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Dale D. Tang and Jian Tan

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Abstract—A pool of actin monomers is induced to polymerize into actin filaments during contractile stimulation of smooth muscle. The inhibition of actin dynamics by actin polymerization inhibitors depresses active force generation in smooth muscle. In this study, we hypothesized that Crk-associated substrate plays a role in the regulation of contraction and actin dynamics in vascular smooth muscle. Antisense or sense oligodeoxynucleotides for Crk-associated substrate were introduced into carotid smooth muscle tissues by chemical loading. The treatment of smooth muscle strips with antisense oligodeoxynucleotides inhibited the expression of Crk-associated substrates; it did not influence the expression of actin, myosin heavy chain, and paxillin. Sense oligodeoxynucleotides did not affect the expression of these proteins in smooth muscle tissues. Force generation in response to stimulation with norepinephrine or KCl was significantly lower in antisense-treated muscle strips than in sense-treated strips or in muscle strips not treated with oligodeoxynucleotides. The downregulation of Crk-associated substrate did not attenuate increases in phosphorylation of the 20-kDa regulatory light chain of myosin in response to stimulation with norepinephrine. The increase in F-actin/G-actin ratio during contractile stimulation was significantly inhibited in antisense-treated smooth muscle strips. Contractile activation of smooth muscle increased the association of profilin with actin monomers; the depletion of Crk-associated substrate inhibited the increases in the profilin-actin complex in response to contractile stimulation. These results suggest that Crk-associated substrate is a necessary molecule of signaling cascades that regulate active force generation in smooth muscle. This molecule may regulate actin dynamics in smooth muscle in response to contractile stimulation.

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Key Words: phosphorylation • muscle, smooth vascular • myosin • norepinephrine

Although the role of vasoconstrictors in the pathogenesis of essential hypertension has been well recognized,1–3 the molecular mechanisms by which vasoconstrictors regulate force generation in vascular smooth muscle are not completely understood. Until recently, smooth muscle contraction has been thought to depend solely on the regulation of actomyosin crossbridge cycling. There is considerable evidence that a pool of monomeric globular (G) actin is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000085333.76141.33

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From the Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Ind.

Correspondence to Dale D. Tang, Department of Cellular and Integrative Physiology, Indiana University School of Medicine, 635 Barnhill Dr, Indianapolis, IN 46202. E-mail dtang@iupui.edu

Our recent studies have shown that profilin is necessary for actin filament polymerization in carotid smooth muscle in response to stimulation with agonists.17

Signaling pathways by which contractile agonists regulate the function of profilin in smooth muscle are currently unknown. Studies on nonmuscle cells and in vitro experiments indicate that the adapter protein p130 Crk-associated substrate (CAS) may be an upstream regulator of profilin.18–20 In addition, contractile stimulation of vascular smooth muscle cells induces CAS tyrosine phosphorylation.21

In this report, we used antisense oligodeoxynucleotides to inhibit the expression of CAS protein in canine carotid smooth muscle strips and then evaluated the effects of CAS downregulation on tension development, MLC phosphorylation, the ratio of F-actin/G-actin, and the association of profilin with G-actin. We found that the downregulation of CAS inhibited force generation in response to contractile stimulation; however, CAS depletion did not influence phosphorylation of the 20-kDa regulatory light chain of myosin, indicating that CAS does not involve the regulation of contractile protein activation. The inhibition of CAS expression decreases the ratio of F-actin/G-actin, an index of actin...
polymerization. Additionally, norepinephrine (NE) stimulation of smooth muscle resulted in an increase in the binding of profilin to actin monomers. CAS downregulation inhibited the association of profilin with G-actin in smooth muscle stimulated by NE. These findings demonstrate that CAS is required for active force development in smooth muscle. CAS may play an essential role in the regulation of actin dynamics and may be an upstream regulator of profilin in smooth muscle.

Methods

Preparation of Smooth Muscle Tissues

Mongrel dogs (20 to 25 kg) were anesthetized with sodium pentobarbital (30 mg/kg IV) and quickly exsanguinated. All procedures were performed according to the guideline of Institutional Animal Care and Use Committee, Indiana University School of Medicine. A 6- to 8-week-old tracheal muscle strip was immediately removed and immersed in physiological saline solution (PSS) at 22 °C (composition in mmol/L): 110 NaCl, 3.4 KCl, 2.4 CaCl2, 0.8 MgSO4, 25.8 NaHCO3, 1.2 KH2PO4, and 5.6 glucose). The solution was aerated with 95% O2–5% CO2 to maintain a pH of 7.4. Circumferential strips of smooth muscle (3 mm wide, 8 to 10 mm long, and 0.2 mm thick) were dissected from the carotid after removal of the endothelial, intimal, and adventitial layers. The use of an appropriately sized strip was critical for maintaining muscle contractility during the incubation period and for the successful introduction of oligonucleotides throughout the muscle strip.

