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Biochemistry

Fimbrin is a cytoskeletal protein that crosslinks F-actin *in vitro*

(microfilaments/brush border/microvilli/membrane ruffles)

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ABSTRACT **Fimbrin is a cytoskeletal protein associated with microfilaments in microvilli, microspikes, stereocilia, membrane ruffles, and cell–substratum attachment sites. Fimbrin purified from intestinal epithelial cell brush borders was found to be a monomeric protein of molecular weight 68,000. In a sedimentation assay, fimbrin bound to F-actin in a salt-dependent manner, with binding being optimal in 30 mM KCl and inhibited in >100 mM KCl. In 50 mM KCl, which allows efficient polymerization of actin, the interaction was stabilized by the presence of polyethylene glycol. Under these conditions, binding was unaffected by the inclusion of up to 5 mM Ca^{2+} but was inhibited by >0.5 mM Mg^{2+}. Electron microscopy revealed that fimbrin crosslinked F-actin into relatively straight bundles with shorter bundles being formed at high fimbrin-to-actin ratios. The results suggest that fimbrin crosslinks F-actin in such a way as to confer some rigidity on the bundle formed. This proposed function for fimbrin is consistent with its *in vivo* localization in straight, highly organized, microfilament bundles such as microvilli, microspikes, and stereocilia.**

Microfilaments are ubiquitous structures of nonmuscle cells that perform many motile functions as well as being intimately involved in the three-dimensional organization of the cytoskeleton (for a review, see ref. 1). One approach to understanding microfilament organizations at the molecular level is to isolate defined microfilament arrangements and to determine the specific role of each component. Because the microfilament core bundle of intestinal microvilli can be isolated ultrastructurally intact and in sufficient quantities for biochemical analysis (2), it has been of interest to elucidate the molecular function of its components and to try to relate these findings to microfilament organizations in other nonmuscle cells.

The isolated microvillus core has four major proteins associated with the bundle of F-actin filaments (3–6). Two of these, villin (M_r, 95,000) and fimbrin (M_r, 68,000), have been suggested as internal core proteins and two, the 110,000 polypeptide and calmodulin (M_r, 17,000), as components of the regular array of crossfilaments that link the core laterally to the membrane in the intact microvillus (3). Villin has been shown to be an F-actin crosslinking protein *in vitro* at free calcium levels <0.1 μM; however, at free calcium >1.0 μM, villin restricts F-actin to short polymers (7, 8). Little is known about fimbrin except that it is a major protein of the microvillus cytoskeleton and is present in a wide variety of cells (4). With the use of antibodies to fimbrin in immunofluorescence microscopy, fimbrin was found to be present in all highly organized microfilament bundles so far examined, such as microvilli, microspikes, virus-induced microvilli, and stereocilia, and in membrane ruffles and cell–substratum attachment sites. On the other hand, little association with the stress fiber systems of cultured cells was found.

Here I describe the purification and biochemical characterization of fimbrin isolated from intestinal epithelial cell brush border fragments and show that it crosslinks F-actin *in vitro*. I discuss the role fimbrin may play in the cytoskeleton of the intestinal microvillus and the other structures in which it is known to be present. A summary of this work has appeared (6).

