

Fimbrin, a New Microfilament-associated Protein Present in Microvilli and Other Cell Surface Structures

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ABSTRACT A 68,000 mol wt polypeptide has been identified as one of the few major proteins in the microfilament bundles of the microvilli present on intestinal epithelial cells. Antibodies against the purified protein have been used in indirect immunofluorescence microscopy on several cultured cells. The protein is found particularly prominent in membrane ruffles, microspikes, and microvilli.

Microfilaments, one of the three cytoskeletal filamentous systems of eucaryotic cells, have been implicated both in the maintenance of cell shape and in several cellular motile phenomena (for a review, see reference 10). Over the last five years, several proteins have been identified by immunofluorescence microscopy as components of the microfilament bundles of tissue culture cells. Antibodies to actin (15), myosin (25), tropomyosin (12), α -actinin (14), and filamin (7) have shown these proteins as being present along microfilament bundles. Recently, a new protein, vinculin, was found to be enriched in the areas of the cell where these bundles attach to the substratum (6). Some of these proteins show a restricted distribution; for example, myosin and tropomyosin cannot be detected in the highly motile cellular areas, such as the ruffles, whereas actin, α -actinin, and filamin are all prominent components (7, 12, 13). In addition, besides actin, none of these proteins has been found in the microvilli of the intestinal epithelial cells (1, 5, 20). Although the limited sensitivity of the immunofluorescence technique does not rule out the possible presence of small amounts of some of these components in the membrane ruffle and intestinal microvillus, it strongly suggests that different arrangements of microfilaments with different associated proteins exist. In the case of the microvillus, the results are strengthened by biochemical studies (2, 3, 16) which were unable to detect myosin, tropomyosin, filamin, or α -actinin in isolated microvilli. Indeed, it is to be expected that different microfilament arrangements exist in view of the wide variety of functions attributed to these structures in nonmuscle cells (10). All the microfilament-associated proteins discussed above were initially identified by raising antibodies to proteins isolated from muscle tissues. It seems likely, therefore, that new proteins will be found in nonmuscle cells associated with those microfilaments which provide structural support or which are involved in motile processes not based on the actomyosin

interaction. Presently, it is not easy to isolate intact microfilaments from tissue culture cells in order to establish which proteins are associated with which system of microfilaments. We have chosen to study the core bundle of the microvilli present on intestinal epithelial cells as a model system for one type of microfilament arrangement in nonmuscle cells.

Cells of the intestinal epithelium each have about one thousand microvilli on their apical surface as part of a structure known as the brush border. Nearly twenty years ago, Miller and Crane (17) developed a method for the rapid isolation of intestinal brush borders. Since then, a method has been developed for the purification of microvilli from disrupted brush borders (2), so allowing a biochemical and ultrastructural analysis of the cytoskeleton of the microvillus. The core of the microvillus is a highly ordered bundle of microfilaments attached to the membrane both at the tip of the microvillus and laterally down its length by a series of regularly spaced cross-filaments (3, 4, 19, 21). This simple structure contains a number of proteins besides actin (2, 16). Recently we described a major protein (mol wt 95,000) of the microvillus core, which we called villin (3) and which we have so far been unable to detect in tissue culture cells. Here we describe a second major protein (mol wt 68,000) from the microvillus core which is also present in membrane ruffles of tissue culture cells and in structures containing highly ordered microfilament bundles. As this protein appears to be preferentially associated with surface structures of cells, we propose the name fimbrin (from the Latin "fimbria," meaning border, fringe).

MATERIALS AND METHODS

Isolation of Fimbrin

Highly purified brush borders (2) were extracted for 30 min at 0°C in 0.6 M KCl, 1 mM CaCl₂, 0.2 mM ATP, 20 mM Tris-HCl, pH 7.8. The extracted material

was recovered as the supernatant fraction after centrifugation at 100,000 g for 1 h. The extract was then dialyzed for 3 h against 51 of the G-actin buffer of Spudich and Watt (24), during which time much of the myosin precipitated and was subsequently removed by centrifugation. This low salt extract was fractionated by adding 45% ammonium sulfate which precipitated most of the cytoskeletal proteins with the exceptions of fimbrin, and some of the villin and actin. Fimbrin was isolated from the 45% ammonium sulfate soluble material by preparative SDS gel electrophoresis (15). During the isolation of fimbrin, all buffers included 0.25 mM phenylmethylsulfonyl fluoride to reduce proteolysis.

