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Villin: The major microfilament-associated protein of the intestinal microvillus

(brush border/membrane attachment/α-actinin/immunofluorescence microscopy/immunoferritin label)

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ABSTRACT **The major protein associated with actin in the microfilament core of intestinal microvilli has been purified. This protein, for which we propose the name villin, has a polypeptide molecular weight of approximately 95,000. Two arguments suggest that villin may be the microvillus crossfilament protein that links the microfilament core laterally down its length to the cytoplasmic side of the plasma membrane. First, electron microscopy shows that crossfilaments stay attached to isolated membrane-free microvillus cores. Calculation of the expected abundance of the crossfilament protein shows that only villin is present in sufficient quantity to account for these structures. Second, decoration of microvillus cores by antibodies to either actin or villin, followed by ferritin-labeled second antibody in a sandwich procedure, results in specific labeling of the cores in both cases. The antivillin decoration, however, gives rise to a greater increase in diameter, in agreement with a model in which villin projects from the F-actin microfilament core. Villin is distinct from α-actinin, a protein suggested to be involved in membrane anchorage of microfilaments in nonmuscle cells. The two proteins differ in molecular weight. Specific antibodies against villin and α-actinin show no immunological crossreactivity. Immunofluorescence microscopy reveals that villin is located in the microvilli of the brush border whereas α-actinin is absent from the microvilli but is found in the terminal web. In addition, villin is not found in microfilament bundles of tissue culture cells, which are rich in α-actinin. Thus, villin and α-actinin appear to be immunologically and functionally different proteins.**

Electron microscopy has amply documented that microfilaments, one of the major cytoskeletal elements of eukaryotic cells, often appear to be anchored to the cytoplasmic membrane (for a review, see ref. 1). Because of the complexity of elucidating the molecular mechanism of this membrane anchorage in tissue culture cells, interest has focused on suitable model systems. Thus Mooseker and Tilney have elegantly investigated the structure and membrane anchorage of microfilaments in the microvilli of the intestinal brush border (1–4). Here each microvillus contains a compact core of about 20 highly ordered parallel microfilaments that insert and terminate in the cell body as part of a structure called the terminal web (3, 5, 6). Extensive electron microscopy has documented that the core microfilaments of the microvilli are attached to the membrane in two ways. First, they are connected at the microvillus tip in an unknown manner (1, 3); second, they are linked laterally down the length of the microvillus to the inner side of the membrane by a regular array of crossfilaments (3, 6). Several indirect arguments led Mooseker and Tilney (3) to propose that these crossfilaments are composed of α-actinin, a protein found at the Z-line of skeletal muscle, where it is believed to anchor thin filaments (7). Supporting evidence for this hypothesis came from the finding that the crossfilaments have molecular dimensions similar to purified muscle α-actinin (8), together with a brief report on the presence of α-actinin in microvilli as judged by immunofluorescence microscopy (9), as well as the presence in the brush border of a polypeptide with a molecular weight similar to that of α-actinin—i.e., 100,000 (4). In addition, indirect evidence from other systems has suggested that α-actinin may be involved in microfilament-membrane anchorage (9–11). However, because α-actinin has never been purified from nonmuscle tissues and its presence has only been inferred by immunological techniques, there exists no direct evidence for its role in microfilament-membrane attachment.

In order to investigate the problem of membrane anchorage of microfilaments in the microvillus in more detail, we have developed a purification procedure for the microfilament cores of intestinal microvilli (12). Electron microscopy revealed the ordered microfilaments of the core with projecting crossfilaments. Sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel analysis of the purified microvillus cores showed the presence of two major proteins, actin and a polypeptide of 95,000 daltons, the protein with a molecular weight similar to that of muscle α-actinin. Thus we were surprised to find that immunofluorescence microscopy with antibodies to α-actinin did not stain the microvilli but did stain the terminal web of intestinal epithelial cells (13). This result suggested that either the protein of molecular weight 95,000 is inaccessible to the antibody or that this protein is immunologically distinct from α-actinin.

