Critical Role of Vimentin Phosphorylation at Ser-56 by p21-activated Kinase in Vimentin Cytoskeleton Signaling*

Qing-Fen Li, Amy M. Spinelli, Ruping Wang, Yana Anfinogenova, Harold A. Singer, and Dale D. Tang

From the Center for Cardiovascular Sciences, Albany Medical College, Albany, New York 12208

Phosphorylation and spatial reorganization of the vimentin network have been implicated in mediating smooth muscle contraction, cell migration, and mitosis. In this study, stimulation of cultured smooth muscle cells with 5-hydroxytryptamine (5-HT) induced PAK1 phosphorylation at Thr-423 (an indication of p21-activated kinase (PAK) activation). Treatment with PAK led to disassembly of wild-type (but not mutant S56A) vimentin filaments as assessed by an in vitro filament assembly assay. Furthermore, stimulation with 5-HT resulted in the dissociation of Crk-associated substrate (CAS; an adapter protein associated with smooth muscle force development) from cytoskeletal vimentin. Expression of mutant S56A vimentin in cells inhibited the increase in phosphorylation at Ser-56 and in the ratios of soluble to insoluble vimentin (an index of vimentin disassembly) and the dissociation of CAS from cytoskeletal vimentin in response to 5-HT activation compared with cells expressing wild-type vimentin. Because CAS may be involved in PAK activation, PAK phosphorylation was evaluated in cells expressing the S56A mutant. Expression of mutant S56A vimentin depressed PAK phosphorylation at Thr-423 induced by 5-HT. Expression of the S56A mutant also inhibited the spatial reorientation of vimentin filaments in cells in response to 5-HT stimulation. Our results suggest that vimentin phosphorylation at Ser-56 may inversely regulate PAK activation possibly via the increase in the amount of soluble CAS upon agonist stimulation of smooth muscle cells. Additionally, vimentin phosphorylation at this position is critical for vimentin filament spatial rearrangement elicited by agonists.

The disassembly and spatial reorganization of the vimentin network may regulate the translocation of certain molecules (7, 10, 11). The adapter protein p130 Crk-associated substrate (CAS; an adapter protein associated with smooth muscle force development) from cytoskeletal vimentin is the most abundant intermediate filament protein in various cell types, including smooth muscle cells (2, 5, 6). Vimentin phosphorylation in association with vimentin disassembly and spatial reorganization occurs during mitosis or in response to extracellular stimulation (8, 9). In cultured smooth muscle cells, contractile stimulation triggers vimentin phosphorylation at Ser-56 concurrently with vimentin partial disassembly and spatial reorientation (6).

The disassembly and spatial reorganization of the vimentin network may regulate the translocation of certain molecules (7, 10, 11). The adapter protein p130 Crk-associated substrate (CAS)2 has been shown to participate in the signaling processes that regulate smooth muscle contraction and cell migration (12–14). Our recent study has suggested that vimentin phosphorylation and disassembly are related to CAS redistribution during contractile activation of smooth muscle (10). In addition, external stress initiates Rho kinase redistribution associated with vimentin depolymerization in fibroblasts and the translocation of Ca2+/calmodulin-dependent protein kinase II in differentiated smooth muscle cells, which may be an important event for cell signaling (7, 11).

p21-activated kinase (PAK) may be an upstream regulator of the vimentin network (6, 9). In cultured smooth muscle cells, agonist-mediated vimentin phosphorylation at Ser-56 and spatial reorientation of the vimentin network are inhibited by silencing of PAK1, a dominant isoform in smooth muscle (6, 15). Additionally, PAK has been implicated in modulating smooth muscle contraction; introduction of an active PAK isoform into smooth muscle potentiates force development at constant intracellular calcium (16). Expression of an inactive PAK1 mutant attenuates migration of cultured smooth muscle cells in response to platelet-derived growth factor (15).