Measurement of Force Development in Smooth Muscle

Each muscle strip was placed in PSS at 37 °C in a 25-mL organ bath and attached to a Grass force transducer that had been connected to a Gould recorder. Smooth muscle strips were allowed to equilibrate at 37 °C for 30 minutes and were then stimulated with NE. Oligodeoxynucleotides (ODNs) dissolved in Tris-EDTA buffer were introduced into muscle strips according to experimental procedures described previously.22,23 Muscle strips were then incubated for 2 days with ODNs in Dulbecco’s Modified Eagle Medium (DMEM). The strips were returned to PSS at 37 °C in 25-mL organ baths and attached to Grass force transducers for the measurement of isometric force. Unstimulated or NE-stimulated strips were frozen with liquid N2–cooled tongs for biochemical analysis.

Loading of Oligodeoxynucleotides and Organ Culture

Antisense ODNs with the following sequence were designed to selectively suppress CAS expression in canine carotid smooth muscle: 5'-GGTACGCTGGATGCTGCTGG-3'. The sequence for sense ODNs was 5'-CCAGCAGCATCCAGTCAC-3'. According to sequence matching results obtained from The National Center for Biotechnology Information, these sequences are not homologous to sequences of any other contractile proteins, cytoskeletal proteins, or actin-associated proteins. Phosphorothioate ODNs were synthesized and purified by Invitrogen Corporation. A homologous region was confirmed for canine CAS mRNA by using reverse transcription polymerase chain reaction (RT-PCR) to amplify a 240-bp fragment from canine carotid mRNA. The 5'-primer used for RT-PCR was 5'-CTGCTGAAGGAGCTGTTCC-3'. The 3'-primer used for RT-PCR was 5'-AGTGTGATACTGACACGAC-3'. The ODNs were introduced into carotid smooth muscle strips by chemical loading we have previously used for the introduction of FAK and paxillin antisense into tracheal muscle strips.22,23 After determination of contractile response, each muscle strip was attached to a metal mount at 8- to 9-mm length. The strips were placed into autoclaved test tubes and incubated successively in each of the following solutions: solution 1 (600 µL, at 4 °C for 120 minutes), containing 10 mmol/L EGTA, 5 mmol/L Na2 ATP, 120 mmol/L KCl, 2 mmol/L MgCl2, and 20 mmol/L N-tris (hydroxyethyl) methyl-2-aminoethanesulfonic acid (TES); solution 2 (600 µL, at 4 °C overnight), containing 0.1 mmol/L EGTA, 5 mmol/L Na2ATP, 120 mmol/L KCl, 2 mmol/L MgCl2, 20 mmol/L L-TES, and 8 mmol/L antisense or sense ODNs; and solution 3 (600 µL, at 4 °C for 30 minutes), containing 0.1 mmol/L EGTA, 5 mmol/L Na2ATP, 120 mmol/L KCl, 10 mmol/L MgCl2, 20 mmol/L L-TES. The strips were then transferred to 25-mL organ baths containing solution 4 (at 22 °C for 60 minutes, 110 mmol/L NaCl, 3.4 mmol/L KCl, 0.8 mmol/L MgSO4, 25.8 mmol/L NaHCO3, 1.2 mmol/L KH2PO4, and 5.6 mmol/L dextrose). Solutions 1 to 3 were maintained at pH 7.1 and aerated with 100% O2. Solution 4 was maintained at pH 7.4 and was aerated with 95% O2–5% CO2. After 30 minutes in solution 4, CaCl2 was added gradually to reach a final concentration of 2.4 mmol/L. The strips were then incubated for 2 days in DMEM containing 5 mmol/L Na2ATP, 100 U/mL penicillin, 100 µg/mL streptomycin, and 8 mmol/L antisense or 8 mmol/L sense oligonucleotides, which were kept at 37 °C. 5% CO2. Chemical loading is believed to load large amounts of ODNs into smooth muscle strips.22–24 The addition of ODNs in media was to compensate for the degradation of oligonucleotides in cells.22,23,25,26 ODNs can be uptaken into cells by endocytosis, maintaining appropriate intracellular ODN concentration.23,27