MATERIAL AND METHODS

Purification of Fimbrin. Brush borders were isolated from the small intestines of chickens (2, 7), treated with 5 mM diisopropyl fluorophosphate for 30 min at 4°C, and washed extensively in solution I (75 mM KCl/0.1 mM $MgCl_2$/1 mM EGTA/10 mM imidazole·HCl, pH 7.3). Throughout the purification all solutions contained 0.5 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. Fimbrin was extracted by adding 5 mM $CaCl_2$ to brush borders in solution I and stirring the suspension on ice for 15 min. The extract was clarified by centrifugation and made 40% in ammonium sulfate. The insoluble material was removed, and the soluble fraction was made 65% in ammonium sulfate. Fimbrin was contained in the harvested 65% ammonium sulfate pellet which was then dissolved in a small volume of solution C (0.2 M KCl/1 mM CaCl/10 mM Tris·HCl, pH 7.2). This solution was clarified by centrifugation and applied to an affinity column to which pancreatic DNase I was covalently bound. As described (7), actin and villin bind tightly to DNase I in the presence of Ca^{2+}, so the unbound material eluted with buffer C was highly enriched in fimbrin. Eluant fractions were monitored for protein by A_{280}; those containing protein were pooled and dialyzed overnight against 50 mM NaCl/10 mM imidazole, pH 6.8. After dialysis, the material was applied to a DE-52 ion exchange column equilibriated with 50 mM NaCl/10 mM imidazole, pH 6.8, and the column was developed with a linear gradient of 50–250 mM NaCl in 10 mM imidazole (pH 6.8). Fimbrin eluted at about the middle of the gradient. Villin was recovered from the DNase I column as described (7). Fimbrin could be stored for 48 hr at 4°C without noticeable proteolysis; storage for a week or more resulted in detectable proteolytic degradation.

Other Procedures. Rabbit skeletal muscle actin was purified by a minor modification of the method of Spudich and Watt (9). The F-actin sedimentation binding assay was performed as described (7) except that the proteins were dialyzed against buffer P (0.1 mM $MgCl_2$/0.1 mM ATP/10 mM imidazole, pH 7.2) prior to use in the assay.

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$, negative stain analysis with uranyl acetate, tannic acid/glutaraldehyde fixation, and preparation of thin sections for electron microscopy have been described (7).

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Abbreviation: PEG, polyethylene glycol.

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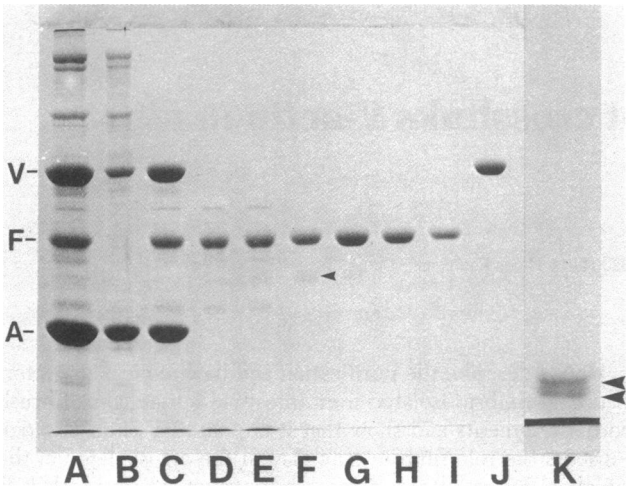

FIG. 1. Purification of fimbrin: 8.5% NaDodSO$_4$/polyacrylamide gel electrophoresis of sequential products. A, material solubilized from brush borders by calcium extraction; B, 40% ammonium sulfate-insoluble material; C, material precipitated by increasing the ammonium sulfate to 65%, which was then dissolved and applied to a DNase I affinity column; D, pooled material that did not bind to the DNase I affinity column (notice that the villin and actin have been quantitatively removed); E, material applied to the DE-52 ion-exchange column; F–I, peak fractions of the fimbrin eluting from the ion exchange column. Note the tail end of fimbrin degradation products (arrowhead) which elute just ahead of fimbrin; J, villin recovered from the DNase I column by elution with a buffer containing ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (for details, see ref 7); K, lower half of a 6.5% NaDodSO$_4$ gel of fimbrin, showing the two fimbrin species (arrowheads). V, villin; F, fimbrin; A, actin.

RESULTS

Purification and Properties of Fimbrin. Because treatment of microvillus cores *in vitro* with Ca^{2+} at >10 μM results in the solubilization of essentially all the fimbrin and villin together with some of the actin and calmodulin (5, 10), we used calcium extraction of intestinal brush borders as the first step in the purification of fimbrin. Homogeneous fimbrin and villin were then easily purified from this extract. The polypeptide composition at each step during the purification procedure is shown in Fig. 1.

