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Organization of F-Actin by Fesselin (avian smooth muscle synaptopodin 2)

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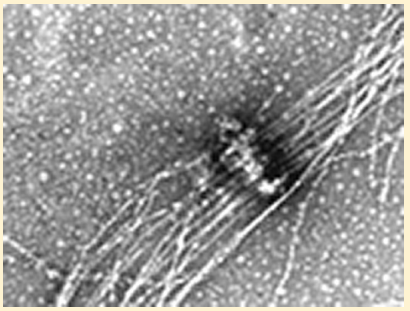
ABSTRACT: Fesselin or avian synaptopodin 2 is a member of the synaptopodin family of actin binding proteins. Fesselin promotes G-actin polymerization and the formation of large actin complexes that can be collected by low-speed centrifugation. Because of the potential role of fesselin in some cancers and its effects on actin, we further investigated the effect of fesselin on actin. Fesselin initiated actin polymerization under a variety of conditions, including the virtual absence of salt. Actin filaments formed at low salt concentrations in the presence of fesselin were similar to filaments polymerized in the presence of 100 mM KCl. In both cases, the filaments were long and straight with a common orientation. Highly ordered actin bundles formed with increasing times of incubation. Blockers of actin growth at the barbed end (cytochalasin D and CapZ) did not prevent fesselin from polymerizing actin. Low concentrations of fesselin increased the critical concentration of actin. Both observations are consistent with preferential growth at the pointed end of actin filaments. These results indicate a role of fesselin in organizing cellular actin. These and other results indicate that fesselin is part of a cellular actin organizing center.



Actin is a major component of muscles and forms the thin filaments over which myosin moves. Actin is also present in non-muscle cells where it performs diverse critical functions, including actin-directed and actin−myosin-driven movements. The many roles of actin are in part the result of the different structures that actin can form in conjunction with the cadre of cellular actin accessory proteins.[1] Monomeric actin is stimulated to polymerize by nucleating factors. Some actin binding proteins induce actin to form various types of bundles or networks. Other proteins stabilize monomeric actin, cap actin filaments, or sever actin filaments. The synaptopodin family of actin binding proteins nucleates actin filament formation and produces higher-order structures that have not been identified. This work describes the structure of the actin−fesselin complex at the level of electron microscopy and explores the possibility that fesselin affects the two ends of actin filaments differently.

The synaptopodin family[2] consists of proline-rich basic proteins that bind to actin.[3−5] Three members of the synaptopodin family have been identified, and each has multiple splice forms.[2] Fesselin, or avian synaptopodin 2,[6,7] is the most readily purified member of the synaptopodin family[5,8] and is the subject of this work. Fesselin consists of a mixture of two polypeptides with molecular masses of 79 and 102 kDa. Both polypeptides contain the myopodin core region common to all synaptopodin 2 family members.[2]

Antibodies directed against synaptopodin 2 decorate Z-lines of rat skeletal and human heart muscle[4] and dense bodies of smooth muscle cells.[9] These locations suggest that one function of synaptopodin 2 is to participate in the organization of highly organized cellular actin structures.[2]

The known functions of fesselin or synaptopodin 2 are consistent with a close association with actin. In particular, fesselin facilitates actin polymerization by accelerating the rate of nucleation.[10] That activity is inhibited by Ca^{2+}-calmodulin.[11] We now show that actin filaments formed by the addition of fesselin to G-actin are unremarkable in appearance at the level of electron microscopy.

Upon standing, mixtures of fesselin and F-actin become turbid, and fesselin−actin complexes can be collected by low-speed sedimentation.[5] We show here that these complexes consist of tightly packed and ordered bundles with uniform polarity.

Our earlier study suggested that fesselin did not alter the critical concentration of actin.[10] However, at lower concentrations of fesselin, we now see an increase in the critical concentration. Other evidence will be shown that fesselin facilitates growth preferentially at the pointed or slow-growing end of actin filaments. This preferential growth might explain the order and uniform polarity of fesselin-induced actin bundles.

