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Avian Synaptopodin 2 (Fesselin) Stabilizes Myosin Filaments and Actomyosin in the Presence of ATP

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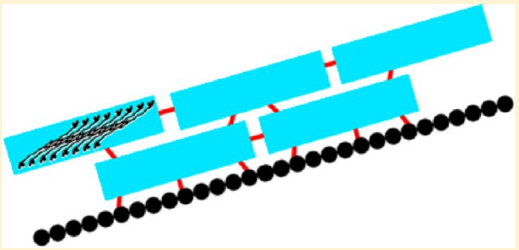
ABSTRACT: Smooth muscle cells maintain filaments of actin and myosin in the presence of ATP, although dephosphorylated myosin filaments and actin−myosin interactions are unstable under those conditions *in vitro*. Several proteins that stabilize myosin filaments and that stabilize actin−myosin interactions have been identified. Fesselin or synaptopodin 2 appears to be another such protein. Rapid kinetic measurements and electron microscopy demonstrated that fesselin, isolated from turkey gizzard muscle, reduced the rate of dissociation of myosin filaments. Addition of fesselin increased both the length and thickness of myosin filaments. The rate of detachment of myosin, but not heavy meromyosin, from actin was also greatly reduced by fesselin. Data from this study suggest that fesselin stabilizes myosin filaments and tethers myosin to actin. These results support the view that one role of fesselin is to organize contractile units of myosin and actin.



Fesselin is an actin binding protein that was first isolated from avian smooth muscle.[1] Fesselin was later shown to be the avian form of synaptopodin 2,[2] a member of the synaptopodin family of proteins.[3,4] This work uses avian muscle as its source, so we will use the term fesselin to describe our studies. However "fesselin" from mammalian sources will be called synaptopodin 2.

Fesselin/synaptopodin 2 is an actin binding protein that is found in smooth,[1] cardiac, and skeletal muscle.[5,6] Fesselin is localized primarily in the dense bodies of smooth muscle[7] and Z-lines of striated muscle.[5] Synaptopodin 2 has been identified in several cell lines where it appears to shuttle between the nucleus and cytoplasm.[5] Fesselin/synaptopodin 2 is primarily known as an actin binding protein.[1,5] Fesselin binds to four actin protomers of an actin filament with an affinity of 0.5 μM but with small positive cooperativity ($\omega = 1.7$), giving an overall affinity for a singly contiguous site of 0.39 μM.[1] Binding to F-actin results in the formation of actin aggregates[1] or bundles.[8] These bundles are ordered with a uniform polarity.[9] Fesselin also binds to G-actin and stimulates actin polymerization.[10] Ca^{2+}-calmodulin inhibits the ability of fesselin to stimulate G-actin polymerization.[11] Ca^{2+}-calmodulin does not affect binding of fesselin to F-actin.[11,12] Therefore, Ca^{2+}-calmodulin regulates fesselin-mediated actin polymerization but not the subsequent bundling of actin filaments. These functions are consistent with its cellular colocalization with actin.[13]

Fesselin is intrinsically disordered[14] and, like many such proteins, has multiple binding partners, including α-actinin,[15] calmodulin,[11,16] zyxin,[17] and myosin.[18] The interaction with myosin is interesting because of the potential for fesselin to polymerize actin and bundle it into filaments and then hold those filaments in the proximity with myosin.

Previous research showed that fesselin binds to smooth muscle myosin with an affinity of 0.5 μM with an apparent stoichiometry of one fesselin per myosin head.[12] Fesselin inhibits activation of myosin S1 ATPase activity by actin in a concentration-dependent manner.[12] That inhibition appears to result from competition with S1-ATP for binding to actin.

Because fesselin polymerizes and organizes actin, we determined if fesselin has similar functions for myosin. We show evidence from pre-steady state kinetics and electron microscopy that fesselin decreases the rate of disassembly of myosin filaments in the presence of high levels of ATP. Several other proteins have been identified with this activity, including kinase-related protein[19] and caldesmon.[20] Those proteins stabilize dephosphorylated myosin filaments under cellular conditions.

