Role of the Adapter Protein Abi1 in Actin-associated Signaling and Smooth Muscle Contraction*[S]

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Signaling and Smooth Muscle Contraction*

Background: Neuronal Wiskott-Aldrich syndrome protein (N-WASP) regulates smooth muscle contraction by affecting actin polymerization.

Results: Abi1 activated by c-Abl regulates N-WASP and smooth muscle contraction, and reciprocally controls c-Abl activation.

Conclusion: The Abi1-mediated actin cytoskeleton process is a novel mechanism for the regulation of smooth muscle contraction.

Significance: The Abi1-mediated actin cytoskeleton process is a novel mechanism for the regulation of smooth muscle contraction.

Actin filament polymerization plays a critical role in the regulation of smooth muscle contraction. However, our knowledge regarding modulation of the actin cytoskeleton in smooth muscle just begins to accumulate. In this study, stimulation with acetylcholine (ACh) induced an increase in the association of the adapter protein c-Abl interactor 1 (Abi1) with neuronal Wiskott-Aldrich syndrome protein (N-WASP) (an actin-regulatory protein) in smooth muscle cells/tissues. Furthermore, contractile stimulation activated N-WASP in live smooth muscle cells as evidenced by changes in fluorescence resonance energy transfer efficiency of an N-WASP sensor. Abi1 knockdown by lentivirus-mediated RNAi inhibited N-WASP activation, actin polymerization, and contraction in smooth muscle. However, Abi1 silencing did not affect myosin regulatory light chain phosphorylation at Ser-19 in smooth muscle. In addition, c-Abl tyrosine kinase and Crk-associated substrate (CAS) have been shown to regulate smooth muscle contraction. The interaction of Abi1 with c-Abl and CAS has not been investigated. Here, contractile activation induced formation of a multiprotein complex including c-Abl, CAS, and Abi1. Knockdown of c-Abl and CAS attenuated the activation of Abi1 during contractile activation. More importantly, Abi1 knockdown inhibited c-Abl phosphorylation at Tyr-412 and the interaction of c-Abl with CAS. These results suggest that Abi1 is an important component of the cellular process that regulates N-WASP activation, actin dynamics, and contraction in smooth muscle. Abi1 is activated by the c-Abl-CAS pathway, and Abi1 reciprocally controls the activation of its upstream regulator c-Abl.

Smooth muscle contraction plays an essential role in regulating the functions of respiratory and cardiovascular systems. Aberrant smooth muscle contraction contributes to the pathogenesis of many diseases such as asthma and hypertension. Despite its important role, the mechanisms that regulate smooth muscle contraction are not completely understood.

Myosin activation by phosphorylation is a key cellular process that regulates force development in smooth muscle. Myosin light chain phosphorylation at Ser-19 by myosin regulatory light chain kinase activates myosin ATPase, and induces sliding of contractile filaments and smooth muscle contraction (1, 2). In addition, recent studies have shown that a pool of actin monomers is assembled onto actin filaments in smooth muscle in response to contractile stimulation. Inhibition of actin polymerization by pharmacological tools and molecular approach attenuates smooth muscle contraction without affecting myosin phosphorylation (3–6). Dynamic changes in the actin cytoskeleton may allow smooth muscle cells to adjust their contractile status upon changes in external environments (3). These studies suggest that both myosin activation and reorganization of the actin cytoskeleton are necessary for force development in smooth muscle. Myosin may serve as an “engine” for smooth muscle contraction whereas the actin cytoskeleton may function as a “transmission system” in smooth muscle. There is considerable information about the regulation of myosin activation (1, 2, 7). In contrast, our knowledge regarding modulation of the actin cytoskeleton in smooth muscle is limited.