Immunoblot Analysis

Pulverized muscle strips were mixed with extraction buffer containing 10% glycerol, 2 mmol/L EDTA, 20 mmol/L Tris-HCl at pH 7.6, 10 mmol/L DTT, 2% SDS, phosphatase inhibitors (2 mmol/L sodium orthovanadate, 2 mmol/L molybdate, and 2 mmol/L sodium pyrophosphate), and protease inhibitors (2 mmol/L benzamidine, 0.5 mmol/L PMSF, 1 mmol/L aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). Each sample was kept on ice for 1 hour and then centrifuged for the collection of supernatant. Blots of muscle extracts were cut into several parts for immunoblotting of different proteins. Monoclonal antibodies against CAS (clone 24, BD Biosciences Pharmingen), α-actin (clone 1A4, Sigma), myosin heavy chain (clone hSM-V, Sigma), and paxillin (clone 349, BD Biosciences Pharmingen) were used to probe each of proteins followed by HRP-conjugated antigoat IgG (Amersham). Proteins were visualized by enhanced chemiluminescence and quantified by scanning densitometry. Densitometric values of CAS, actin, myosin heavy chain, and paxillin were determined for sense-treated and antisense-treated strips and normalized to those of no ODN–treated strips. The ratios of these proteins were calculated to verify that changes in protein expression were selective for CAS.

Analysis of MLC Phosphorylation

MLC phosphorylation was determined by immunoblot analysis of urea glycerol gels described previously.22,23,28

Analysis of F-Actin/G-Actin Ratio

The concentration of F-actin and G-actin in smooth muscle tissues was measured by use of a G-actin/F-actin in vivo assay kit (BK 037) from Cytoskeleton Inc. Briefly, each of carotid smooth muscle strips was homogenized in 200 µL F-actin stabilization buffer (50 mmol/L PIPES, pH 6.9, 50 mmol/L NaCl, 5 mmol/L MgCl2, 5 mmol/L EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P40, 0.1% Tween 20, 0.1% B-mercapto-ethanol, 0.001% antifoam, 1 mmol/L ATP, 1 µg/mL peptatin, 1 µg/mL leupeptin, 10 µg/mL benzamidine, 500 µg/mL soybean trypsin inhibitor). Supernatants of the protein extracts were collected after centrifugation at 100 000g for 60 minutes at 30 °C. The pellets were resuspended in ice-cold dH2O plus 1 µmol/L cytochalasin D and then incubated on ice for 1 hour to dissociate F-actin. The resuspended pellets were gently mixed every 15 minutes. Equal amounts of protein for the supernatant (G-actin) and pellet (F-actin) fractions were subjected to analysis of immunoblot with the use of actin antibody. The ratio of F-actin to G-actin was determined by scanning densitometry.
Analysis of the Association of Profilin With G-Actin
G-actin fractions and F-actin fractions of carotid smooth muscle strips were separated by the methods described above. G-fractions were incubated with monoclonal antibody against α-actin (clone 1A4, Sigma) for 1 hour and then incubated with a 10% suspension of protein A-Sepharose beads conjugated to rabbit anti-mouse IgG for 2 hours. Immunocomplexes were washed 4 times in Tris-buffered saline and then subjected to analysis of SDS-PAGE. Blots of actin immunoprecipitates were detected with profilin antibody (Cytoskeleton Inc), stripped, and reprobed with actin antibody. The ratio of profilin/G-actin was determined by densitometry.

Statistical Analysis
All statistical analysis was performed with the use of SigmaStat software. Comparison among multiple groups was performed by 1-way ANOVA or Kruskal-Wallis 1-way ANOVA. Differences between pairs of groups were analyzed by Student-Newman-Keuls test or the Dunn method. Values of n refer to the number of experiments used to obtain each value. A value of P<0.05 was considered to be significant.

Results
Downregulation of CAS Protein by CAS Antisense ODNs
We introduced CAS antisense or sense oligodeoxynucleotides into smooth muscle strips by a method of chemical loading.22–24 Protein extracted from smooth muscle tissues that had been treated with antisense or sense ODNs or not treated with ODNs was analyzed by Western blot to compare the expression of CAS with that of actin, myosin heavy chain (MHC) and the cytoskeletal protein paxillin.