Our studies also suggest that fesselin decreases the rate of dissociation of actin−myosin complexes by ATP. We propose that fesselin tethers smooth muscle myosin to actin. Such tethering has been observed with other proteins, including caldesmon[21,22] and C-protein.[23,24] Stabilization of myosin and the actin−myosin complex may be important for organizing contractile units in smooth muscle cells.

EXPERIMENTAL PROCEDURES

Proteins. Myosin and heavy meromyosin (HMM) were prepared from turkey gizzards.[25] Myosin was stored in 0.5 M NaCl, 10 mM MOPS, 2 mM $MgCl_2$, and 0.1 mM dithiothreitol.

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Actin was prepared from rabbit erector spinae muscle[26] and stored in 4 mM MOPS and 2 mM $MgCl_2$. Tropomyosin was prepared from turkey gizzards[27] and labeled with acrylodan.[28] Fesselin was prepared from frozen turkey gizzards[1] and stored in 94 mM NaCl, 10 mM MOPS, 2 mM $MgCl_2$, and 0.1 mM dithiothreitol (pH 7). Protein concentrations were determined by absorbance measurements using the following extinction coefficients ($\varepsilon^{0.1\%}$ at 280 nm): 1.15 for actin, 0.5 for myosin, 0.22 for tropomyosin, and 1.0 for fesselin. The molecular weights of the proteins were assumed to be 42000 for actin, 480000 for myosin, 350000 for HMM, 68000 for tropomyosin, and 102000 for fesselin.

Transient Kinetics. The rate of dissociation of myosin from actin following rapid mixing with ATP was measured by light scattering on a SF20 sequential mixing stopped-flow spectrometer (Applied Photophysics Ltd., Leatherhead, Surrey, United Kingdom). The temperature was controlled with a circulating water bath. The excitation wavelength was set with a monochromator using slit widths of either 0.5 or 1 mm. Emission wavelengths were selected with high-pass filters. Acrylodan fluorescence was measured with excitation at 391 nm with emission regulated by a Schott (Duryea, PA) GG 455 high-pass filter with a 455 nm midpoint. MANT fluorescence changes were monitored via excitation at 366 nm and emission measured with an Oriel model 51270 high-pass filter. Light scattering measurements were taken by increasing the emission wavelength above the working range of the excitation filter (600 nm for the 455 nm high-pass filter).

Single-turnover studies were conducted like earlier studies,[29,30] but with a sequential mixing stopped flow using MANT-ATP as a fluorescent indicator of ATP and ADP dissociation. MANT-ATP was synthesized as described by Hiratsuka.[31] Myosin was rapidly mixed with MANT-ATP using the sequential mixing stopped flow. After 0.1 s, the myosin−MANT-ATP complex was rapidly mixed with a 100-fold excess of ATP to compete with the MANT-ATP and MANT-ADP that dissociated from myosin.

Electron Microscopy. Protein filaments were visualized using a negative staining methodology. Carbon-coated Formvar nickel grids (200 mesh) were negatively charged using an EMS 100 Glow Discharge unit immediately prior to applying protein solutions. Solutions of myosin, actin, and fesselin were prepared by adding concentrated stocks to 94 mM NaCl, 10 mM MOPS, 2 mM $MgCl_2$, and 0.1 mM dithiothreitol (pH 7). After incubation for 30 s on the grid, the solutions were removed by touching the edge of the grid to Whatman #1 filter paper. Rinsing was accomplished by positioning the grid against a piece of filter paper and applying 50 μL of 0.1 M ammonium acetate in 1 mM sodium phosphate buffer (pH 7) to the grid. In this manner, the rinse solution flowed across the grid into the filter paper. Subsequently, 10 μL of 1% aqueous uranyl acetate was applied in the same manner before an additional 10 μL of uranyl acetate was applied and allowed to incubate on the grid for 45 s. Excess uranyl acetate was removed with filter paper and the grid allowed to air-dry. Grids were examined in a JEOL 1200EX transmission electron microscope at an accelerating voltage of 80 kV. Images were recorded using a SIS ImageView III CCD camera (Olympus, Tokyo, Japan).