Actin polymerization in smooth muscle may be regulated by neuronal Wiskott-Aldrich syndrome protein (N-WASP),2 a member of the WASP/WAVE protein family (8, 9). In the unstimulated state, N-WASP exists in an autoinhibited conformation, wherein the verprolin-cofilin-acidic domain in the C terminus of N-WASP is masked by an intramolecular interaction with the N-terminal GTPase-binding domain (9). Contractile stimulation induces a conformational change, resulting in the release of the C-terminal part of N-WASP, which activates actin polymerization and branching mediated by the Arp2/3 complex (6, 8–10).

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References:
1. The abbreviations used are: N-WASP, neuronal Wiskott-Aldrich syndrome protein; Abi1, c-Abl interactor 1; ACh, acetylcholine; Arp2/3, actin-related protein 2/3; c-Abl, Abelson tyrosine kinase; CAS, Crk-associated substrate; CFP, cyanine fluorescence protein; HASM, human airway smooth muscle; KD, knockdown; YFP, yellow fluorescence protein.

2. The abbreviations used are: N-WASP, neuronal Wiskott-Aldrich syndrome protein; Abi1, c-Abl interactor 1; ACh, acetylcholine; Arp2/3, actin-related protein 2/3; c-Abl, Abelson tyrosine kinase; CAS, Crk-associated substrate; CFP, cyanine fluorescence protein; HASM, human airway smooth muscle; KD, knockdown; YFP, yellow fluorescence protein.
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c-Abl interactor 1 (Abi1) is an adapter protein that has been implicated in the regulation of actin dynamics. In vitro biochemical studies suggest that Abi1 directly binds to N-WASP, which activates the N-WASP and Arp2/3-dependent actin polymerization (11). Moreover, Abi1 has been shown to modulate cell adhesion and migration, which are associated with dynamic changes in the actin cytoskeleton (12, 13). Additionally, c-Abl tyrosine kinase regulates smooth muscle force development by controlling actin dynamics (8, 14, 15). Furthermore, CAS (Crk-associated substrate) has been shown to participate in the regulation of smooth muscle contraction and signaling (8, 16–19). However, the interaction of Abi1 with c-Abl and CAS has not been investigated.

The objective of this study was to evaluate the role of Abi1 in N-WASP activation, actin polymerization, and contraction in smooth muscle. Furthermore, we also assessed whether c-Abl and CAS regulate the activation of Abi1, or vice versa in smooth muscle in response to contractile activation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human airway smooth muscle (HASM) cells were prepared from human airway smooth muscle tissues that were obtained from the International Institute for Advanced Medicine. Human tissues were non-transplantable and consented for research. This study was approved by the Albany Medical College Committee on Research Involving Human Subjects. Briefly, muscle tissues were incubated for 20 min with dissociation solution (130 mM NaCl, 5 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 10 mM HEPES, 130 mM NaCl, 5 KCl, 10 glucose, 1 CaCl₂, 1 MgCl₂, washed with HEPES-buffered saline solution (composition in enzymes were obtained from Sigma. The tissues were then papain (type IV), 1 mg/ml of BSA, and 1 mM dithiothreitol). All dissociation solution (130 mM NaCl, 5 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 10 mM HEPES, 130 mM NaCl, 5 KCl, 10 glucose, 1 CaCl₂, 1 MgCl₂, 0.25 EDTA, 10 mM D-glucose, 10 mM taurine, pH 7, 4.5 mg of collagenase (type I), 10 mg of papain (type IV), 1 mg/ml of BSA, and 1 mM dithiothreitol). All enzymes were obtained from Sigma. The tissues were then washed with HEPES-buffered saline solution (composition in mm: 10 HEPES, 130 NaCl, 5 KCl, 10 glucose, 1 CaCl₂, 1 MgCl₂, 0.25 EDTA, 10 taurine, pH 7). The cell suspension was mixed with Ham’s F-12 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin). Cells were cultured at 37 °C in the presence of 5% CO₂ in the same medium. The medium was changed every 3–4 days until the cells reached confluence, and confluent cells were passaged with trypsin/EDTA solution (20–23). Smooth muscle cells within passage 5 were used for the studies.

