The Adapter Protein CrkII Regulates Neuronal Wiskott-Aldrich Syndrome Protein, Actin Polymerization, and Tension Development during Contractile Stimulation of Smooth Muscle*

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Actin polymerization has been shown to occur in tracheal smooth muscle tissues and cells in response to contractile stimulation, and there is evidence that the polymerization of actin is required for contraction. In tracheal smooth muscle, agonist-induced actin polymerization is mediated by activation of neuronal Wiskott-Aldrich syndrome protein (N-WASp) and the Arp (actin-related protein) 2/3 complex, and activation of the small GTPase Cdc42 regulates the activation of N-WASp. In the present study, the role of the adapter protein CrkII in the regulation of N-WASp and Cdc42 activation, actin polymerization, and tension development in smooth muscle tissues was evaluated. Stimulation of tracheal smooth muscle tissues with acetylcholine increased the association of CrkII with N-WASp. Plasmids encoding wild type CrkII or a CrkII mutant lacking the SH3 effector-binding ability, CrkII SH3N, were introduced into tracheal smooth muscle tissues, and the tissues were incubated for 2 days to allow for protein expression. Expression of the CrkII SH3N mutant in smooth muscle tissues inhibited the association of CrkII with N-WASp and the activation of Cdc42. The CrkII SH3N mutant also inhibited the increase in the association of N-WASp with Arp2, a major component of the Arp2/3 complex, in response to contractile stimulation, indicating inhibition of N-WASp activation. Expression of the CrkII SH3N mutant also inhibited tension generation and actin polymerization in response to contractile stimulation; however, it did not inhibit myosin light chain phosphorylation. These results suggest that CrkII plays a critical role in the regulation of N-WASp activation, perhaps by regulating the activation of Cdc42, and that it thereby regulates actin polymerization and active tension generation in tracheal smooth muscle. These studies suggest a novel signaling pathway for the regulation of smooth muscle tension development in smooth muscle tissues.

CrkII is a member of a family of adapter proteins, which are composed of one Src homology 2 (SH2)† and various numbers of SH3 domains and have no functional motifs (1, 2). The SH2 domain of these proteins recognizes tyrosine-phosphorylated proteins, and the SH3 domain associates with poly-proline (PXXP) containing motifs (3). These adaptor proteins, which also include the proteins Nck and Grb2, are thus capable of forming selective multiprotein complexes that couple distinct signal transduction proteins to protein complexes that mediate core functions of the cell (4). The formation of specific protein complexes mediated by CrkII has been associated with the regulation of actin stress fiber organization, cell migration, and mitogenesis in some non-muscle cells including fibroblasts and NBT-II cells (5–7).

Globular (G)-actin is stimulated to polymerize onto filamentous (F)-actin in various smooth muscle tissues and cultured smooth muscle cells in response to contractile stimulation (8–15). Inhibition of actin polymerization by cytochalasin or latrunculin depresses force development in smooth muscle without affecting myosin light chain phosphorylation (8, 16–18). These studies suggest that both actin polymerization and contractile protein activation are required for tension development in smooth muscle.

The actin-related protein (Arp) 2/3 complex, a 7-component protein complex, promotes the nucleation of new actin filaments in vitro and in a number of motile cell types (19–21). In response to external stimulation, the Arp2/3 complex is activated by N-WASp, which is a ubiquitously expressed member of the Wiskott-Aldrich syndrome protein (WASP) family of proteins that interacts with the Arp2/3 complex and G-actin to stimulate actin polymerization (19, 22–24). We have found that contractile stimulation of smooth muscle increases the association of N-WASp with Arp2, a major component of the Arp2/3 complex, and that the activation of the Arp2/3 complex by N-WASp regulates actin polymerization in smooth muscle (15, 25).

The small GTPase Cdc42 can regulate the activation of N-WASp in vitro. The GTP-bound form of Cdc42 interacts with N-WASp and initiates actin filament formation mediated by the Arp2/3 complex (19, 26, 27). The activation of Cdc42 in response to contractile stimulation can regulate the association of N-WASp with the Arp2/3 complex and regulates actin polymerization in smooth muscle tissues (25).

