

# Smitin, a novel smooth muscle titin-like protein, interacts with myosin filaments *in vivo* and *in vitro*

Kyoungtae Kim and Thomas C.S. Keller III

Department of Biological Science, Florida State University, Tallahassee, Florida 32306-4370

Smooth muscle cells use an actin–myosin II-based contractile apparatus to produce force for a variety of physiological functions, including blood pressure regulation and gut peristalsis. The organization of the smooth muscle contractile apparatus resembles that of striated skeletal and cardiac muscle, but remains much more poorly understood. We have found that avian vascular and visceral smooth muscles contain a novel, megadalton protein, smitin, that is similar to striated muscle titin in molecular morphology, localization in a contractile apparatus, and ability to interact with myosin filaments. Smitin, like titin, is a long fibrous molecule with a globular domain on one end. Specific reactivities of an anti-smitin polyclonal antibody and an anti-titin monoclonal antibody suggest that smitin and titin are distinct proteins rather than differentially spliced isoforms encoded by the same gene. Smitin immu-

nofluorescently colocalizes with myosin in chicken gizzard smooth muscle, and interacts with two configurations of smooth muscle myosin filaments *in vitro*. In physiological ionic strength conditions, smitin and smooth muscle myosin coassemble into irregular aggregates containing large sidepolar myosin filaments. In low ionic strength conditions, smitin and smooth muscle myosin form highly ordered structures containing linear and polygonal end-to-end and side-by-side arrays of small bipolar myosin filaments. We have used immunogold localization and sucrose density gradient cosedimentation analyses to confirm association of smitin with both the sidepolar and bipolar smooth muscle myosin filaments. These findings suggest that the titin-like protein smitin may play a central role in organizing myosin filaments in the contractile apparatus and perhaps in other structures in smooth muscle cells.

## Introduction

Vertebrate smooth muscle is composed of highly elongated, spindle-shaped cells that wrap circularly, longitudinally, or at various angles around visceral and vascular tubular structures (Small, 1995). Contractile force produced by smooth muscle cells contributes to visceral and vascular functions such as gut peristalsis and blood pressure maintenance. Smooth muscle cells generate contractile force through an actin–myosin II (hereafter referred to as myosin) sliding filament mechanism similar to that of striated (skeletal and cardiac) muscle (Bagby, 1986).

Certain features of the overall organization of the actin–myosin contractile apparatus in smooth and striated muscle cells are similar. The contractile apparatus of striated muscle is composed of contractile unit structures known as sarcomeres. Sarcomeres share Z-disk structures on each end with adjacent sarcomeres to form long linear myofibrils. Actin filaments attached to each Z-disk project toward the center of each sarcomere. Each pole of a myosin bipolar

thick filament interdigitates with actin filaments from one Z-disk. Energy-dependent crossbridge interactions between myosin heads and the actin filaments produce force for contraction of the sarcomere. Long fibrous molecules of the protein titin tether each pole of the myosin bipolar thick filaments to one of the Z-disks (Wang, 1982; Labeit et al., 1992; Keller, 1995; Labeit and Kolmerer, 1995; Labeit et al., 1997; Gregorio et al., 1999; Trinick and Tskhovrebova, 1999). These titin filaments appear to play an important organization role in assembling the sarcomere structure during myofibrillogenesis (Fulton and Isaacs, 1991; McElhinny et al., 2000; Sanger et al., 2000). In mature striated muscle, the elastic nature of the titin molecules maintains the thick filaments in the center of the sarcomere during contraction and provides passive resistance to overstretch of the sarcomere structure (Labeit et al., 1992, 1997; Labeit and Kolmerer, 1995; Linke et al., 1996; Horowitz, 1999). All three major types of filaments are oriented parallel to the long axis of the muscle cell. The regimented alignment of sarcomeres in adjacent myofibrils across the cell gives the muscle its striated appearance.

Smooth muscle cells appear “smooth” because the contractile units are oriented at oblique angles to the longitu-

Address correspondence to Tom Keller, Dept. of Biological Science, Florida State University, Tallahassee, FL 32306-4370. Tel.: (850) 644-5572. Fax: (850) 644-0481. E-mail: tkeller@bio.fsu.edu

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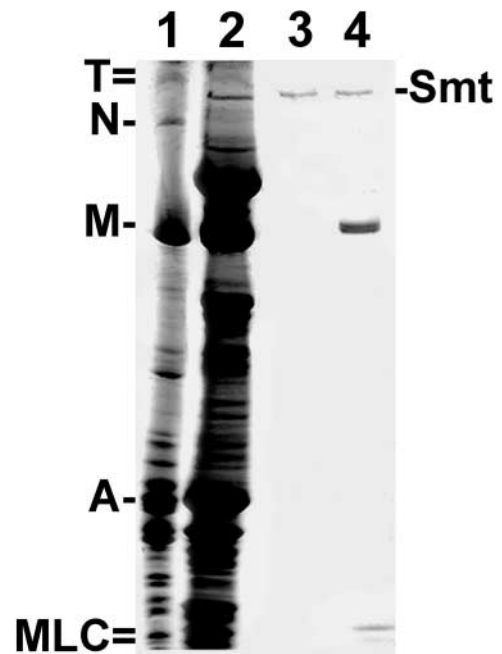
dinal axis of the cells and not regularly aligned across the cell. This distinctive organization contributes to the ability of smooth muscle cells to produce constant contractile force throughout extensive cell length changes, and to maintain the contracted state with little expenditure of energy (Marston, 1989; Stull et al., 1991; Murphy and Walker, 1998; Somlyo et al., 1998). However, this lack of structural alignment and regimentation has made it difficult to discern the organization of the smooth muscle actin–myosin filament contractile system. Current models of smooth muscle cell organization exhibit certain similarities between smooth muscle contractile unit structures and striated muscle sarcomeres (Bagby, 1986; Small, 1995; Somlyo, 1997). Smooth muscle contractile units are bounded on each end by dense plaques anchored to the cell membrane, or by dense body structures distributed throughout the cell. These structures appear to function like the Z-disks of striated muscle sarcomeres as force-coupling anchorage sites for actin filament that project toward the center of the contractile unit. The smooth muscle myosin thick filaments, which are sidepolar rather than bipolar as in striated muscle, interact with the actin filaments projecting from two dense plaques/bodies. Sliding force produced by the myosin filaments on the actin filaments pulls the attachment sites closer together.

The structural analogies between the smooth and skeletal muscle contractile apparatuses raise the possibility that smooth muscles also contain a protein that functions like striated muscle titin in contributing to the structural integrity and function of the contractile apparatus. In accord with this possibility, a protein with an amino acid composition similar to that of skeletal muscle titin was reportedly isolated from chicken gizzard smooth muscle (Maruyama et al., 1977), but this protein has remained uncharacterized. We report here the identification and initial characterization of a novel titin-like protein in chicken gizzard smooth muscle. We refer to this protein as smitin to reflect its smooth muscle origin and titin-like characteristics, including interaction with myosin filaments. Smitin exhibits an unexpected versatility in interacting with different forms of smooth muscle myosin filaments, which may reflect an ability of smitin and smooth muscle myosin to form different structural organizations *in vivo*.