Immunoblot Analysis—Cells were lysed in SDS sample buffer composed of 1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.01% bromphenol blue. The lysates were boiled in the buffer for 5 min and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked with bovine serum albumin or milk for 1 h and probed with the use of primary antibody followed by horseradish peroxidase-conjugated secondary antibody (Fisher Scientific). Proteins were visualized by enhanced chemiluminescence (Fisher Scientific) using the LAS-4000 Fuji Image System. Abi1 antibody was purchased from Sigma. Antibodies against N-WASP, phosphomyosin light chain (Ser-19), myosin light chain, c-Abl, and phospho-Abl (Tyr-412) were purchased from Santa Cruz Biotechnology. CAS antibody was purchased from BD Biosciences and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Fitzgerald (Acton, MA). The levels of total protein or phosphoprotein were quantified by scanning densitometry of immunoblots (Fuji Multigauge Software). The luminescent signals from all immunoblots were within the linear range.

Co-immunoprecipitation Analysis—Protein-protein interactions and protein complex formation were evaluated by co-immunoprecipitation analysis as previously described (8, 21, 24) with minor modifications. Briefly, cell extracts were incubated overnight with corresponding antibodies and then incubated for 2–3 h with 125 μl of a 10% suspension of protein A-Sepharose beads. Immunocomplexes were washed four times in buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100. The immunoprecipitates were separated by SDS-PAGE followed by transfer to nitrocellulose membranes. The membranes of immunoprecipitates were probed with the use of corresponding antibodies.

Lentivirus-mediated RNAi in Cells—For Abi1 knockdown (KD), lentiviruses encoding Abi1 shRNA or control shRNA were purchased from Santa Cruz Biotechnology. HASM cells were infected with control shRNA lentivirus or Abi1 shRNA lentivirus for 12 h. They were then cultured for 3–4 days. Positive clones expressing shRNAs were selected by puromycin. Immunoblot analysis was used to determine the expression levels of Abi1 in these cells. Abi1 KD cells and cells expressing control shRNA were stable at least five passages after the initial infection. The experimental procedures for generating c-Abl KD cells were previously described (22, 23).

Construction of N-WASP Sensors and Plasmid Purification—Plasmid encoding N-WASP (Addgene) was used for PCR to generate cDNA of N-WASP. The 5′-primer sequence was 5′-TATACCTGATTTTCCATTTCCCAGGAAGAAA-3′. The 3′-primer sequence was 5′-TGTACAGCTAGCTCGGGAAGAGGATCCAAATAC-3′. The resulting product was ligated with EYFP cDNA, which was then subcloned into pECFP (Clone Tech) followed by bacterial transformation. Plasmid purification was performed using the Maxiprep kit from Qiagen. The QuikChange II site-directed mutagenesis kit (Stratagene) was used to generate mutant N-WASP sensors. For generating the V215G mutant, the 5′-primer sequence was 5′-CCAGCACATTGGACATGCGAGCTTGGGCATCCGAAATAC-3′; the 3′-primer sequence was 5′-GAATTGGATCCACCGTGCCCAGGATCTGG-3′. The resulting product was ligated with the EYFP cDNA, which was then subcloned into pECFP (Clone Tech) followed by bacterial transformation.
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power level, detector gain and amplifier gain) was used to minimize the potential bleed-through. The same microscope setting was used for the experiments. Thus, if any YFP emission occurs, the majority of the fluorescence signals should stem from FRET (25, 26). For quantification of FRET efficiency, the region of interest of cells were positioned and the fluorescent intensity of each channel was measured by Zeiss Analysis software. CFP/YFP fluorescent ratios were used to assess the FRET efficiency. For in vitro analysis, extracts of HASM cells transfected with plasmids for the N-WASP sensor were collected 48 h after transfection. Cdc42 and Abi1 proteins were produced as previously described (20, 27). Cell extracts were treated with 10 μg/ml of Abi1 or Cdc42 plus GTP (100 μM), or were left untreated. Cell extracts were placed in a PTI fluorospectrometer for measurement of the emissions of CFP and YFP.