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EXPERIMENTAL PROCEDURES

Protein Preparations. Fesselin was purified from turkey gizzards by our published method[5] with additional gel permeation chromatography on a Superose 6 (GE Healthcare, Piscataway, NJ) column. Actin was purified from rabbit back muscle.[12,13] In some studies, F-actin was modified with *N*-(1-pyrenyl)-iodoacetamide (Molecular Probes) at pH 7.6.[14] G-Actin was prepared by exhaustive dialysis of F-actin against G-actin buffer [5 mM Tris (pH 8.0), 0.1 mM $CaCl_2$, 0.2 mM ATP, and 1 mM dithiothreitol]. The G-actin was purified by gel filtration chromatography over Superdex 200 or Superose 6. Skeletal myosin was prepared from rabbit back muscle.[15] Myosin S1 was made by digesting myosin with chymotrypsin (Worthington Biochemical, Freehold, NJ).[16] Heterodimeric CapZ with the $\alpha_1\beta_1$ subunits was expressed in BL21(DE3) bacteria from a pET3d plasmid, a gift of J. Cooper (Washington University, St. Louis, MO) (Addgene plasmid 13451). Expression and purification of CapZ were performed according to the methods of Soeno et al.[17]

Preparation of Samples for Electron Microscopy. Column-purified G-actin was flash-frozen and stored at −80 °C in small aliquots. Directly before being used, the aliquots were thawed and centrifuged for 45 min at 4 °C and 100000 rpm in a TLA 120.1 rotor. The upper 60% of the supernatants was used for polymerization. Bound Ca^{2+} in G-actin was exchanged with Mg^{2+} [18] immediately before the sample was mixed with fesselin at different ratios that was in a buffer containing 10−100 mM NaCl, different buffers (pH 7.0−7.2), and 1 mM dithiothreitol. The mixture was loaded onto glow-discharged, carbon-coated grids. The sample was blotted off after the appropriate incubation time, and the specimens were negatively stained with 2% uranyl acetate.[19] Images were obtained on a T-12 (FEI) electron microscope with an accelerating voltage of 80 kV, at a magnification of 30000×. Electron micrographs were scanned with a Nikon-Super Coolscan-9000 digital camera.

Critical Concentration Measurements. Freshly column-purified G-actin (20% pyrene-labeled) was polymerized. Following ATP removal, the F-actin was serially diluted into G-actin buffer in the absence or presence of fesselin. The concentrations of both actin and fesselin were varied to keep the free fesselin concentration constant in each experiment. Reaction mixtures were incubated at 20 °C overnight. Fluorescence measurements were taken on an Aminco Bowman II luminescence spectrometer (Thermo Electron, Madison, WI) at 25 °C with excitation at 365 nm (bandpass of 1 nm) and emission at 407 nm (bandpass of 4 nm). All fluorescence measurements were taken in triplicate. The measured fluorescence was plotted against the used actin concentration. The abrupt change in slope gave the critical concentration.

RESULTS

Figure 1A shows the time course of pyrene-labeled G-actin fluorescence at very low ionic strengths. In the absence of fesselin, G-actin was stable under these conditions and no observable increase in fluorescence occurred over 150 s. Addition of fesselin to a final concentration of 0.075 μM resulted in a rapid increase in pyrene fluorescence that indicates actin polymerization.[20] Figure 1B shows an experiment performed in the presence of 100 mM KCl. Under these conditions, G-actin was polymerized at a slow rate (bottom curve). Fesselin accelerated polymerization 4- and 11-fold at 0.075 and 0.27 μM, respectively. Higher concentrations of fesselin were required in the presence of KCl presumably because of a reduced affinity of fesselin for actin.