The main problem encountered during purification was proteolysis of fimbrin, which was greatly reduced by treating the brush borders with diisopropyl fluorophosphate prior to calcium extraction and by including phenylmethylsulfonyl fluoride in all solutions. Despite these precautions, proteolytic fragments having M_r of about 55,000 (Fig. 1, lane F), which cross-reacted with fimbrin antibody in gel overlay experiments (not shown), still appeared during the purification procedure. These products were separated from the bulk of the fimbrin during ion-exchange chromatography in which they eluted at a slightly lower salt concentration than fimbrin (Fig. 1, lanes F–I).

Fimbrin migrated in NaDodSO$_4$/polyacrylamide gel electrophoresis as a polypeptide of apparent M_r 68,000. Under optimal conditions, fimbrin was resolved into two species of slightly different apparent M_rs (Fig. 1, lane K). No carbohydrate could be detected associated with fimbrin when gels were stained with the periodic acid-Schiff reagent. On isoelectric focusing gels, fimbrin migrated as a single species with an isoelectric point intermediate between that of actin and that of villin (see figure 6 in ref. 10). On a Sephacryl S200 gel filtration column in solution I, fimbrin eluted between aldolase (M_r, 158,000; Stokes radius, 48 Å) and ovalbumin (M_r, 43,000; Stokes radius, 30.5 Å) with a Stokes radius of about 38 Å. Taken together, the data suggest that isolated fimbrin exists in solution as a monomeric protein with M_r 68,000.

Fimbrin Crosslinks F-Actin *in Vitro*. The interaction of fimbrin with F-actin was investigated by using a sedimentation assay (7) in which fimbrin and G-actin were mixed, KCl was added, the mixture was incubated for 2 hr at 22°C to allow polymerization of actin, and the resulting polymers were collected by centrifugation at 100,000 × *g*. The pellet and supernatant fractions were then analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2) and the gels were scanned to estimate