Analysis of F-actin/G-actin Ratios—The content of F-actin and G-actin in smooth muscle was measured using a method as previously described (8, 10, 28). Briefly, smooth muscle cells were treated with F-actin stabilization buffer (50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 1 mM ATP, 1 μg/ml of pepstatin, 1 μg/ml of leupeptin, 10 μg/ml of benzamidine). The supernatants of the protein extracts were collected after centrifugation at 151,000 × g for 60 min at 37 °C. The pellets were resuspended in ice-cold H₂O plus 1 mm 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 16,100 × g for 2 min at 4 °C. Equal volumes of the first (G-actin) or second (F-actin) supernatant were subjected to immunoblot analysis using α-actin antibody. The amount of F-actin and G-actin was determined by scanning densitometry.

Measurement of Smooth Muscle Contraction—Human bronchial rings (diameter, 5 mm) were placed in physiological saline solution at 37 °C in a 25-ml organ bath and attached to a Grass force transducer connected to a computer with A/D converter (Grass). For lentivirus-mediated RNAi in tissues, the epithelium layer of human bronchial rings was removed by using forceps. They were then transduced with lentivirus encoding Abi1 shRNA or control shRNA for 3–4 days. Force development in response to contractile activation was compared before and after lentivirus transduction. For biochemical analysis, human tissues were frozen using liquid nitrogen and pulverized as previously described (16, 29).

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 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 Activation Increases the Association of Abi1 with N-WASP in HASM Cells/Tissues—N-WASP is known to regulate force development in smooth muscle in response to contractile activation (8, 9, 28). The role of Abi1 in smooth muscle has not been investigated. We hypothesized that contractile stimulation may enhance the interaction of Abi1 with N-WASP, which may facilitate the activation of N-WASP in smooth muscle. To test this, HASM cells were treated with acetylcholine (ACh) or left unstimulated. Extracts of smooth muscle cells were immunoprecipitated with the use of Abi1 antibody, and blots of the precipitates were probed using antibodies against N-WASP and Abi1. In unstimulated cells, the amount of N-WASP in Abi1 immunoprecipitates was relatively low. In contrast, the amount of N-WASP in the precipitates was increased in response to activation with ACh. The ratios of N-WASP/Abi1 were higher in ACh-stimulated cells than in control cells (Fig. 1A). In addition, we found that N-WASP was not detected in Abi1 precipitates from Abi1 KD cells (See below), suggesting the specificity of the assay (Fig. 1B).

To verify this, we used reverse co-immunoprecipitation analysis. Cell extracts were immunoprecipitated using N-WASP antibody, and blots of the immunoprecipitates were probed using antibodies against Abi1 and N-WASP. The level of Abi1 in the precipitates from stimulated cells was higher as compared with unstimulated cells (Fig. 1C).

Furthermore, we evaluated the effects of contractile activation on the interaction of Abi1 with N-WASP at the tissue level. Human bronchial rings were treated with ACh, and Abi1 immunoprecipitates of tissue extracts were subjected to immunoblot analysis. The level of N-WASP in Abi1 precipitates was higher in stimulated tissues than in unstimulated tissues (Fig. 1D).

Effects of ACh on N-WASP Activation in Live Smooth Muscle Cells—To monitor N-WASP activation in live cells, we constructed a fluorescence resonance energy transfer (FRET)-based N-WASP sensor. We fused CFP to the N terminus lacking the first 30 amino acids, and YFP to the C terminus of N-WASP (Fig. 2A) (30). Because Cdc42 is known to activate N-WASP in vitro studies and in smooth muscle (9, 10), we evaluated the basal and Cdc42-treated FRET signal of the sensor. Extracts of unstimulated HASM cells expressing the N-WASP sensor were treated with Cdc42 plus GTP, or left untreated. The emission ratios of YFP/CFP were measured using a spectrofluorometer. The basal FRET signal of the sensor was higher, whereas the Cdc42-mediated FRET efficiency was lower (Fig. 2B). The results demonstrate that changes in the FRET signal represent the activation state of N-WASP.