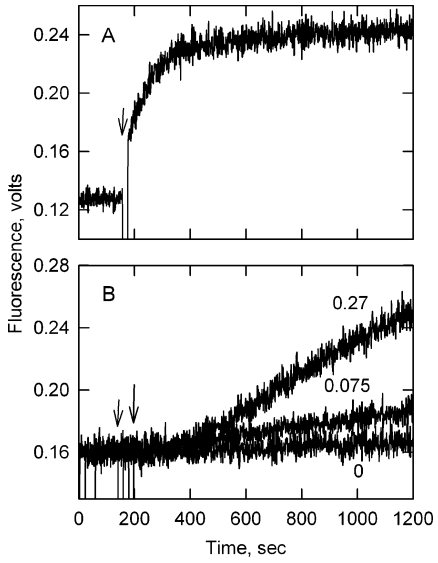

Figure 1. Fesselin-induced actin polymerization at very low (A) and moderate (B) ionic strengths. Pyrene fluorescence was monitored for 2 μM Mg-G-actin (10% pyrene-labeled) at 25 °C. (A) Conditions: 5 mM imidazole (pH 7.0), 0.2 mM ATP, and 0.005 mM $MgCl_2$. After 150 s, fesselin was added (indicated by an arrow) to a final concentration of 0.075 μM. (B) Conditions: 100 mM KCl added to the buffer used for panel A. Fesselin was added as indicated to give a final concentration of either 0.075 μM (short arrow) or 0.27 μM (long arrow).

The structure of actin filaments polymerized with fesselin was determined by electron microscopy. Panels A and B of Figure 2 show filaments formed at very low ionic strengths and prepared 30 s and 2 min, respectively, after fesselin had been added. The incubation times were measured after the protein mixture was spotted onto the microscopy grids. Actin filaments and short bundles were observed at 30 s. Long actin filaments and larger bundles were seen at 2 min.

Panels C and D of Figure 2 show actin filaments formed in the presence of 100 mM KCl. After 2 min, many long actin filaments were seen, and in many cases, the filaments formed bundles. At longer times (Figure 2D), the number of individual actin filaments decreased as the number of large actin bundles increased. Exceptionally well ordered bundles such as those shown in Figure 2D were common. Decoration of actin filaments by fesselin was not visible.

Complexes of actin and moderate concentrations of fesselin (0.02−0.2 μM) were often found as loosely packed parallel filaments (Figure 3A). Those loose bundles show evidence of nonrandom initiation points. An increasing level of order was seen with increasing incubation times (Figure 3B). Figure 3C shows well ordered bundles in addition to free actin filaments. Densely stained regions, from which actin filaments extended, were occasionally observed when the ratio of fesselin to actin was high. Figure 4 shows one view of such an organization center at two magnifications. Actin filaments were parallel as they passed over the aggregate, suggesting an organization similar to that described for the Z-disk by Tskhovrebova.[21]

Actin bundles formed in the presence of fesselin were decorated with S1 to determine the orientation of the actin filaments. Figure 5 shows that all of the visible actin filaments within a bundle have the same polarity. That is, the S1 "arrowheads" point in the same direction, as indicated in the figure, and are always directed to central dense regions of the bundles.

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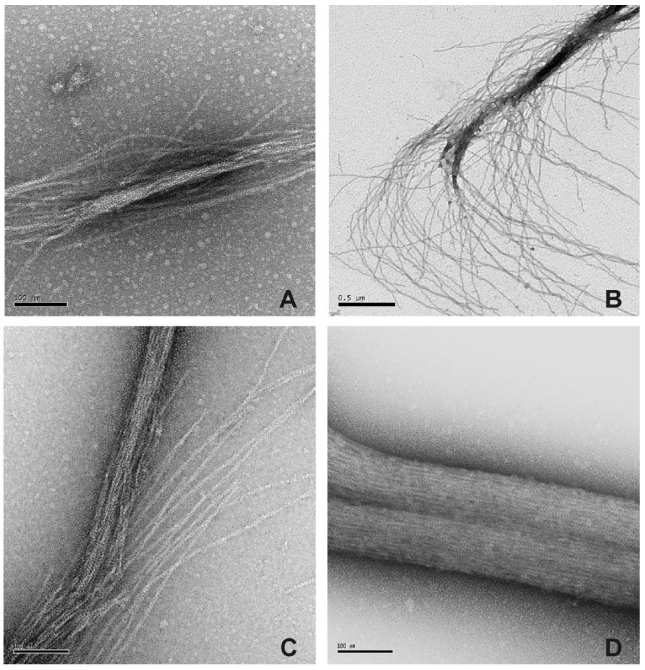