## Results

### Purification of Smitin—a high molecular mass polypeptide from chicken gizzard smooth muscle

To investigate whether smooth muscle contains a titin-like protein that contributes to the organization and function of the contractile apparatus, we extracted chicken gizzard smooth muscle with a solution containing 0.5 M KCl and 4 mM ATP. SDS-PAGE analysis revealed that in addition to actin and myosin, the extract contained an extremely high molecular mass polypeptide (Fig. 1). This smooth muscle polypeptide migrates somewhat faster than the ~3,000-kD pectoralis muscle titin standard, but significantly slower than the ~800-kD pectoralis nebulin standard. Linear regression analysis of smitin migration rate compared with that of pectoralis muscle titin T1, titin T2, and nebulin indicates that the smooth muscle protein has an apparent molecular mass of



**Figure 1. Purification of chicken gizzard smooth muscle smitin and myosin.** SDS-PAGE analysis of samples from steps in the purification of smitin and myosin: 0.5 M KCl + 4 mM ATP extract of isolated smooth muscle myofibrils (lane 2); Sephacryl S-1000 fraction containing smitin alone (lane 3); Sephacryl S-1000 column fraction containing smitin and smooth muscle myosin II (lane 4). SDS extract of chicken pectoralis muscle used as a marker for titin and myosin heavy chain migration in the 4–20% polyacrylamide gradient gel (lane 1). A, actin; M, myosin heavy chain; MLC, myosin light chains; N, nebulin; Smt, smitin; T, striated muscle titin.

~2,000 kD. We refer to this protein as smitin because of its smooth muscle origin and similarities to skeletal muscle and cellular titins described below.

We isolated smitin from the gizzard extract (Fig. 1, lane 2) by Sephacryl S-1000 gel filtration chromatography. Fractions containing partially purified smitin eluted near the void volume of the column (Fig. 1, lane 3), ahead of fractions that contained both smitin and smooth muscle myosin but no other proteins (Fig. 1, lane 4). Subsequent Hydroxyapatite or DEAE-cellulose column chromatography was used to concentrate smitin, but neither of these columns effectively separated myosin from smitin when both proteins were present in the fractions. In most of the following experiments we used samples from Sephacryl S-1000 fractions containing both proteins without further purification to characterize the interaction of smitin and myosin.

### Molecular morphology of smitin

We determined the molecular morphology of smitin by using platinum to rotary replicate molecules in samples of column-purified smitin. Electron microscopy of the replicas revealed numerous long (750–900 nm), fibrous molecules with single globular heads (Fig. 2). The molecular morphology of smitin is similar to that of striated muscle titin and brush border cellular titin, although the length of smitin is somewhat shorter than that of muscle titin, in accord with its apparent lower molecular mass. A few of the smitin molecules we observed appeared to contain a region of overlap between two smitin molecules

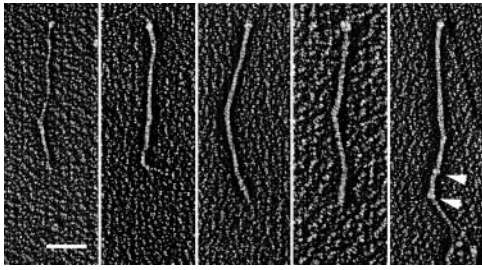


Figure 2. **Molecular morphology of native smitin.** Sephacryl S-1000 column-purified smitin was sprayed onto newly cleaved mica and rotary replicated with platinum. Selected individual smitin molecules ranging in length from 750–900 nm are shown. Arrowheads in last panel indicate region of possible overlap of smitin molecule with another molecule of smitin or another protein. Bar, 200 nm.

(Fig. 2, last panel, arrowheads). However, no image we obtained provided unambiguous confirmation that both molecules are smitin. The interpretation that this represents end on association of smitin molecules therefore remains tentative.

### Distinctive immunoreactivity of smitin and striated muscle titin

To investigate the relationship between smitin and striated muscle titin and to determine the localization of smitin in smooth muscle cells, we raised an anti-smitin rabbit polyclonal antibody using SDS-PAGE-purified chicken gizzard smitin as the antigen. The specificity of the antigen for smitin was confirmed by Western blot analysis (Fig. 3 A). The antibody reacted specifically with the smitin band in a crude gizzard extract (Fig. 3 A, lanes 2 and 4) and in a Sephacryl column-purified smitin sample containing myo-

sin (unpublished data). The anti-smitin antibody failed to react with chicken pectoralis muscle titin (Fig. 3 A, lanes 1 and 3), whereas the 9D10 monoclonal antibody that reacts with striated muscle titin from a variety of vertebrate species, including chicken pectoralis muscle (Fig. 3 A, lane 5), failed to react with smitin (Fig. 3 A, lane 6). The anti-smitin polyclonal antibody also failed to react with the c-titin in a sample of isolated chicken intestinal epithelial cell brush borders (Fig. 3 B, lanes 2 and 4).

### Smitin expression in visceral and vascular smooth muscle

Western analysis using the anti-smitin polyclonal antibody demonstrated smitin expression in a variety of chicken smooth muscles. The antibody detected similar high molecular mass proteins in the gizzard (Fig. 3 C, lanes 2 and 6) and intestinal (Fig. 3 C, lanes 3 and 7) visceral smooth muscles and in aorta (Fig. 3 C, lanes 4 and 8) vascular smooth muscle, in which smitin appears as a tight doublet. In some blots, a high molecular mass reactive band was also present in the pectoralis striated muscle sample (present in Fig. 3 C, lane 5, but not in Fig. 3 A, lane 3). When present, this reactive band is distinguishable from the T1 (higher) and T2 (lower) titin bands by their respective migration positions on the blot. Immunolocalization data shown below (see Fig. 5) suggests that the variable presence of smitin in the pectoralis muscle samples could arise from smooth muscle contamination of the samples by arteries in the muscle.

### Immunolocalization of smitin and myosin in gizzard

We used our anti-smitin polyclonal antibody and a myosin monoclonal antibody to immunolocalize smitin and myo-

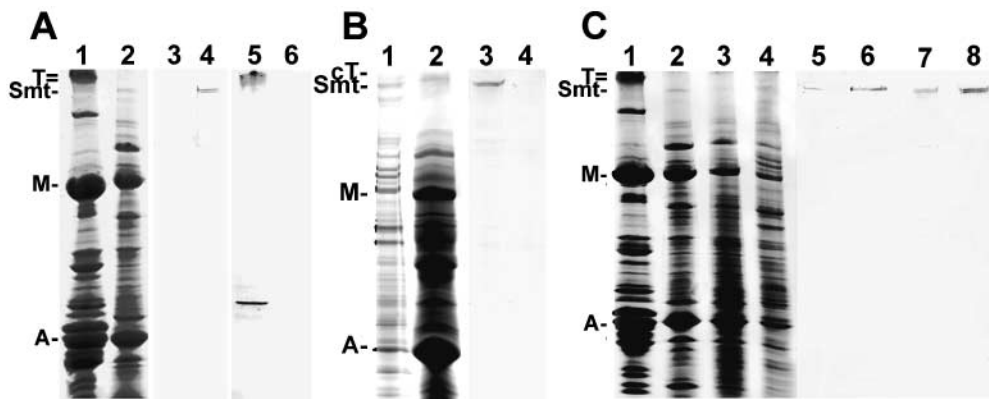
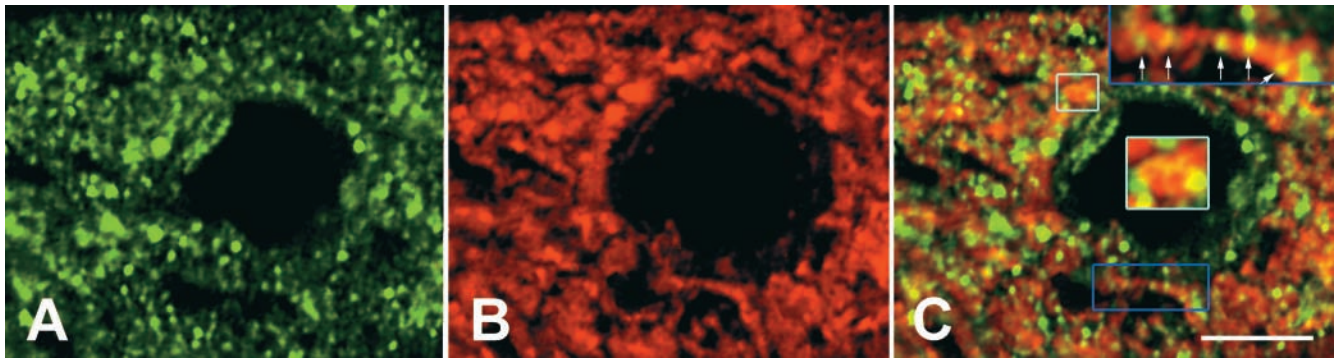