Here we describe the isolation of the 95,000 molecular weight protein, which we call villin, and show that it is immunologically and functionally distinct from α-actinin. In addition, we present evidence that villin may be the crossfilament protein that links the microfilament core to the inner side of the microvillus membrane.

MATERIALS AND METHODS

Purification of Brush Border, Microvilli, and Microvillus Cores, and Isolation of Villin. Highly purified chicken intestinal brush borders, microvilli, and the demembranated cores of microvilli were prepared as described (12). The pelleted cores were dispersed and extracted for 3 hr at 4°C in 2 mM Tris·HCl, pH 7.6/0.2 mM $CaCl_2$/1 mM ATP/1 mM dithiothreitol. The extract was clarified at 100,000 × *g* for 1 hr at 4°C. After lyophilization the extracted proteins were separated by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis. The villin band was excised and the protein was eluted and recovered (14). Chicken gizzard and chicken skeletal muscle α-actinin were purified by an unpublished modification of the procedure of Arakawa *et al.* (15). The electrophoretically homogeneous

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

α-actinins (Fig. 1 slot G) showed the typical rod-like appearance (8) in negative staining analysis.

Immunological Techniques. Antibodies to electrophoretically purified villin and to native chicken gizzard α-actinin were elicited in rabbits. Specific IgGs were selected from total IgGs by affinity chromatography on the corresponding antigen covalently coupled to Sepharose 4B (16). Indirect immunofluorescence microscopy was performed on mouse intestinal epithelial cells (13). The rabbit antibody against actin has been described (14), and was used after affinity chromatography on actin covalently coupled to Sepharose 4B. The α-actinin antibody has been described (13) and specifically stained the Z-lines of isolated myofibrils, as judged by immunofluorescence microscopy. First antibodies were used at 50 μg/ml in phosphate-buffered saline. The second antibody, fluorescein-coupled monospecific sheep anti-rabbit IgGs, was used at the same concentration. Immune replicas of proteins separated by NaDodSO$_4$/polyacrylamide gels were performed as reported (17).

Electron Microscopy. Thin sectional analysis and negative staining of microvilli and microvillus cores were performed essentially as described (12), although staining was with 2% phosphotungstic acid (pH 7.0) for 5 min. Microvillus cores to be decorated with antibodies were prepared at room temperature in buffer C (12) containing 5% polyethylene glycol (buffer CP) and 0.8% Triton X-100. Phalloidin, a drug that stabilizes F-actin filaments (18), was included at 20 μg/ml. The cores were then incubated with first antibody (final concentration 25 μg/ml) for 30 min at 14°C. After a 1:3 dilution with buffer CP, the solution was layered onto 5 ml of buffer CP containing 5% sucrose, and the decorated cores were sedimented onto a Formvar-coated electron microscope grid at 20,000 rpm for 1 hr at 14°C in a Beckman SW50 rotor. Sample and sucrose solution were carefully removed and the grid was washed in buffer CP. The grid was inverted onto a drop of ferritin-labeled (19) sheep anti-rabbit IgG (60 μg/ml) in buffer CP for 15 min at 14°C and then thoroughly washed in buffer CP. After fixation for 5 min in buffer CP containing 2% glutaraldehyde, the grid was washed in water and stained with 1% aqueous uranyl acetate for 1 min. After a final wash, the grid was examined in a Philips 301 electron microscope.

RESULTS AND DISCUSSION

Isolation of Villin. Microfilament cores were prepared from purified chicken intestinal microvilli and analyzed by polyacrylamide gel electrophoresis in the presence of NaDodSO$_4$. This analysis revealed the presence of only two major proteins: actin and a protein that we call villin (Fig. 1 slot B). Villin was purified by extracting the microvillus cores in low salt and subjecting the resulting soluble extract to preparative gel electrophoresis. The excised protein band was eluted to give a homogeneous pure villin preparation (Fig. 1 slot D). Villin has a polypeptide molecular weight of approximately 95,000. It shows a faster electrophoretic mobility than muscle α-actinin (molecular weight 100,000) (Fig. 1 slot F), from which it can easily be separated on either NaDodSO$_4$ (Fig. 1 slot E) or NaDodSO$_4$/urea/polyacrylamide gels (not shown).