The SH2/SH3 adapter proteins Nck and Grb2 have been implicated in the regulation of N-WASp activation in reconstituted in vitro systems (28, 29). Addition of Nck SH3 domains

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‡ The abbreviations used are: SH2, Src homology 2; N-WASp, neuronal Wiskott-Aldrich syndrome protein; Arp, Cdc42, and N-WASp.
into an in vitro system in the presence of purified N-WASP initiates the nucleation of actin filaments mediated by purified Arp2/3 complex (29). Grb2 has also been shown to act as an N-WASP effector in systems of purified proteins in vitro, leading to actin assembly via the N-WASP Cdc42. N-WASP activation in this tissue by regulating the activation of actin polymerization by external stimuli in smooth muscle. Our results demonstrate that CrkII plays an essential role in regulating N-WASP activation, actin polymerization, and force generation in smooth muscle tissues. We hypothesized that CrkII may couple phosphorylated paxillin to N-WASP and thereby provide a mechanism for the initiation of actin polymerization by external stimuli in smooth muscle. Our results demonstrate that CrkII plays an essential role in regulating N-WASP activation, actin polymerization, and tension development during the contractile stimulation of smooth muscle tissues with acetylcholine. We also found that CrkII regulates the activation of Cdc42 and the association of Cdc42 with N-WASP. These observations suggest that CrkII may regulate N-WASP activation in this tissue by regulating the activation of Cdc42.

EXPERIMENTAL PROCEDURES

Preparation of Smooth Muscle Tissues—Mongrel dogs (20–25 kg) were anesthetized with pentobarbital sodium (30 mg/kg, intravenously) and euthanized by exsanguination. A 12- to 14-cm segment of extra-thoracic trachea was immediately removed and immersed in physiological saline solution at 22 °C (composition in mM: 110 NaCl, 3.4 KCl, 2.4 CaCl₂, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 glucose). The solution was aerated with 95% O₂ and 5% CO₂. After 30 min in solution 1 (at 4 °C overnight) containing 5 mM NaCl, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml streptomycin, the trachea was immediately removed and immersed in physiological saline solution at 37 °C and aerated with 95% O₂. DNA (50 μg/ml) was then added to the tissue and incubated at 37 °C for 20 min. The tissue was then transferred to a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂. After 30 min in solution 4, the tissue was washed extensively and incubated in a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂ for 120 min. The tissue was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min. The tissue was then homogenized and centrifuged for the collection of supernatant, and the supernatant was mixed with lysis buffer containing 25 mM Tris (pH 7.5), 5 mM MgCl₂, 0.1 mM NaCl, 1% Nonidet P-40, 5% sucrose, 50% glutathione beads for 15 min at 4 °C, and centrifuged. The supernatant was washed with a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂ for 120 min. The tissue was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min. The tissue was then homogenized and centrifuged for the collection of supernatant, and the supernatant was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min.

Toxin and Measurement of Muscle Force—Pulverized tissue strips were treated with various toxins and/or inhibitors. The strips were then incubated in a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂. After 30 min in solution 4, the tissue was washed extensively and incubated in a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂ for 120 min. The tissue was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min. The tissue was then homogenized and centrifuged for the collection of supernatant, and the supernatant was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min.

Assessment of Cdc42 Activation—The activation of Cdc42 was determined by using an assay kit (BK034) from Cytoskeleton, Denver, CO. Pulverized muscle tissues were treated with various toxins and/or inhibitors. The strips were then incubated in a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂. After 30 min in solution 4, the tissue was washed extensively and incubated in a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂ for 120 min. The tissue was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min. The tissue was then homogenized and centrifuged for the collection of supernatant, and the supernatant was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min.

Analysis of Protein Expression—Pulverized muscle strips were treated with various toxins and/or inhibitors. The strips were then incubated in a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂. After 30 min in solution 4, the tissue was washed extensively and incubated in a solution of 20 mM Tris, pH 7.4, 2% Triton X-100, 0.5% SDS, 200 μg/ml EDTA, and 0.5% sodium deoxycholate. The solution was maintained at pH 7.4 and aerated with 95% O₂ and 5% CO₂ for 120 min. The tissue was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min.
4 K-gCa^2+–EGTA, 1 DTT, 10 sodium creatine phosphate, 20 imidazole, 8.9 magnesium acetate, 100.5 potassium acetate, and 1 mg/ml creatine phosphokinase (pH 7.1).

**Analysis of Myosin Light Chain Phosphorylation**—Muscle strips were rapidly frozen at desired time points after contractile stimulation and then immersed in acetone containing 10% (v/v) trichloroacetic acid and 10 mM DTT that was precooled with dry ice. Strips were thawed in acetone-trichloroacetic acid-DTT at room temperature and then washed 4 times with acetone-DTT. Proteins were extracted for 60 min in 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM DTT. Myosin light chains (MLCs) were separated by glycerol-urea polyacrylamide gel electrophoresis and transferred to nitrocellulose. The membranes were blocked with 5% milk and incubated with polyclonal affinity-purified rabbit MLC20 antibody. The primary antibody was reacted with horse-radish peroxidase-conjugated anti-rabbit IgG (ICN). Unphosphorylated and phosphorylated bands of MLCs were visualized by ECL and quantified by scanning densitometry. MLC phosphorylation was calculated as the ratio of phosphorylated MLCs to total MLCs.