RESULTS

The ability of myosin to bind to different effectors could be a function of the physical state of myosin. Smooth muscle myosin monomers exist as a mixture of compactly folded and inactive (10S) and extended and active (6S) forms.[32,33] We determined the state of the unphosphorylated smooth muscle myosin under the conditions used in this study by examining the rate of product release. Myosin was mixed with the fluorescent ATP derivative (MANT-ATP) and allowed to react long enough (100 ms) to bind and to reach equilibrium between the MANT-ATP and MANT-ADP-P_i states. This complex was then rapidly mixed with an excess of unlabeled ATP to displace the fluorescent nucleotides. The decay was monoexponential with a rate constant of 0.66 s^{-1} in a buffer containing 500 mM KCl (not shown). This is consistent with stabilization of the active, 6S state of dephosphorylated myosin at 0.6 M KCl.[29,34] Under the conditions used in our studies (94 mM KCl), we observed a biexponential release of the products with rate constants of 0.6 and 0.036 s^{-1} consistent with equilibrium between the 6S and 10S, inactive, states of myosin (Figure 1, inset). This is typical of smooth muscle myosin, with the 10S state increasingly favored as the salt concentration is decreased.

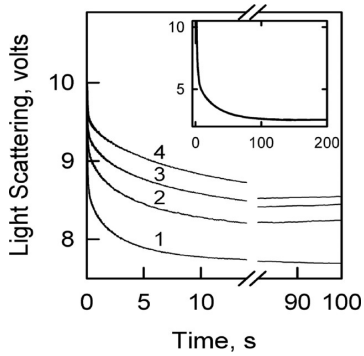

Figure 1. Fesselin reduces the rate of ATP-induced, smooth muscle myosin dissociation. Biexponential fits to the light scattering transients are superimposed on the data. In the absence of fesselin (curve 1), k_1 = 1.1 s^{-1} and k_2 = 0.10 s^{-1}. In the pesence of 0.05 μM fesselin (curve 2), k_1 = 0.51 s^{-1} and k_2 = 0.05 s^{-1}. In the presence of 0.1 μM fesselin (curve 3), k_1 = 0.42 s^{-1} and k_2 = 0.04 s^{-1}. In the presence of 0.2 μM fesselin (curve 4), k_1 = 0.35 s^{-1} and k_2 = 0.03 s^{-1}. Conditions: 0.05 μM smooth muscle myosin, 94 mM NaCl, 10 mM MOPS, 2 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0, and 10 °C. The inset shows MANT fluorescence as a function of time (seconds) as the products (MANT-ATP and MANT-ADP) detach from smooth muscle myosin under the conditions described above (k_1 = 0.6 s^{-1}, and k_2 = 0.036 s^{-1}).

We examined the possibility that binding of fesselin to myosin affects the stability of myosin filaments. The time course of disassembly of filamentous dephosphorylated smooth muscle myosin by ATP was measured. ATP binding is expected to change myosin filaments to inactive folded myosin monomers. Figure 1 shows that the transition was biexponential, indicating the presence of at least one intermediate in the disassembly process. Myosin alone exhibited two apparent rate constants of 1.1 and 0.1 s^{-1}. Fesselin decreased both rate constants by similar fractions. At 0.2 μM fesselin, the rate constants were reduced to 0.35 and 0.03 s^{-1}. This suggests that fesselin stabilized the myosin filaments, preventing dissociation upon addition of ATP.

A decrease in light scattering indicates a reduction in filament size, and it is known from earlier work that this is due to the disassembly of myosin filaments. We examined the effect of fesselin on the structure of myosin filaments to confirm the stabilizing effect of fesselin and to determine the specific structural changes caused by fesselin. Figure 2A shows electron

