Ca^{2+}−Calmodulin Regulates Fesselin-Induced Actin Polymerization[†,‡]

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ABSTRACT: Fesselin is a proline-rich actin-binding protein that was isolated from avian smooth muscle. Fesselin bundles actin and accelerates actin polymerization by facilitating nucleation. We now show that this polymerization of actin can be regulated by Ca^{2+}−calmodulin. Fesselin was shown to bind to immobilized calmodulin in the presence of Ca^{2+}. The fesselin−calmodulin interaction was confirmed by a Ca^{2+}-dependent increase in 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) fluorescence upon addition of fesselin to MIANS-labeled wheat germ calmodulin. The affinity was estimated to be $\sim 10^9$ M^{-1}. The affinity of Ca^{2+}−calmodulin to the fesselin F-actin complex was approximately 10^8 M^{-1}. Calmodulin binding to fesselin appeared to be functionally significant. In the presence of fesselin and calmodulin, the polymerization of actin was Ca^{2+}-dependent. Ca^{2+}-free calmodulin either had no effect or enhanced the ability of fesselin to accelerate actin polymerization. Ca^{2+}−calmodulin not only reversed the stimulatory effect of fesselin but reduced the rate of polymerization below that observed in the absence of fesselin. While Ca^{2+}−calmodulin had a large effect on the interaction of fesselin with G-actin, the effect on F-actin was small. Neither the binding of fesselin to F-actin nor the subsequent bundling of F-actin was greatly affected by Ca^{2+}−calmodulin. Fesselin may function as an actin-polymerizing factor that is regulated by Ca^{2+} levels.

Actin is a widely distributed protein that has many cellular functions. The polymerized form of actin, F-actin, participates in myosin-mediated motility and is an important component in the cytoskeleton of eukaryotic cells. Polymerized actin can exist as sheets, bundles, or cross-linked networks (*1*). The transition from monomeric G-actin to F-actin can itself produce amoeboid-type locomotion. Because of the diverse and critical role of actin in cells, the various transformations of actin are regulated by a large number of accessory proteins (*1*). Fesselin may be another of these actin accessory proteins.

Fesselin appears to be structurally similar to the murine and human proteins synaptopodin (*2*) and myopodin (*3*). Fesselin was first isolated by Leinweber et al. (*4*) from avian smooth muscle. Fesselin is proline-rich, and the available sequence has greater homology to myopodin than to synaptopodin. All three proteins have similar high isoelectric points and similar mobilities on SDS−polyacrylamide gels. It is not clear whether fesselin is the avian form of muscle myopodin or whether it is another member of the same protein family.

Fesselin has properties that suggest that it could participate in cellular actin structural changes. Fesselin induces actin bundling (*4*) and stimulates polymerization by increasing the rate of nucleation (*5*). Arp2/3, ENA/VASP, and formins (*6*, *7*) are among the other proteins that nucleate actin polymerization.

The ability of caldesmon (*8*), myelin basic protein (*9*, *10*), and MARCKS[1] (*11*, *12*) to stimulate actin polymerization is regulated by calmodulin. Because of the likelihood that the activities of fesselin are also regulated, we examined the interaction of fesselin with calmodulin. We observed that fesselin binds tightly to calmodulin. The fesselin−calmodulin complex polymerized actin in a Ca^{2+}-dependent manner. Although calmodulin modulated the interaction of fesselin with G-actin, the interaction with F-actin was not greatly affected. Neither the binding of fesselin to actin nor the cross-linking of actin filaments by fesselin were particularly sensitive to Ca^{2+}−calmodulin. These data are further evidence that fesselin plays a role in actin filament structure, dynamics, or both.

MATERIALS AND METHODS

Protein Preparations. Fesselin was purified from turkey gizzards using the method of Leinweber et al. (*4*). The fesselin concentration was determined by the Lowry assay using bovine serum albumin as standard. Recombinant (rat) calmodulin (pT7-7-Cam clone from Dr. Madeline Shea) was expressed in *Escherichia coli* BL21 (DE3:pLysS) cells and

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[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; MARCKS, myristoylated alanine-rich C-kinase substrate; MBP, myelin basic protein; pyrene, *N*-(1-pyrene)iodoacetamide; $calmodulin_{rec}$, recombinant rat calmodulin.

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prepared as described by Pedigo and Shea (*13*). Wheat germ calmodulin was prepared according to Anderson (*14*). The single thiol residue of wheat germ calmodulin was modified with MIANS according to Mills et al. (*15*). Rabbit muscle actin was purified using the method of Spudich and Watt (*16*) with modifications (*17*). Actin was modified with pyrene iodoacetamide at pH 7.6 by the method of Brenner and Korn (*18*) and Kouyama and Mihashi (*19*). The reaction was stopped with dithiothreitol and the solution was centrifuged in a 50Ti rotor for 20 min at 30 000 rpm to remove insoluble probe. The supernatant was centrifuged at 45 000 rpm for 1 h. The actin pellet was swollen in buffer, homogenized, and dialyzed with several changes.

Binding of Fesselin to Calmodulin. Binding was measured using affinity chromatography and fluorescence changes. Calmodulin was coupled to Affi-Gel 10 (BioRad) according to the manufacturer's directions using a buffer system containing 100 mM MOPS (pH 7.5) and 80 mM $CaCl_2$. Fesselin was loaded onto the affinity column in a buffer containing 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 2 mM dithiothreitol and washed extensively with the same buffer. Fesselin was eluted with a buffer containing 30 mM Tris-MES (pH 7.0), 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 2 mM dithiothreitol.