Figure 3. **Immunoblot analysis of smitin relatedness to titin and c-titin and smitin expression in smooth muscles.** (A) Reactivities of titin and smitin with a polyclonal antibody raised against gizzard smitin and a monoclonal antibody raised against mammalian striated muscle titin. SDS-extracts of chicken pectoralis striated muscle (lanes 1, 3, and 5) and chicken gizzard smooth muscle (lanes 2, 4, and 6) were subjected to SDS-PAGE. One pair of lanes was stained with Coomassie blue (lanes 1 and 2). Two other sets of lanes were electroblotted to nitrocellulose (lanes 3–6). One blotted set of lanes was incubated with a rabbit polyclonal antibody raised against injected chicken gizzard smitin and developed (lanes 3 and 4). The other blotted set of lanes was incubated with the 9D10 mouse monoclonal antibody reactive against vertebrate striated muscle titin and developed (lanes 5 and 6). The blots were developed using Western analysis with an alkaline phosphatase-conjugated secondary antibody. (B) Reactivities of smitin and chicken intestinal epithelial cell c-titin with the anti-smitin polyclonal antibody. SDS extracts of chicken aorta smooth muscle (lanes 1 and 3) and chicken intestinal epithelial cell brush borders (lanes 2 and 4) were subjected to SDS-PAGE. Lanes from the gel were stained with Coomassie blue (lanes 1 and 2) or electroblotted to nitrocellulose, incubated with the anti-smitin polyclonal antibody, and developed for Western analysis (lanes 3 and 4). (C) SDS extracts of chicken pectoralis striated muscle (lanes 1 and 5), gizzard smooth muscle (lanes 2 and 6), intestinal smooth muscle (lanes 3 and 7), and aorta smooth muscle (lanes 4 and 8) were subjected to SDS-PAGE. The gel was stained with Coomassie blue (lanes 1–4) or blotted to nitrocellulose (lanes 5–8). The blot was probed with an anti-smitin polyclonal antibody by Western analysis. A, actin; M, myosin heavy chain; Smt, smitin; T, titin.





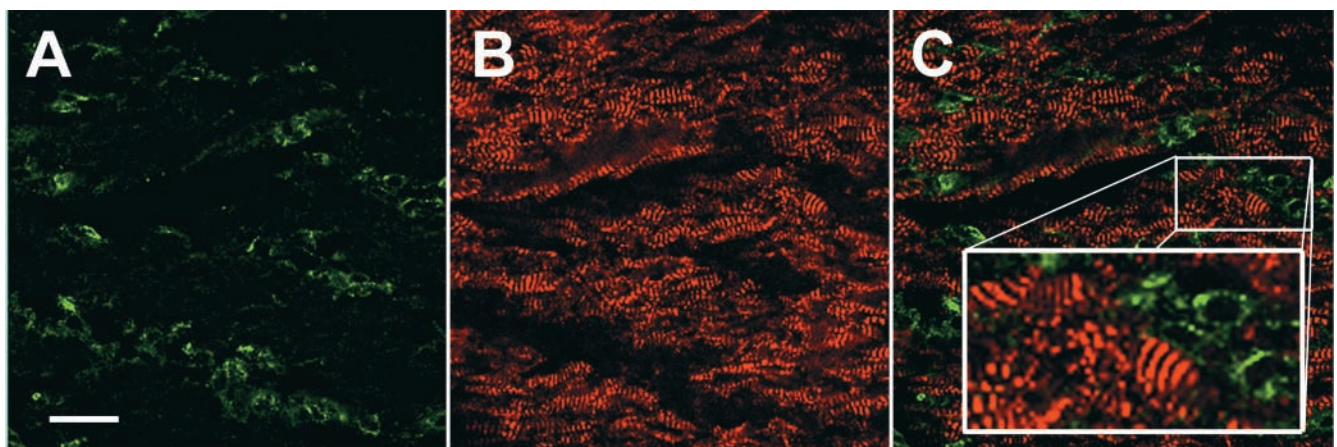
**Figure 4. Immunofluorescence localization of smitin and myosin in cryosections of chicken gizzard smooth muscle.** A cryosection of chicken gizzard muscle was double-labeled with an anti-smitin polyclonal antibody (A, green) and an anti-myosin monoclonal antibody (B, red) and imaged with laser scanning confocal microscopy. A single 0.55- $\mu\text{m}$  thick optical section is shown. Panel C is a merged image with insets of two areas (blue and turquoise boxes) of the image magnified to twice the original size. One inset (blue boxes) shows what appears to be a smooth muscle myofibril with the anti-smitin label (green) distributed at regular intervals along a strand stained continuously along its length for myosin (red). The other inset (turquoise boxes) shows what appears to be a cross-sectional area of a strand containing both smitin (green) and myosin (red) in close association (yellow). The large open area in the center of the section is a cross section of a blood vessel through the smooth muscle. Bar, 10  $\mu\text{m}$ .

sin in cryosections of gizzard smooth muscle. Immunofluorescent labeling of 10  $\mu\text{m}$ -thick cryosections of gizzard muscle revealed characteristic and distinctive patterns for smitin and myosin localization (Fig. 4). In a 0.55- $\mu\text{m}$  thick optical section of the preparation obtained with laser confocal microscopy, the myosin appears to be organized into long wavy filamentous structures (Fig. 4, B and C, red). The continuous distribution of staining along these structures is consistent with the possibility that the myosin is present in myofibril-like structures containing unregimented arrays of sidepolar myosin filaments, none of which is resolved individually in the image.

The smitin appears to be associated primarily with these myosin-containing structures, but the smitin and myosin staining patterns are clearly distinguishable. Whereas the myosin staining is continuous along the structures, the smitin staining is localized more discretely

to distinct spots along the structures (Fig. 4, A and C, green). The relationship of smitin and myosin localization along individual structures is particularly apparent at high magnification (Fig. 4, blue box inset). The space between clearly visible spots appears to be  $\sim 1 \mu\text{m}$ . It is possible that regions of the smitin that interact directly with the myosin filaments are inaccessible to the anti-smitin antibody and not labeled in these preparations. Therefore, the anti-smitin staining might only localize regions of the smitin molecules that project from the myosin filaments.

Certain smitin–myosin-containing structures appear to be imaged in cross section (Fig. 4 C, turquoise box inset). In these structures, the smitin appears to be closely associated with the myosin in the periphery of structures (Fig. 4 C, turquoise box inset, yellow overlap of green and red staining) but absent from the myosin core (red).



**Figure 5. Immunofluorescence localization of smitin and titin in cryosections of chicken cardiac striated muscle.** A 10- $\mu\text{m}$  thick cryosection of chicken cardiac muscle was double labeled with an anti-smitin polyclonal antibody and anti-rabbit Ig secondary antibody (A, green) and the 9D10 anti-titin monoclonal antibody and anti-mouse IgM secondary antibody (B, red). A single 0.4- $\mu\text{m}$  thick optical section is shown. Panel C contains a merged image with an inset displaying a region of the original image (small box) magnified 2.5 $\times$  (large box). Bar, 10  $\mu\text{m}$ .