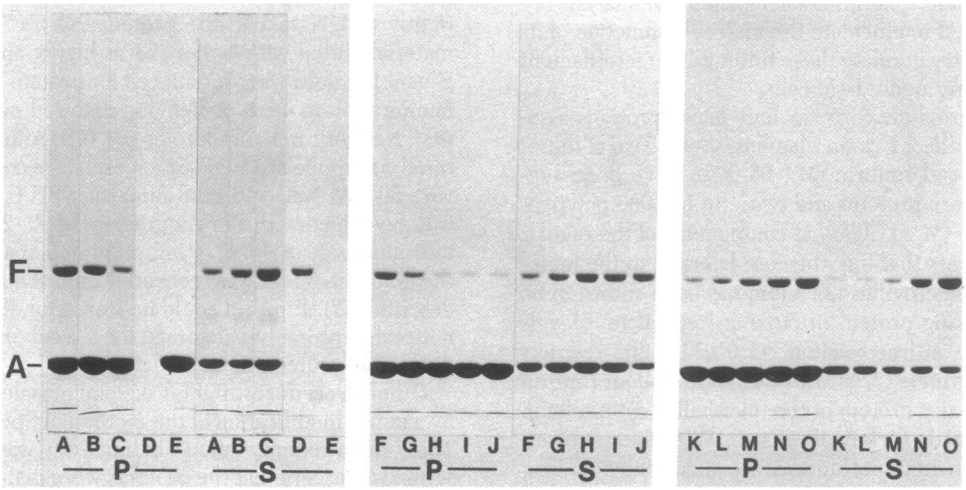

FIG. 2. Interaction between F-actin and fimbrin, shown by 7.5% NaDodSO$_4$/polyacrylamide gel electrophoresis. G-actin or fimbrin or both were mixed in 100 μl of buffer P, agents were added to initiate polymerization of actin, the mixture was incubated for 2 hr at 22°C, and the samples were centrifuged at 100,000 × *g* for 20 min to give pellet (P) and supernatant (S) fractions. The fractions were prepared for gel electrophoresis and one-quarter of the sample was loaded on the gel. Lanes A–C, G-actin and fimbrin were mixed to give concentrations of 0.5 and 0.2 mg/ml, respectively, in buffer P and polymerization was induced by 50 mM KCl/4% PEG (A), 30 mM KCl (B), 60 mM KCl (C); D and E, fimbrin alone (D) and actin alone (E) in solution P and 50 mM KCl/4% PEG. Lanes F–J, effect of Mg^{2+} on the fimbrin–F-actin interaction. G-actin and fimbrin were mixed to give concentrations of 0.5 and 0.16 mg/ml, respectively, and polymerization was induced by adding 50 mM KCl/4% PEG to samples containing Mg^{2+} at 0.1 mM (F), 0.3 mM (G), 0.5 mM (H), 1 mM (I), or 5 mM (J). Lanes K–O, binding capacity of F-actin for fimbrin. G-actin was mixed with fimbrin to give an actin concentration of 0.5 mg/ml and approximate molar ratios of G-actin to fimbrin of 10:1 (K), 7:1 (L), 5:1 (M), 3:1 (N), or 2:1 (O). Polymerization was induced by the addition of 50 mM KCl/4% PEG. F, fimbrin; A, actin.

the percentage of each protein sedimented. When 30 mM KCl was added to a mixture of G-actin (0.5 mg/ml) and fimbrin (0.2 mg/ml) in buffer P (0.1 mM $MgCl_2$/0.1 mM ATP/10 mM imidazole·HCl, pH 7.2) to induce polymerization of actin, about 65% of the fimbrin could be cosedimented with F-actin (lane B). This cosedimentation of fimbrin was greatly decreased when actin polymerization was induced by 60 mM KCl (lane C) and abolished when polymerization was induced by >100 mM KCl. Because the cosedimentation of fimbrin with F-actin was somewhat variable in 30 mM KCl, partly because this salt concentration is in the minimum range needed to polymerize actin (11), we investigated the possibility of stabilizing the interaction with polyethylene glycol (PEG) at higher salt concentrations. When the assay was performed with 50 mM KCl in the presence of 4% (wt/vol) PEG, >75% of the fimbrin and about 90% of the actin were sedimented (lane A). Fimbrin bound to F-actin when the assays were performed at 4°C, 22°C, or 37°C (buffer pH 7.2 at 4°C) or in the pH range 6.6–7.6 (at 22°C), so all further assays were performed at pH 7.2 at 22°C. The inhibitory effects of higher KCl concentrations were also found in the presence of PEG because about 30% of the fimbrin cosedimented with F-actin in 100 mM KCl (not shown). I therefore chose buffer P containing 50 mM KCl and 4% PEG as the standard condition in which to investigate the interaction further. Under these conditions fimbrin alone did not sediment (lane D), whereas actin alone polymerized and sedimented (lane E). This binding did not require fimbrin to be present during the polymerization of actin, because identical cosedimentation results were obtained when fimbrin was added to prepolymerized F-actin.

The effects of divalent cations on the binding were examined. Inclusion of up to 5 mM Ca^{2+} had no detectable effect on the binding of fimbrin to F-actin. By contrast, the inclusion of Mg^{2+} at >0.5 mM inhibited the binding of fimbrin to F-actin (Fig. 2, lanes F–J). On the other hand, no requirement for divalent cations was found: inclusion of either EDTA (1 mM) or ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (1 mM) did not decrease or enhance the amount of fimbrin cosedimenting with F-actin.

An attempt was made to determine whether the binding of fimbrin to F-actin could be saturated. A series of assays were performed in which increasing concentrations of fimbrin were mixed with a fixed final concentration of actin (Fig. 2, lanes K–O). Gel scans of the pellet and supernatant fractions in samples in which the ratio of G-actin to fimbrin ranged from 10:1 to 5:1 revealed that >70% of the fimbrin cosedimented with F-actin. Increasing the starting ratio to 3:1 or 2:1 resulted in a significant percentage of the fimbrin in the supernatant, giving a ratio of about 1 fimbrin molecule per 3.5 actin monomers in the pellet fraction at the highest ratio tested. The data indicate that F-actin could be saturated with fimbrin and that the stoichiometry at saturation would be at least 1 fimbrin molecule per 3.5 actin monomers.