Equilibrium binding of fesselin to MIANS-labeled wheat germ calmodulin was measured with a Spex 2 spectrofluorometer with 1.25 mm slits, excitation at 320 nm, and emission measured over the range 360−500 nm. Binding was measured at 25 °C in a thermostated cell with constant stirring. The solution composition is given in the figure legends. The fluorescence of the MIANS probe has been shown to be related to the fraction of calmodulin bound to ligand (*20*). The fraction of calmodulin with bound fesselin at some concentration i of fesselin was calculated from $(F_{max} - F_i)/(F_{max} - F_{min})$, where F_{max}, F_i, and F_{min} are values of fluorescence at saturating fesselin concentrations, at a fesselin concentration of i M and in the absence of fesselin, respectively. The concentration of bound fesselin was equal to the product of fraction of bound calmodulin, total calmodulin concentration, and the ratio of fesselin to calmodulin in the fully bound complex. The free fesselin concentration was determined from the conservation of mass. To obtain all positive values of the free fesselin concentration, it was necessary to assume that each molecule of fesselin could bind to two molecules of calmodulin.

Pre-steady-state binding was measured using a DX17.MV/2 sequential stopped-flow spectrofluorometer (Applied Photophysics, Leatherhead, U.K.). The excitation wavelength was set at 320 nm with a monochromator using either 0.5 or 1.0 mm slits. Emission was measured through a Schott GG 395 long pass filter. All measurements were made at 15 °C. Protein concentrations given in figure legends are the initial concentrations in the reaction cell at the instant after mixing in the stopped-flow apparatus.

Binding of Fesselin to G-Actin. Binding to G-actin was determined by affinity chromatography. Actin was coupled to Affi-Gel 10 according to Miller et al. (*21*) except that the gel filtration resin was omitted. G-actin affinity columns were used on the day of preparation. Fesselin and calmodulin were loaded together in a buffer containing 130 mM KCl, 50 mM HEPES−KOH (pH 7.5), 10% glycerol, 0.1% protease inhibitor cocktail (SIGMA), 2 mM dithiothreitol, and either 0.5 mM $CaCl_2$ or 1 mM EGTA. Columns were washed with the same buffer. Column fractions were electrophoresed on SDS−polyacrylamide gels and stained with Coomassie blue.

Binding of Fesselin to F-Actin. Binding to F-actin was measured by changes in fluorescence and by a sedimentation assay. Binding to pyrene-labeled F-actin was measured in the stopped-flow device with excitation at 341 nm and emission measured through an Oriel 366 nm high pass filter (no. 51265). One micromolar pyrene−actin and varying amounts of calmodulin were mixed with 0.75 μM fesselin at 25 °C in a buffer containing 100 mM potassium propionate, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and 1 mM dithiothreitol.

Measurement of F-actin-binding by a high-speed sedimentation assay was performed as described by Leinweber et al. (*4*). The assay was performed with Ca^{2+}−calmodulin in a buffer containing 80 mM potassium propionate, 8 mM imidazole (pH 7.0), 1 mM Tris-HCl, 10 mM NaCl, 1 mM dithiothreitol, and either 0.2 mM $CaCl_2$ or 1.8 mM $MgCl_2$, 0.05 mM EDTA, and 0.05 mM EGTA. Five micromolar F-actin was incubated for 1 h on ice with or without 1 μM fesselin and with calmodulin ranging from 0 to 25 μM. The samples were centrifuged for 1 h at 45 000 rpm in a type 50Ti rotor at 4 °C. The sedimented proteins were dissolved in protein loading buffer (National Diagnostics), and 10% of each sample was loaded on 12% SDS−polyacrylamide gels for electrophoresis.

Actin Bundling. Bundling was examined by low-speed sedimentation of the bundled actin complex (*22*) using 10 μM actin. The amount of actin that sedimented at low speed was determined by SDS−polyacrylamide gel electrophoresis.

The Rate of Actin Polymerization. The polymerization rate was measured by the time course of the increase in fluorescence of pyrene-labeled actin as described earlier (*23*). Pyrene-labeled actin was depolymerized by dialysis against G-actin buffer according to Pardee and Spudich (*24*) (0.2 mM ATP, 2 mM Tris (pH 8.0), 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.005% NaN_3) for 3−4 days with fresh buffer each day. The actin was centrifuged in a type 50Ti rotor at 45 000 rpm for 1 h to remove filamentous actin. The concentration of G-actin was determined by a Lowry assay or by absorbance at 290 nm using an extinction coefficient of 0.638 M^{-1} cm^{-1} measured against buffer containing ATP.

The Mg^{2+}-bound form of G-actin was used in all polymerization assays. G-actin was made 125 μM in EGTA and 50 μM in $MgCl_2$ at least 5 min before the initiation of the assay to exchange Ca^{2+} in the binding site with Mg^{2+} (*25*). Polymerization was initiated by adding 0.04 mL of 50 mM $MgCl_2$ together with a variable amount of fesselin to a solution containing 3.6 μM pyrene actin, variable amounts of calmodulin, 15 mM NaCl, 0.1 mM ATP, 0.05 mM $CaCl_2$, 2.5 mM Tris-HCl (pH 7.8), and 1 mM imidazole (pH 7.0). The final volume was 2.0 mL. The pCa was 4.3 in the experiments with Ca^{2+}−calmodulin and 7.8 in the low-Ca^{2+} experiments. The solution was stirred constantly during the reaction. Fluorescence was measured on a Fluorolog 2 spectrofluorometer (Spex) at 25 °C with excitation at 365 nm and emission at 407 nm using 0.5 mm slits.