Immunological Techniques

Antibodies to fimbrin were elicited in rabbits. Affinity purified antibodies were prepared from the positive sera using a Sepharose column to which fimbrin was covalently attached. The affinity purified antibodies to actin and chicken gizzard α -actinin have been described (26). Fluorescein labeled goat-anti-rabbit IgGs were purchased from Miles-Yeda, Israel and preabsorbed extensively on methanol-fixed chick embryo fibroblasts before use at a final concentration of ~0.4 mg/ml. Indirect immunofluorescence microscopy on intestinal epithelial cells has been described (1). Immunofluorescence microscopy on tissue culture cells was performed on cells fixed and made permeable by a 5-min incubation in -20°C methanol. Cells attached to coverslips were then immediately washed in phosphate-buffered saline and processed for immunofluorescence microscopy (15).

Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE) was performed as described (11). The immune replica method was performed using ¹²⁵I-labeled protein A to detect immune complexes (23).

RESULTS

The Brush-border 68,000 Protein is a Component of the Microvillus Core

Highly purified brush borders can readily be prepared from the small intestine of the chicken (19). Removal of the membrane by treatment with Triton X-100 reveals the brush-border cytoskeleton (18) which contains about half of the total brush-border protein. Because ~15 mg of brush-border cytoskeletal protein can be isolated from a single bird, the brush border provides a rich source of any particular cytoskeletal protein. The polypeptide composition of brush borders and their cytoskeletons has been documented in part elsewhere (2, 16, 18); here we summarize what is known about the distribution of the major proteins between the microvilli and the terminal web region.

The polypeptide composition of purified brush borders as analyzed by SDS PAGE is shown in Fig. 1. Cytoskeletons recovered after treatment of brush borders with Triton X-100 (track B) appear to be intact because essentially none of their major components are found in the Triton X-100 soluble material (track C) and morphological studies have shown that they retain their prominent ultrastructural features (18). Analysis of the protein components of the brush-border cytoskeleton reveals several proteins having polypeptide mol wt above 40,000. Prominent species of 280,000, 250,000, 200,000, 145,000, 110,000, 95,000, 68,000, and 43,000 are easily detected. The 250,000, 200,000, 145,000, and 68,000 proteins can sometimes be resolved into more than one species. Of these cytoskeletal proteins, the 200,000 species has been identified as the heavy chain of myosin (20) and the 43,000 species as a mixture of β and γ nonmuscle actin (2). Higher cross-linked gels (see, for example, references 2, 16, and 18) reveal in addition some lower molecular weight components, among which is a doublet at 30,000, identified as typical nonmuscle tropomyosin (2, 18).

Purified microvilli (tract D) do not contain the 280,000, 250,000, 200,000, 145,000, or 30,000 (not shown) polypeptides.

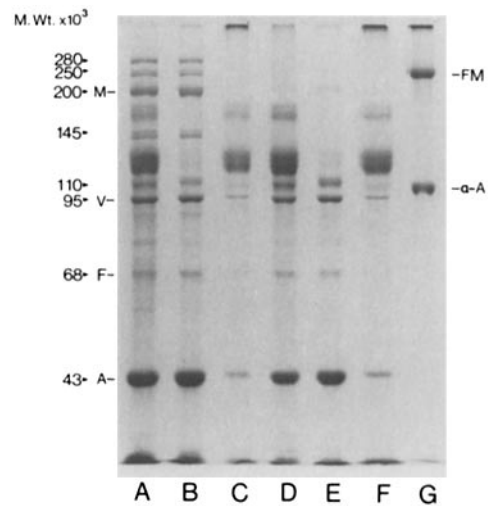


FIGURE 1 7.5% SDS PAGE of the polypeptide components of brush borders, microvilli and their cytoskeletons. (A) Purified brush borders, (B) brush-border cytoskeletons made by removing the cytoplasmic membrane with Triton X-100 (18), (C) Triton X-100 soluble material of brush borders, (D) purified microvilli, (E) microvilli cytoskeletons, (F) Triton X-100 soluble material of microvilli, (G) two molecular weight markers, chicken gizzard filamin (250,000) and chicken gizzard α -actinin (100,000). Approximate molecular weights are shown at left. M, myosin heavy chains; V, villin; F, fimbrin; A, actin; FM, filamin; α -A, α -actinin.