In response to external stimulation, PAK undergoes autophosphorylation at Thr-423, which increases PAK activity for substrates (17, 18). In addition to the small GTPases Cdc42 and Rac1, the activity of PAK may be regulated by the paxillin kinase linker/PIX (PAK-interacting exchange factor; guanine nucleotide exchange factor) (19–21). CAS has been shown to interact with the paxillin kinase linker/PIX via CrkII and paxillin (21–

1 To whom correspondence should be addressed: Center for Cardiovascular Sciences, Albany Medical College, 47 New Scotland Ave., MC-8, Albany, NY 12208. Tel.: 518-262-6416; Fax: 518-262-8101; E-mail: tangd@mail.amc.edu.
2 The abbreviations used are: CAS, Crk-associated substrate; PAK, p21-activated kinase; 5-HT, 5-hydroxytryptamine; EGFP, enhanced green fluorescent protein.
Thus, CAS could be involved in the regulation of PAK activation.

The aim of this study was to test the hypothesis that vimentin phosphorylation at Ser-56 may play a critical role in regulating PAK activation and the spatial reorientiation of the vimentin network. Our results demonstrate that PAK-mediated vimentin phosphorylation at Ser-56 leads to increases in vimentin disassembly and CAS dissociation from the vimentin network during 5-hydroxytryptamine (5-HT; serotonin) stimulation. Expression of a non-phosphorylatable vimentin mutant (S56A) attenuates the increase in PAK activation and the structural rearrangement of vimentin filaments upon agonist stimulation.

**EXPERIMENTAL PROCEDURES**

**Smooth Muscle Cell Culture**—Primary canine tracheal smooth muscle cells were prepared and cultured according to the methods described previously (6). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum or bovine growth serum at 1:10, v/v) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B). Cells within passage 4 were then serum-deprived 24 h before biochemical and immunofluorescence or fluorescence experiments. These smooth muscle cells in culture express high levels of smooth muscle-specific α-actin as determined by immunoblot analysis.

**Assessment of Protein Phosphorylation**—Smooth muscle cells were treated with SDS sample buffer containing 1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.01% bromophenol blue. Cells were boiled in the buffer for 4 min and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, after which the membranes were blocked with 2% bovine serum albumin. To analyze PAK phosphorylation, membranes were reacted with anti-phospho-PAK1 (Thr-423)/PAK2 (Thr-403) antibody (Cell Signaling Biotechnology, Inc.), followed by horseradish peroxidase-conjugated anti-rabbit IgG (ICN Biomedicals). Proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce) using a Fuji LAS-3000 imaging system. The membranes were stripped and reprobed with anti-PAK1 antibody (Cell Signaling Biotechnology, Inc.). For determination of vimentin phosphorylation, membranes were probed with a phosphorylation site-specific antibody for Ser-56 (anti-phosphovimentin (Ser-56) antibody), stripped, and reprobed with anti-vimentin monoclonal antibody (clone RV202, BD Biosciences), followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences) (6). Phosphoprotein and total protein were quantified by scanning densitometry of immunoblots (Fuji MultiGauge software). Changes in protein phosphorylation were expressed as the magnitude increase over the phosphorylation levels in unstimulated cells. The luminescent signals from all immunoblots were within the linear range.

**Preparation of Recombinant Proteins**—PCR-mediated mutagenesis was carried out on pEGFP-vimentin (kindly provided by Dr. Robert D. Goldman, Northwestern University, Chicago, IL) to generate mutant S56A vimentin. DNA sequencing was used to confirm the mutation of vimentin. The PCR product and wild-type vimentin cDNA were subcloned into pGEX-4T at the BamHI and EcoRI sites, followed by transformation of *Escherichia coli* BL21. Recombinant proteins were harvested and purified according to the manual accompanying the glutathione S-transferase gene fusion system of Amersham Biosciences. Thrombin (Amersham Biosciences) was used to release the recombinant protein from glutathione-Sepharose beads and was then removed by incubation with *p*-aminobenzamidine-agarose beads (Sigma).

**In Vitro Kinase Assay**—Purified wild-type or mutant S56A vimentin (0.1 mg/ml) was incubated with 2 μg/ml activated PAK (Upstate) for 10 or 30 min in kinase buffer containing 20 mM HEPES (pH 7.5), 60 mM NaCl, 2 mM MgCl2, 5 mM EGTA, and 100 μM ATP. The reaction mixture was boiled in SDS sample buffer for 5 min, and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-vimentin antibody.