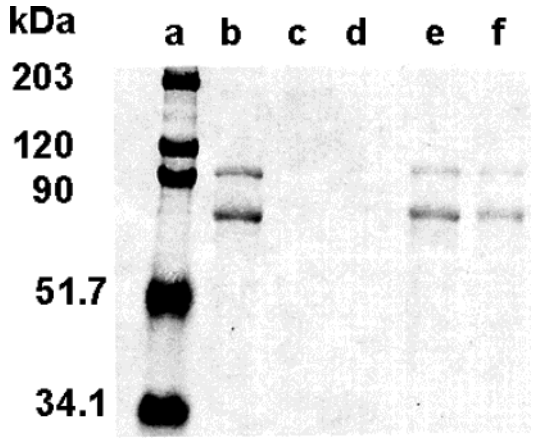

FIGURE 1: Binding of fesselin to immobilized calmodulin$_{rec}$. The Coomassie-stained SDS−polyacrylamide 12% gel electrophoresis shows that fesselin bound to immobilized calmodulin in 25 mM Tris (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM dithiothreitol. The column was washed with the same buffer, and fesselin was eluted with 30 mM Tris-MES (pH 7.0), 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol. Molecular weight standards are in lane a, original fesselin in lane b, flow through in lane c, wash in lane d, and elution in lanes e and f.

RESULTS

Ca^{2+}−Calmodulin Binds to Fesselin. Fesselin was found to bind to calmodulin that was coupled to Affi-Gel 10. When 0.12 mg of fesselin was loaded onto a 0.2 mL calmodulin affinity column, virtually all of the fesselin remained attached to the column even with extensive washing. Fesselin was eluted with a similar buffer in which Ca^{2+} was replaced with 1 mM EGTA. The eluted fesselin consisted of two bands corresponding to the 79 and 103 kDa polypeptides. There was no change in the distribution of the two bands following affinity chromatography (Figure 1). We observed that some fesselin remained bound to the column following a wash with a Ca^{2+}-free buffer and a further wash with the same buffer plus 4 M urea. The residual fesselin could be removed by boiling the resin with the SDS gel loading buffer.

The fesselin−calmodulin interaction was confirmed by fluorescence spectroscopy. Figure 2A shows that mixing fesselin with MIANS-labeled calmodulin produced a rapid increase in fluorescence. This change was not observed with mixing unlabeled calmodulin with fesselin nor with mixing MIANS−calmodulin with buffer. The fluorescence change seen in Figure 2A was dependent on Ca^{2+}; no signal was observed when Ca^{2+} was replaced with EGTA. Thus the MIANS probe reflected an event that was associated with the fesselin−calmodulin interaction. The smooth curve through the data is a biexponential fit of the data. The binding curves were in general poorly described by a single-exponential function under pseudo-first-order conditions. Thus the interaction may be described by a model having at least two steps.

Figure 2B shows a plot of the sum of the observed apparent rate constants against the concentration of free fesselin and calmodulin at equilibrium. The slope gives an estimate of the second-order rate constant of binding of 10^8 $M^{-1}s^{-1}$. That is, the first step of the binding reaction appears to be diffusion-limited.

The fluorescence change associated with binding of fesselin to MIANS−calmodulin was reversible. The lower

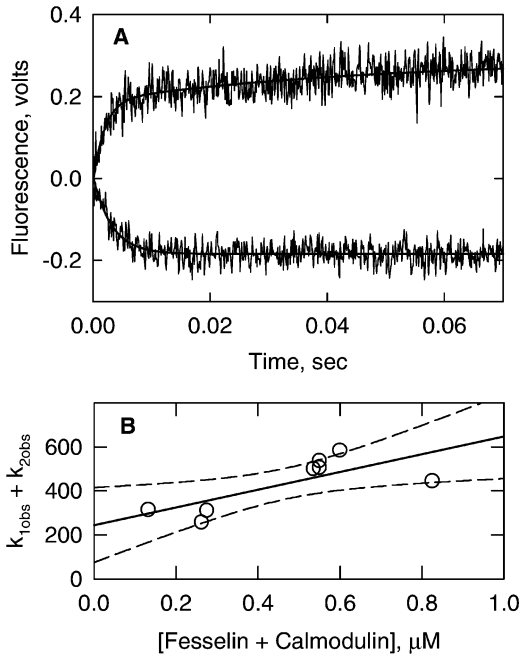

FIGURE 2: Kinetics of fesselin binding to MIANS-labeled wheat germ calmodulin. Binding was measured on a stopped-flow fluorometer at 15 °C in a buffer containing 200 mM KCl, 10 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 0.5 mM $CaCl_2$. Panel A shows examples of binding (upper curve) and dissociation (lower curve). Binding was measured with 0.5 μM fesselin and 0.05 μM MIANS−calmodulin. The smooth curve is a biexponential fit to the data with observed rate constants $k_{1_{obs}} = 516$ and $k_{2_{obs}} = 22$ s^{-1}. Dissociation was measured by mixing the fesselin−MIANS−calmodulin complex with an excess of unlabeled calmodulin. The final protein concentrations were 0.5 μM fesselin, 0.05 μM MIANS−calmodulin, and 5 μM unlabeled wheat germ calmodulin. Panel B shows the sum of the two observed rate constants for the binding reaction as a function of the total protein concentration. The slope gives the second-order rate of the reaction (approximately 10^8 M^{-1} s^{-1}).

curve in Figure 2A shows the dissociation of MIANS−calmodulin from the MIANS−calmodulin−fesselin complex. The signal was reversed with a half-life of about 30 ms. The fluorescence change was also reversed upon rapidly sequestering the Ca^{2+} with a large excess of EGTA (data not shown). This Ca^{2+}-dependent change occurred with a half-life of 35 ms.