As discussed previously (2), these species are located in the terminal web region of the brush border. Prominent components of the microvillus cytoskeleton (track E) include actin (43,000) and the 110,000, 95,000 (villin), and 68,000 (fimbrin) polypeptides. We have described villin as the major protein associated with actin in the microvillus core (3). In agreement with others (5, 16), we now find the 110,000 protein to be a relatively major component. Previously we did not detect it as a major species because of its high protease sensitivity, especially in the presence of polyethylene glycol which we used to stabilize the microvillus cores (2). In addition to these higher molecular weight components, Howe and Mooseker (9) recently reported that calmodulin is a component of the microvillus core. We can confirm this result. We have found that purified microvillus cores contain a 16,500 polypeptide that comigrates by SDS PAGE with calmodulin, and that immunofluorescence microscopy using calmodulin antibody reveals this protein as a component of the microvilli of the brush border.

Antibody to Fimbrin

Having identified fimbrin as a component of the microvillus core, the protein was isolated from brush borders (Fig. 2). The brush borders were extracted with a 0.6 M KI buffer that solubilizes most of the cytoskeletal proteins. The salt was removed by dialysis against the G-actin buffer of Spudich and Watt (24), during which time most of the myosin precipitates. The clarified extract was then subjected to ammonium sulfate fractionation, rendering most of the cytoskeletal proteins insoluble, and leaving fimbrin and some of the actin and villin in the soluble fraction. Fimbrin was then isolated from the supernate by preparative SDS PAGE.

Antibodies to fimbrin were elicited in two rabbits. Both animals were found to be positive by immunodiffusion analysis

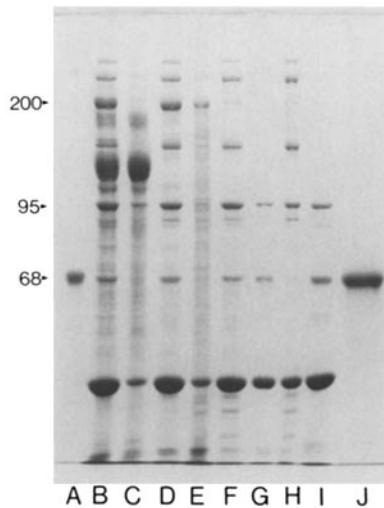


FIGURE 2 7.5% SDS PAGE of the fractionation steps used in the isolation of fimbrin. (A) Bovine serum albumin molecular weight marker (68,000), (B) purified brush borders, (C) material insoluble in 0.6 M KI buffer, (D) material extracted in 0.6 M KI buffer, (E) precipitate formed during dialysis to remove the high salt, (F) extract soluble in low salt, (G and I) 45% ammonium sulfate soluble material, (H) material precipitated by 45% ammonium sulfate, and (J) fimbrin isolated from the material shown in lane I by preparative gel electrophoresis. Approximate molecular weights ($\times 10^3$) are shown at left.

against the isolated protein. The sera were further characterized by the immune replica method, where the immunological reaction between a serum and proteins separated by SDS PAGE is determined (23). Both sera reacted strongly with a polypeptide of 68,000 mol wt present in the microvillus core and in cultured chick embryo fibroblasts (Fig. 3). Antibodies specific for fimbrin were isolated by affinity chromatography on a column to which the isolated protein was covalently bound. Either these affinity purified antibodies (at 0.05 mg/ml), or a 100-fold dilution of the positive sera, were used in indirect immunofluorescence microscopy. Essentially identical results were obtained using the different antibody preparations.

Localization of Fimbrin in Nonmuscle Cells

Immunofluorescence microscopy on chicken, mouse, or guinea pig intestinal epithelial cells visualizes fimbrin in the microvilli of the brush border (Fig. 4). Owing to the intense fluorescence from the microvilli, it is not possible to determine

independently at the light microscope level whether fimbrin is also present in the terminal web region of the brush border. Control experiments in which nonimmune first antibody was used in place of the fimbrin antibody showed only weak background fluorescence (not shown, but see controls in reference 1).

In tissue culture cells, structures containing fimbrin could best be seen as cells reattached after replating. Fig. 5 shows chick embryo fibroblasts (8) 2.5 h after replating and viewed in immunofluorescence microscopy after decoration with either antibody to fimbrin (A-C) or to actin (D). Fimbrin can be seen to be present in all membrane ruffles, in the microfilament meshwork which lies between the cell body and the ruffling membrane, in surface microvilli, and in microspikes. Weak staining of stress fibers was also seen, particularly in the regions