**Immunofluorescence Molecular Imaging**—We developed a molecular immunostaining technique to visualize vimentin filament assembly *in vitro*. Briefly, purified wild-type and mutant S56A vimentin (0.2 mg/ml) was placed in kinase buffer in the presence or absence of PAK for 30 min. NaCl (150 mM) was then added to the reaction mixture, followed by incubation at 37 °C for 1 h to initiate filament formation. The mixture was treated with 0.1% glutaraldehyde at room temperature for 30 min to cross-link vimentin filaments and plated on slides, followed by fixation with 4% paraformaldehyde for 15 min. The purified molecules were reacted with anti-vimentin monoclonal antibody, followed by incubation with Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR). Vimentin filament formation was examined and analyzed under a Zeiss laser scanning confocal microscope.

**Analysis of the Ratios of Soluble to Insoluble Protein in Cells**—The amounts of soluble and insoluble vimentin were evaluated by the method described previously (4, 6). Smooth muscle cells were washed with ice-cold Hanks’ balanced salt solution and then scraped, collected, and centrifuged at 8000 × g for 5 min. The resulting pellets were incubated at 37 °C for 30 min in buffer containing 1% Nonidet P-40, 10% (v/v) glycerol, 20 mM HEPES (pH 7.6), 150 mM NaCl, 2 mM sodium orthovanadate, 2 mM molybdate, 2 mM sodium pyrophosphate, and protease inhibitors (2 mM benzamidine, 0.5 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The soluble (disassembled) and insoluble (assembled) fractions were collected after centrifugation at 2100 × g for 30 min at 4 °C and assessed by immunoblot analysis using anti-vimentin antibody. The soluble/insoluble vimentin ratios were determined after scanning densitometry of the immunoblots. To assess the ratios of soluble to insoluble (vimentin-associated) CAS and the ratios of insoluble CAS to cytoskeletal vimentin, the membranes were stripped and reprobed with anti-CAS antibody (clone 24, BD Biosciences).

**Cell Transfection and Assessment of Protein Expression**— Cultured smooth muscle cells were transfected with plasmids encoding enhanced green fluorescent protein (EGFP)-tagged wild-type and mutant S56A vimentin using Lipofectamine™ and PLUS™ transfection kits (Invitrogen). After 48 h, the cells were incubated in serum-free medium for 1 day. Cells were...
Vimentin Phosphorylation Modulates PAK Activation

treated with 1× SDS sample buffer to extract and denature proteins, after which the proteins separated by SDS-PAGE and transferred to nitrocellulose membranes. To assess the expression of recombinant proteins, the membranes were detected with anti-EGFP antibody (clone GFP02, Lab Vision Corp.) or anti-vimentin antibody.

Cell Fluorescence Analysis—After transfection with plasmids containing EGFP-labeled proteins, cells on coverslips were fixed with paraformaldehyde and mounted on slides using Fluromount-G (Fisher). Cells were also stained with 4’,6-diamidino-2-phenylindole to visualize nuclei. Cell images were viewed under a confocal fluorescence microscope. Cells with reorganized vimentin filaments were determined and quantitatively analyzed by the method described previously (6).

Statistical Analysis—All statistical analysis was performed using Prism Version 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 post test. Differences between pairs of groups were analyzed by the Student-Newman-Keuls test or Dunn’s test. Values of n refer to the number of experiments performed to obtain each value. p < 0.05 was considered to be significant.

RESULTS

Activation with 5-HT Induces PAK Phosphorylation in Smooth Muscle Cells—PAK has been implicated in the modulation of smooth muscle functions (6, 15, 16). To evaluate whether agonist stimulation activates PAK, cultured tracheal smooth muscle cells were treated with 10 μM 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phospho-PAK1 (Thr-423) antibody, stripped, and reprobed with anti-PAK1 antibody.

The activation of smooth muscle cells with 5-HT led to the enhancement of PAK phosphorylation at Thr-423. As shown in Fig. 1A, PAK phosphorylation at Thr-423 was increased 5 min after stimulation with 5-HT and was sustained for the 15-min duration. The phosphorylation levels in smooth muscle cells were increased by 1.5-fold 10–15 min after activation with 5-HT (n = 4) (Fig. 1B).