The amount of CAS protein in antisense-treated tissues was lower than that in tissues treated with sense ODNs or not treated with ODNs (Figure 1A). However, the treatment with antisense ODNs did not inhibit the expression of actin, MHC, and paxillin. Protein expression in sense-treated and antisense-treated strips was normalized to that in strips not treated with ODNs. The expression of CAS relative to that of actin, MHC, and paxillin was significantly lower in antisense-treated strips than in strips not treated with ODNs or in sense-treated strips (Figure 1B, n=5, P<0.01). This indicates that the decrease in CAS expression was a specific effect of the antisense treatment and that it did not result from general deterioration of the tissue during the incubation period.

Effect of CAS Depletion on Force Development and MLC Phosphorylation in Response to Contractile Stimulation
We assessed isometric force development in response to NE in muscle strips treated with no ODNs, CAS sense, or antisense. Tension development in response to 10−3 mol/L NE was compared before and after 2-day incubation period (Figure 2A). Without ODN treatment, contractile force in response to stimulation with NE for 5 minutes was 96.5±7.1% of the preincubation force (n=8). Force in CAS sense–treated tissues in response to NE stimulation was 99.4±9.5% of preincubation force (n=8). Contractile response in antisense-treated tissues was significantly reduced to 12.3±6.7% (n=8, P<0.01). In addition, KCl-induced forces (40 mmol/L, 5 minutes) were 102.1±9.1% and 99.7±8.3% of preincubation force in tissues not treated with ODNs and sense-treated strips (n=8). The KCl-induced force was 13.6±6.4% in antisense-treated strips (n=8, P<0.01). Tensions before NE or KCl stimulation were not significantly different among these 3 groups (Figure 2A, n=8, P>0.05). MLC phosphorylation is a major regulatory event during smooth muscle contraction.23–26 To determine the role for CAS in the control of contractile protein activation, smooth
muscle strips that had been incubated with CAS antisense or sense ODNs or without ODNs were stimulated with 10^{-5} mol/L NE for 5 minutes and then frozen for the analysis of MLC phosphorylation. Although active force was significantly depressed, NE stimulation resulted in increase in MLC phosphorylation in tissues treated with CAS antisense. The average increases in MLC phosphorylation during contractile stimulation in strips not treated with ODNs, sense-treated, and antisense-treated tissues were not significantly different (Figure 2B). The levels of MLC phosphorylation before contractile stimulation were not significant different in no-ODN-treated, sense-treated, and antisense-treated tissues (Figure 2B, P > 0.05).

**F-Actin/G-Actin Ratio in CAS-Deficient Smooth Muscle Strips**

We evaluated the role of CAS in the regulation of actin polymerization by determining the ratio of F-actin/G-actin in CAS-depleted muscle tissues. Carotid smooth muscle strips that had been treated with CAS antisense or sense ODNs or not treated with ODNs were stimulated with 10^{-5} mol/L NE for 5 minutes and then frozen for analysis of the F-actin/G-actin ratio.

In muscle strips not treated with ODNs, NE stimulation enhanced the ratio of F-actin to G-actin (Figure 3). The treatment of muscle strips with CAS sense did not affect NE-induced increases in the F-actin/G-actin ratio (n = 4, P > 0.05), whereas treatment with CAS antisense inhibited the increases in the F-actin/G-actin ratio induced by NE (Figure 3, n = 4, P < 0.05). CAS sense or antisense did not influence the ratio of F-actin/G-actin before NE stimulation (n = 4, P > 0.05).

**Effect of CAS Depletion on the Association of Profilin With G-Actin During Contractile Activation**

G-actin fractions and F-actin fractions were collected from carotid smooth muscle tissues (see Methods section). Blots of the G-actin fractions and F-actin fractions were probed with profilin antibody to evaluate whether profilin associates with G-actin and/or F-actin. The amount of profilin in G-actin fractions was abundant, whereas the content of profilin was very low in F-actin fractions (Figure 4A). This demonstrates that profilin primarily interacts with G-actin in carotid smooth muscle tissues.

Carotid smooth muscle strips not treated with ODNs or treated with CAS sense or antisense were stimulated with 10^{-5} mol/L NE for 5 minutes or were unstimulated. Actin was immunoprecipitated from supernatant (G-actin fractions) of muscle extracts, and blots of actin immunoprecipitates were probed with profilin antibody, stripped, and reprobed with actin antibody. The ratio of profilin/G-actin in the immunoprecipitates was then determined by densitometry.