**Assessment of Paxillin Tyrosine Phosphorylation**—Paxillin tyrosine phosphorylation was determined by immunoblot analysis using antiphosphotyrosine antibody (ICN) (33, 34).

**Analysis of F-actin/G-actin Ratio**—The concentration of F-actin and G-actin in smooth muscle tissues was measured using an assay kit from Cytoskeleton Inc. (12, 15, 42–44). Briefly, each of the 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 P40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% antifoam C, 1 mM ATP, 1 μg/ml peptatin, 0.15 μl leupeptin, 0.01 μl benzamidine, 0.004 mM tosyl arginine methyl ester). The supernatants of protein extracts were collected after centrifugation at 151,000 x g for 60 min at 30 °C. The pellets were resuspended in ice-cold distilled H2O plus 10 μM cytochalasin D and then incubated on ice for 1 h to dissociate F-actin. The resuspended pellets were gently mixed every 15 min. The supernatant of the resuspended pellets was collected after centrifugation at 5,000 rpm, 2 min at 4 °C. Equal volumes of the first supernatant (G-actin) or second supernatant (F-actin) were subjected to analysis by immunoblot using anti-actin antibody. The amount of F-actin and G-actin was determined by scanning densitometry.

**Assessment of Protein Interactions by Co-immunoprecipitation**—Muscle extracts containing equal amounts of protein were precleared for 30 min with 50 μl of 10% protein A-Sepharose (Sigma). The precleared extracts were centrifuged at 14,000 rpm for 2 min. The pellets were resuspended in 2–3 h with goat polyclonal antibody against N-WASP (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated for 2 h with 125 μl of a 10% suspension of protein A-Sepharose beads conjugated to rabbit anti-goat Ig. Immunocomplexes were washed four 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. The immunoprecipitates were separated by SDS-PAGE followed by transfer to nitrocellulose membranes. The nitrocellulose membranes were divided into two parts; the lower part was probed with monoclonal antibody for CrkII (BD Biosciences), stripped, and reprobed with polyclonal Arp2 antibody (Santa Cruz Biotechnology) or monoclonal Cdc42 antibody (BD Biosciences). The upper part was probed with N-WASP antibody. Proteins were quantitated by scanning densitometry of immunoblots.

**Statistical Analysis**—All statistical analysis was performed using Prism 4 software (GraphPad Software, San Diego, CA). Comparison among multiple groups was performed by one-way analysis of variance followed by post test (Tukey’s multiple comparison test). Differences between pairs of groups were analyzed by Student-Newman-Keuls test or Dunn’s method. Values of n refer to the number of experiments used to obtain each value. p < 0.05 was considered to be significant.

**RESULTS**

**Contractile Stimulation Increases the Interaction of CrkII with N-WASP in Smooth Muscle Strips**—We evaluated the effect of contractile stimulation on the interaction of CrkII with N-WASP in smooth muscle tissues. Tracheal smooth muscle strips were stimulated with 10−5 M ACh for 5 min, or they were unstimulated. Extracts of these smooth muscle strips were immunoprecipitated with N-WASP antibody. Blots of N-WASP immunoprecipitates and corresponding supernatants were probed using CrkII antibody, stripped, and reprobed with N-WASP antibody. Our previous studies have demonstrated that the other signaling molecules protein kinase Cα and Rho are not found in the N-WASP immunoprecipitates, indicating that the immunoprecipitates have relatively high selectivity for CrkII (25).

Stimulation with ACh resulted in an increase in the association of CrkII with N-WASP in smooth muscle tissues. As shown in Fig. 1A, the amount of CrkII that co-immunoprecipitated with N-WASP was significantly greater in the ACh-stimulated tissues than in unstimulated strips. The ratios of CrkII to N-WASP in the pellet or supernatant of ACh-stimulated tissues was calculated against the corresponding value obtained from unstimulated strips. Values represent means ± S.E. (n = 3). The asterisk indicates significant different ratios of CrkII/N-WASP in the stimulated strips relative to the ratio in unstimulated tissues (p < 0.05). C, representative immunoblot illustrating that the density of N-WASP blotting for whole homogenates was similar between unstimulated and ACh-stimulated muscles. The blot is representative of three identical experiments.

![Fig. 1. Increase in the association of CrkII with N-WASP in smooth muscle tissues in response to stimulation with ACh.](image)

The upper part was probed with N-WASP antibody. The primary antibody was reacted with horse-radish peroxidase-conjugated anti-rabbit IgG (ICN). Unphosphorylated and phosphorylated bands of MLCs were visualized by ECL and quantified by scanning densitometry. MLC phosphorylation was calculated as the ratio of phosphorylated MLCs to total MLCs.