PAK Catalyzes Vimentin Phosphorylation at Ser-56 in Vitro—To determine whether PAK directly catalyzes vimentin phosphorylation, we evaluated the state of vimentin phosphorylation at Ser-56 mediated by PAK in vitro. Recombinant wild-type vimentin was incubated with active PAK for 10 or 30 min in kinase buffer. Vimentin phosphorylation at Ser-56 was assessed by immunoblot analysis using the vimentin phosphorylation site-specific antibody (6). Incubation of purified wild-type vimentin with PAK resulted in the enhancement of vimentin phosphorylation at Ser-56; the levels of vimentin phosphorylation were dramatically increased 30 min after treatment with PAK (n = 4) (Fig. 2, A and B).

To assess whether Ser-56 is a major phosphorylation site on vimentin mediated by PAK, phosphorylation of mutant S56A vimentin was also evaluated. The phosphorylation levels of the S56A mutant were relatively lower compared with those of wild-type vimentin, and they were not significantly increased after the addition of PAK (n = 4; p < 0.05) (Fig. 2).

PAK Induces Disassembly of Wild-type (but Not S56A Mutant) Vimentin Filaments in Vitro—Vimentin phosphorylation is associated with vimentin disassembly in smooth muscle cells upon agonist activation (6). To assess whether vimentin phosphorylation at Ser-56 by PAK modulates its assembly/disassembly, we developed an in vitro filament assembly assay. Soluble wild-type vimentin and its S56A mutant were treated with or without activated PAK, after which 150 mM NaCl was added to initiate vimentin filament formation. These proteins were plated on slides and immunostained with anti-vimentin antibody.

In the absence of PAK, wild-type vimentin was filamentous, and a portion of the vimentin filaments was assembled into a supercoiled structure (Fig. 3a). The formation of supercoiled vimentin filaments might be due to lack of tension imposed on the filaments. Treatment with PAK led to the disassembly of wild-type vimentin intermediate filaments (Fig. 3b). Likewise, mutant S56A vimentin displayed a filamentous structure when it was not treated with PAK (Fig. 3c). However, incubation of the mutant with PAK did not trigger the disassembly of mutant S56A vimentin filaments (Fig. 3d). These results suggest that vimentin phosphorylation at Ser-56 by PAK leads directly to vimentin filament disassembly.

The Amount of CAS in Insoluble Vimentin Fractions Is Reduced in Smooth Muscle Cells Stimulated with 5-HT—The vimentin framework binds to certain signaling partners such as Ca2+/calmodulin-dependent protein kinase II and Rho kinase (7, 11). The adapter protein CAS and the small GTPase Cdc42 have been shown to regulate active force development in smooth muscle (12, 14, 24, 25). To determine whether CAS and Cdc42 associate with the vimentin network, soluble and insol-
Vimentin Phosphorylation Modulates PAK Activation

The insoluble/soluble CAS ratios elicited by 5-HT stimulation were lower compared with those in unstimulated smooth muscle cells \((n = 5; p < 0.05)\) (Fig. 4D).

Vimentin Phosphorylation Modulates PAK Activation—Thus far, our results suggested that the PAK-catalyzed vimentin phosphorylation and disassembly were associated with the redistribution of CAS upon agonist stimulation of smooth muscle cells. To determine whether vimentin phosphorylation and disassembly influence CAS redistribution, we attempted to express the S56A mutant in smooth muscle cells to block vimentin phosphorylation and disassembly and then evaluated CAS redistribution compared with that in cells expressing wild-type vimentin.

We first assessed whether vimentin phosphorylation in response to 5-HT stimulation is depressed in cells expressing mutant S56A vimentin. pEGFP plasmid alone or plasmid encoding wild-type or mutant S56A vimentin was transfected into smooth muscle cells. Immunoblot analysis showed that EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.