Figure 2. Electron micrographs of actin filaments formed in the presence of fesselin at very low ionic strengths. Mg-G-actin (2 μM) was placed on a carbon-coated, glow-discharged grid and mixed with fesselin. For panels A and B, the ionic strength was 12 mM and the final concentrations of actin and fesselin were 1.5 and 0.075 μM, respectively. The reactions were terminated by adding staining solution (2% uranyl acetate) after 30 s in panel A and after 2 min in panel B. The scale bars represent 100 nm in panel A and 500 nm in B. In panels C and D, the salt concentration was increased to 100 mM KCl and the final concentrations of actin and fesselin were 5 and 0.01 μM, respectively. The reactions were stopped after 2 min in panel C and 10 min in panel D. The scale bars in panels C and D represent 100 nm.

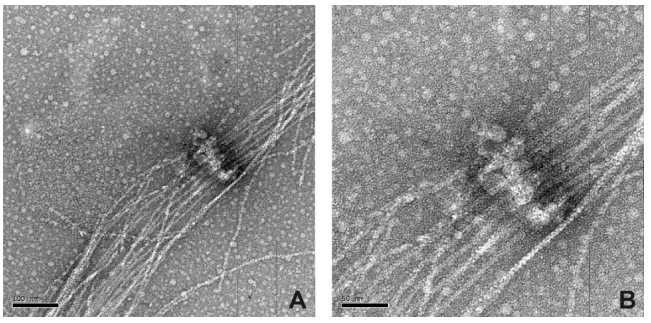

Figure 4. Fesselin complexes form organizing centers for actin. Polymerization of 1.5 μM Mg-G-actin was induced by 0.075 μM fesselin. The ionic strength of the reaction mixture was 12 mM. The reaction was stopped after 30 s. The scale bar in panel A represents 100 nm, and that in B represents 50 nm. The conditions were the same as those for panels A and B of Figure 2.

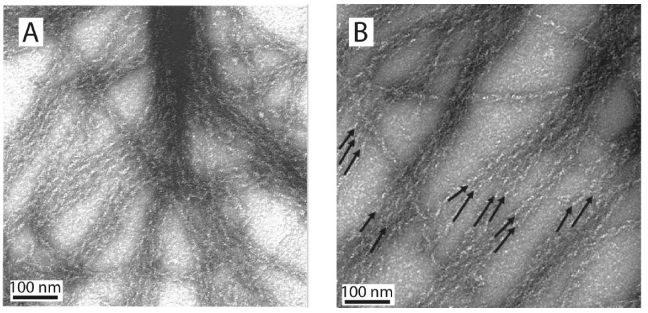

Figure 5. S1 decoration of actin within a fesselin–actin bundle showing that actin filaments in a bundle have the same polarity. G-Actin (2 μM) was mixed with 0.04 μM fesselin in 40 mM KCl, 1 mM $MgCl_2$, and 5 mM potassium phosphate buffer (pH 7.0). After incubation in the tube for 2–3 min, the sample was applied to the grid. The complex was washed with 1–2 drops of 1 μM S1 in the same buffer. Panels A and B are different fields of the same grid. Arrows on panel B show the polarity.

To address the reason for the common start–termination points of actin filaments within a fesselin-induced bundle, we explored the possibility that fesselin blocks one end of an actin filament. We determined if fesselin could promote actin filament growth in the presence of cytochalasin B, a known barbed end blocker.[22]