The polymers formed between F-actin and fimbrin were adsorbed to a grid, negatively stained, and examined by electron microscopy. At low fimbrin-to- F-actin ratios, some bundling was evident (Fig. 3*B*) and at higher ratios, thick bundles of filaments were seen (Fig. 3 *C–E*). These bundles were rather straight over short distances (Fig. 3, *C–E*) and, at high fimbrin-to-F-actin ratios, shorter bundles were formed (Fig. 3*D*). In addition, the bundles frequently had kinks and breaks in them (Fig. 3*C* and *E*), which may suggest that they are rather rigid and brittle.

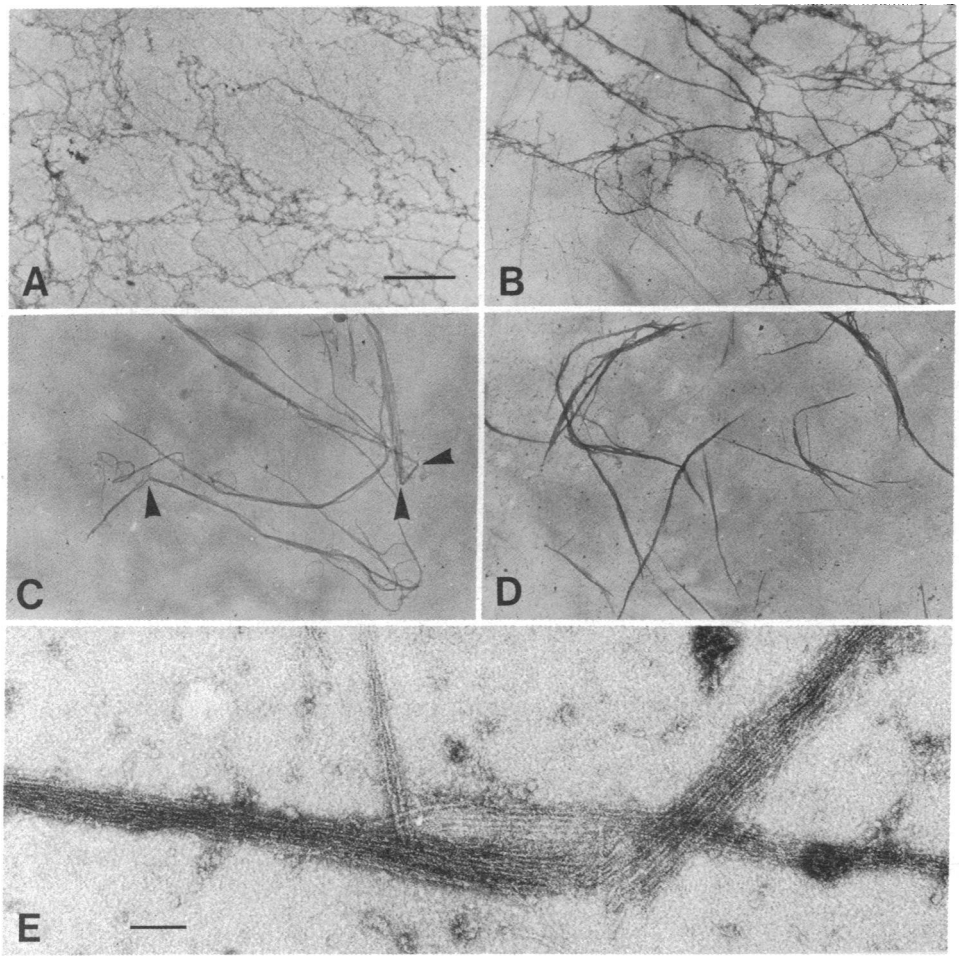

FIG. 3. Electron micrographs of negatively stained preparations of F-actin (*A*) and of F-actin and fimbrin mixtures (*B–E*). Actin (*A*) was polymerized alone or in the presence of increasing amounts of fimbrin (*B–D*) in buffer P and 50 mM KCl/4% PEG. The G-actin/fimbrin molar ratios in the samples were approximately 10:1, (*B*), 5:1 (*C*), and 3:1 (*D*). (*E*) G-Actin and fimbrin polymerized in the presence of solution P and 30 mM KCl at a molar ratio of approximately 3:1. Note the kinks in the bundle shown in *C* (arrowheads) and the break in the straight bundle shown in *E*. (*A–D* are at the same low magnification; bar in *A* is 1 μm. Bar in *E* is 0.1 μm.)