To assess whether Abi1 directly regulates N-WASP activation, extracts of unstimulated cells expressing the N-WASP probe were treated with Abi1. The emission ratios of YFP/CFP were then determined. Treatment with Abi1 reduced FRET efficiency of the biosensor, suggesting an important role of Abi1 in activating N-WASP in vitro (Fig. 2B).

We then evaluated the effects of contractile stimulation on N-WASP activation in live cells. HASM cells expressing the N-WASP sensor were treated with ACh, and emissions of CFP and YFP were monitored live by laser-scanning confocal microscopy. Before stimulation, the emission ratios of CFP/YFP were relatively lower. In response to ACh stimulation, the CFP signal was increased, whereas the YFP signal was decreased (Fig. 2C). The CFP/YFP ratios gradually increased
during the course of contractile activation (Fig. 2D). These results suggest that contractile stimulation activates N-WASP in live smooth muscle cells. In addition, the time course of N-WASP activation is consistent with that of F/G-actin ratios and contraction (supplemental Fig. S1).

To further characterize the biosensor, cells expressing single-probe constructs (CFP-tagged construct or YFP-tagged construct) were treated with ACh and emission of CFP and YFP were evaluated using the confocal microscope under the same experimental condition. For cells expressing the CFP construct, CFP emission was high, whereas YFP emission was undetected. In cells expressing the YFP construct, neither CFP nor YFP emission was detected. In addition, ACh stimulation did not alter CFP/YFP ratios of V215G N-WASP mutant (to disrupt Cdc42 binding) or P320L/P330L N-WASP mutant (to impair Abi1 binding) (supplemental Figs. S2 and S3). The results validate the selectivity and sensitivity of the biosensor.

**KD of Abi1 Attenuates Activation of N-WASP in Smooth Muscle Cells/Tissues**—To reveal the functional role of Abi1 in N-WASP activation, we evaluated the effects of Abi1 KD on the FRET signal in smooth muscle cells. Stable Abi1 KD cells were generated by using lentivirus-mediated RNAi. Immunoblot analysis showed that the protein level of Abi1 in cells infected with virus for Abi1 shRNA was lower compared with control cells. However, the expression level of GAPDH was similar in these cells. Ratios of Abi1/GAPDH were lower in Abi1 KD cells than in control cells (Fig. 3A).

Stable Abi1 KD cells and cells expressing control shRNA were transfected with plasmids encoding the N-WASP sensor. The effects of ACh stimulation on N-WASP activity in these
cells were determined 2–3 days after transfection. In cells expressing control shRNA, the ratios of CFP/YFP were increased in response to stimulation with ACh. However, the ACh-induced ratios of CFP/YFP were reduced in Abi1 KD cells (Fig. 3B).

We also evaluated the effects of Abi1 KD on Arp2/N-WASP ratios (another indication of N-WASP activation) (8, 9) in tissues. We developed a lentivirus-mediated RNAi to inhibit the expression of Abi1 in smooth muscle tissues. Briefly, human bronchial rings were transduced with lentivirus encoding control shRNA or Abi1 shRNA for 3–4 days. Immunoblot analysis confirmed lower Abi1 expression in these tissues (Fig. 4A). More importantly, the levels of Arp2 in N-WASP precipitates in response to ACh stimulation were lower in tissues infected with virus encoding Abi1 shRNA than in uninfected tissues and tissues transduced with virus encoding control shRNA (Fig. 4B).

Abi1 KD Inhibits Increases in F/G-actin Ratios, but Not Myosin Light Chain Phosphorylation at Ser-19 elicited by ACh—Because both actin polymerization and myosin phosphorylation are critical cellular mechanisms that regulate smooth muscle contraction (1–3, 6, 28), we evaluated the effects of Abi1 knockdown on actin dynamics and myosin activation by using the fractionation assay and immunoblot analysis, respectively. The increase in F/G-actin ratios in response to ACh stimulation was reduced in Abi1 KD cells compared with uninfected cells and cells expressing control shRNA (Fig. 5A). However, myosin light chain phosphorylation at Ser-19 was not different among uninfected cells, cells infected with virus for control shRNA, and Abi1 KD cells (Fig. 5B).