Curve 1 of Figure 6A shows that actin polymerization was very slow at low ionic strengths with 1 mM Mg^{2+} and that the rate was reduced to 75% of the initial rate by the addition of 0.093 μM cytochalasin B (curve 2). Upon addition of fesselin to G-actin in the absence of cytochalasin B, there was a 16-fold increase in the initial rate of polymerization (curve 6). Addition of 0.04 μM cytochalasin B had no effect on the polymerization curve of the actin–fesselin complex [overlap with curve 6 (not shown)]. Higher concentrations of cytochalasin B (0.093 μM for curve 5 and 0.264 μM for curve 3) produced a more pronounced flattening of the polymerization curve but did not affect the initial slope of the fluorescence change versus time. Figure 6B shows that fesselin was able to stimulate actin polymerization when the barbed, fast-growing end was blocked with a concentration of cytochalasin B 1000-fold greater than that in Figure 6A. Figure 6C shows that at higher ionic strengths fesselin continued

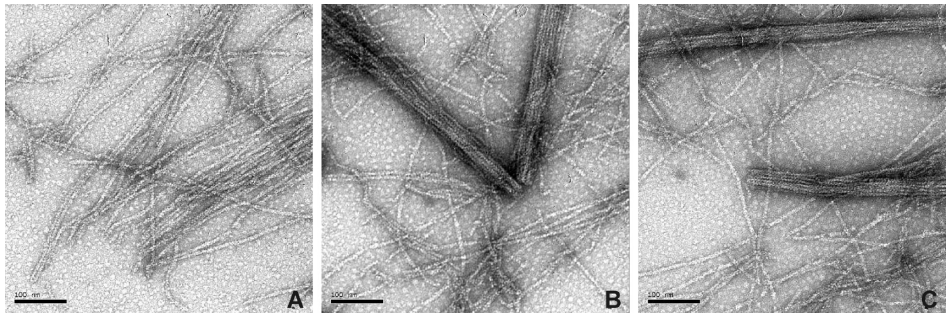

Figure 3. Images of bundle formation formed with 0.02 μM fesselin and 2 μM Mg^{2+}-G-actin. Fesselin and actin were mixed in a buffer containing 5 mM phosphate (pH 7.2), 0.2 mM $MgCl_2$, 0.2 mM EGTA, and 0.2 mM dithiothreitol. KCl was added after 1 min to a final concentration of 100 mM. After an additional 1 min, the samples were spotted onto grids. The samples were incubated on the grids for 20 s before being blotted and fixing. The scale bar represents 100 nm.

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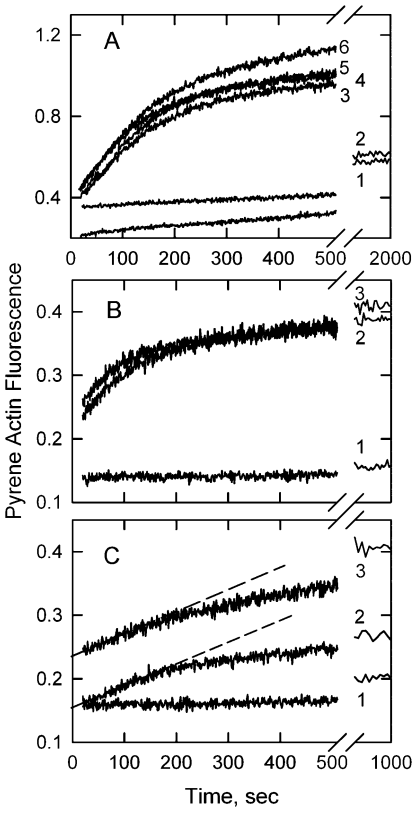

Figure 6. Effect of cytochalasin B on fesselin-stimulated actin polymerization at 25 °C. Polymerization was initiated by adding $MgCl_2$ to actin. In some experiments, G-actin was preincubated with cytochalasin for 5 min before polymerization was started. (A) G-Actin (2 μM, 5% pyrene-labeled) was polymerized in a buffer that consisted of 15 mM KCl, 2.8 mM Tris-HCl (pH 8), 1.5 mM imidazole (pH 7.0), 0.1 mM ATP, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM dithiothreitol. Concentrations of fesselin and cytochalasin B as indicated:

curve	1	2	3	4	5	6
[fesselin] (μM)	0	0	0.15	0.15	0.15	0.15
[cytochalasin B] (μM)	0	0.093	0.246	0.164	0.093	0

(B) G-Actin (2 μM, 5% pyrene-labeled) with 0 μM (curve 1) or 0.1 μM fesselin (curves 2 and 3). A high concentration of cytochalasin B (0.29 mM) was present in experiment 3. The buffer was similar to that in panel A. (C) Polymerization with the KCl concentration increased to 39 mM. Curve 1 is for 2 μM G-actin. Curve 2 is for 2 μM G-actin with 0.1 μM fesselin. Curve 3 is for 2 μM G-actin with 0.29 μM cytochalasin B and 0.1 μM fesselin.

to induce polymerization in the presence of a very high concentration of cytochalasin B.