### Distinct localization of smitin and titin in cryosections of cardiac striated muscle

To further investigate the origin of smitin detected by Western analysis of striated muscle extracts (Fig. 3 C, lane 5) and confirm the distinctiveness of titin and smitin, we immunolocalized smitin and titin in cryosections of chicken cardiac muscle (Fig. 5). The 9D10 anti-titin monoclonal antibody demonstrated a distinctive sarcomere arrangement of titin throughout the cardiac muscle cells (Fig. 5, B and C, red). In contrast, the anti-smitin polyclonal antibody demonstrated that smitin is excluded from the sarcomeres and appears to be confined to structures outside the striated muscle cells (Fig. 5, A and C, green). In certain regions of the sections, the smitin containing structures appear tubular in cross section (Fig. 5, C inset, green). These tubular structures are the size and morphology expected of arterioles through the striated muscle. Distribution of smitin as discrete spots in the walls of these structures is consistent with its punctate distribution in gizzard smooth muscle (Fig. 4).

### Coassembly of smitin and myosin

We investigated the possibility that smitin interacts directly with myosin filaments using *in vitro* reconstitution coassembly assays with Sephacryl S-1000 column-purified mixtures of smitin and myosin. At physiologic levels of ionic strength (coassembly buffer containing 150 mM KCl), the smooth muscle myosin assembled into 1–2  $\mu\text{m}$  long sidepolar filaments (Fig. 6, A). When assembled in the presence of smitin, the sidepolar myosin filaments associated with each other in side-by-side arrays (Fig. 6, B–D). The myosin filaments in these arrays are various lengths; therefore, the arrays had little apparent long-range periodicity or regimentation in the alignment of myosin filaments. However, some localized alignment of the side polar filaments is apparent in the alignment of the myosin heads across several filaments in some of the coassembly structures (Fig. 6 D).

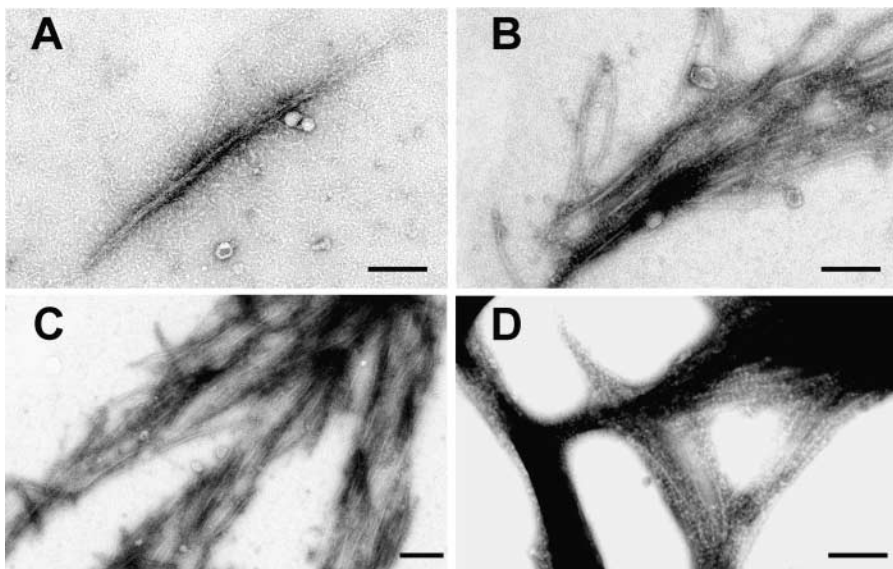
In low ionic strength (coassembly buffer containing 50 mM KCl), smooth muscle myosin assembled into minibipolar filaments, which are  $\sim 400$  nm long. When assembled in

the presence of smitin, these filaments formed large, highly ordered arrays (Fig. 7). In some of the supramolecular arrays, the myosin filaments were associated end-to-end and aligned side-by-side (Fig. 7, B). The end-to-end abutment of myosin filaments give these arrays a filament alignment periodicity of  $\sim 400$  nm. We also found coassembly structures in which small groups of myosin minibipolar filaments were associated end-to-end, but at acute angles to each other in geometric patterns (Fig. 7, C and D). It appears from our images that these filament arrays formed as three-dimensional structures, but collapsed onto the grid during sample preparation.

### Immunolocalization of smitin in smitin-smooth muscle myosin coassembly structures

We confirmed association of smitin with the coassembly structures by immunofluorescence and immunogold localization analysis. Smitin–myosin coassembly structures formed in the low ionic strength coassembly condition were double labeled with the anti-smitin rabbit polyclonal antibody and an anti-myosin mouse monoclonal antibody (Fig. 8, A–C). The smitin staining is distributed in a punctate pattern of spots along linear structures in the coassemblies. Spaces between these spots along the structures ranges from  $\sim 0.4$   $\mu\text{m}$  (Fig. 8, A, bracket) up to  $\sim 1$   $\mu\text{m}$  (Fig. 8 C, boxed inset region, green spots). This may indicate that these structures can be extended by forces generated during application of the structures to the poly-lysine-coated slides.

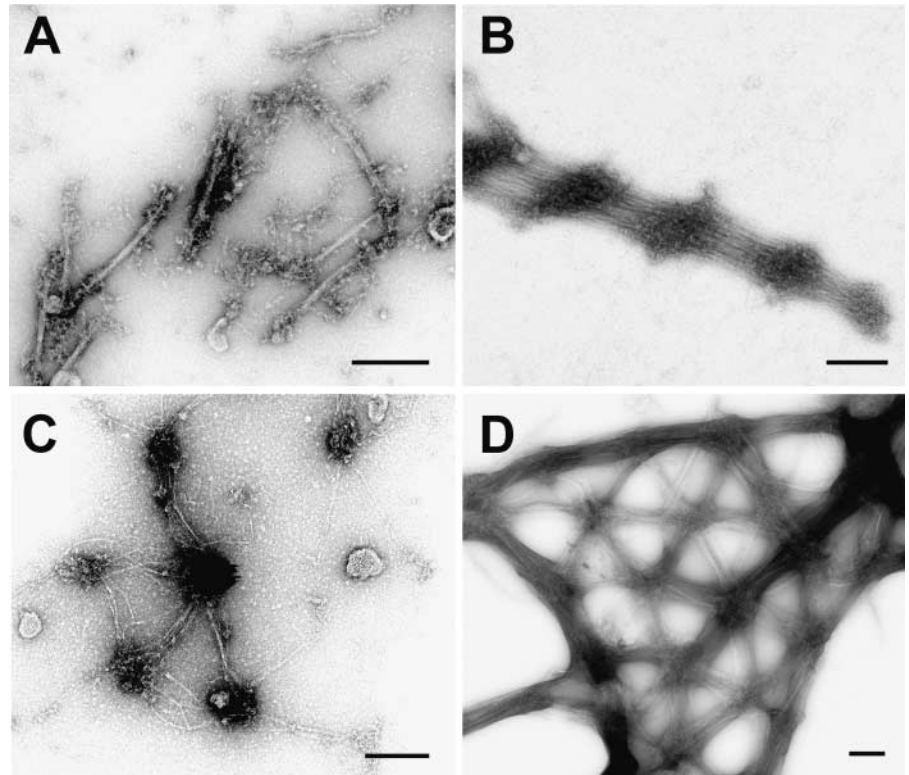
Although more diffuse than the smitin staining, the myosin staining also appears to be distributed in discrete regions along the smitin-containing structures. The myosin regions are roughly complementary to the smitin staining spots (Fig. 8, B and C, red). This colocalization of the smitin and myosin in the same coassembly structures confirms association of smitin with these structures. The distribution of smitin and myosin staining in the coassembly structures is consistent with these structures being composed of myosin filaments aligned end-to-end and side-by-side in regular arrays, as revealed by electron microscopy of negatively stained samples (Fig. 7).