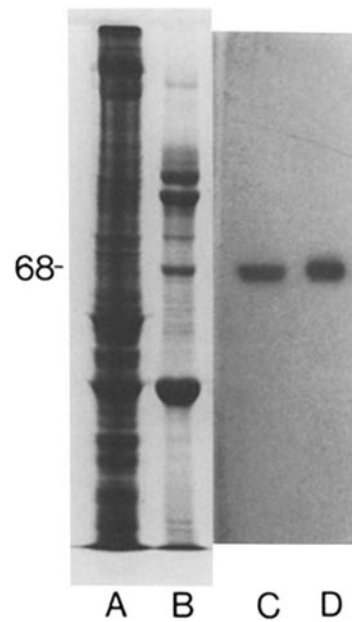
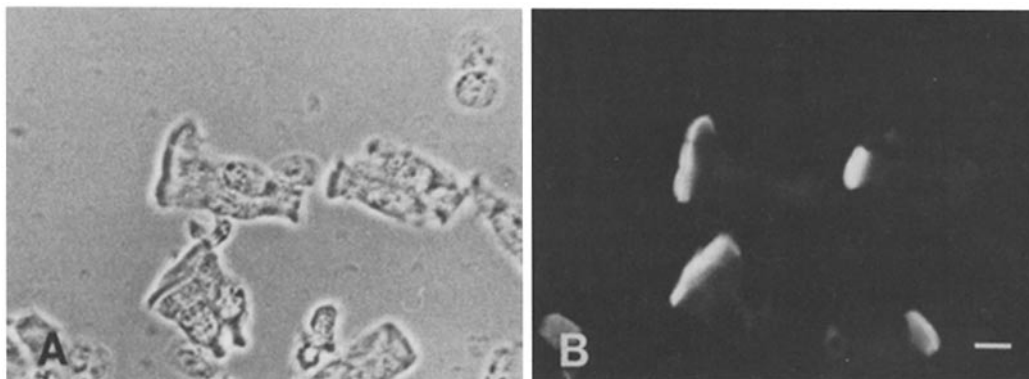


FIGURE 3 Immune replica showing the immunological reactivity of one of the antifimbrin sera to proteins separated by 8.5% SDS PAGE. (A and B) Coomassie Blue-stained gels of (A) total proteins of chick embryo fibroblasts, and (B) microvillus cytoskeletons. (C and D) Autoradiographs showing the immunological reactivity of the anti-fimbrin serum with (C) total proteins of chick embryo fibroblasts shown in lane A, and with (D) the microvillus cytoskeletal proteins shown in B. Note that the antiserum reacts with a 68,000 protein present in both microvilli and chick embryo fibroblasts.



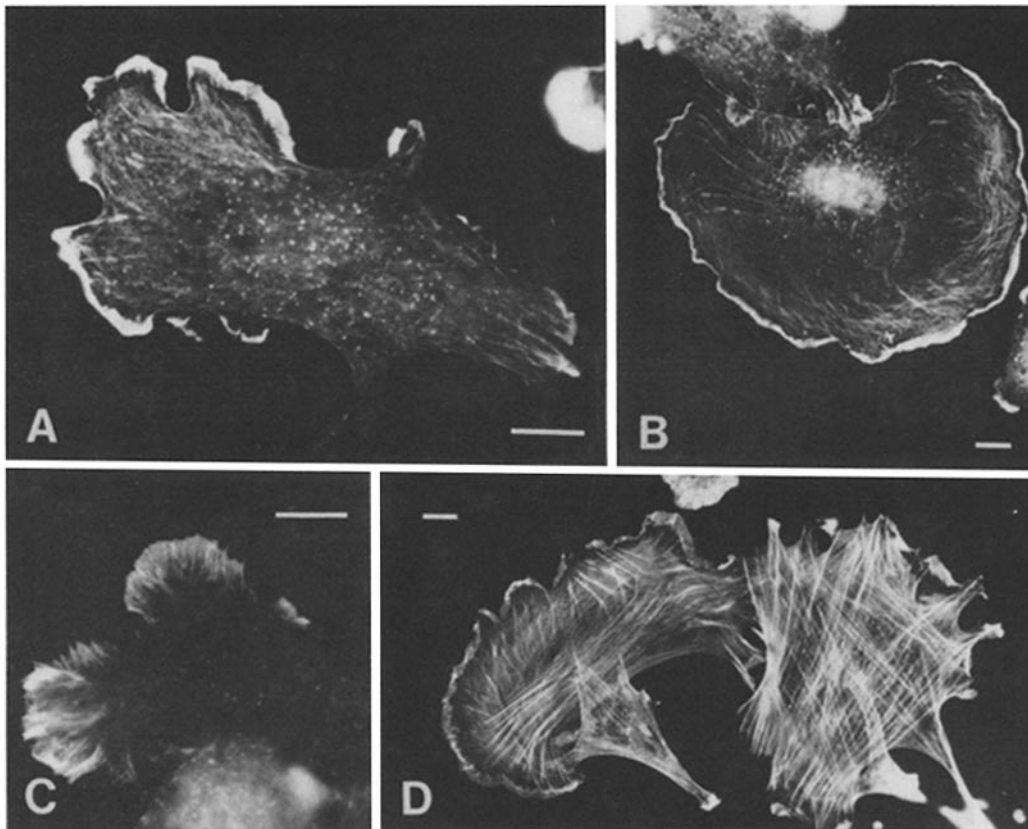
FIGURES 4 Phase (A) and fluorescence (B) micrographs of the same mouse intestinal epithelial cells stained with fimbrin antibody. Note that the microvilli of the brush border are heavily decorated. Bar, 10 μ m. $\times 470$.