Stimulation with 5-HT resulted in increases in vimentin phosphorylation at Ser-56 in cells transfected with plasmid encoding wild-type or mutant S56A vimentin. EGFP-labeled recombinant vimentin was highly expressed; \(\approx 90\%\) of the total vimentin was recombinant vimentin in the transfected cells (Fig. 5, A and B). Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 \(\mu M\) 5-HT for 5–15 min or left unstimulated. Blots of protein extracts from these cells were probed using anti-phosphovimentin (Ser-56) antibody, stripped, and reprobed with anti-EGFP antibody.
encoding wild-type vimentin (Fig. 5C). The phosphorylation levels in cells expressing wild-type vimentin were increased by 1.7-fold after stimulation with 5-HT for 10–15 min (Fig. 5D), which is similar to our previous results in untransfected cells (6). However, the phosphorylation levels in response to 5-HT stimulation in cells expressing the S56A mutant were significantly lower compared with cells expressing wild-type vimentin ($n = 4; p < 0.05$) (Fig. 5, C and D).

Stimulation with 5-HT Does Not Lead to an Increase in the Ratios of Soluble to Insoluble Vimentin in Cells Expressing S56A Mutant Vimentin—To assess whether vimentin phosphorylation at Ser-56 affects vimentin disassembly in vivo, smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 $\mu$M 5-HT for 5–15 min or left unstimulated. The soluble/insoluble vimentin ratios were then determined.

Activation with 5-HT increased the amount of soluble vimentin in cells expressing wild-type vimentin; however, the 5-HT-mediated increase in the level of soluble vimentin was attenuated in cells expressing the S56A mutant (Fig. 6A). The ratios of soluble to cytoskeletal vimentin in response to 5-HT stimulation were lower in cells expressing mutant S56A vimentin than in cells expressing wild-type vimentin ($n = 4; p < 0.05$) (Fig. 6B).

Redistribution of CAS Elicited by 5-HT Is Depressed in Cells Expressing S56A Mutant Vimentin—To evaluate whether vimentin phosphorylation at Ser-56 is required for CAS redistribution, we assessed the ratios of vimentin-associated (insoluble) CAS to cytoskeletal vimentin in unstimulated and stimulated (10 $\mu$M 5-HT, 15 min) smooth muscle cells expressing wild-type or mutant S56A vimentin. Expression of the S56A mutant suppressed the decrease in the CAS/vimentin ratios in response to 5-HT stimulation. The ratios of insoluble CAS to cytoskeletal vimentin elicited by 5-HT were significantly decreased in cells expressing wild-type vimentin compared with unstimulated cells. In contrast, the ratios of insoluble CAS to cytoskeletal vimentin were not significantly different in unstimulated and 5-HT-stimulated cells expressing mutant S56A vimentin ($n = 7; p < 0.05$) (Fig. 7A).

Moreover, we assessed the effects of mutant S56A vimentin on the vimentin-associated/soluble CAS ratios. The insoluble/soluble CAS ratios were decreased in 5-HT-stimulated cells expressing wild-type vimentin compared with unstimulated cells. However, the ratios of insoluble to soluble CAS in

FIGURE 4. CAS is dissociated from cytoskeletal vimentin upon 5-HT stimulation. A, CAS is associated with cytoskeletal vimentin. Blots of vimentin supernatant (S) and pellet (P) fractions from cultured tracheal smooth muscle cells were probed for CAS, Cdc42, and vimentin. CAS (but not Cdc42) was found in the cytoskeletal (pellet) vimentin fraction. B, the amount of CAS on the immunoblots from each fraction was quantified after scanning densitometry analysis. The relative portion of soluble CAS is expressed as (soluble CAS/total CAS (soluble + insoluble CAS)) × 100; the relative portion of insoluble CAS is expressed as (insoluble CAS/total CAS) × 100 ($n = 8$). C, blots of equal amounts of vimentin from unstimulated and 5-HT-stimulated (10 $\mu$M, 15 min) cells were probed with anti-CAS and anti-vimentin antibodies. The inset is an immunoblot showing the effects of 5-HT stimulation on the amount of CAS in cytoskeletal vimentin. The CAS/vimentin ratios in stimulated cells were normalized to those in unstimulated cells. *, significantly lower CAS/vimentin ratios in stimulated cells compared with unstimulated cells ($n = 5; p < 0.05$).