Equilibrium fluorescence measurements were made to determine the amplitude of the fluorescence change or extent of binding as a function of the fesselin concentration. Figure 3A shows that the fluorescence amplitude increased with the amount of added fesselin and the increment of the fluorescence change decreased with increasing fesselin concentrations. The maximum signal at saturating fesselin was about 2.3 times that of the original fluorescence. Figure 3B shows a plot of the fraction of calmodulin with bound fesselin against the free fesselin concentration at ~105 mM ionic strength. The solid line is a theoretical curve for an association constant of 1.2 nM with two molecules of calmodulin bound per fesselin. It was not possible to fit the data with the assumption of a 1:1 stoichiometry of fesselin to calmodulin.

Calmodulin Modulates the Actin Polymerization Activity of Fesselin and Binding to G-Actin. We determined earlier that fesselin accelerates the rate of actin polymerization and bundles actin filaments. These activities were used as the

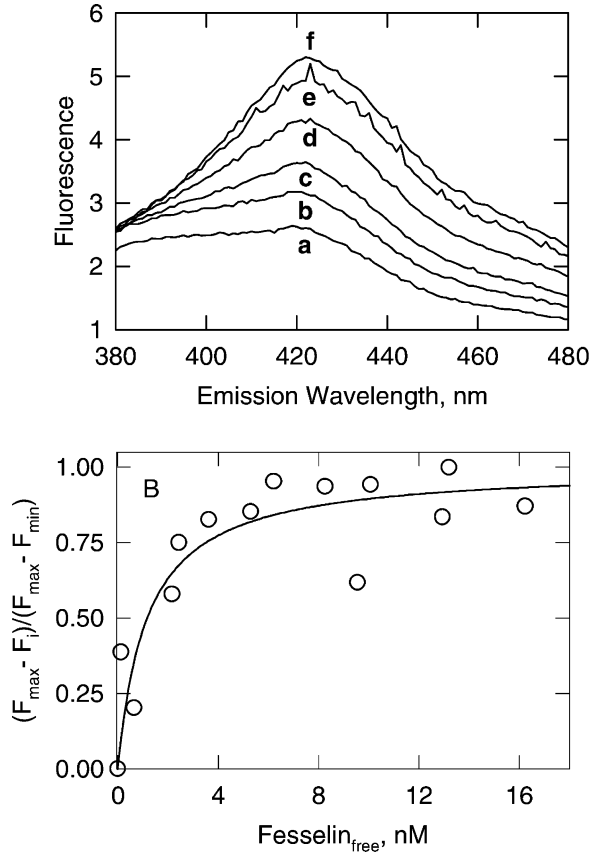

FIGURE 3: Panel A shows the emission spectrum of 50 nM MIANS-labeled calmodulin in the absence of fesselin (a) and at fesselin concentrations of 5.7 (b) 11.3 (c), 25 (d), 50 (e), and 100 nM (f). Panel B shows the fraction of calmodulin with bound fesselin as a function of the free fesselin concentration in the presence of either 20 or 50 nM total MIANS-labeled calmodulin at 25 °C. F_{max}, F_{min}, and F_i are values of fluorescence in the presence of saturating fesselin, no fesselin, and intermediate concentrations of fesselin, respectively. Binding was measured in a buffer containing 100 mM potassium propionate, 10 mM imidazole-HCl (pH 7.0), 0.2 mM $CaCl_2$, and 1 mM dithiothreitol. The solid line is a theoretical curve for an association constant of 1.2 nM with two molecules of calmodulin bound per fesselin.