In smooth muscle tissues not treated with ODNs or treated with CAS sense, NE stimulation increases the ratio of profilin/G-actin. However, the increases in the profilin/G-actin ratio in response to stimulation with NE were significantly lower in muscle strips treated with CAS antisense.
Approximately 30% of the total actin in unstimulated smooth muscle exists in the form of monomeric actin, which consists of free G-actin and G-actin that is sequestered by the proteins such as profilin and thymosin. Our present results showed that the F-actin/G-actin ratio in unstimulated muscle strips is 1.9, thus 35% of total actin is G-actin (Figure 3). A pool of monomeric actin is induced to assemble into F-actin during contractile activation of smooth muscle. The disruption of actin dynamics in smooth muscle inhibits active force development. CAS may play a role in mediating actin polymerization in nonmuscle cells such as fibroblasts, osteoclasts, and RatER cells. Our present results demonstrate that CAS is required for normal actin dynamics in vascular smooth muscle. In addition, our previous study has also shown that the depletion of the focal adhesion protein paxillin attenuates agonist-induced contraction and actin polymerization in smooth muscle. Since both CAS/paxillin-mediated process and MLC phosphorylation are required for smooth muscle contraction, we speculate that CAS/paxillin-mediated cytoskeletal remodeling may facilitate the transmission of MLC phosphorylation-regulated force to extracellular matrix. Further studies are needed to define the quantitative importance of CAS/paxillin in the regulation of smooth muscle contraction independent of MLC phosphorylation.

Our previous studies have shown that profilin may mediate actin dynamics in carotid smooth muscle. In the present study, we found that contractile stimulation of smooth muscle increases the binding of profilin to actin monomers. The increases in the profilin-actin complex may promote actin polymerization; the binding of the profilin-actin complex to N-WASP (Neuronal Wiskott Aldrich Syndrome Protein) may deliver actin monomers to the Arp2/3 (actin-related protein) complex nucleation site or the barbed end of a growing filament. Studies on nonmuscle cells and in vitro experiments indicate that the adapter protein CAS may be an upstream regulator of profilin. CAS regulates the activity of profilin, possibly by interacting with the SH2/SH3-containing protein CrkII and the small GTP-binding protein Cdc42. Our results demonstrate that the association of profilin with G-actin is depressed during stimulation of CAS-deficient smooth muscle, supporting the hypothesis that CAS mediates the function of profilin in smooth muscle.

**Discussion**

CAS is a 130-kDa adapter protein that has been implicated in regulating the migration of nonmuscle cells including CHO cells, COS-7 cells, and pancreatic carcinoma cells. Our results demonstrate that CAS plays an essential role in the cellular mechanisms that mediate active force generation in smooth muscle. The treatment of smooth muscle tissues with CAS antisense oligodeoxynucleotides selectively downregulates the expression of CAS protein (Figure 1). The down-regulation of CAS protein dramatically inhibits force generation in smooth muscle in response to NE and KCl stimulation.

Smooth muscle contraction is initiated by phosphorylation of the 20-kDa regulatory light chain of myosin by Ca²⁺/calmodulin-regulated myosin light chain kinase. We considered that in carotid smooth muscle, the depression of agonist-induced force generation in CAS-deficient tissues could be due to the inhibition of contractile protein activation. However, our results exclude the possibility; MLC phosphorylation is normal in CAS-depleted smooth muscle in response to stimulation with NE (Figure 3).

**Figure 4.** CAS depletion inhibits increases in association of profilin with G-actin in response to stimulation with NE. A, Profilin primarily binds to G-actin in smooth muscle tissues. G-actin fractions (supernatant) and F-actin fractions (pellet) were collected from carotid smooth muscle tissues. Blots of G-actin fractions and F-actin fractions were probed with actin antibody, stripped, and reprobed with actin antibody. B, Carotid smooth muscle strips not treated with ODNs (No ODNs), or strips treated with CAS sense or antisense ODNs (CAS Sense or CAS Antisense) were stimulated with 10⁻⁵ mol/L NE for 5 minutes (black bars) or were unstimulated (open bars). Profilin/G-actin ratios of these strips were then determined. NE-induced profilin/G-actin ratio in antisense-treated strips is significantly lower than ratio for stimulated strips not treated with ODNs (n=4, P<0.05).
smooth muscle. The downregulation of CAS depresses actin polymerization in response to contractile stimulation. CAS may regulate actin dynamics by mediating the formation of the profilin-actin complex. Further studies are needed to understand signaling pathways by which CAS mediates the function of profilin in smooth muscle.

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