**Analysis of Myosin Light Chain Phosphorylation**—Muscle strips were stimulated with 10−5 M ACh for 5 min, or they were unstimulated. N-WASP was immunoprecipitated from muscle extracts with N-WASP antibody. Blots of the immunocomplex and corresponding supernatants were detected by use of CrkII antibody, stripped, and reprobed with N-WASP antibody. A representative immunoblot illustrating the increase in the interaction of CrkII with immunoprecipitated N-WASP in response to ACh stimulation. The amount of CrkII in the supernatant was reduced in Ach-stimulated muscles as compared with unstimulated tissues. B, the ratio of CrkII to N-WASP in the pellet or supernatant of ACh-stimulated tissues was calculated against the corresponding value obtained from unstimulated strips. Values represent means ± S.E. (n = 3). The asterisk indicates significant different ratios of CrkII/N-WASP in the stimulated strips relative to the ratio in unstimulated tissues (p < 0.05). C, representative immunoblot illustrating the density of N-WASP blotting for whole homogenates was similar between unstimulated and ACh-stimulated muscles. The blot is representative of three identical experiments.
stimulated tissues (Fig. 1, A and B, n = 4, p < 0.05). The amount of N-WASp in whole homogenates from unstimulated and ACh-stimulated muscles was not significantly different when evaluated by densitometric analysis of N-WASp immunoblots (Fig. 1C), confirming that ACh stimulation does not affect the accessibility of the antibody to the N-WASp.

Expression of Recombinant CrkII Proteins in Smooth Muscle Tissues—Crk-associated signaling events may depend on the ability of this protein to couple to downstream effectors via its NH2-terminal SH3 domain (35). N-WASp protein possesses a proline-rich domain that may associate with SH3-containing proteins (26). To determine whether the association of CrkII with N-WASp plays a role in regulating tension development in smooth muscle, we introduced plasmids encoding wild type CrkII or the CrkII mutant SH3N (a mutant that does not bind downstream effectors via its NH2-terminal SH3 domains) into smooth muscle strips by reversible permeabilization. These strips were then incubated for 2 days to allow for protein expression. Expression of these untagged wild type and mutant CrkII proteins in smooth muscle strips was assessed by immunoblot analysis.

The protein levels of CrkII were higher in muscle strips transfected with plasmids encoding recombinant proteins than in strips not treated with plasmids (Fig. 2A). To ensure the accuracy of protein loading, we also assessed the protein levels of the cytoskeletal proteins vinculin and metavinculin, a muscle isoform of vinculin present in smooth muscle. The levels of metavinculin/vinculin were similar in untransfected strips, strips expressing wild type CrkII, and tissues expressing SH3N CrkII mutant (Fig. 2A). The ratios of CrkII to metavinculin/vinculin were 2-fold higher in muscle strips expressing wild type or SH3N CrkII than in muscle tissues not treated with plasmids (Fig. 2B, n = 4, p < 0.05).

The SH3N CrkII Mutant Inhibits the Association of N-WASp with CrkII and Arp2—We evaluated whether expression of the SH3N CrkII mutant affects the increase in the association of CrkII with N-WASp and the activation of N-WASp, as indicated by the association of N-WASp with Arp2, a protein constituent of the Arp2/3 complex. Tracheal smooth muscle strips that had been incubated with plasmids encoding wild type CrkII or plasmids for the SH3N CrkII mutant were stimulated with ACh for 5 min, or they were not stimulated. Untransfected muscle strips were also subjected to the same treatments. The association of N-WASp with CrkII or Arp2 was then determined by co-immunoprecipitation analysis.

In untransfected tissues, the amount of CrkII and Arp2 that associated with N-WASp immunoprecipitates was increased in response to ACh stimulation (Fig. 3A). However, in muscle strips expressing the CrkII SH3N mutant, ACh stimulation of smooth muscle did not markedly increase the amount of CrkII and Arp2 that co-immunoprecipitated with N-WASp. ACh stimulation of tissues expressing wild type CrkII increased the

![Fig. 2. Expression of wild type and mutant CrkII proteins in smooth muscle tissues. A, blots of smooth muscles strips that had been treated with plasmids encoding wild type CrkII (WT) or SH3N CrkII dominant negative mutant (SH3N), or strips that had not treated with plasmids (NP) were probed with CrkII antibody, stripped, and reprobed with metavinculin (Meta)Vinculin (Vin) antibody. B, the ratios of CrkII to metavinculin/vinculin in muscle strips expressing wild type CrkII or SH3N CrkII mutant were normalized to that in muscle strips not treated with plasmids. Values are means ± S.E. *, significantly higher ratios in muscle strips expressing wild type CrkII or SH3N CrkII mutant than the value for muscles not treated with plasmids (p < 0.05, n = 4).