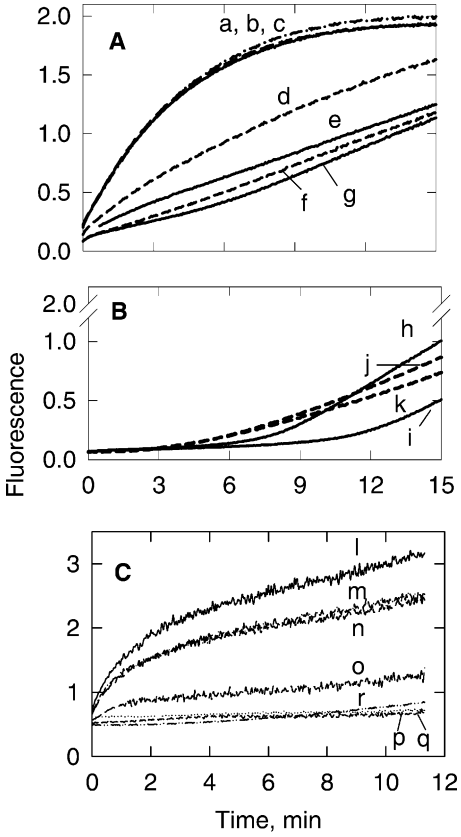

FIGURE 4: Fesselin-induced pyrene−actin polymerization at various Ca^{2+}−calmodulin concentrations measured by fluorescence changes at 25 °C. Panels A and B show polymerization of 3.6 μM pyrene-labeled skeletal muscle actin by 0.19 μM fesselin using increasing concentrations of Ca^{2+}−$calmodulin_{rec}$. Time courses (curves a and b) without calmodulin and with 0.04 (c), 0.12 (d), 0.19 (e), 0.27 (f), 0.41 (g), 0.94 (h), and 1.9 μM $calmodulin_{rec}$ (i) are presented. Controls with neither fesselin nor calmodulin are shown as dashed lines (j and k). Panel C shows polymerization of 3.5 μM pyrene-labeled skeletal muscle actin by 0.14 μM fesselin with increasing concentrations of MIANS-labeled wheat germ calmodulin. Time courses (l) without calmodulin and with 0.06 (m), 0.03 (n), 0.24 (o), 0.98 (p), and 1.0 μM wheat germ calmodulin (q) are presented. Curve r is a control with neither fesselin nor calmodulin. Buffer included 2.5 mM Tris-HCl (pH 7.8), 1 mM imidazole (pH 7.0), 15 mM NaCl, 0.1 mM ATP, 1 mM dithiothreitol, 0.05 mM $CaCl_2$ and 1 mM $MgCl_2$.

basis for examining the functional consequences of calmodulin binding.

Figure 4A,B shows a series of polymerization time courses with varied concentrations of calmodulin and with two preparations of fesselin. In the absence of fesselin, the polymerization of 3.6 μM G-actin occurred slowly with a noticeable lag phase (curves k and j). Addition of 0.19 μM fesselin produced a large enhancement of the polymerization rate with elimination of the lag (curves a and b). Increasing concentrations of Ca^{2+}−calmodulin reversed the accelerating effect of fesselin (curves a−g). At concentrations of calmodulin >0.94 μM, the lag phase of polymerization was more pronounced than in the absence of fesselin (curves h and i). MIANS-labeled wheat germ calmodulin was also active in regulating the polymerization of actin by fesselin (Figure 4C). At a concentration of 1 μM MIANS-labeled wheat germ Ca^{2+}−calmodulin, the addition of fesselin had no effect on the rate of actin polymerization.

Figure 5 shows that the effects of Ca^{2+}−calmodulin on fesselin-induced actin polymerization are complex. The actin polymerization curve in the presence of fesselin with Ca^{2+}−calmodulin (b) showed the same type of lag seen in the absence of fesselin or calmodulin (a), but the elongation phase seemed to be slightly faster. In contrast to this, the addition of Ca^{2+}-free calmodulin (d) often had a positive effect on the fesselin-induced actin polymerization (compare to curve c with fesselin and Ca^{2+} but no calmodulin). Calmodulin increased the range of polymerization rates so that in the presence of Ca^{2+} the rate was almost as slow as in the absence of fesselin while in the absence of Ca^{2+} the rate was generally greater than in the presence of fesselin and no calmodulin.

The reversal of fesselin-induced polymerization by Ca^{2+}−calmodulin could result from a decrease in the affinity of fesselin for G-actin, from a change in conformation of the fesselin−actin complex, or from a combination of effects. We studied the effect of calmodulin on fesselin binding to G-actin as an approach to this question. Figure 6 shows that when fesselin and calmodulin were added together to an immobilized G-actin column less fesselin was retained in the presence of Ca^{2+}. Lanes b and b′ show the effluent prior to loading fesselin and calmodulin. A small amount of actin

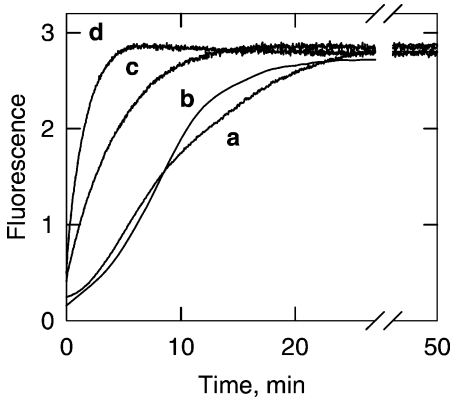

FIGURE 5: Effect of Ca^{2+} on the polymerization of actin in the presence of fesselin and calmodulin. Five micromolar pyrene-labeled Mg^{2+}−G-actin was polymerized in the absence of both fesselin and calmodulin (a), in the presence of 0.5 μM fesselin, 1 μM MIANS-labeled wheat germ calmodulin, and 0.05 mM $CaCl_2$ (b), 0.5 μM fesselin and $CaCl_2$ (c), and 0.5 μM fesselin with 1 μM MIANS−calmodulin and no $CaCl_2$ (d). Conditions for the time courses (a−c) were 2.5 mM Tris-HCl (pH 7.8), 1 mM imidazole (pH 7.0), 15 mM NaCl, 0.1 mM ATP, 1 mM dithiothreitol, 0.05 mM $CaCl_2$, and 1 mM $MgCl_2$. In time course d, Ca^{2+} was replaced with 0.5 mM EGTA.