of the cell where they are attached to the substratum ("focal points"). For comparison, two cells stained with antibody to actin (*D*) are shown; here both the membrane ruffles and stress fibers are strongly stained, giving rise to a staining pattern characteristically different from that obtained with fimbrin antibody.

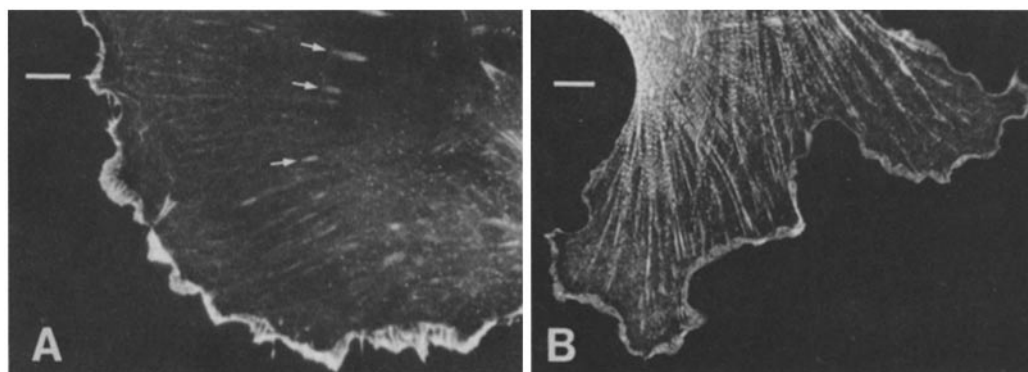
Fimbrin can also be detected in a wide variety of cultured mammalian cells. Fig. 6 shows the edges of two fully spread rat mammary cells (22) in culture, one stained with antibody to fimbrin and, for comparison, one with antibody to α -actinin. Again the fimbrin antibody stained the membrane ruffle and

microspikes, as well as the substratum attachment points of the stress fibers (arrows). As described for this and other cell lines (15, 22, 25), α -actinin is present in the membrane ruffle, attachment points of the stress fibers, and in the stress fibers themselves.

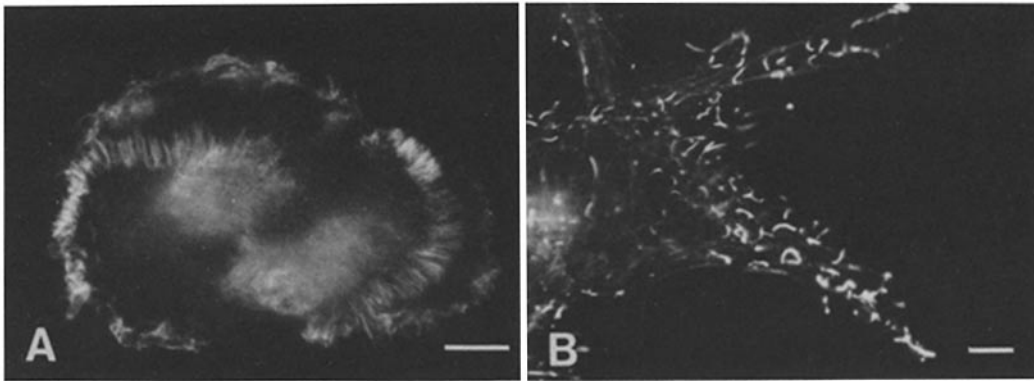
Other cell surface structures were examined to see if they also contained fimbrin. Fig. 7*A* shows a rat mammary cell stained with fimbrin antibody 2.5 h after replating. The cell has two rows of large surface microvilli which are probably derived from two large membrane ruffles which passed back on the body of the cell from the periphery. Fimbrin is a