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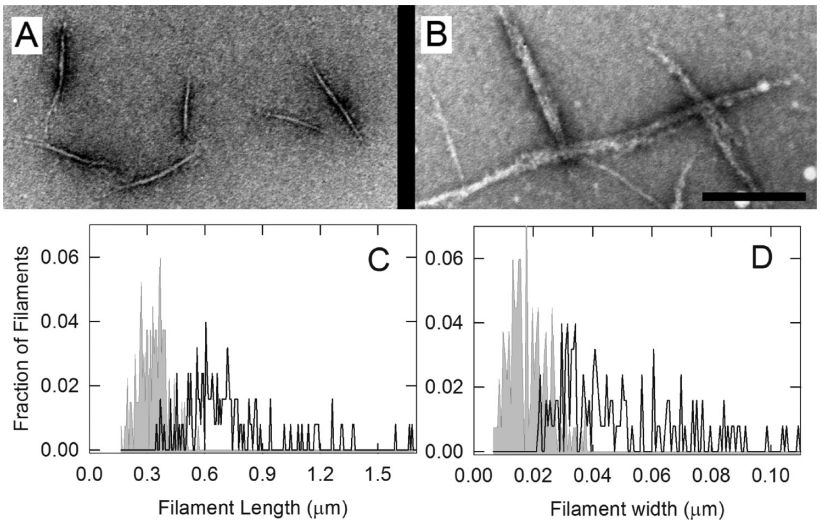

Figure 2. Electron microscopic analysis shows fesselin increases the length and width of smooth muscle myosin filaments. Panels A and B are representative electron micrographs of 0.05 μM smooth muscle myosin in the absence (A) and presence (B) of 0.1 μM fesselin. Distributions of filament lengths (C) and widths (D) were determined by measuring the filaments with NIH ImageJ. These measurements were ordered by size and plotted as size vs the fraction of filaments at that size. One hundred thirty-three filaments were measured in the absence of fesselin, and 126 filaments were measured in the presence of fesselin. Gray shaded regions are in the absence of fesselin, with an average filament length of 0.32 μm and a width of 0.017 μm. Black lines are in the presence of fesselin, with an average filament length of 0.72 μm and a width of 0.048 μm.

micrographs of dephosphorylated myosin filaments in the absence of fesselin. Filaments were short and consistent with the bipolar structure typical of smooth muscle myosin under these conditions. Addition of fesselin increased the size of the myosin filaments (Figure 2B), although the gross appearance of the filaments appeared to be the same. Furthermore, myosin filaments tended to cluster together in the presence of fesselin.

Panels C and D of Figure 2 show the length and width distributions of myosin filaments (gray lines) and myosin filaments with fesselin (black lines). Fesselin increased the average myosin filament length by 2.2-fold and the average filament width by 2.8-fold. The distribution curves for myosin with fesselin were skewed to the right, indicating the presence of very long and very thick filaments. The increase in both length and width suggests that lateral and tandem associations among myosin filament units are equally favored by fesselin. This indicates that in the rapid kinetic measurements of Figure 1 the initial states were different for curves 1 and 2, with larger filaments existing when fesselin was present (curve 2).

Figure 2 shows the state of myosin filaments prior to the addition of ATP. Figure 3 shows the state of myosin filaments 30 s after addition of ATP (ATP was added 30 s after myosin had been loaded onto the grid). No filaments were observed in the absence of fesselin (Figure 3A). Filaments were seen in samples containing fesselin (Figure 3B). Those filaments were similar in size to those observed with myosin alone in the absence of ATP. These images along with those in Figure 2 suggest that disassembly of the large filaments formed in the presence of fesselin occurs in steps, with very large filaments disassembling to form normal myosin filaments.

We next addressed the possibility that fesselin can cross-link myosin to actin. Initial studies examined changes in light scattering after mixing of the actin−myosin complex with ATP. Binding of ATP to myosin decreases its affinity for actin by several orders of magnitude and results in detachment of myosin from actin. Figure 4A shows that fesselin had a strong inhibitory effect on the dissociation of actin from myosin. The dissociation curve in the absence of fesselin (curve 1) was