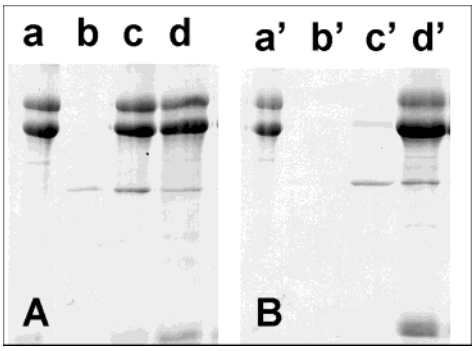

FIGURE 6: Fesselin binding to an Affi-Gel 10-G−actin affinity column. Fesselin was loaded onto the column together with 1 μM calmodulin in the presence of either Ca^{2+} (panel A) or EGTA (panel B) and washed with the same buffer. Coomassie blue-stained 12% SDS polyacrylamide gels are shown of a fesselin standard (lanes a and a′), the effluent prior to loading fesselin and calmodulin (lanes b and b′), the initial fractions after loading (lanes c and c′), and the first wash with buffer (lanes d and d′). Conditions were 50 mM HEPES−KOH (pH 7.5), 130 mM KCl, 10% glycerol, 0.1% protease inhibitor cocktail, 2 mM dithiothreitol, and either 0.5 mM $CaCl_2$ (panel A) or 1 mM EGTA (panel B).

was present in this and subsequent fractions. Lanes c and c′ show the fesselin and calmodulin that were present in the initial fraction collected after loading these proteins. Less fesselin was retained on the column in the presence of Ca^{2+} (more fesselin is present in the flow-through of lane c than in c′). With continued washing of the column, additional fesselin was eluted in both the presence and absence of Ca^{2+}.

Calmodulin Has Little Effect on the Interaction of Fesselin with F-Actin. The experiments reviewed above show that fesselin binds to G-actin and that this interaction is weakened by Ca^{2+}−calmodulin. Fesselin also binds to F-actin and induces the formation of actin bundles. We explored the possibility that the interaction of fesselin with F-actin is also modulated by Ca^{2+}−calmodulin.

The time course of binding of 0.38 μM fesselin to 0.5 μM pyrene-labeled F-actin was followed by the change in pyrene actin fluorescence (Figure 7A). Curve c shows the

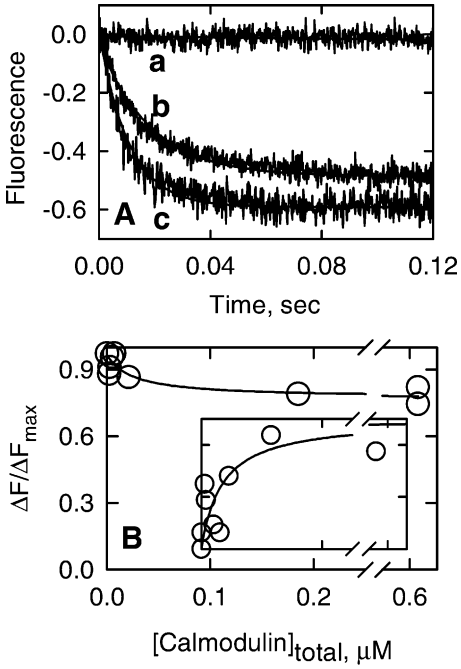

FIGURE 7: Effect of Ca^{2+}−$calmodulin_{rec}$ on the rate of binding of fesselin to pyrene-labeled F-actin at 25 °C. Panel A shows time courses of the pyrene fluorescence change for the following conditions: 0.5 μM actin and 0.62 μM recombinant calmodulin in the absence of fesselin (a); 0.5 μM actin, 0.38 μM fesselin, and 0.62 μM $calmodulin_{rec}$ (b); 0.5 μM actin, 0.38 μM fesselin, and 0.003 μM $calmodulin_{rec}$ (c). Buffer contained 100 mM potassium propionate, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and 1 mM dithiothreitol. Panel B shows the change in fluorescence amplitude as a function of the total $calmodulin_{rec}$ concentration. The inset shows $\Delta F_{max}/\Delta F$ (to give the form of a binding curve) as a function of the free $calmodulin_{rec}$ concentration assuming a 1:1 binding of calmodulin to fesselin. The solid line is the theoretical curve for binding of Ca^{2+}−$calmodulin_{rec}$−fesselin to F-actin with an association constant of 1.4×10^8 M^{-1}.

time course for binding in the presence of 2.5 nM calmodulin. At that concentration, the time course was identical to that in the absence of calmodulin (not shown). In the presence of 620 nM calmodulin, curve b, there was a slight decrease in amplitude but no change in the rate of binding. At saturating amounts of calmodulin, the amplitude was reduced by only 20%. High concentrations of calmodulin (620 nM in curve a) did not affect F-actin fluorescence in the absence of fesselin. The magnitude of the effect of Ca^{2+}−calmodulin on the binding of fesselin to F-actin can be seen more clearly in Figure 7B where the difference in fluorescence amplitude is plotted as a function of the total calmodulin concentration.

The inset to Figure 7B shows the fractional change in fluorescence amplitude as a function of the free calmodulin concentration. This curve can be interpreted as the binding of calmodulin to a stable fesselin−F-actin complex. The solid line in the inset to Figure 7B is a theoretical curve for binding of Ca^{2+}−calmodulin to fesselin−F-actin with an affinity of 1.4×10^8 M^{-1}. An alternative explanation of the fluorescence change is that there are two populations of fesselin with 20% detaching from F-actin upon binding to Ca^{2+}−calmodulin. This possibility is less likely since Ca^{2+} did not appear to cause significant detachment of fesselin from F-actin in other experiments as described below.