FIGURES 5 Respreding chick embryo fibroblasts processed for immunofluorescence microscopy after staining with antibody to fimbrin (*A–C*) and to actin (*D*). Note the intense staining found in the membrane ruffles and surface microvilli (*A* and *B*) and in microspikes (*C*). Very little stain is seen associated with the stress fibers, yet they are visible when cells are stained with actin antibody (*D*). All bars, 10 μ m. (*A* and *C*) \times 950, (*B*) \times 480, (*C*) \times 460.



FIGURES 6 Fluorescence micrographs of the leading edges of two rat mammary cells stained with antibody to (*A*) fimbrin and (*B*) α -actinin. Note the strong stain seen in the membrane ruffle in *A* and staining of substratum attachment points (arrows). α -Actinin is also present in the membrane ruffle, substratum attachment points and, in addition, in the stress fiber system. Bars, 10 μ m. (*A*) \times 620, (*B*) \times 550.



FIGURES 7 Localization of fimbrin in two types of microvilli: in *A* a respreading rat mammary cell with two rows of large microvilli probably derived from membrane ruffles is stained with antibody to fimbrin; in *B* a vaccinia virus infected chick embryo fibroblast, having many virus-induced microvilli (8), is also stained with antibody to fimbrin. Bars, 10 μ m. (A) \times 820, (B) \times 580.

prominent component of these large microvilli. Recently, Hiller et al. (8) described virus-induced microvilli found on chick embryo fibroblasts infected with vaccinia virus and reported that these induced microvilli contain both actin and α -actinin. Fig. 7*B* shows part of an infected cell covered with virus-induced microvilli and viewed in immunofluorescence microscopy after decoration with fimbrin antibody. These microvilli as well contain fimbrin as a prominent component.

DISCUSSION

We have identified the 68,000 mol wt polypeptide of the brush border as a major component of the microvillus core. Indirect immunofluorescence microscopy with antibody to this protein reveals it as a prominent component of a number of microfilament-containing surface structures of nonmuscle cells. These include the membrane ruffle, intestinal epithelial cell microvilli, microspikes, both the small surface microvilli and the larger microvilli derived from folded-back membrane ruffles, and microvilli induced after infection of fibroblasts with vaccinia virus. In addition, the 68,000 protein is present in the microfilament bundle of the stereocilia found on sensory hair cells of the inner ear (A. Bretscher and Å. Flock, unpublished results). It is clearly a protein, therefore, which is associated with both highly ordered microfilament arrangements, such as microvilli, microspikes, and stereocilia, as well as being associated with the fine microfilament network of the membrane ruffle. It is the first microfilament-associated protein described that is much more prominent in the peripheral microfilament-containing structures of the cell than in the stress fiber system. For this reason we propose the name "fimbrin," which is derived from the Latin "fimbria," meaning border or fringe.

What is the function of fimbrin? No obvious function is suggested by its distribution between various cell structures. It is most prominent in those structures that appear to lack myosin and tropomyosin (such as membrane ruffles, microspikes, microvilli [1, 7, 12, 13, 14, 20]) and present in structures that either contain filamin and α -actinin (membrane ruffles [7, 14]) or appear to lack these proteins (microvilli of the intestinal epithelium [1]). It is abundant in the highly ordered microfilament arrangements so far examined, with the exception of stress fibers. To understand the function of fimbrin, its biochemical properties, and its role in at least one of these structures will have to be elucidated. At present, the microfilament core of the microvillus of the intestinal epithelium provides the one highly ordered microfilament arrangement available ultra-

structurally intact and in sufficient quantities for biochemical analysis (2). This structure contains, in addition to actin and fimbrin, at least three other proteins (16). We have recently shown (manuscript in preparation) that one of these, villin, has the ability to bundle F actin in vitro and so is probably a microfilament cross-linking protein and not the microfilament-membrane cross-filament protein as previously suggested (3). Another microvillus protein, the 110,000 polypeptide, has been suggested by Matsudaira and Burgess (16) to be the true cross-filament protein based on their ultrastructural studies of microvillus cores extracted under various conditions. The fifth protein known to be present in the microvillus core is calmodulin (9), although its role there is not yet understood. We are currently attempting to determine how these proteins are assembled into the microvillus core and we anticipate that these experiments will give some insight into the function of fimbrin.

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