D, insoluble/soluble CAS ratios in cells stimulated by 5-HT activation were normalized to those in unstimulated cells. *, significantly lower ratios in stimulated cells compared with unstimulated cells ($n = 5; p < 0.05$). Values are the means ± S.E.
response to 5-HT stimulation were not reduced in cells expressing the S56A mutant compared with unstimulated cells (n/H11005/10; p/H11021/0.05) (Fig. 7B).

Activation of PAK1 Elicited by 5-HT Stimulation Is Suppressed in Cells Expressing S56A Mutant Vimentin—Because the dissociation of CAS from the vimentin network is regulated by vimentin phosphorylation and because CAS may affect PAK activity (19, 22), we determined whether vimentin phosphorylation at Ser-56 reciprocally regulates PAK1. Smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 100 μM 5-HT for 15 min or left unstimulated. PAK1 phosphorylation in these cells was evaluated by immunoblot analysis.

Phosphorylation of PAK1 at Thr-423 in response to 5-HT stimulation was enhanced in cells expressing wild-type vimentin. Vimentin phosphorylation in response to 5-HT stimulation was reduced in cells expressing the S56A mutant compared with cells expressing wild-type vimentin.

Fusion proteins in these cells. Blots are representative of four identical experiments. B, protein extracts of untransfected cells or cells transfected with empty vector or plasmid encoding wild-type or mutant S56A vimentin were immunoblotted with anti-vimentin monoclonal antibody. In cells transfected with the wild-type or mutant S56A vimentin construct, ~90% of the total vimentin was the tagged recombinant proteins. Blots are representative of four identical experiments. C, smooth muscle cells expressing wild-type or mutant S56A vimentin were stimulated with 10 μM 5-HT for 5–15 min or left unstimulated. Vimentin phosphorylation in response to 5-HT stimulation was reduced in cells expressing the S56A mutant compared with cells expressing wild-type vimentin. D, vimentin phosphorylation was normalized to the level in unstimulated cells expressing wild-type vimentin. *, significantly lower values for each time point in cells expressing the S56A mutant than in cells expressing wild-type vimentin (p < 0.05). Values are the means ± S.E. (n = 4–5).
Vimentin Phosphorylation Modulates PAK Activation

FIGURE 7. Expression of the non-phosphorylatable vimentin mutant attenuates the redistribution of CAS stimulated with 5-HT. A, smooth muscle cells expressing recombinant proteins were stimulated with 10 μM 5-HT for 15 min or left unstimulated. The insoluble CAS/cytoskeletal vimentin ratios were then determined. Ratios were normalized to the level in unstimulated cells transfected with plasmid encoding wild-type (WT) vimentin. The decrease in the CAS/vimentin ratios elicited by 5-HT was inhibited in cells expressing mutant S56A vimentin compared with cells expressing wild-type vimentin (n = 7). B, insoluble/soluble CAS ratios were assessed for unstimulated and 5-HT-stimulated cells expressing recombinant proteins. The decrease in the insoluble/soluble CAS ratios elicited by 5-HT was suppressed in cells expressing the S56A mutant compared with cells expressing wild-type vimentin (n = 10), * significantly lower CAS/vimentin ratios or soluble/insoluble CAS ratios in stimulated cells than in the corresponding unstimulated cells (p < 0.05). Values are the means ± S.E.

FIGURE 8. Increase in PAK phosphorylation elicited by 5-HT is diminished in cells expressing mutant S56A vimentin. Smooth muscle cells expressing wild-type (WT) or mutant S56A vimentin were exposed to 10 μM 5-HT for 15 min or left unstimulated. Blots of these cells were probed with anti-phospho-PAK1 (Thr-423) antibody, stripped, and reprobed with anti-PAK1 antibody. PAK phosphorylation was normalized to the level obtained in unstimulated cells transfected with wild-type vimentin. *, significantly lower phosphorylation levels in stimulated cells containing the S56A mutant than in stimulated cells expressing wild-type vimentin (p < 0.05). Values are the mean ± S.E. (n = 5).