We studied the ability of fesselin to stimulate actin polymerization with another barbed end blocker, CapZ (β-actinin), using electron microscopy. G-Actin was incubated with CapZ and then mixed with fesselin. Actin remained primarily in the globular state in the presence of CapZ and in the absence of fesselin (Figure 7A). However, the addition of fesselin resulted in rapid polymerization and bundle formation (Figure 7B). We observed that although fesselin induced actin growth in the presence of CapZ, higher concentrations were required than in the absence of this blocker.

The previous results indicate that fesselin is able either to displace very high concentrations of agents that block the fast-growing end of actin filaments or to stimulate growth from the

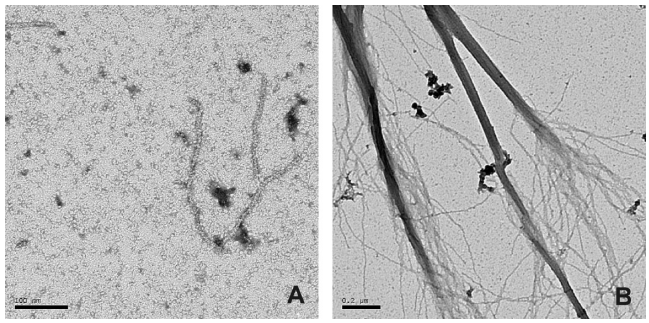

Figure 7. Synaptopodin 2 polymerized actin even in the presence of the barbed end blocker CapZ. (A) Actin polymerization in the presence of CapZ after incubation for 3 min. The concentrations of Mg-G-actin and CapZ were 1.5 and 0.075 μM, respectively. (B) Polymerization of 1.5 μM Mg-G-actin in the presence of 0.075 μM CapZ and 0.075 μM fesselin after incubation for 3 min. The ionic strength of the mixture was 90 mM.

slow-growing, pointed end. The prediction for the latter case is that the critical concentration for filament growth should increase.

The critical concentration of actin was measured at low fesselin concentrations where bundling of actin filaments was not observed. Figure 8 shows critical concentration determinations

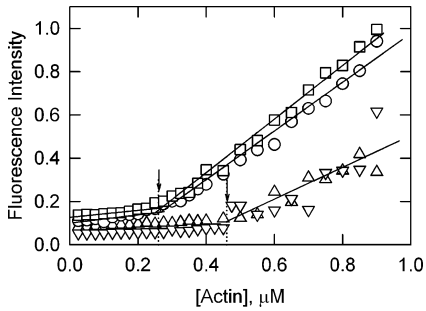

Figure 8. Synaptopodin 2 increases the critical concentration of actin. Two experiments are shown in the absence of fesselin (○ and □) and two in the presence of fesselin (△ and ▽). Varying concentrations of column-purified G-actin, 20% pyrene-labeled, were mixed with avian fesselin, keeping the free fesselin concentration constant at either 0.01 μM (△; θ = 0.018) or 0.007 μM (▽; θ = 0.013). The reaction conditions included 2 mM $MgCl_2$, 100 mM KCl, 1.5 mM imidazole (pH 7.0), 3.3 mM Tris (pH 8.0), 0.066 mM $CaCl_2$, and 1 mM dithiothreitol. The critical concentration for actin alone (○ and □) was 0.26 μM. The critical concentration for actin in presence of fesselin (△ and ▽) was between 0.43 and 0.46 μM.

made with pyrene-labeled actin to monitor actin filament formation. The concentration of fesselin was adjusted at each actin concentration to give a constant free fesselin concentration. The concentration of free fesselin was calculated from the McGhee–von Hippel equation[23] using MLAB (Civilized Software, Bethesda, MD) and the following constants: single-site association constant of 2×10^6 M^{-1}, cooperativity parameter of 1.7, and four actin protomers covered by a single fesselin molecule.[5] Under these conditions, fesselin increased the critical concentration of actin from ~0.26 to ~0.44 μM, a value closer to that for growth at the pointed end.