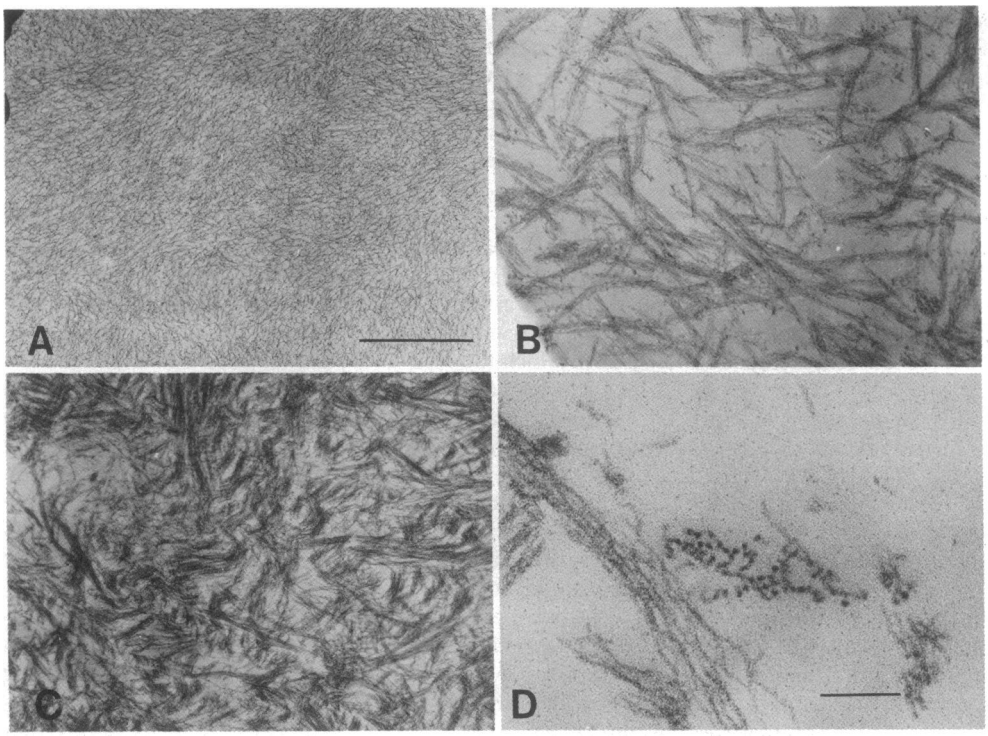

FIG. 4. Electron micrographs of thin sections of F-actin polymerized alone (*A*) or in the presence of fimbrin (*B* and *D*) or villin (*C*) at a G-actin/fimbrin or villin molar ratio of about 3:1. Polymerization was induced in buffer P by adding 50 mM KCl/4% PEG. (*A–C* are at the same magnification; bar in *A* is 1 μm. Bar in *D* is 0.1 μm.)

Examination of thin sections of fimbrin–F-actin polymers by electron microscopy also revealed bundles of filaments (Fig. 4*B*). When these bundles were compared with those formed by villin and F-actin under the same conditions (Fig. 4*C*; see also ref. 7), the fimbrin–F-actin structures appeared more uniform and relatively straight. In fimbrin–F-actin bundles sectioned transversely, it was also observed that the filaments often had the appearance of being crosslinked (Fig. 4*D*). The spacing between these filaments was variable but in cases in which a clearer crosslink was seen, the center-to-center spacing of the filaments was approximately 10–13 nm.

DISCUSSION

In this report we describe the purification of fimbrin from intestinal brush borders. It makes use of the Ca^{2+}-mediated solubilization of fimbrin from the brush border cytoskeleton, together with other cytoskeletal proteins. Fimbrin was then easily purified to homogeneity. Purified fimbrin was found to be a monomeric protein of M_r 68,000, although it is not known whether other solution conditions, or the F-actin crosslinking activity discussed below, induce oligomerization of the molecule.