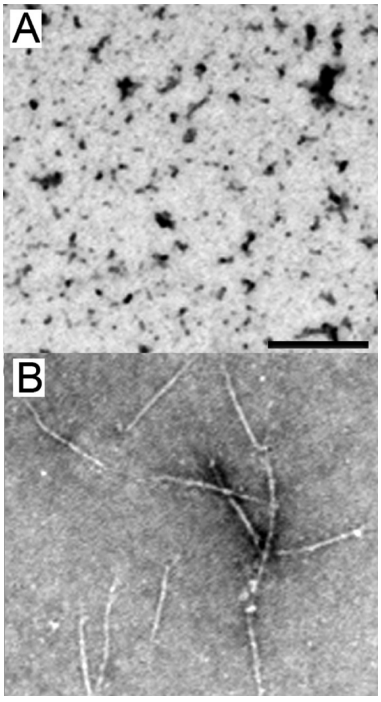

Figure 3. ATP resistance of fesselin−myosin filaments shown using electron microscopy. (A) Smooth muscle myosin with ATP. (B) Smooth muscle myosin−fesselin filaments with ATP. Conditions: 0.05 μM smooth muscle myosin, 0.1 μM fesselin, 2 mM ATP, 94 mM NaCl, 10 mM MOPS, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.0, and 10 °C. ATP was added to myosin immediately before it was added to the grid.

biphasic with a k_1 of 4.2 s^{-1} and a k_2 of 0.24 s^{-1}. Addition of 0.05 μM fesselin (curve 4) reduced the observed rate constants to 1.6 and 0.16 s^{-1}, respectively, or ~40% of their original values. Fesselin also reduced the amplitude, indicating the possibility of multiple populations of actin−fesselin−myosin filaments: one that dissociated at a rate that was 40% of the rate

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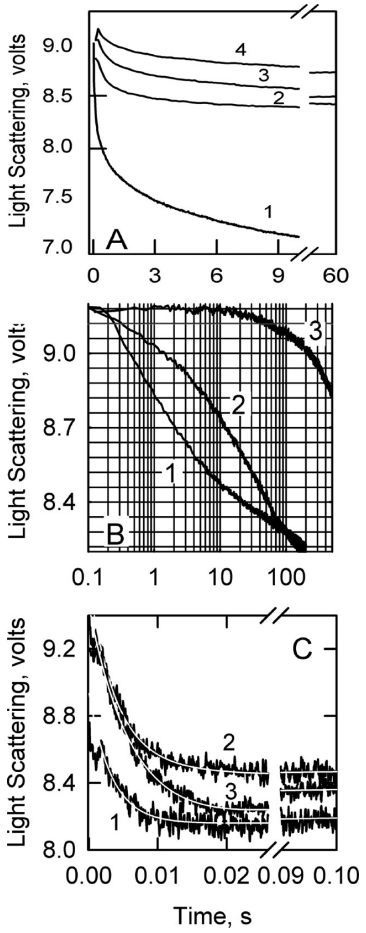

Figure 4. Fesselin increased the resistance of actin–myosin filaments toward dissociation by 2 mM ATP. (A) Light scattering transients of 0.2 μM phalloidin–actin filaments and 0.05 μM smooth muscle myosin. In the absence of fesselin (curve 1), k_1 = 4.2 s^{-1} and k_2 = 0.24 s^{-1}. In the presence of 0.0125 μM fesselin (curve 2), k_1 = 2.3 s^{-1} and k_2 = 0.23 s^{-1}. In the presence of 0.025 μM fesselin (curve 3), k_1 = 1.7 s^{-1} and k_2 = 0.13 s^{-1}. In the presence of 0.05 μM fesselin (curve 4), k_1 = 1.6 s^{-1} and k_2 = 0.16 s^{-1}. The reduced amplitude of curve 4 suggests that most actin–fesselin–myosin complexes were stable on this time scale. Conditions: 94 mM NaCl, 10 mM MOPS, 2 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0, and 10 °C. (B) Fesselin was more effective than caldesmon in reducing the rate of dissociation. Note the logarithmic time axis: 0.05 μM myosin and 0.2 μM phalloidin–actin with no addition (curve 1), with 1 μM caldesmon (curve 2), and with 1 μM fesselin (curve 3). Conditions were the same as those for panel A except with 0.2 mM ATP. (C) Light scattering transients of actin and smooth muscle HMM. In the absence of fesselin (curve 1), k_1 = 320 s^{-1}. In the presence of 0.05 μM fesselin (curve 2), k_1 = 230 s^{-1}. In the presence of 0.2 μM fesselin (curve 3), k_1 = 280 s^{-1}. Conditions were the same as those for panel A.

of actin–myosin filaments and another population that did not disassemble on the experimental time scale. Figure 4B compares the effects of 2 μM fesselin or caldesmon on the rate of dissociation at long time scales. The semilogarithmic plot shows that fesselin is much more potent than caldesmon in reducing the rate of dissociation of actin–myosin filaments. The time for reduction of the signal to 50% was increased by 10-fold by caldesmon and by several hundred-fold in the presence of fesselin.