**Figure 6. Electron micrographs of smitin and smooth muscle myosin sidepolar filament interaction.** Shown are a smooth muscle myosin sidepolar filament (A) and smitin–myosin coassembly structures (B–D) formed in physiological ionic strength (150 mM KCl, pH 7.0) buffer. Bars, 200 nm.



**Figure 7. Electron micrographs of smitin and smooth muscle myosin minibipolar filament interaction.** Shown are unassociated smooth muscle myosin minibipolar filaments (A) and smitin–myosin coassembly structures (B–D) formed in low ionic strength (50 mM KCl, pH 7.0) buffer. Bars, 200 nm.



The immunofluorescence localization analysis also confirmed association of smitin with the sidepolar filaments formed by smooth muscle at physiological ionic strength. These coassemblies display smitin and myosin staining patterns consistent with structures composed of aligned myosin sidepolar filaments (Fig. 8, D–E). These coassembly structures revealed a periodic staining pattern with an interval of  $\sim 400$  nm for smitin staining distribution. Higher magnification images of these structures reveals an almost lattice-like pattern, with smitin staining evident as cross striations across the width of the coassembly structures as well as distributed along the length of the structures (Fig. 8 F, inset, green). This staining could be revealing regions of the smitin molecules that project from the myosin filaments and interact side-on as well as end-on with smitin molecules aligned in a similar manner along the structures.

We extended these studies of smitin and myosin localization in *in vitro* reconstituted coassembly structures by using EM-immunogold localization to map more precisely the localization of smitin in these coassemblies. After deposition on a grid, the coassemblies were incubated with the anti-smitin polyclonal antibody followed by an anti-rabbit IgG conjugated to a 10-nm gold particle. In the low ionic strength coassemblies containing the minibipolar myosin filaments, most of the 10-nm gold particles labeling smitin were found near the myosin head regions (Fig. 9, A and B). Some of the gold label was found associated with amorphous protein protrusions from these coassembly structures. Likewise, immunogold localization with the anti-smitin antibody also confirmed the presence of smitin associated along the length of the long sidepolar myosin filaments (Fig. 9 C). A distinct lattice-like periodicity for the gold label distribution was less evident than in the immunofluorescent labeling

of these structures. Smitin in samples lacking myosin aggregated into linear bundles that also bound gold label, but these also lacked a distinct periodicity in the distribution of gold label (Fig. 9 D).

#### **Stoichiometry of smitin–myosin coassembly interactions—sucrose density gradient analysis**

We used sucrose density gradient centrifugation analysis and SDS-PAGE to determine the stoichiometric ratio of smitin binding to myosin sidepolar and bipolar filaments. We found that smitin–myosin coassemblies containing myosin bipolar filaments formed in low ionic strength buffer (50 mM KCl), and that those containing myosin sidepolar filaments formed in physiological ionic strength buffer (150 mM KCl); both were sedimented to similar positions near the bottom of 5–60% sucrose gradients overlaying pads of 65% sucrose (Fig. 10, A and B). Samples of fractions from each of the gradients were analyzed by scanning densitometry of Coomassie blue–stained SDS gels. This analysis revealed that the mean optical density ratio for myosin heavy chain to smitin in both coassembly configurations was  $\sim 17:1$ . Assuming a molecular mass of 2,000 kD for a molecule of smitin and 400 kD for the two heavy chains in a molecule of myosin, we estimate the myosin to smitin molecular ratio in both the bipolar and sidepolar coassemblies to be  $\sim 85:1$ . This is most consistent with a ratio of  $<1$  titin molecule per bipolar myosin filament, because each myosin filament appears to contain  $<85$  myosins. If at least one smitin molecule associates with each myosin bipolar filament, the amount of Coomassie blue stain bound by smitin in SDS gels may underestimate the amount of smitin protein present in the coassemblies. Myosin in excess of that coassembled with the

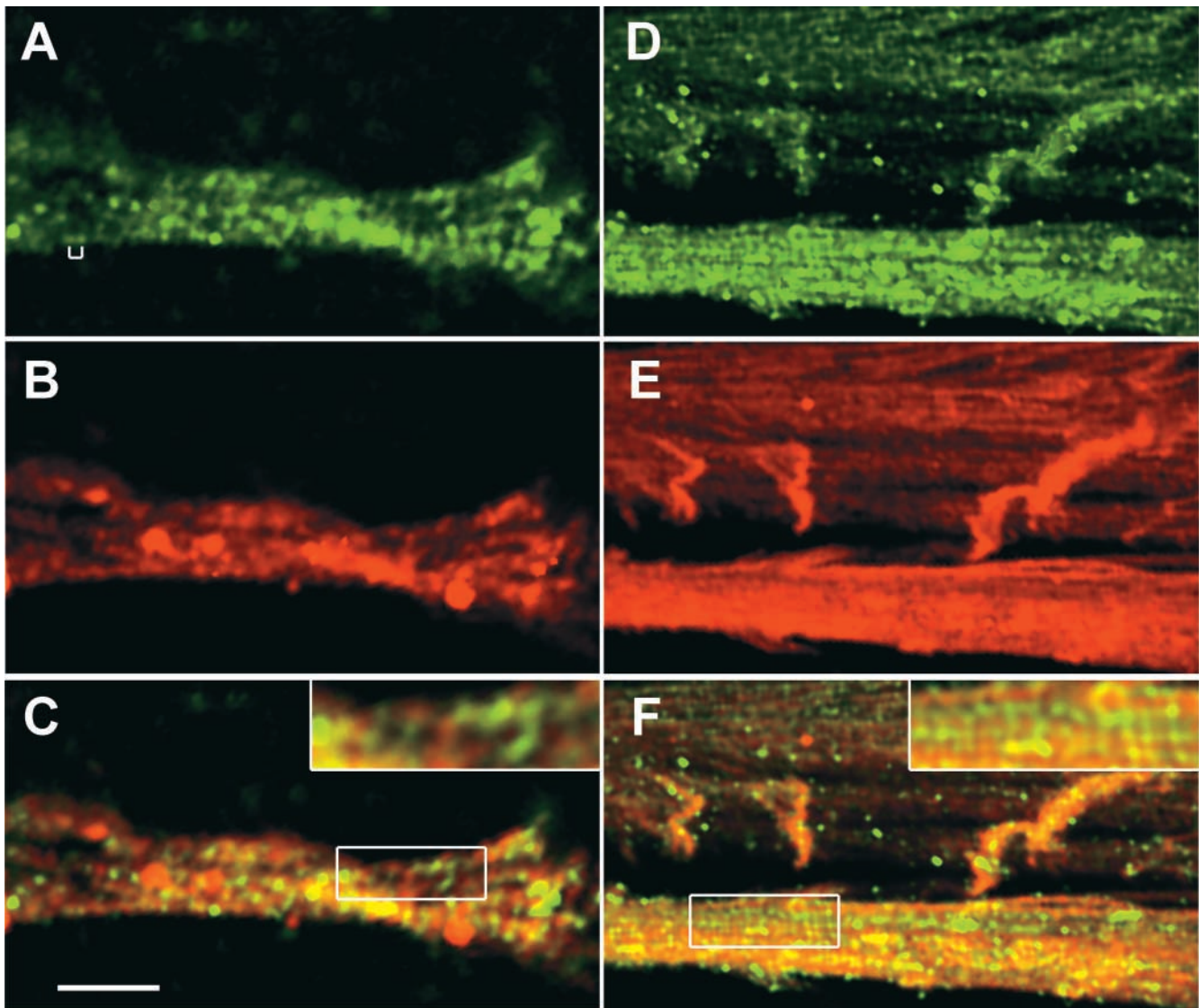


Figure 8. **Confocal microscopy images of smitin and myosin in vitro reconstituted coassembly structures.** Mixtures of smitin and smooth muscle myosin were dialyzed into assembly buffers containing 50 mM KCl (A–C) to form coassembly structures containing minibipolar filaments of myosin, or 150 mM KCl to form coassembly structures containing sidepolar filaments of myosin (D–F). The coassembled structures were adhered to poly-L-lysine-coated coverslips and double labeled with an anti-smitin polyclonal antibody (green) and an anti-myosin monoclonal antibody (red). Merged images of A and B (C) and D and E (F) contain insets of the boxed regions that are magnified 2 $\times$ . Bar, 5  $\mu$ m.

smitin forms filaments that sediment higher in the gradient (Fig. 10, A and B, fractions 8–10).