![Fig. 3. Effects of SH3N CrkII mutant on the interaction of N-WASp with CrkII and Arp2 in response to contractile stimulation. A, tracheal smooth muscle strips that had been treated with plasmids encoding wild type CrkII (WT) or SH3N CrkII mutant (SH3N) were stimulated with 10−5 M ACh for 5 min, or they were not stimulated. Blots of N-WASp immunoprecipitates from these tissues were probed with N-WASp antibody, stripped, and reprobed with antibodies against CrkII and Arp2. Ratios of CrkII/N-WASp (B) and ratios of Arp2/N-WASp (C) in muscle strips expressing wild type CrkII or SH3N CrkII mutant are normalized to corresponding ratios in unstimulated strips not treated with plasmids (NP). Values represent means ± S.E. (n = 4). The asterisk indicates significant higher corresponding protein ratios in the stimulated strips relative to the ratio in unstimulated tissues (p < 0.05).]
amount of CrkII and Arp2 that co-immunoprecipitated with N-WASp (Fig. 3A). The ratios of CrkII to N-WASp and Arp2 to N-WASp were significantly lower in muscle tissues expressing the SH3N CrkII mutant than in untransfected tissues or muscle strips expressing wild type CrkII (Fig. 3, B and C, n = 4, p < 0.05). This indicates that the SH3N CrkII inhibits the association of endogenous CrkII with N-WASp, and impairs the activation of N-WASp.

CrkII SH3N Mutant Inhibits Cdc42 Activation and Its Association with N-WASp—Our previous studies have shown that the activation of N-WASp in tracheal smooth muscle requires the activation of Cdc42 and its association with N-WASp (25). We therefore evaluated the effect of the SH3N CrkII mutant on the association of Cdc42 with N-WASp and on Cdc42 activation.

The interaction between Cdc42 and N-WASp was assessed by co-immunoprecipitation analysis after smooth muscle strips expressing wild type CrkII or the SH3N CrkII mutant were stimulated with 10^-5 M ACh for 5 min, or they were not stimulated. The amount of Cdc42 that co-immunoprecipitated with N-WASp was lower in muscle strips expressing wild type CrkII compared with muscle strips expressing wild type CrkII (Fig. 4, n = 4).

We assessed the effect of SH3N CrkII mutant on Cdc42 activation in response to contractile stimulation. Tracheal muscle strips expressing wild type CrkII or SH3N CrkII mutant were stimulated with 10^-5 M ACh for 5 min. Extracts of these muscle strips were mixed with GST-PBD (p21-activated kinase binding domain) that selectively binds to active Cdc42. The active Cdc42-GST-PBD complex was separated using glutathione affinity beads. The amount of active Cdc42 was determined by immunoblot analysis using Cdc42 antibody.

The amount of Cdc42 in the GST complex precipitates was lower in tissues expressing SH3N CrkII mutant than in muscle strips treated with wild type CrkII or untransfected tissues (Fig. 5A). The extent of Cdc42 activation in muscle strips treated with SH3N CrkII mutant was significantly lower than that in tissues treated with wild type CrkII or untransfected tissues (Fig. 5B, n = 4, p < 0.05). The results suggest that the SH3N CrkII mutant inhibits the activation of Cdc42 in response to contractile stimulation.

Inactive Asn-17 Cdc42 Mutant Does Not Inhibit the Association of N-WASp with CrkII or Paxillin Tyrosine Phosphorylation.—We have previously shown that Asn-17 Cdc42 mutant inhibits the activation of N-WASp in smooth muscle tissues (25). To determine whether the activation of Cdc42 affects the interaction of CrkII with N-WASp, tracheal muscle tissues expressing Asn-17 Cdc42 or wild type Cdc42 were stimulated with ACh and the interaction between CrkII and N-WASp was analyzed by co-immunoprecipitation.

In smooth muscle tissues expressing wild type Cdc42, in untransfected tissues, and in muscle strips expressing Aas17 Cdc42, the amount of CrkII that co-immunoprecipitated with N-WASp was increased in response to ACh stimulation. The increase in the amount of CrkII that co-immunoprecipitated with N-WASp in response to ACh stimulation was not significantly different among the three groups of tissues (Fig. 6, n = 4, p < 0.05).

We also examined paxillin tyrosine phosphorylation in smooth muscle tissues expressing Aas17 Cdc42 mutant or wild type Cdc42. The increase in paxillin tyrosine phosphorylation was determined by anti-phosphotyrosine antibody in response to ACh stimulation was significantly increased in muscle strips expressing Aas17 Cdc42 mutant, muscle tissues expressing wild type Cdc42, and untransfected tissues. There were no
significant differences in the increase in paxillin tyrosine phosphorylation among the three groups of tissues (Fig. 7, \( n = 4 \), \( p < 0.05 \)).