Heretofore, the role of fimbrin in the microvillus core was uncertain. However, because it has proved possible to extract microvillus cores selectively and leave a bundle of microfilaments containing only actin, villin, and fimbrin as major components (3), it seemed likely that fimbrin bound directly to F-actin, to villin, or to both. Here it is shown that purified fimbrin binds and crosslinks F-actin *in vitro*. Experiments reported elsewhere (6) show that fimbrin and villin bind independently to different sites on F-actin.

Fimbrin bound F-actin *in vitro* under special conditions. It cosedimented with F-actin in 30 mM KCl, yet in more concentrated KCl (>100 mM) very little fimbrin cosedimented. The inclusion of PEG stabilized the interaction in 50 mM KCl. Because 30 mM KCl is on the borderline of the salt concentration needed to polymerize actin (11), in general the former conditions were used to investigate the interaction. Under these conditions, the binding of fimbrin to F-actin was sensitive to Mg^{2+} >0.5 mM but insensitive to Ca^{2+} up to 5 mM. It is conceivable that the KCl and Mg^{2+} sensitivities of the interaction may be because optimal binding conditions were not achieved or because muscle actin was used in these studies, as opposed to the cytoplasmic actin present in the microvillus (2), although these two actins only differ slightly in amino acid sequence (12). Alternatively, the sensitivities of the binding may reflect an *in vivo* mechanism for the regulation of bundle formation and disassembly.

Examination of the fimbrin–F-actin polymers by electron microscopy revealed that fimbrin crosslinked F-actin into compact, rather straight bundles, particularly by comparison with the bundles formed between villin and F-actin. These observations suggest that fimbrin not only crosslinks F-actin but also may confer some rigidity on the bundle formed.

Is fimbrin related to other F-actin crosslinking proteins? It is clearly different from both α-actinin and filamin which are large dimeric proteins in solution (M_r 200,000 and 500,000, respectively) (13, 14) but which have not been characterized extensively with respect to their ability to cause F-actin to form bundles. Fimbrin probably most closely resembles fascin, a protein of M_r 58,000 that crosslinks F-actin in sea urchin microvilli (15, 16). However, fascin–F-actin bundles show a striking 11-nm pattern which has not yet been observed in fimbrin–F-actin bundles.

What is the role of fimbrin in the microvillus cytoskeleton, where it is found together with villin, another F-actin crosslinking protein? Villin crosslinks F-actin filaments in the absence of Ca^{2+} but also severs them when the free Ca^{2+} level is increased above 1 μM (7, 8), leading to partial disassembly of the core. By contrast, fimbrin crosslinks F-actin filaments to provide the core bundle with some rigidity in a calcium-insensitive interaction. Although the properties of villin and fimbrin seem to explain some of the major features of the microvillus core, we do not know exactly how the structure is built and, in particular, whether fimbrin and villin crosslink the same or different F-actin filaments.

Fimbrin is also found in many other microfilamentous struc-

tures (4). The suggestion that fimbrin is involved in crosslinking F-actin and confers some rigidity on the bundle formed is consistent with its known cellular locations as a component of microvilli, microspikes, and stereocilia. Of particular interest is the stereocilium of the inner ear, a rigid structure that contains a microfilament bundle which will break rather than bend when manipulated *in vitro* (17). In this bundle, fimbrin has been detected by immunofluorescence microscopy, but not α-actinin, filamin, or villin, the other known F-actin cross-linking proteins of higher cells (unpublished data). It seems likely therefore that fimbrin is a major F-actin crosslinking protein in the stereocilium and contributes to the rigidity of the structure.

The data presented here indicate that fimbrin crosslinks F-actin *in vitro* to form bundles of filaments and that this interaction is sensitive to KCl and Mg^{2+} in the physiologically important concentration range. The exciting possibility that this phenomenon is involved in the assembly and disassembly of transient structures known to contain fimbrin, such as the microvilli and microspikes of cultured cells, remains to be investigated.

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