The effect of calmodulin on the ability of fesselin to bundle F-actin is shown in Figure 8 (lanes 1−7). Bundling was

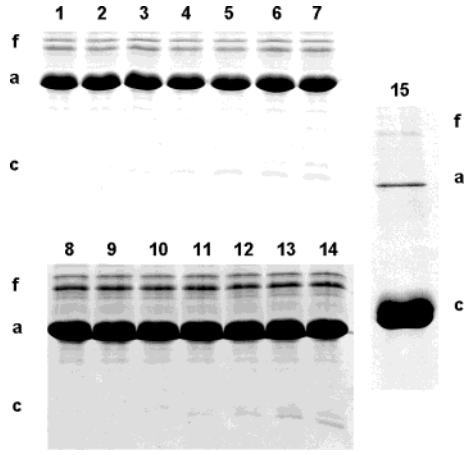

FIGURE 8: The binding of fesselin to F-actin is independent of the Ca^{2+}−calmodulin concentration. Either 5 or 10 μM F-actin and 1 μM fesselin were mixed with varied calmodulin concentrations in a buffer containing 1 mM Tris-HCl (pH 7.8), 8 mM imidazole (pH 7.0), 80 mM potassium propionate, 10 mM NaCl, 1 mM dithiothreitol, and 0.2 mM $CaCl_2$. Each sample was centrifuged at either a low or high centrifugal force, and the pellets were analyzed on 12% SDS polyacrylamide gels to determine the amount of bound fesselin. Lanes 1−7 show the low centrifugal force study with calmodulin concentrations of 0, 1, 5, 10, 15, 20, and 25 μM and 10 μM actin. Lanes 8−14 show the high force study with calmodulin conentrations of 0, 1, 5, 10, 15, 20, and 25 μM and 5 μM actin. Lane 15 shows the supernatant from the actin-binding assay corresponding to 5 μM F-actin, 1 μM fesselin, and 25 μM Ca^{2+}−calmodulin. The positions of fesselin, actin, and calmodulin are indicated by f, a, and c, respectively.

defined by the ability of fesselin to form complexes that sedimented under low radial forces and low times of centrifugation. Lanes 1−7 show the pellets formed after centrifuging 10 μM F-actin, 1 μM fesselin, and 0−25 μM Ca^{2+}−calmodulin. Even the highest concentration of calmodulin did not lessen the bundling of F-actin by fesselin as seen by the constant actin (a) band. The amount of fesselin (f) in the pellet was also constant.

Lanes 8−14 show the pellets with calmodulin concentrations increasing from 0 to 25 μM following sedimentation at a force that would sediment 5 μM F-actin whether or not it was bundled. The amount of fesselin in the high-speed actin pellet did not change even at the highest calmodulin concentration. Lane 15 shows the supernatant corresponding to 25 μM Ca^{2+}−calmodulin. This means that Ca^{2+}−calmodulin failed to reverse either the bundling of F-actin or the binding of fesselin to F-actin. Calmodulin was observed in the pellets along with fesselin and actin when the added calmodulin concentration was high. This suggests the possibility that Ca^{2+}−calmodulin was bound to the complex of fesselin and F-actin. Note the large amount of calmodulin and the paucity of actin and fesselin in the supernatant.

DISCUSSION

We observed fesselin binding to Ca^{2+}−calmodulin by affinity chromatography, fluorescence spectroscopy, and effects on fesselin activity. Binding to calmodulin affinity columns was tight with a fraction being eluted with EGTA while the remainder was resistant to 4 M urea and was only eluted by boiling in SDS buffer. Binding of fesselin to MIANS−calmodulin was similar to that of other calmodulin binding proteins. The fluorescence of the MIANS probe increased 2.3 times upon binding to fesselin. This is in the range of the 2-fold change reported for caldesmon and the 4.6-fold change reported for MLCK (*15*). The fluorescence change was dependent on Ca^{2+} and was reversible. The second-order rate constant of binding (10^8 M^{-1} s^{-1}) is probably diffusion-limited. The rate of reversal of the fluorescence change was rapid (300 s^{-1}).

The affinity of fesselin for MIANS−calmodulin was ~1.2 nM at 105 mM ionic strength assuming that each fesselin bound to two molecules of Ca^{2+}−calmodulin. The data could not be fitted with the assumption of a 1:1 association. At present, we have not been able to verify the stoichiometry of binding, so the value of the affinity must be considered to be an estimate. We were also able to obtain an estimate of the affinity Ca^{2+}−calmodulin for fesselin bound to pyrene-labeled actin. Figure 7 shows the change in pyrene fluorescence when $calmodulin_{rec}$ was added to a complex of fesselin and pyrene-labeled actin. The fluorescence change could not be attributed to displacement of fesselin from F-actin (Figure 8, lanes 8−14). Therefore, the fluorescence change was probably due to the binding of Ca^{2+}−calmodulin to the fesselin−F-actin complex. If that was the case then Ca^{2+}−calmodulin bound to fesselin−F-actin with about 1/10th the affinity as to fesselin alone with an association constant of approximately 1.4×10^8 M^{-1} or to 7 nM.