Force Development in Response to ACh Stimulation Is Reduced in Abi1-deficient Bronchial Rings—We assessed the role of Abi1 in smooth muscle contraction. Briefly, the contractile responses of human bronchial rings were determined,
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FIGURE 3. Abi1 knockdown inhibits N-WASP activation in smooth muscle cells. A, KD of Abi1 in cells by lentivirus-mediated RNAi. Blots of extracts from uninfected (UI) cells, cells transduced with lentivirus encoding control (CTL) shRNA or Abi1 shRNA were probed with use of antibodies against Abi1 and GAPDH. Abi1/GAPDH protein ratios in cells expressing control or Abi1 shRNA are normalized to uninfected cells (n = 3, *p < 0.05). B, cells stably expressing control shRNA or Abi1 shRNA were transfected with plasmids encoding N-WASP sensor. These cells were stimulated with ACh (100 μM) 2–3 days after transfection. The fluorescence of CFP/YFP was captured live using a laser confocal microscope. The ratios of CFP/YFP fluorescence were determined using the method described under "Experimental Procedures." Values represent mean ± S.E. (n = 15). *, significantly different at each time point between cells producing control shRNA and Abi KD cells (p < 0.05).

FIGURE 4. Abi1 KD attenuates increases in the association of Arp2 with N-WASP during ACh stimulation in human bronchial tissues. A, immunoblot analysis showing the effects of lentivirus encoding Abi1 shRNA on Abi1 protein expression in human bronchial rings. Protein ratios of Abi1/GAPDH in tissues transduced with lentivirus encoding Abi1 shRNA were significantly lower than those in uninfected (UI) cells and tissues expressing control (con) shRNA (p < 0.05, n = 3). B, representative immunoblots illustrating the effects of Abi1 KD on the association of Arp2 with N-WASP in human tissues. Uninfected bronchial rings and bronchial tissues that had been infected with lentivirus encoding control shRNA (Con shRNA) or Abi shRNA were stimulated with ACh or left unstimulated. Blots of N-WASP immunoprecipitates from these tissues were probed with use of antibodies against N-WASP and Arp2. Ratios of Arp2/N-WASP in various treatments are normalized to uninfected and unstimulated tissues. *, significantly lower ACh-induced Arp2/N-WASP ratios in Abi1 KD cells as compared with UI cells and cells producing control shRNA (p < 0.05). Values represent mean ± S.E. (n = 3).

Slighting of Abi1 by co-immunoprecipitation analysis. The interaction of c-Abl with CAS (another index of c-Abl activation) were determined to assess this, the effects of Abi1 KD on the coupling of c-Abl with CAS sense (Fig. 8). To determine whether CAS controls the activation of Abi1, we assessed the effects of CAS antisense (17, 18) on the activation of Abi1. The association of Abi1 with N-WASP in response to ACh stimulation was lower in CAS KD cells than in untreated cells and cells treated with CAS sense (Fig. 8).

Silencing of Abi1 Attenuates c-Abl Phosphorylation and the Association of c-Abl with CAS—It has been suggested that Abi1 is a target of c-Abl in vitro studies and NIH 3T3 cells (32, 33). To assess whether Abi1 conversely affects c-Abl activation, we assessed the effects of Abi1 KD on c-Abl phosphorylation at Tyr-412 (an indication of c-Abl activation) (8, 34) by immunoblot analysis. c-Abl phosphorylation during contractile activation was lower in Abi1 KD cells than in uninfected cells and cells expressing control shRNA (Fig. 9A). Immunoblot analysis showed that the phospho-Abl band was barely detected in Abl KD cells, suggesting the specificity of the phospho-Abl antibody under the experimental condition (Fig. 9B). To further investigate this, the effects of Abi1 KD on the coupling of c-Abl with CAS (another index of c-Abl activation) were determined by co-immunoprecipitation analysis. The interaction of c-Abl with CAS in response to contractile activation was also reduced by Abi1 KD (Fig. 9C).