DISCUSSION

The cellular localization of avian fesselin (synaptopodin 2) in Z-disks and dense bodies together with its stimulating effect on

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actin polymerization suggests that fesselin is part of an actin organization nucleation complex. These results support that idea.

Actin filaments formed in the presence of fesselin had the typical appearance of actin filaments polymerized in the presence of higher concentrations of metal ions. The filaments were unbranched and otherwise unremarkable. Although binding studies show fesselin to be bound along the filaments,[5] it was invisible in the low-resolution electron microscope. Actin filaments formed with fesselin have a strong tendency to form large ordered bundles with a uniform polarity. High concentrations of fesselin tended to produce short thick bundles.

G-Actin requires bound metals for polymerization.[24] The high-affinity site in the actin cleft between subdomains 2 and 4[25] has a K_d of 2.6 nM for Ca^{2+} and a K_d of 7.3 nM for Mg^{2+}. The approximately nine low-affinity sites have K_d values of 0.15 mM for Ca^{2+} and Mg^{2+} and 10 mM for K^+.[26] The high-affinity site affects the nucleation rate (Mg^{2+} is faster than Ca^{2+}), but the low-affinity sites must normally be occupied for G-actin to polymerize to F-actin.[24] Fesselin induced actin polymerization at a total Mg^{2+} concentration (5 μM) far below that required to saturate the low-affinity sites essential for polymerization. The images of filaments formed under these conditions were similar to those of actin polymerized in the presence of 100 mM KCl. Metal ions induce conformational changes in G-actin that make it polymerization competent.[27,28] Fesselin appears to have the same effect on actin. Several mechanisms of this effect are possible such as charge neutralization (fesselin is positively charged at intracellular pH) and a conformational change induced by fesselin binding.

Actin filaments in fesselin-induced bundles had a constant orientation as determined by decoration with S1. One way for this to occur is for actin filaments to grow from a common initiation point. Such ordered growth would also explain the common observation of blunt ends of fesselin–actin bundles. Fesselin tends to aggregate in solution, and it is possible that parallel actin filaments grow from these large complexes. Fesselin was not visible under the conditions presently used for electron microscopy. However, at high fesselin concentrations, we sometimes observed dense aggregates from which parallel actin bundles radiated. These structures are intriguing because of the presence of fesselin in smooth muscle dense bodies and other synaptopodin 2 members in Z-lines of striated muscle. Both structures also contain α-actinin, so it will be interesting to examine actin filaments formed in the presence of both fesselin and α-actinin.

Fesselin-induced actin filament growth appeared to occur primarily from the pointed end of actin filaments. That is, actin growth was not inhibited by barbed end blockers. Furthermore, fesselin increased the critical concentration for polymerization in a manner that suggested that barbed ends of actin filaments were capped. Thus, fesselin has the appearance of a protein that is a component of an actin nucleation center and could therefore be essential for the formation of dense bodies in smooth muscle or their skeletal muscle equivalent, the Z-lines. Figure 4 shows that fesselin–actin complexes sometimes resemble those structures.

Each end of an actin filament has its own critical concentration, and the observed value measured at equilibrium is the average of that from the pointed and barbed ends. The critical concentration for the pointed end is generally ~0.6 μM,[29] while that at the barbed end is ~0.1 μM. The effect of totally blocking growth at barbed ends of actin filaments is to increase the observed equilibrium critical concentration to a value equal to that for the pointed end alone. Figure 8 shows that fesselin nearly doubled the critical concentration of actin, indicating preferential growth at the pointed end.