Fesselin had a minimal effect on dissociation of HMM from actin (Figure 4C). A single-exponential process with a rate constant of 320 s^{-1} was observed without fesselin. Addition of 0.05 μM fesselin reduced the rate constant to 230 s^{-1}, while 0.2 μM fesselin reduced the rate constant to 280 s^{-1}. Those observations suggest that fesselin does not alter the rate constants of binding of ATP to myosin or the subsequent breaking of bonds between myosin and actin. Rather, it suggests that fesselin tethers actin to the light meromyosin portion of myosin.

The conformation of this tethering came from the minimal effect that fesselin had on a probe that is sensitive to the specific interaction of actin with myosin. Acrylodan-labeled tropomyosin undergoes a decrease in fluorescence as myosin rapidly detaches from actin following an ATP chase.[35] Figure 5A shows

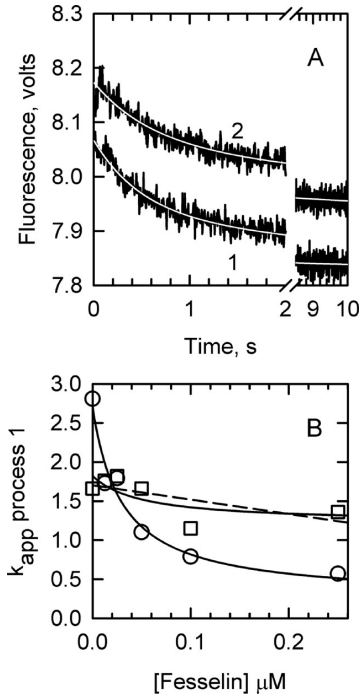

Figure 5. Fesselin had little effect on acrylodan tropomyosin fluorescence following the rapid addition of ATP to the actin–myosin complex. (A) Acrylodan fluorescence transients in the absence of fesselin (for curve 1, k_1 = 1.6 s^{-1} and k_2 = 0.17 s^{-1}) and with 0.25 μM fesselin (for curve 2, k_1 = 1.4 s^{-1} and k_2 = 0.15 s^{-1}). The fitted curves are shown as white lines on the data traces. (B) Plots of the apparent rate constant for the rapid phase, k_1, vs fesselin concentration for light scattering (○) and acrylodan fluorescence (□). Hyperbolic fits are shown for both processes. The best straight line fit to acrylodan fluorescence is also shown as a dashed line. Conditions: 94 mM NaCl, 10 mM MOPS, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.0, 10 °C, and 0.05 μM smooth muscle myosin.

the biphasic decreases in acrylodan tropomyosin fluorescence when ATP was rapidly mixed with the myosin–actin complex in the presence and absence of fesselin. The apparent rate constants were 1.6 and 0.17 s^{-1} without fesselin and 1.4 and 0.15 s^{-1} with 0.25 μM fesselin. The lack of change argues against an effect of fesselin on the specific ATP-dependent interaction between myosin and actin. Figure 5B shows the dependencies of light scattering and acrylodan tropomyosin fluorescence on fesselin concentration. Fesselin decreased the rapid phase of light scattering to 20% of its initial value. However, the rapid rate constant for acrylodan fluorescence decreased to only 80% of its initial value. That is, although

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fesselin had a large effect on the breakdown of the actin−myosin protein complex, fesselin had little effect on the ATP specific interaction between actin and myosin.

The formation and stabilization of the actin−myosin complex in the presence or absence of fesselin were evaluated by negative staining of filaments and examination in the electron microscope to confirm stopped-flow observations. Figure 6 shows that myosin applied to grids containing actin filaments was removed when the grids were washed with a solution of ATP. Inclusion of fesselin with myosin resulted in retention of myosin filaments that were located in the proximity of actin filaments.