Expression of the SH3N CrkII Mutant Depresses Tension Development in Smooth Muscle—We assessed the effect of the expression of wild type or mutant CrkII on contractile force by evaluating ACh-induced contraction in muscle strips transfected with plasmids encoding wild type CrkII and SH3N CrkII mutant or without plasmids. Contractile force in strips expressing wild type CrkII was slightly higher than that in strips treated without plasmids. Expression of the CrkII mutant inhibited contractile force. B, mean active force in response to \( 10^{-5} \) M ACh is quantified as percentage of ACh-induced force in each strip before treatment. Values are mean \( \pm \) S.E., * significantly lower response compared with muscles without plasmids (\( n = 10 \), \( p < 0.05 \)).

Depletion of CrkII Protein Suppresses the Interaction of N-WASp with Arp2 and Inhibits Tension Development Stimulated by Acetylcholine—We depleted CrkII protein in muscles tissues using antisense ODNs to verify that the inhibition of ACh-induced N-WASp activation and tension development by the SH3N CrkII mutant does not result from other effects of overexpression of the mutant CrkII protein. Protein extracted from smooth muscle strips that had been treated with antisense or sense ODNs or with no ODNs for 2 days was analyzed by Western blot. Endogenous CrkII expression was

with ACh was \( \sim 20\% \) of the preincubation force (Fig. 8B, \( n = 10 \), \( p < 0.05 \)).

Depletion of CrkII Protein Suppresses the Interaction of N-WASp with Arp2 and Inhibits Tension Development Stimulated by Acetylcholine—We depleted CrkII protein in muscles tissues using antisense ODNs to verify that the inhibition of ACh-induced N-WASp activation and tension development by the SH3N CrkII mutant does not result from other effects of overexpression of the mutant CrkII protein. Protein extracted from smooth muscle strips that had been treated with antisense or sense ODNs or with no ODNs for 2 days was analyzed by Western blot. Endogenous CrkII expression was
lower in muscle strips treated with antisense ODNs than in strips treated with sense ODNs or no ODNs (Fig. 9A). Meta-
vinculin/vinculin was similar in sense-treated and antisense-
treated strips and in muscle tissues not treated with ODNs (Fig. 9A). The ratio of CrkII versus metavinculin/vinculin in antisense-treated tissues was significantly lower than that in sense-treated or no-ODN-treated strips (Fig. 9B, n = 4, p < 0.05).

A significant increase in the ratio of Arp2 to N-WASp in N-WASp immunoprecipitates was observed in response to ACh stimulation in untreated and sense-treated muscle tissues. In contrast, ACh did not induce a significant increase in the ratio of Arp2/N-WASp in CrkII antisense-treated tissues (Fig. 9C, n = 4, p < 0.05). Similarly, ACh-stimulated tension development in antisense-treated strips was significantly inhibited compared with that in sense-treated muscle tissues or muscle tissues not treated with ODNs (Fig. 9D, n = 8, p < 0.05).

Expression of the SH3N CrkII Mutant Inhibits Increases in the F-actin/G-actin Ratio Stimulated by ACh in Smooth Muscle Tissues—We evaluated whether the SH3N CrkII mutant affects actin polymerization by assessing the effects of the CrkII mutant SH3N on the F-actin/G-actin ratio in smooth muscle. Smooth muscle strips treated with plasmids encoding wild type CrkII and the CrkII mutant SH3N were stimulated with 10^{-5} M ACh for 5 min for the analysis of F-actin and G-actin. The ratio of F-actin/G-actin was analyzed by fractionation followed by Western blotting as described under “Experimental Procedures.”

In the extracts of muscle tissues not treated with plasmids, the ratio of F-actin to G-actin was 4.09 ± 0.53 in unstimulated strips and 9.18 ± 1.29 in stimulated strips after 5 min stimulation with ACh (Fig. 10, p < 0.05, n = 4). Contractile stimulation led to an increase in the ratio of F-actin/G-actin in smooth muscle tissues expressing wild type CrkII; however, contractile stimulation did not significantly increase the ratio of F-actin/G-actin in strips expressing SH3N CrkII mutant (Fig. 10, p > 0.05, n = 4).

