues demonstrated that the paxillin did not redistribute to the membrane in response to contractile stimulation; thus the LIM3 domain of paxillin is critical for the targeting of paxillin to the smooth muscle cell periphery during stimulation with ACh. The relocalization of vinculin to the membrane in response to contractile stimulation was also prevented by expression of the mutant paxillin LIM3 dl.

The expression of paxillin LIM3 dl also dramatically inhibited tension development in smooth muscle tissues. However, it had no effect on MLC phosphorylation and it did not prevent a rise in intracellular Ca^{2+} stimulated by ACh. Thus the recruitment of paxillin and vinculin to the cell periphery appears to be critical for the development of active tension in smooth muscle. These observations suggest that tension development may involve the regulation of cytoskeletal linkages between actin filaments and transmembrane molecules and that paxillin and vinculin may play a critical role in the formation of these linkages.

Molecular regulation of adhesion plaque formation. The molecular mechanisms that regulate the recruitment and assembly of focal adhesion plaques in cultured cells are controversial. Vinculin can exist in two conformations. In its inactive conformation, an intramolecular interaction between its head and tail regions masks binding sites for talin, α-actinin, and F-actin. In this condition vinculin cannot form a link between actin filaments and the integrin-binding proteins talin or α-actinin. The binding of phosphatidylinositol 4,5-bisphosphate (PIP_2) to vinculin results in a conformational change that exposes binding sites for α-actinin, talin, and F-actin, enabling vinculin to promote connections between actin filaments and transmembrane integrins (53). This discovery has led to the proposal that the rho-dependent synthesis of PIP_2 stimulates the recruitment of vinculin to the membrane initially via an interaction between membrane phospholipids and the COOH terminus of vinculin, which inserts into the membrane (9, 20, 53). The active form of vinculin has been proposed to recruit talin and actin and thereby promote the assembly of focal adhesions.

Evidence that talin is recruited to the membrane first (11) led to the proposal that vinculin is recruited to adhesion plaques by binding to talin. The talin binding site on vinculin is localized to the NH_2-terminal 258 amino acids within the head region of vinculin (21, 22). Paxillin binds selectively to the rod domain of vinculin (47). Thus an alternative possibility for the localization of vinculin to focal adhesions is that it is recruited by binding to paxillin. Wood et al. (54) identified two regions near the COOH terminus of the vinculin molecule that were required for its binding to paxillin and for focal adhesion targeting. Although these regions were not identical, they were adjacent, raising the possibility that they are the same. Subsequently, Brown et al. (4) demonstrated that paxillin can target to focal adhesions independently of its interactions with either vinculin or FAK and that the principal mechanism of targeting paxillin to focal adhesions is through its LIM3 domain.

We found that vinculin coprecipitated with endogenous, wild-type, and mutant paxillin that was immunoprecipitated from unstimulated tracheal smooth muscle strips; thus neither the LIM3 domain of paxillin nor its localization to the membrane was required for the association of paxillin with vinculin. These observations are consistent with evidence that vinculin binding motifs on paxillin are near its NH_2 terminus within a contiguous stretch of 21 amino acids (44) that is distinct from the LIM domains located toward the COOH terminus of the molecule (4). Our finding that the LIM3 domain of paxillin was required for the recruitment of paxillin to the membrane during smooth muscle contraction is also consistent with the observa-
tion that this domain of paxillin is required for its recruitment to focal adhesions in cultured fibroblasts. Our results clearly showed that the redistribution of vinculin to the membrane in response to contractile stimulation in smooth muscle was inhibited in cells expressing the paxillin LIM3 dl mutant, as well as in cells depleted of paxillin by antisense. These data indicate that the translocation of vinculin to the smooth muscle cell periphery in response to contractile stimulation is dependent on the recruitment of paxillin. The paxillin binding site on vinculin is exposed when vinculin is in the inactive state (9); thus paxillin and vinculin may interact before their localization at the membrane and be recruited as a complex through the recruitment of paxillin. Paxillin itself may bind directly to β-integrins (35).

In conclusion, our results demonstrate that the cytoskeletal proteins vinculin, talin, paxillin, and FAK are recruited to the smooth muscle cell periphery in response to the stimulation of freshly dissociated smooth muscle cells with ACh. We also find that the recruitment of vinculin and paxillin to the membrane during contractile stimulation is necessary for tension development in smooth muscle. These results suggest that these proteins play a critical role in regulating cytoskeleton-transmembrane linkages in response to contractile stimulation and the regulation of these linkages is essential for force transmission. We also show that the recruitment of vinculin to the cell membrane in smooth muscle depends on the recruitment of paxillin and that paxillin recruitment is mediated by the LIM3 domain of the paxillin molecule. Thus the ability of paxillin to target to the membrane and recruit vinculin is an essential step in the regulation of tension development in smooth muscle. These observations suggest a mechanism for the dynamic regulation of the cytoskeletal organization of smooth muscle cells during active contraction.

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