We previously were unable to observe a change in the critical concentration of actin in response to fesselin.[10] Our earlier critical concentration measurements were determined with higher fesselin concentrations where actin bundling occurred. We now used much lower fesselin concentrations and maintained a constant free fesselin concentration within each series of measurements. Furthermore, we now increased the time of equilibration. These changes resulted in a more reliable determination of the critical concentration.

Figure 9 shows our tentative view of fesselin stimulation of actin filament and bundle formation. Fesselin is intrinsically

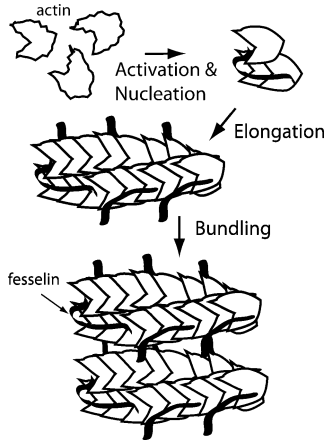

Figure 9. Hypothetical model for fesselin binding, polymerization, and bundling of actin. Actin monomers are drawn with barbed and pointed ends to illustrate the asymmetry of the two ends of actin filaments. Fesselin binds to approximately four actin protomers; nucleation is shown as a complex of fesselin with three actin monomers. Actin and actin–fesselin bundles add rapidly to nuclei to form filaments. These filaments bundle, perhaps as a result of fesselin spanning parallel filaments.

disordered when free in solution but may fold when bound to a target ligand.[30] A single molecule of fesselin binds to approximately four actin monomers.[5] The nucleus is shown with a 1:3 binding stoichiometry as this is generally considered to be the nucleus size. The G-actin is initially shown in a polymerization incompetent state such as that which exists in the absence of divalent metals. Fesselin induces a change similar to that caused by metal binding possibly by neutralizing charges or by altering actin structure through the energy of binding. Fesselin-initiated nucleation is inhibited by calmodulin bound to calcium.[11] The ability of fesselin to stimulate filament growth in the presence of barbed end blockers suggests that fesselin promotes growth from the pointed end and may itself block the barbed end. Blocking the barbed end is consistent with critical concentration measurements. Actin monomers rapidly add to the pointed end of actin filaments. At sufficiently high fesselin concentrations, the entire length of actin may be covered by fesselin. Because the actin–fesselin bundle is able to rapidly bind to actin nuclei, the binding of fesselin to existing filaments may also occur in a way that leaves the barbed end free. The region of fesselin that normally binds to the barbed end of actin might then associate elsewhere such as with an adjacent actin–fesselin unit, with actin on a parallel filament, or with a fesselin molecule on a parallel actin filament. We have not found experimental evidence of these various types of association. However, because fesselin is

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intrinsically disordered, it is able to bind to several different ligand proteins.[2] As a result, fesselin may have multiple ways of binding to actin. Actin bundle formation occurs after both polymerization and the lateral binding of fesselin to actin.

There are several possible reasons for the actin bundle formation observed. Actin bundle formation can result from tethering by proteins that have multiple sites such as gelsolin[31] or those that bundle actin by virtue of formation of dimers such as is the case for α-actinin.[32] Linnemann et al. found evidence of the presence of several actin binding sites in human myopodin,[33] a close relative of fesselin. The region containing residues 269−521 can dimerize under nonreducing conditions and bundle actin.[33] Therefore, bundling by binding of fesselin to parallel actin filaments could occur. Basic actin binding proteins may promote bundling by charge neutralization.[34] Fesselin is basic with a pI of 9.3,[5] so this mechanism is also possible. This is an interesting possibility because actin bundles formed with fesselin (i.e., Figure 2D) resemble those found with polylysine.[35]

The results shown here together with the localization of avian fesselin (synaptopodin 2) in Z-disks and dense bodies and its stimulating effect on actin polymerization suggest that fesselin is part of an actin organization nucleation complex.

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Notes

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ABBREVIATIONS

S1, myosin subfragment 1; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pyrene actin, actin modified with *N*-(1-pyrenyl)iodoacetamide.

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