**SH3N CrkII Mutant Does Not Affect Myosin Light Chain Phosphorylation That Occurs in Response to Contractile Stimulation**—Smooth muscle strips treated with plasmids encoding wild type CrkII, SH3N CrkII mutant, or with no plasmids were frozen for the analysis of myosin light chain phosphorylation 5 min after contractile activation with acetylcholine. Force and myosin light chain phosphorylation in response to ACh stimulation are at a steady state by this time (39, 45). Although force production was dramatically depressed (Fig. 8), the increase in myosin light chain phosphorylation in strips expressing SH3N CrkII mutant was similar to that of the muscle strips not treated with plasmids (Fig. 11). The mean increases in myosin and antisense-treated strips are normalized to ratios obtained in no ODNs-treated muscle strips (no ODNs). Values represent mean ± S.E. (n = 4). *, significantly lower ratios in antisense-treated strips relative to no ODNs-treated and sense-treated muscle strips (p < 0.05). C: smooth muscle strips that had been treated with or without ODNs were stimulated with 10^{-5} M ACh for 5 min, or they were not stimulated. Blots of N-WASp immunoprecipitates from these tissues were probed with N-WASp antibody, stripped, and reprobed with Arp2 antibody. Ratios of Arp2/N-WASp are normalized to the ratio in unstimulated strips not treated with ODNs. Values represent means ± S.E. (n = 4). *, significantly higher corresponding protein ratios in the stimulated strips relative to the ratio in unstimulated tissues (p < 0.05). D: smooth muscle strips were contracted with 10^{-5} M ACh before and after treatment with or without ODNs. Contractile force in strips depleted of CrkII was lower than that in strips treated with sense ODNs or strips not treated with ODNs. Mean active force is quantified as the percentage of ACh-induced force in each strip before treatment. *, significantly lower response compared with muscles not treated with ODNs (n = 8, p < 0.05).
Contractile stimulation initiates actin polymerization in tracheal smooth muscle tissues (8, 15, 40). Contractile stimulation also initiates the interaction of N-WASp with the Arp2/3 complex in this tissue, and this requires activation of the small GTPase Cdc42 (25). Activation of the Arp2/3 complex by N-WASp is required for actin polymerization and tension development in response to contractile stimulation with acetylcholine in tracheal smooth muscle tissues (15). Our present results demonstrate that the coupling of CrkII to N-WASp is also required for N-WASp activation, actin polymerization, and tension development in smooth muscle. Furthermore, we find that CrkII regulates the activation of Cdc42 and the interaction of CrkII with N-WASp. These results suggest a novel mechanism for the regulation of N-WASp, actin dynamics, and active tension generation in smooth muscle. In tracheal smooth muscle, contractile stimulation triggers paxillin tyrosine phosphorylation and tyrosine-phosphorylated paxillin increases its binding to CrkII (12, 32, 33). The expression of non-phosphorylatable paxillin mutants inhibits the increase in the association of paxillin with CrkII and also inhibits actin polymerization (12). In the present study, contractile stimulation also increased the association of CrkII with N-WASp, suggesting that paxillin phosphorylation may regulate N-WASp activation via its coupling to CrkII. To evaluate the role of CrkII in regulating N-WASp activation in smooth muscle, we introduced plasmids...
encoding wild type CrkII and the CrkII mutant SH3N, which contains a point mutation within the SH3 domain that inhibits SH3 domain binding (35), into canine tracheal smooth muscle tissues by a method of reversible permeabilization (12, 15, 25, 41). Whereas expression of wild type CrkII in smooth muscle did not inhibit N-WASp activation, expression of the SH3N CrkII mutant inhibited the activation of N-WASp, as indicated by its coupling to Arp2. These results suggest that paxillin-CrkII-N-WASp complex formation is a critical step that is necessary for N-WASp activation in smooth muscle in response to stimulation with acetylcholine.

We recently demonstrated that activation of the small GTPase Cdc42 is required for the activation of N-WASp and acetylcholine-induced actin polymerization in smooth muscle; expression of the dominant-negative Cdc42 mutant Asn-17 in this tissue inhibits the activation of N-WASp (25). In the present report, expression of the CrkII SH3N mutant inhibited the association of N-WASp with Cdc42. Moreover, the CrkII SH3N mutant also inhibited the activation of Cdc42 in response to contractile activation. These results indicate that CrkII regulates both the activation of Cdc42 and the function of N-WASp in smooth muscle. Expression of the Cdc42 mutant Asn-17 did not inhibit the association of CrkII with N-WASp or the increase in paxillin tyrosine phosphorylation in response to acetylcholine stimulation. Because the Asn-17 Cdc42 mutant inhibits the interaction of endogenous Cdc42 with N-WASp and depresses N-WASp activation (25), the observations indicate that the association of both Cdc42 and CrkII with N-WASp are required for N-WASp activation during contractile stimulation of smooth muscle.

The role of Cdc42 in activating WASp family proteins is well documented (46, 47). Both WASp and N-WASp contain a G-protein binding domain, which includes a Cdc42/Rac interactive binding motif that binds to Cdc42 in its GTP-bound form (47–51). The binding of Cdc42 to N-WASp is believed to result in a conformational change in the structure of N-WASp that exposes the N-terminal domains required for N-WASp coupling to the Arp2/3 complex and G-actin, enabling the initiation of actin polymerization (19, 20).