Proteins that are regulated by Ca^{2+}−calmodulin usually have dissociation constants in the high picomolar to low nanomolar range (*26*). For example, myosin light chain kinase has a K_d of 9 nM (*15*). On the other hand, caldesmon has a K_d of 250 nM (*15*), and there is uncertainty about the role of calmodulin in the regulation of caldesmon activity. Therefore the association of Ca^{2+}−calmodulin with fesselin is typical of other calmodulin binding proteins.

Calmodulin binding altered the interaction of fesselin with G-actin. Ca^{2+}−calmodulin reduced the rate of fesselin-dependent actin polymerization in a concentration-dependent manner. This inhibition could have been the result of a decreased affinity of calmodulin−fesselin to actin, of a decreased rate of nucleation or elongation from the Ca^{2+}−calmodulin−fesselin−G-actin complex, of a decreased rate of elongation of growing Ca^{2+}−calmodulin−fesselin−F-actin filaments, or of a combination of effects. It seems certain that at least part of the effect is due to competitive binding. We do know from affinity chromatography (Figure 6) that Ca^{2+}−calmodulin decreased the affinity of fesselin for G-actin. It is likely, from consideration of detailed balance, that the affinity of Ca^{2+}−calmodulin for fesselin is weakened when fesselin is bound to F-actin; the effect is probably greater with G-actin.

Some of our data point to additional effects that cannot be explained by a competitive binding mechanism. In the presence of fesselin, high concentrations of calmodulin produced a lag in binding that exceeded those observed with pure actin (absence of fesselin). The addition of Ca^{2+}−calmodulin to G-actin, in the absence of fesselin, did not reduce the rate of polymerization. Therefore, Ca^{2+}−calmodulin−fesselin must interact to some extent with G-actin and decrease the reactivity of actin to which it is bound. Furthermore, despite the exaggerated lag, the rate of elongation appeared to be greater than that with pure actin. Our

prediction is that the Ca^{2+}−calmodulin−fesselin complex binds to actin and adds to growing actin filaments but is unable to nucleate polymerization.

In the absence of Ca^{2+}, the addition of calmodulin usually accelerated the rate of polymerization by fesselin. Because calmodulin had no direct effect on the polymerization of actin in the absence of fesselin, it is likely that calmodulin binds to fesselin in the absence of Ca^{2+}. This was substantiated by the partial elution of fesselin from a G-actin affinity column by Ca^{2+}-free calmodulin (data not shown). Calmodulin has both positive and negative effects on the rate of fesselin-induced actin polymerization. Ca^{2+}−calmodulin may act as not only a switch but also an amplifier of the effects of fesselin.

The results of polymerization studies gave an indirect indication that calmodulin−fesselin binds to G-actin in both the presence and absence of Ca^{2+}. Fesselin is not unique in its ability to bind to calmodulin in the absence of Ca^{2+}. For example, myelin basic protein binds to calmodulin in both the presence and absence of Ca^{2+}, although the former is more stable (*27*).

Ca^{2+}−calmodulin has been reported to alter actin polymerization activities of other proteins. Ca^{2+}−calmodulin is inhibitory to the activity of caldesmon (*8*, *28*) and myelin basic protein (*9*), just as we have shown with fesselin. In contrast, the activity of an unidentified protein increased the rate of polymerization in the presence of Ca^{2+}−calmodulin (*29*).

The interactions of several actin-binding proteins with actin are inhibited by Ca^{2+}−calmodulin. In most cases, Ca^{2+}−calmodulin affects interactions with both G- and F-actin. For example, Ca^{2+}−calmodulin inhibits the abilities of caldesmon (*8*), MARCKS (*11*), and MBP (*9*, *30*) to bind to F actin, bundle actin, or polymerize G-actin. Fesselin differs from these proteins in that only interactions with G-actin are inhibited by Ca^{2+}−calmodulin. As noted previously, Ca^{2+}-free calmodulin can stimulate the polymerization of actin by fesselin, so the effects of calmodulin and fesselin on actin polymerization are complex.

Equally complex are the effects of Ca^{2+} on the actin cytoskeleton. Janmey (*31*) pointed out that elevated Ca^{2+} concentrations often have a negative effect on the actin cytoskeleton. Actin cross-linking proteins such as α-actinin and fimbrin are inhibited, and proteins that sever or depolymerize actin fibers such as gelsolin, villin, and scinderin are activated. The Ca^{2+}-dependent severing of actin by gelsolin and villin is thought to increase net actin polymerization by increasing the number of nucleating sites. However, the cellular Ca^{2+} concentration may not be high enough to stimulate actin severing under normal physiological conditions (*32*, *33*). Elevated Ca^{2+} also leads to the formation of puncta (*34*) and formation of stress fibers (*35*). If the role of fesselin is to stimulate actin filament formation then Ca^{2+} may have both positive and negative effects on actin filament formation.

Until now Arp2/3 (*36*), formins (*37*), and ENA/VASP (*38*) were the only known factors capable of initiating cellular actin nucleation. Fesselin can initiate nuclei formation in solution (*5*), and we now show that this activity is regulated by Ca^{2+}−calmodulin. However, the cellular function of fesselin is unknown. Fesselin shares some structural features with the synaptopodin family of proteins. One member of that family, myopodin, binds to cellular actin and induces bundle formation (*3*). The known properties of fesselin and its analogy with myopodin suggest that the function of fesselin is related to the control of cellular actin dynamics and structure.

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