## Discussion

Vertebrate smooth muscle cells employ an actin–myosin-based contractile system to generate force for a variety of vascular and visceral organ functions. Members of the titin family of proteins play important roles in the structure and function of analogous actin–myosin contractile systems in vertebrate striated muscles. The possibility that smooth muscles also contain a titin or titin-like protein was suggested by a report that an otherwise uncharacterized avian gizzard smooth muscle protein has an amino acid composition similar to that of striated muscle titin (Maruyama et al., 1977), but the existence of a smooth muscle titin has

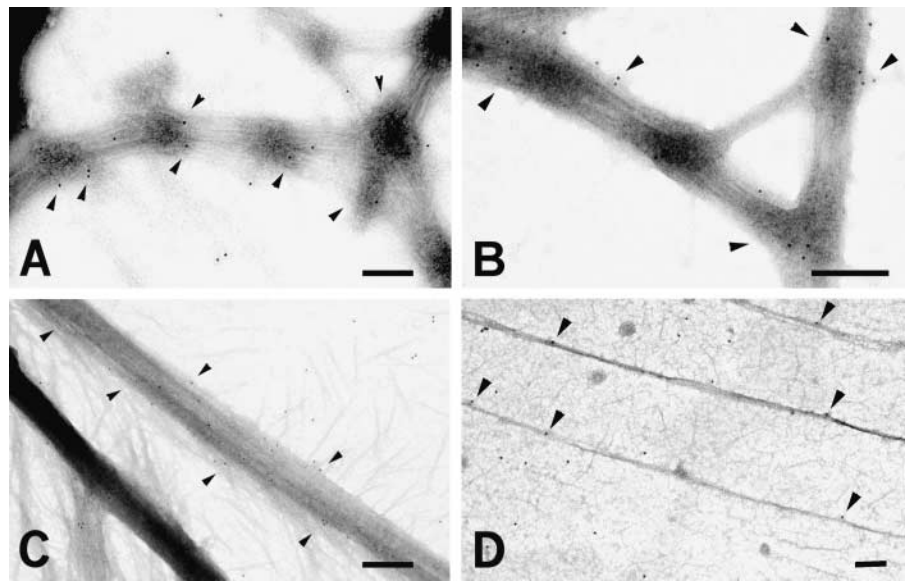
remained unsubstantiated and generally unrecognized. Indeed, none of the current models of smooth muscle structure incorporates titin as a structural or functional component (Bagby, 1986; Small, 1995; Somlyo, 1997). Our discovery and initial characterization of the novel protein smitin provides the first direct evidence for a titin-like protein in smooth muscles. Our initial characterization of smitin presented here begins to address roles smitin might play in the structure and function of the smooth muscle contractile system.

### Smitin resembles striated muscle and c-titins in size and molecular morphology

We discovered smitin as a very high molecular mass protein in extracts of chicken gizzard smooth muscle, and found that smitin has a molecular morphology similar to that of striated

**Figure 9. Immunogold localization of smitin in smitin–myosin coassemblies.**

Mixtures of smitin and myosin were dialyzed into buffers containing 50 mM KCl (A and B) or 150 mM KCl (C). Coassembly structures were collected on formvar-coated EM grids. The grids were incubated with the anti-smitin polyclonal antibody, washed, incubated with 10-nm gold particle–conjugated secondary antibodies, and negatively stained with uranyl acetate. Many of the immunogold particles bound to the coassembly structures containing the bipolar filaments in the regions of the structures near the myosin filament head domains (A and B, arrowheads). Immunogold particles bound along the length of the myosin sidepolar filaments formed in 150 mM KCl (C, arrowheads). Purified smitin dialyzed into coassembly buffer containing 150 mM KCl but no myosin aggregated into threads that labeled with anti-smitin antibody (D, arrowheads). Bars, 200 nm.



muscle titin and nonmuscle cell titin-like protein (c-titin). Like striated muscle titin and c-titins (Eilertsen and Keller, 1992; Nave et al., 1989), smitin has a globular domain on one end and a long fibrous tail. The length of the smitin tail domain ( $\sim 800$  nm) appears to be on average a little shorter than the tail domains of the striated muscle titin and c-titins (900–1,000 nm long), but significantly longer than the  $\sim 250$ -nm tail of invertebrate minititin (Nave et al., 1991).

This shorter morphology is consistent with the apparently lower molecular mass of the smooth muscle protein compared with muscle titins and c-titins. Based on its migration in SDS-PAGE, we estimate that the smitin polypeptide molecular mass is  $\sim 2,000$  kD, compared with estimates of  $\sim 3,000$  kD for the molecular masses of chicken striated muscle titin and c-titins. Although the 2,000-kD form of the smooth muscle protein could be a proteolytic fragment of a larger polypeptide, we have been unable to confirm existence of a larger version of the protein in various types of gizzard smooth muscle preparations.

Despite the apparent differences in size, smitin could be a splice variant isoform encoded by the striated muscle titin gene or c-titin gene. However, lack of crossreactivity of our anti-smitin polyclonal antibody with either striated muscle titin or intestinal epithelial cell c-titin suggests that the smitin is a distinct protein and unlikely to be a splice variant encoded by the striated muscle or c-titin genes. We speculate that the three apparent forms of titin or titin-like proteins found in striated muscle, smooth muscle, and nonmuscle cells are encoded by distinct genes or gene families, as are the three major forms of myosin II found differentially expressed in striated muscle, smooth muscle, and nonmuscle cells (Goodson, 1994; Mooseker and Cheney, 1995; Sellers et al., 1996; Berg et al., 2001). Moreover, each of the titin or titin-like protein genes may encode multiple isoforms, as has been shown for human striated muscle titin gene (Centner et al., 2000).

### Colocalization of smitin and myosin in vivo

The relatively unrigimented alignment of structural components in the smooth muscle contractile apparatus has made it

difficult to discern its three-dimensional organization (Bagby, 1986; Small, 1995). A current model of smooth muscle cell organization proposes that the cytoplasm is composed of two distinct but connected structural and functional domains: a “contractile” domain, which produces force for contraction, and a “cytoskeletal” domain, which may help to maintain the organization of the cell during contraction and extension (Small, 1995). The contractile domain contains wavy myofibril-like arrangements of sarcomere-like contractile unit structures. Like the Z-lines in striated muscle sarcomeres, the dense plaques associated with the cell membrane and the dense bodies spread throughout the cytoplasm provide anchorage sites for oppositely oriented actin thin filaments at each end of the smooth muscle contractile unit. Large sidepolar myosin filaments that are interdigitated with these actin filaments in the center of the contractile unit produce the force on the actin filaments that pulls the dense plaques/bodies closer together. In contrast to striated muscle, the long axis of each smooth muscle contractile unit is oriented at an angle of several degrees off the long axis of the cell in extended muscle. This angle becomes more oblique as the muscle contracts. The cytoskeletal domain contains bundles of nonmuscle  $\beta$ -actin filaments and intermediate filaments, which run the length of the cell and interact with the dense bodies (Draeger et al., 1990).