The regulation of the activation of N-WASp by the SH2/SH3 adaptor protein CrkII has not been described; however, the SH2/SH3 adapter proteins Nck and Grb2 have been implicated in regulating N-WASp activation (19, 28, 29). Both of these proteins can bind to the proline-rich region of N-WASp via their SH3 domains (52). In reconstituted actin polymerization systems in vitro, Nck SH3 domains and PIP2 (phosphatidylinositol 4,5-bisphosphate), which binds to conserved sequences near the Cdc42/Rac interactive binding motif of N-WASp, were found to act synergistically to activate N-WASp independently of Cdc42 (29). In contrast, Grb2 bound to N-WASp simultaneously with Cdc42 and enhanced N-WASp-mediated actin polymerization synergistically in an in vitro assay system (28).

In the present study, we found that the interaction of CrkII with N-WASp was not sufficient to stimulate actin polymerization when the activation of Cdc42 was inhibited. These results suggest that the interaction of CrkII with N-WASp is necessary for Cdc42 to bind to and activate N-WASp.

The mechanisms by which CrkII might regulate Cdc42 and N-WASp activation are not known; however, observations of the role of intersectin-1 in the regulation of N-WASp and Cdc42 activation provide a possible model for the function of CrkII in regulating the Cdc42-N-WASp complex (53). Intersectin-1 is a scaffolding protein with an SH3 domain that has guanine nucleotide exchange factor activity toward Cdc42 (53). In cultured cells, the direct binding of N-WASp to intersectin-1 up-regulates its guanosine nucleotide exchange factor activity enabling it to catalyze the generation of GTP-bound Cdc42; the GTP-bound Cdc42 then activates N-WASp (53).

The amino-terminal SH3 domain of CrkII can bind to members of the DOCK180 family of proteins; DOCK180 proteins can function as critical regulators of the small GTPases Rac and Cdc42 by activating the exchange of GDP for GTP (54). In smooth muscle tissues, CrkII may bind to and activate a guanine nucleotide exchange factor such as DOCK180 that regulates the activation of Cdc42, thus enabling the activation of N-WASp.

In the present study, filamentous actin increased by ~10% in response to contractile stimulation, which is consistent with our previous observations in this tissue (8, 12, 15, 25, 40). The mechanism by which a relatively small change in the amount of actin might regulate tension development remains speculative. In motile cells, WASp family proteins bind to the membrane upon activation where they activate the Arp2/3 complex to catalyze the nucleation of actin filaments that branch from existing actin filaments (19, 22, 27). The actin filaments of smooth muscle cells attach to the membrane at dense plaques (55), which are structurally similar to focal adhesion sites of cultured cells (56, 57). At these sites, the cytoplasmic domains of transmembrane β-integrins link to actin filaments via linker proteins, and extracellular regions of integrins engage with extracellular matrix. Thus, it is possible that the actin polymerization mediated by CrkII and Cdc42 occurs at dense plaques of smooth muscle cells during contractile stimulation. The branching of the cortical actin filaments might serve to strengthen the connections of actin filaments to extracellular matrix at dense plaques and thereby regulate tension development and cytoskeletal organization.

We also considered the possibility that the inhibition of tension development in smooth muscle in response to ACh by the SH3N CrkII mutant was caused by an inhibition of intracellular Ca²⁺ or a depression of myosin light chain phosphorylation. A rise in intracellular Ca²⁺ and the phosphorylation of the regulatory light chain of myosin are recognized as primary mechanisms for the activation of crossbridge cycling and tension development during contractile stimulation (58, 59). However, we found that the expression of the SH3N CrkII mutant protein had no effect on myosin light chain phosphorylation in smooth muscle.
response to ACh stimulation, indicating that this was unlikely to be the mechanism for its inhibition of active tension development in these tissues. These observations are consistent with our previous findings that the inhibition of Cdc42 does not inhibit myosin light chain phosphorylation (25). Furthermore, the inhibition of tension development in tissues expressing the SH3N CrkII mutant proteins could not be reversed when the tissues were permeabilized with α-toxin and stimulated with Ca2+, indicating that suppression of the intracellular Ca2+ transient was unlikely to be the mechanism for the depression of tension development caused by the mutant CrkII protein.

In summary, contractile stimulation with acetylcholine increases the association of CrkII with N-WASp in tracheal smooth muscle tissues (Fig. 13). The expression of a CrkII lacking effector binding ability in smooth muscle tissues inhibits the association of CrkII with N-WASp and inhibits the activation of Cdc42 and N-WASp. The depression of CrkII-mediated N-WASp activation by this mutant inhibits tension generation and prevents actin polymerization in response to contractile stimulation without significantly inhibiting myosin light chain phosphorylation. These results suggest that CrkII plays a critical role in the regulation of N-WASp activation, actin polymerization, and active tension generation in tracheal smooth muscle, by regulating the activation of cdc42. These studies suggest a novel signaling pathway for the activation of N-WASp that can regulate active tension development in response to contractile stimulation tracheal smooth muscle tissues.

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