DISCUSSION

Actin polymerization has recently emerged as a critical cellular process that regulates smooth muscle contraction. Our knowledge regarding how actin dynamics is regulated in smooth muscle just begins to accumulate. Our present studies suggest that the adapter protein Abi1 is an essential component of the cellular process that regulates dynamics of the actin cytoskeleton in smooth muscle during contractile activation. Furthermore, we have identified a novel activation loop, in which...
c-Abl tyrosine kinase and CAS modulate the activation of Abi1, and Abi1 conversely affects the activation of c-Abl.

We have previously shown that contractile stimulation of smooth muscle activates N-WASP as evidenced by co-immunoprecipitation (8, 28). To monitor N-WASP activation in live cells, we constructed a FRET-based N-WASP sensor. To characterize the biosensor, we used extracts from unstimulated cells expressing the N-WASP sensor because these cells have a lower activation level of N-WASP (see Fig. 2, C and D). The addition of Cdc42 plus GTP, which is equal to active Cdc42, decreases the YFP/CFP ratios. The results suggest that changes in the FRET signal represent the activation state of N-WASP. In addition, our results suggest that Abi1 activates N-WASP in vitro (Fig. 2B). Although endogenous Abi1 (<1 μg/ml) exists in cell extracts, the addition of a high concentration of Abi1 (10 μg/ml) may easily have access to the biosensor, thus activating N-WASP. Furthermore, our results suggest that Abi1 or Cdc42 itself is sufficient to activate N-WASP in vitro, which is consistent with previous biochemical results published by others (11).

Properties of our N-WASP biosensor are similar to the N-WASP biosensors constructed by others despite minor structural differences (30, 35). We conjugated CFP to the N terminus lacking the first 30 amino acids, and YFP to the C terminus of N-WASP, whereas Ward et al. (30) fused YFP to the N terminus without the first 30 residues, and CFP to the C terminus. In addition, Lorenz et al. (35) used full-length N-WASP for the construction. These independent studies demonstrate that FRET analysis is a novel technology to study N-WASP activation.

In the present study, we provide the first evidence that contractile stimulation activates N-WASP in live smooth muscle cells by using the N-WASP biosensor. The results also provide direct evidence that contractile stimulation is able to induce conformational changes of N-WASP from a “closed” structure to an “open” conformation. When in the closed state, the verprolin-homology domain of N-WASP binds to its GTP-binding domain, masking its binding motif for the Arp2/3 complex and inhibiting the activity of N-WASP. When in the open state, N-WASP exposes the binding motif for the Arp2/3 complex and initiating actin polymerization and branching mediated by the Arp2/3 complex (8, 9, 28).

Abi1 is an adapter protein that has a role in the regulation of cell adhesion, migration, and endocytosis (11, 12). In vitro biochemical studies suggest that the SH3 domain of Abi1 directly binds to the proline-rich domain of N-WASP, activating the N-WASP and Arp2/3-dependent actin polymerization (11). However, the role of Abi1 in smooth muscle contraction has not been investigated. In this report, we provide several lines of evidence to suggest that Abi1 may activate N-WASP in smooth muscle cells/tissues upon contractile activation. First, contractile activation induced an increase in the interaction of Abi1 with N-WASP. Second, Abi1 was able to directly activate N-WASP in vitro. Third, Abi1 knockdown by RNAi attenuated the activation of N-WASP in live smooth muscle cells as evidenced by FRET analysis of the N-WASP biosensor. Fourth, Abi1 knockdown also inhibited the interaction of N-WASP with CAS.
with Arp2 (another indication of N-WASP activation) in human bronchial tissues in response to contractile activation. Contractile activation of smooth muscle induces actin filament polymerization, and inhibition of actin polymerization attenuates smooth muscle force development (3, 4). In this report, we found that silencing of Abi1 diminished actin polymerization and contractile force in smooth muscle, suggesting an important role of Abi1 in controlling actin dynamics and contraction in smooth muscle. Because ACh stimulation increases the association of Abi1 with N-WASP, and Abi1 silencing inhibits both N-WASP activation and actin dynamics, we propose that agonist stimulation may activate Abi1, which subsequently activates N-WASP and induces actin polymerization mediated by the Arp2/3 complex and contraction in smooth muscle. Furthermore, Abi1 KD did not affect increases in myosin light chain phosphorylation upon contractile stimulation, indicating that Abi1 is not involved in the regulation of myosin activation.