Our immunofluorescent localization of smitin and myosin in cryosections of gizzard smooth muscle revealed a significant degree of overlap between distinct smitin and myosin staining patterns. As others have described previously, we found myosin localized continuously along long wavy structures running through the contractile domain of the gizzard smooth muscle cells (Small et al., 1986; North et al., 1994). The smitin appears to be associated with these structures, but its immunofluorescence localization pattern is much more punctate than that of the myosin. Cross-sectional views of these structural elements reveal that the myosin staining is concentrated in the core, but that smitin staining is more prominent on the periphery of the structures and may project away from the myosin filaments. This staining



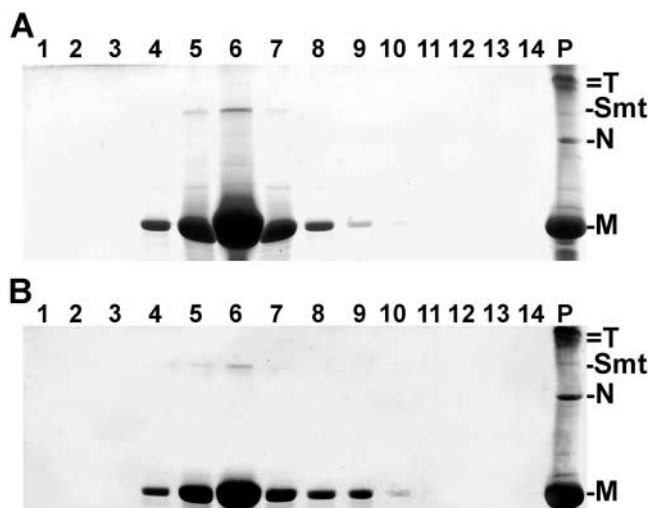


Figure 10. **Stoichiometry of smitin–myosin interaction.** SDS-PAGE analyses of smitin and smooth muscle myosin coassembled in solutions containing 50 mM KCl (A) or 150 mM KCl (B). The samples were subjected to sucrose density gradient centrifugation and fractionated into 1 ml fractions. Lane 1 in each gel corresponds to the fraction from a 1-ml pad of 60% sucrose at the bottom of each tube. Lanes 2–14 are from the bottom to the top of the 5–60% sucrose gradient. Only the upper portion of each gel is shown. M, myosin heavy chain; N, nebulin; P, SDS-extract of chicken pectoralis muscle used as a molecular mass standard for the gel; Smt, smitin; T, titin.

pattern may indicate that smitin interacts only with certain spots on the periphery of the smooth muscle myosin filaments. More likely, it reflects lack of accessible smitin epitopes in regions of smitin–myosin interaction in the filament complex.

Several features of the smooth muscle contractile apparatus make it difficult to apply the striated muscle sarcomere structural paradigm toward understanding smitin organization in smooth muscle cells. In the striated muscle sarcomere, individual molecules of titin span the  $\sim 1\text{-}\mu\text{m}$  distance between the central M-line and the Z-disk, and associate with one pole of the myosin bipolar thick filament in the half sarcomere (Trinick, 1991; Labeit et al., 1992, 1997; Wang et al., 1993; Keller, 1995; Horowitz, 1999). The contractile units of avian gizzard smooth muscle cells appear to be several times longer than the striated muscle sarcomere and contain 3–6- $\mu\text{m}$  long actin filaments (Small et al., 1990). The distance between the myosin thick filament and potential anchoring sites for smitin on dense plaques/bodies could be several micrometers, much longer than the  $\sim 800\text{-nm}$  long smitin molecules, even if they are extended to more than double length. In addition, the gizzard smooth muscle myosin filaments are  $>1.5\text{-}\mu\text{m}$  long (Small et al., 1990), more than 0.5  $\mu\text{m}$  longer than striated muscle myosin filaments, and are side-polar rather than bipolar (Craig and Megerman, 1977; Small, 1977; Hinssen et al., 1978; Cooke et al., 1989). In striated muscle, individual molecules of titin are long enough to associate with one pole of a myosin thick filament from the center of its central bare zone region to the end of the filament, with an additional region of the titin spanning the I band to the Z-line. In the smooth muscle

side-polar filaments, all the myosin molecules along one side of the filament are oriented with one polarity, whereas those along the other side have the opposite polarity. This creates bare zone regions lacking myosin heads on one side of each side-polar filament end (Craig and Megerman, 1977; Small, 1977; Hinssen et al., 1978; Cooke et al., 1989). The lack of a clearly delineated center for the smooth muscle side-polar filament makes it difficult to envision how smitin could interact with only one half of a side-polar filament, but smitin molecules appear to be far too short to interact along the entire length of the side-polar filament and extend beyond the end. Perhaps smitin molecules interact in a staggered fashion along the side-polar filament and project to interact with nearby dense bodies or unidentified structures.

### Smitin–myosin interaction in vitro

Although our immunofluorescence data suggest that smitin and myosin are closely localized in smooth muscle, differences in the organization of the smooth and striated muscle contractile apparatuses raise the questions concerning the ability of smitin to interact directly with smooth muscle myosin and organize its activity in smooth muscle. Our investigations of coassembly of smitin and myosin in vitro address some of these questions. We confirmed association of smitin with smooth muscle side-polar filaments with in vitro coassembly assays using isolated smitin and myosin. In physiological ionic strength conditions, the smooth muscle myosin assembled in vitro into large side-polar filaments similar to those known to exist in vivo (Cooke et al., 1989). In the presence of smitin, the smooth muscle myosin side-polar filaments aggregated into bundles in which the filaments aligned along the long axis of the bundle. Negatively stained preparations of the coassemblies display little apparent regimentation in alignment of the filaments along the bundles. This apparent lack of regimentation is also reflected in the continuous staining for myosin in coassembly structures that were immunofluorescently labeled with an anti-myosin antibody (Fig. 8). The continuous nature of this myosin staining pattern is similar to that of the myosin structures in smooth muscle cells (Fig. 4). In contrast to the myosin staining pattern, the smitin staining pattern associated with the coassembly structures is more discretely punctate and exhibits striations across the coassembly structures. Immunogold localization of smitin confirmed its association with the coassembly structures, but a corresponding periodicity of gold label distribution along the structures is less readily apparent (Fig. 9).

In low ionic strength conditions, smooth muscle myosin assembles into small bipolar filaments (Craig and Megerman, 1977; Trybus and Lowey, 1987). We were surprised to find that in low ionic strength conditions smitin organized the small myosin bipolar filaments side-by-side and end-to-end into highly regimented linear and geometric arrays. This degree of adaptability in smitin interaction with both side-polar and bipolar configurations of smooth muscle myosin filaments is unexpected. The molecular basis for smitin association with the different configurations of smooth muscle myosin filaments remains to be elucidated. Nevertheless, our

cosedimentation results demonstrating that the sidepolar and bipolar myosin filament coassembly structures contain similar ratios of smitin to myosin suggests that fundamental features of the smitin-myosin interaction may be common to both of the coassembly configurations.