DISCUSSION

The intermediate filament protein vimentin undergoes phosphorylation at Ser-56 and partial filament disassembly in cultured smooth muscle cells upon stimulation with 5-HT (6). The results from this study show, first, that PAK is able to directly initiate vimentin disassembly associated with Ser-56 phosphorylation. Second, the adapter protein CAS is dissociated from cytoskeletal vimentin during 5-HT activation. The expression of the non-phosphorylatable vimentin mutant S56A attenuates the dissociation of CAS from cytoskeletal vimentin. Third, the expression of the S56A mutant suppresses PAK activation and the spatial reorganization of vimentin filament framework upon agonist stimulation. These results suggest that vimentin phosphorylation at Ser-56 and its disassembly may play a critical role in regulating PAK activation and the vimentin cytoskeleton.

In cultured smooth muscle cells, PAK silencing attenuates vimentin phosphorylation upon contractile activation (6). In this study, stimulation with 5-HT induced PAK1 autophosphorylation at Thr-423 in smooth muscle cells. In the inactive state, the N-terminal domain of PAK interacts with the C-terminal catalytic motif, forming an intramolecular inhibitory conformation and diminishing enzyme activity. Autophosphorylation at Thr-423 has been proposed to induce a conformational
Vimentin Phosphorylation Modulates PAK Activation

Vimentin molecule (2, 6). In in vitro experiments in this study, treatment of wild-type vimentin with PAK initiated almost complete filament disassembly, whereas substitution of alanine at Ser-56 impaired disassembly. These results suggest that PAK-mediated Ser-56 phosphorylation profoundly influences the assembly status of vimentin filaments. Because the effects of serine phosphorylation of a protein can be mimicked by introduction of a negative charge at the corresponding serine position (27), we speculate that phosphorylation at Ser-56 creates a negative charge in the side chain, which may facilitate changes in vimentin conformation associated with disassembly.

There is evidence that the vimentin filament framework harbors Rho kinase and Ca\(^{2+}\)/calmodulin-dependent protein kinase II, which may be dissociated from the network when vimentin partial disassembly or structural rearrangement occurs (7, 11). The adapter protein CAS may participate in the signaling process that mediates smooth muscle force development (12, 14). In this study, CAS associated with the vimentin cytoskeleton, and agonist activation triggered the dissociation of CAS from cytoskeletal vimentin. The amount of CAS dissociated from cytoskeletal vimentin was ~50% of total CAS associated with vimentin filaments. Furthermore, the expression of mutant S56A vimentin in smooth muscle cells attenuated CAS redistribution during agonist stimulation. Because vimentin phosphorylation at Ser-56 and partial disassembly were also inhibited in cells expressing the S56A mutant, we propose that contractile stimulation of smooth muscle cells leads to vimentin phosphorylation at this position, inducing vimentin disassembly and CAS release from the vimentin filament network.

Changes in the physiological status of a substrate may conversely serve as a regulator of its upstream enzyme, forming a feedback mechanism. As mentioned above, PAK is able to mediate vimentin phosphorylation both in vitro and in vivo. In this study, PAK activation in response to 5-HT stimulation was inhibited in cells expressing mutant S56A vimentin, suggesting that vimentin phosphorylation at Ser-56 reciprocally regulates PAK activity. The positive feedback mechanism may render PAK in an active state during agonist stimulation. The mechanisms by which vimentin phosphorylation at this residue regulates PAK activity are unknown. Because CAS can be dissociated from the vimentin network during contractile activation, it is possible that the increase in soluble CAS mediated by vimentin phosphorylation may facilitate the activation of PAK. When activated, CAS may be able to form a protein complex containing CrkII, paxillin, the p95 paxillin kinase linker, and PAK, which may mediate the activation of PAK (21, 22).

In addition to the regulation of signaling molecule redistribution, vimentin phosphorylation may be associated with spatial restructuring of the vimentin network (6). In this study, 5-HT-stimulated vimentin phosphorylation at position 56 was reduced in cells expressing mutant S56A vimentin. Moreover, spatial reorganization of the vimentin cytoskeleton in response to stimulation with 5-HT was also attenuated in cells expressing the S56A mutant. These results lead us to suggest that phosphorylation at Ser-56 is required for vimentin structural reorganization during agonist activation of smooth muscle cells.