Actin polymerization may facilitate force development by several mechanisms. Actin polymerization may enhance the linkage of actin filaments to integrins strengthening the transduction of mechanical force between contractile units and extracellular matrix (3, 6, 10, 28, 36, 37). In addition, actin polymerization may promote the “latch” formation of contractile elements, supporting force maintenance under the condition of lower cross-bridge phosphorylation (5, 38). Finally, actin filament assembly may also increase the numbers of contractile units and the length of actin filaments, providing more and efficient contractile elements for force development (39).

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FIGURE 7. KD of c-Abl affects the interaction of CAS with Abi1, and Abi1 activation induced by ACh. A, immunoblot analysis showing Abi1 KD by lentivirus-mediated RNAi. Protein ratios of c-Abl/GAPDH in cells infected with lentivirus encoding c-Abl shRNA were significantly lower than those in uninfected (UI) cells and cells expressing scramble (Scr) shRNA (*, p < 0.05, n = 4). B, representative immunoblots illustrating the role of c-Abl in the association of CAS with Abi1. Blots of CAS immunoprecipitates (IP) from uninfected cells, cells expressing scramble shRNA, and c-Abl KD cells treated with or without ACh were probed with use of antibodies against CAS and Abi1. C, representative immunoblots illustrating the role of c-Abl in the interaction of Abi1 with N-WASP (an indication of Abi1 activation). Blots of Abi1 immunoprecipitates from control cells and c-Abl KD cells treated with or without ACh were probed for Abi1 and N-WASP. D, the ratios of Abi1/CAS and N-WASP/Abi1 in treated cells are normalized to corresponding protein ratios in uninfected and unstimulated cells (*, p < 0.05, n = 3).

FIGURE 8. The interaction of Abi1 with N-WASP is regulated by CAS in smooth muscle during contractile activation. Blots of Abi1 precipitates of untreated cells, CAS sense-treated cells, and CAS antisense-treated cells were probed using Abi1 antibody and N-WASP antibody. N-WASP/Abi1 ratios during ACh stimulation were reduced in CAS KD cells as compared with untreated cells and CAS sense-treated cells (p < 0.05, n = 3–4). Data are mean ± S.E. IP, immunoprecipitate.
of c-Abl (an index of c-Abl activation), and the association of c-Abl with CAS (another indication of c-Abl activation). Because Abi1 is activated by the c-Abl-CAS cascade and Abi1 reciprocally affects the activation of c-Abl, we propose that c-Abl, CAS, and Abi1 form a unique activation loop in smooth muscle in response to agonist activation. The presence of Abi1 in the multiprotein complex may stabilize the conformation of c-Abl, rendering c-Abl in active status during agonist stimulation. This novel activation loop may assist smooth muscle cells to efficiently utilize their energy upon external activation.

Thus, we propose that in addition to myosin activation, agonist stimulation induces formation of the multiprotein complex including c-Abl, CAS, and Abi1, which subsequently activates N-WASP, actin polymerization, and smooth muscle contraction. Furthermore, Abi1 may stabilize the conformation of c-Abl, rendering c-Abl active (Fig. 10).

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