Smitin may be unique among the titins or titin-like proteins in ability to interact with large sidepolar filaments of myosin, just as striated muscle titin may be unique in interacting with large bipolar filaments of myosin. However, coassembly of smitin with small bipolar filaments of myosin in linear and geometric arrays is similar to what we have found for coassembly of chicken and human blood platelet c-titin interaction with non-muscle myosin (Eilertsen et al., 1994; Keller et al., 2000). Moreover, rabbit skeletal muscle myosin, perhaps contaminated with skeletal muscle titin, also forms similar linear and geometric arrays of small bipolar filaments (Podlubnaya et al., 1987). This suggests that the ability to associate with and organize small bipolar filaments of myosin is a fundamental property of vertebrate striated muscle titins, c-titins, and smitin.

### Small bipolar filament coassemblies of smitin–smooth muscle myosin—physiologically relevant?

The ability of smitin to organize small bipolar filaments of smooth muscle myosin into linear arrays similar to those found in cytoskeletal structures such as stress fibers may have physiological relevance. Vertebrate smooth muscle cells, especially those surrounding arteries and airway passages, exhibit a remarkable phenotypic plasticity in changing from a contractile to a synthetic phenotype in response to certain stimuli (Halayko and Solway, 2001). The contractile phenotype is characterized by the presence of the extensive contractile system described above, and by a lack of cell motility and proliferation. In response to wounding or other growth stimuli, the cells change to the synthetic phenotype by disassembling the contractile apparatus, assembling cytoskeletal structures such as stress fibers, and becoming motile and proliferative. Transition of smitin–myosin interaction from the organization of large sidepolar filaments in the contractile apparatus of the contractile phenotype to the stress fiber-like arrangement of small bipolar filaments may play a role in the change of the cell to the synthetic phenotype. Alternatively, smitin organization of small bipolar filaments of smooth muscle myosin may play a role in formation of the contractile apparatus during smooth muscle development.

Clearly, further investigation of the molecular characteristics of smitin and its interaction with other smooth muscle components will yield greater insight into the organization and function of smitin in smooth muscle cells. Clarification of the relationship between smitin and striated muscle titin will require analysis of smitin protein sequence, as yet unavailable, for hallmarks of titin protein domain organization and functional domains.

## Materials and methods

### Purification of chicken gizzard smitin and myosin

Smitin was extracted from fresh chicken gizzard by a modification of a method for extracting cardiac muscle titin (Pan et al., 1994). Fresh chicken gizzard smooth muscles were diced and homogenized for 10 s with a

Waring blender in buffer A (2 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 0.2 mM PMSF, and 0.05 mM leupeptin, 10 mM Imidazole, pH 7.0, containing 50 mM KCl). Gizzard myofibrils were pelleted by centrifugation (5,000 g for 10 min, 4°C), washed three times with buffer A, and resuspended in extraction buffer (2 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 0.2 mM PMSF, 0.05 mM leupeptin, 10 mM Imidazole, pH 7.0, containing 0.6 M KCl and 4 mM ATP) for 30 min. The extracted myofibrils were pelleted (15,000 g for 30 min at 4°C) and the supernatant was loaded directly onto a Sephacryl S-1000 column. The Sephacryl S-1000 column was equilibrated and eluted with 0.2 M KCl, 10 mM Imidazole, pH 7.5, 1 mM EGTA, 0.5 mM EDTA, and 0.2 mM DTT. Fractions containing smitin alone or smitin and myosin that lacked actin and other contaminating proteins were pooled and used for some experiments without further purification.

### Gel electrophoresis

Electrophoresis was performed on high-porosity SDS-polyacrylamide gels as described previously (Eilertsen and Keller, 1992).

### Antibodies and Western blotting

A smitin-specific polyclonal antibody was raised in rabbits by injecting SDS-PAGE-purified chicken gizzard smitin. The antigen was prepared by coassembling smitin and myosin in 50 mM KCl, pH 7.0, coassembly buffer to concentrate the smitin before making the gel sample. For antigen injections, the smitin bands were excised from Coomassie blue-stained SDS-polyacrylamide gels, frozen and pulverized in liquid nitrogen, and emulsified in Freund's complete adjuvant. For the subsequent boosts, Freund's incomplete adjuvant was used to emulsify the gel pieces.

The reactivity of anti-smitin was demonstrated by Western blot assay of proteins that were electroblotted to nitrocellulose. To facilitate the transfer of the large proteins, the gel was incubated for 2 min in a trypsin (0.5 µg/ml) solution. We have noticed no negative affect of this trypsin treatment on reactivity of smitin or striated muscle titin with antibodies in Western analysis. Following trypsin digestion, the gel was soaked in transfer buffer for 15 min. The blot was incubated with the anti-smitin polyclonal antibody followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) for 1 h each at room temperature and developed as described previously (Eilertsen and Keller, 1992).

The 9D10 anti-vertebrate striated muscle mouse monoclonal antibody was used for detection of titin in Western blot analysis and immunofluorescence localization analysis. This monoclonal antibody developed by M. Greaser was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA).

### Coassembly of smitin and myosin

Aliquots of Sephacryl S-1000-purified smooth muscle smitin and myosin were dialyzed for 24 h against either of two coassembly buffers, both of which contained 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2 mM DTT, 1 mM PMSF, and 10 mM Imidazole, pH 7.0. The low ionic strength coassembly buffer also contained 50 mM KCl. The physiological ionic strength buffer contained 150 mM KCl.

### Sucrose density gradient ultracentrifugation analysis

Coassembled samples were analyzed by sucrose density gradient analysis. The coassembled samples were sedimented at 118,000 g for 30 h through a 5–60% sucrose gradient layered onto a pad of 65% sucrose as described previously (Eilertsen et al., 1994, 1997). The sucrose gradients were collected as 30-drop fractions from the bottom to the top. The fractions were subjected to SDS-PAGE. The proteins in the gel lanes were quantified by scanning densitometry (pdi, Inc.) of Coomassie blue-stained gels using the Quantity One (Bio-Rad Laboratories) program.

### Immunolocalization of smitin and myosin in cryosections of gizzard smooth muscle

Strips of fresh chicken gizzard smooth muscle or cardiac muscle were excised and immediately fixed for 30 min in 3.7% paraformaldehyde fixation solution containing 0.1% Triton X-100. The fixed muscle strips then were rapidly frozen by immersion in 2-propanol that was precooled with liquid nitrogen. The strips were sectioned transversely and longitudinally at a thickness of 10 µm with a cryostat. The sections were collected on 2% gelatin-coated slides. For double-label immunofluorescence analysis, the gizzard smooth muscle sections were incubated simultaneously in the anti-smitin polyclonal antibody and an anti-pan myosin monoclonal antibody (BAbCO MMS-456S; Innovative Products), and the cardiac muscle was incubated with the anti-smitin polyclonal antibody and the 9D10 anti-titin monoclonal antibody. We used an FITC-conjugated goat anti-rabbit IgG (1:100; Sigma-Aldrich) and a Texas

red-labeled secondary antibody (1:450) for myosin (Jackson ImmunoResearch, Inc.) for detection of the primary antibodies.

### Electron microscopy and immunogold labeling

Molecules of smitin were extended and rotary replicated with platinum as described before (Eilertsen and Keller, 1992). Coassembled protein samples were negatively stained with or without immunogold labeling and examined by electron microscopy as described previously (Eilertsen and Keller, 1992). For immunogold labeling, samples on grids were fixed for 5 min with 1% glutaraldehyde in PBS, pH 7.4, blocked for 5 min on a drop of 0.1 M Tris-HCl, pH 7.0, incubated for 5 min with the anti-smitin antibody (diluted 1:75 in 0.1 M Tris-HCl, pH 7.0), followed by 5 min with anti-rabbit IgG-conjugated 10-nm gold particles (Sigma-Aldrich), and negatively stained. All samples were examined by transmission electron microscopy (1200 EX; JEOL USA) at an accelerating voltage of 80 kV.

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