As mentioned above, PAK treatment of vimentin protein in vitro triggered almost complete filament disassembly, whereas

![Image](https://example.com/image.png)

**Figure 9.** Activation of smooth muscle cells with 5-HT does not induce structural rearrangement of mutant S56A vimentin filaments. A, smooth muscle cells expressing EGFP-tagged wild-type or mutant S56A vimentin were stimulated with 10 μM 5-HT for 15 min or left unstimulated. Images of wild-type or mutant vimentin filaments in these cells were viewed under a confocal fluorescence microscope. In cells expressing wild-type vimentin, stimulation with 5-HT induced the reorientation of vimentin filaments from a curved structure (panel a) to a straight structure (panel b). In contrast, activation with 5-HT did not trigger the structural rearrangement of the mutant S56A vimentin filaments in cells (panels c and d). The 4',6-diamidino-2-phenylindole-stained nuclei are shown in blue. B, vimentin filaments displaying straightness longer than one-quarter of the cell length were considered as straight filaments. Cells with more than four straight wild-type or mutant vimentin filaments were counted as cells with rearranged filaments. The results of quantitative analysis are expressed as (numbers of cells with rearranged vimentin/mutant filaments)/numbers of total cells observed) × 100 (6). *, significantly higher percentage of cells with rearranged vimentin filaments in 5-HT-stimulated cells expressing wild-type vimentin compared with the corresponding unstimulated cells (p < 0.05). Values are the means ± S.E. (n = 4).

change for the activation of PAK (18). Thus, the results suggest that stimulation with 5-HT may increase PAKI activity in smooth muscle cells.

Although the intermediate filament framework in the cell is resistant to extraction with detergents, its assembly/disassembly may be regulated by phosphorylation (1, 2). Contractile stimulation of smooth muscle cells induces phosphorylation at Ser-56, which is located in the globular head domain of the
vimentin filaments underwent partial disassembly and spatial rearrangement in the cells in response to agonist activation. The difference between the *in vitro* and *in situ* studies may stem from the lower extent of vimentin phosphorylation in the cells. Our previous investigation showed that ~65% of soluble vimentin is phosphorylated at Ser-56 in smooth muscle cells stimulated with 5-HT, which is associated with the partial vimentin disassembly as estimated by a cell fractionation assay, and is regulated by PAK (6). Thus, we postulate that smooth muscle cells may possess a limited amount of PAK and/or that PAK has limited access to vimentin. Upon agonist stimulation, vimentin in the cells undergoes partial phosphorylation and partial disassembly, which may facilitate the spatial reorientation of vimentin filaments. In contrast, in the *in vitro* study, a sufficient amount of PAK is added to the reaction system, and/or PAK has full access to vimentin. PAK treatment is able to induce comprehensive filament disassembly.

The biological significance of vimentin filament rearrangement in smooth muscle cells is not fully understood. The spatial reorganization might facilitate contractile element reorganization in desmosomes, which may be involved in cellular processes that affect force development in smooth muscle (5, 6, 28–30). The structural rearrangement of vimentin filaments might also be associated with mitogenic processes in smooth muscle cells induced by 5-HT (6, 31).

Thus, we propose a unique vimentin cytoskeletal signaling mechanism in smooth muscle cells. Activation of PAK by 5-HT stimulation may catalyze vimentin phosphorylation at Ser-56, leading to vimentin disassembly and the dissociation of the adapter protein CAS from the vimentin network. The released CAS may facilitate the formation of a multiprotein complex including CAS and PAK, maintaining PAK in an active state. In addition, vimentin phosphorylation at this position may induce the spatial reorientation of vimentin filaments, which may be associated with contractile element reorganization in response to agonist stimulation (Fig. 10).

**REFERENCES**

Critical Role of Vimentin Phosphorylation at Ser-56 by p21-activated Kinase in Vimentin Cytoskeleton Signaling
Qing-Fen Li, Amy M. Spinelli, Ruping Wang, Yana Anfinogenova, Harold A. Singer and Dale D. Tang

doi: 10.1074/jbc.M607715200 originally published online September 20, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M607715200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 13 of which can be accessed free at
http://www.jbc.org/content/281/45/34716.full.html#ref-list-1