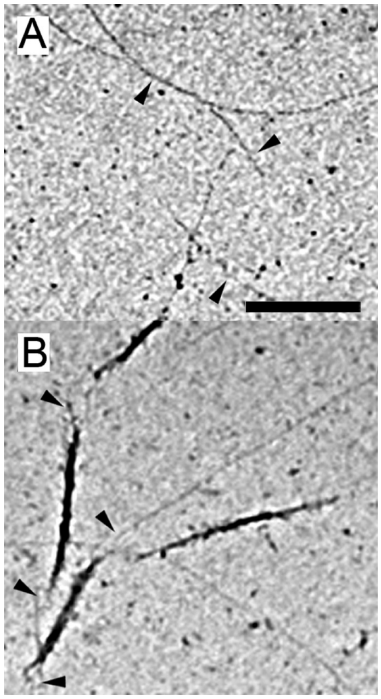

Figure 6. Electron microscopy images showing myosin filaments binding along actin filaments in the presence of ATP only when fesselin is present. Smooth muscle myosin and actin with or without fesselin were added to the grid, and ATP was added directly: (A) actin−myosin complex with ATP and (B) actin−myosin−fesselin complex with ATP. Actin (0.2 μM) was used with 0.05 μM myosin and 0.1 μM fesselin. Conditions: 94 mM NaCl, 10 mM MOPS, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.0, and 10 °C.

DISCUSSION

The structure of smooth muscle myosin is regulated by binding to ATP, by phosphorylation of the regulatory light chain, and by binding to other ligand proteins. Dephosphorylated myosin filaments *in vitro* normally dissociate in the presence of ATP to form folded monomers.[33,36,37] That folded state or 10S state is inactive with Mg-ADP-P_i trapped at the active sites.[29] The weakened ability to bind actin and hydrolyze ATP is the result of a specific type of interaction between the two myosin heads.[38−41] The inability to polymerize may be due to the folding of the myosin tail region, again in a specific manner.[25]

In relaxed smooth muscle, myosin filaments continue to persist[42] in spite of the presence of millimolar concentrations of ATP. The stability of these filaments is thought to be due to other ligand proteins that associate with myosin such as smooth muscle kinase-related protein[43] and caldesmon.[20] The kinase-related protein accomplishes this by promoting the unfolded state of myosin.[44] Fesselin/synaptopodin 2 may also function to stabilize myosin filaments in the relaxed state.

When smooth muscle myosin was mixed rapidly with ATP, there was a biexponential time-dependent decrease in light scattering (Figure 1). Fesselin reduced both apparent rate constants. The transition from myosin filaments to 10S myosin monomers is thought to occur by the scheme shown below:

$$T + M_F \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} TM^*_F \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} TM_F \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} TM_{6S} \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} TM_{10S}$$

Binding of ATP to 6S myosin filaments (M_F) leads to formation of a collision complex (TM^*_F) that isomerizes prior to dissociation to form ATP-bound 6S monomers (TM_{6S}) and finally 10S monomers (TM_{10S}).[45]

Binding of ATP to myosin was much faster than the succeeding transitions, under our conditions, and did not affect the kinetics. In the absence of fesselin, the curve was described well by $k_2 + k_{-2} = 1.2\ s^{-1}$, $k_3 + k_{-3} = 58\ s^{-1}$, and $k_4 + k_{-4} = 0.09\ s^{-1}$, although other combinations of rate constants were possible. That is, our results are consistent with the model of Rosenfeld et al.[45]

Myosin filaments formed in the presence of fesselin tended to be longer and thicker than normal filaments. Fesselin shifted the peaks of the size distribution curves to >2-fold higher values and skewed the distribution to allow the formation of very large filaments. Filaments formed in the presence of fesselin had an average length of 0.72 μm, with lengths ranging from ∼0.34 to 1.7 μm. Filaments formed in the absence of fesselin had a tighter distribution with an average length of 0.32 μm. Native myosin filaments in smooth muscle homogenates had an average length of 1.8 μm, with a range from 0.8 to >3 μm.[46] Fesselin may contribute to both the stability of myosin filaments and the larger size.

The increased resistance to ATP was likely the result of cross-links between individual myosin molecules and between groups of myosin filaments as shown in Figure 7. Cross-links between filaments must occur in both side-by-side and end-to-end manners. Packing is assumed to occur in a manner that maximizes the number of surfaces from adjoining filaments that are in contact with each other. Figure 7 also shows the proposed cross-linking of myosin to actin.

Evidence of cross-linking myosin to actin came from the decreased kinetics of dissociation of the actin−myosin complex upon mixing with ATP. The effects of fesselin on actin−myosin cross-linking were strong; a fraction of the actin−fesselin−myosin complex did not dissociate on the experimental time scale. The observed effects of fesselin were much stronger than those of caldesmon, a known tethering protein.[47] Fesselin did not greatly reduce the rate constant for breaking the association between actin and myosin. That is, fesselin had little effect on the rate of dissociation of actin from HMM. Also, fesselin had little effect on acrylodan tropomyosin fluorescence that changes in response to changes in the attachment of myosin to actin. Rather, fesselin appeared to tether myosin and actin together after the normal bond between myosin and actin was broken. Similar observations have been made for caldesmon,[48,49] calponin,[50] and C protein.[24] Electron micrographs showed a tendency of myosin filaments to be localized along actin filaments in the presence of fesselin and ATP (Figure 6). Fesselin has micromolar affinity for both myosin and actin. That affinity is on the order of 100-fold greater than the actin−myosin affinity during ATP hydrolysis.

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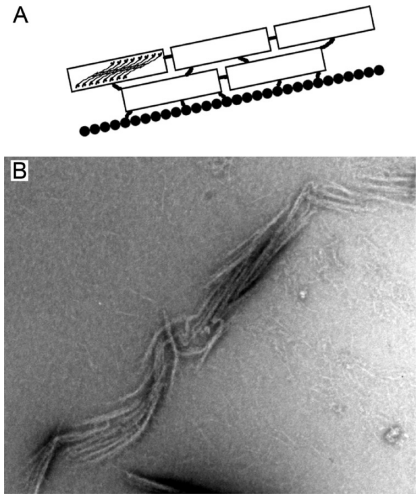

Figure 7. Fesselin organizes myosin and actin−myosin filaments. (A) Schematic showing possible cross-links (black bars) between short myosin filaments (rectangles) and between myosin and actin (circles). An example of an individual myosin filament is shown in the top left rectangle. Cross-links within myosin filaments may also occur. (B) Electron microscopy image of 0.05 μM myosin and 0.05 μM fesselin showing the buildup of a large myosin filament. Conditions were the same as those described in the legend of Figure 2.

This work adds to the list of effects of fesselin/synaptopodin 2 on actin structure. Binding of fesselin to G-actin is followed by rapid polymerization that is probably the result of stabilization of actin nuclei.[10] That nucleation is inhibited by Ca^{2+}-calmodulin.[11] The resulting F-actin forms parallel bundles,[1,8] in the absence of myosin. The bundling is unaffected by Ca^{2+}-calmodulin. These effects are consistent with the localization of fesselin in dense bodies and Z-lines.

We show here that fesselin may have other functions related to stabilization of myosin filaments. The large myosin filaments that formed appeared to be cross-linked with actin based on kinetic studies. Electron micrographs show a close association of myosin with actin filaments, although cross-links were not directly seen. When added to a mixture of both myosin and actin, fesselin appears to preferentially bundle myosin and tether myosin to actin filaments. Fesselin inhibits binding of the S1 portion of myosin to actin with a resulting loss of ATPase activity.[18] However, binding of fesselin to a fraction of the actin sites may be sufficient to cross-link actin to myosin. The remaining actin sites are free to interact normally with myosin and stimulate ATP hydrolysis. Low concentrations of caldesmon enhance the movement of myosin past actin in the *in vitro* motility assay,[51] indicating that tethering need not impede motility. It is possible that fesselin behaves in the same way.

While it is clear that fesselin/synaptopodin 2 has a role in organizing actin, we must consider the possibility that fesselin also organizes myosin filaments. There is no available evidence of fesselin binding to cellular myosin. However, staining of gizzard muscle with anti-fesselin IgG showed diffuse background staining in addition to intense staining of dense bodies.[7] That diffuse staining may have been due to the association of fesselin with smooth muscle myosin.

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ABBREVIATIONS

MOPS, 3-(*N*-morpholino)propanesulfonic acid; MANT-ATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)-ATP.

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