Villin and α-Actinin Are Immunologically Different Proteins. Antibodies to villin were elicited in rabbits by injection of electrophoretically purified villin (see above). The resulting antivillin serum gave rise to a precipitin line in double-diffusion analysis with a low salt extract of microvillus cores (for the strong relative enrichment of villin in this extract see Fig. 1 slot C). The rabbit serum against highly purified chicken gizzard α-actinin (Fig. 1 slot G) was also tested by double-diffusion analysis and gave a precipiting line with gizzard α-actinin. Sera were further characterized by the very sensitive immune replica method of Saltzgaber-Müller and Schatz (17), which allows one to determine immunological reaction of polypeptides separated by electrophoresis in NaDodSO$_4$/polyacrylamide gels. By this technique, the antivillin serum was found to react specifically with villin and no other microvillus protein (Fig. 2 slot D). In addition, the serum did not react with purified α-actinin (Fig. 2 slot E). The anti-α-actinin serum, by contrast, showed the opposite specificity: it reacted with α-actinin but did not react with villin or any other microvillus protein (Fig. 2 slots G and H). These results are not limited to these two sera, since identical data were obtained with a second serum to NaDodSO$_4$-denatured villin as well as a serum to NaDodSO$_4$-denatured gizzard α-actinin and one to chicken skeletal muscle α-actinin. This lack of crossreactivity between villin and α-actinin within the same species (chicken) is not due to tissue specificity (smooth muscle α-actinin compared to intestinal epithelium villin), since in immune replica experiments the serum against chicken gizzard α-actinin specifically reacted with polypeptides of molecular weight 100,000 in a preparation of highly purified brush borders (Fig. 2 slot I). The antiserum reacts with two proteins of very similar molecular weight, which could either be a result of proteolysis of the α-actinin or reflect that nonmuscle α-actinin may be composed of two chains of slightly different molecular weights, as has been suggested (10). This sensitive technique detects α-actinin as a minor protein component of the brush border, as the autoradiograph does not correspond with major bands on the Coomassie blue-stained gel. It does not necessarily follow, however, that brush borders

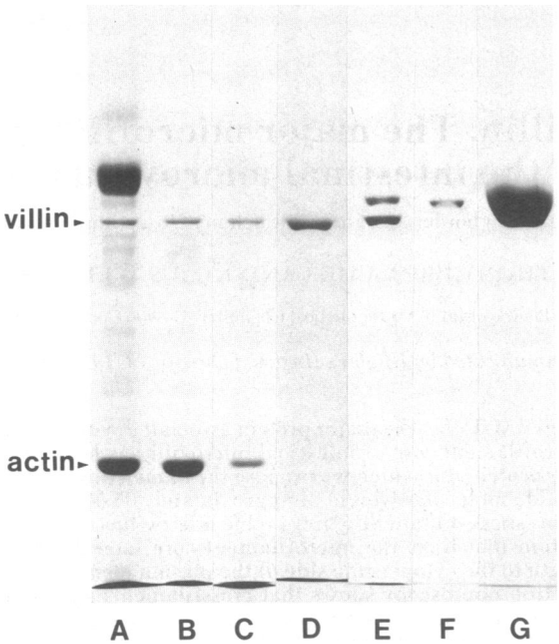

FIG. 1. 7.5% NaDodSO$_4$/polyacrylamide gels (20) of the fractions used to isolate villin, and a comparison of its electrophoretic mobility with that of muscle α-actinin. Slot A, purified intestinal microvilli. Slot B, microvillus cores obtained by treatment of pure microvilli with Triton X-100 in the presence of polyethylene glycol. Slot C, material extracted from cores by low salt buffer, supernatant fraction. Slot D, pure villin after preparative gel electrophoresis of the extract shown in slot C. Slot E, villin mixed with chicken gizzard α-actinin as a molecular weight marker (molecular weight 100,000); note the different electrophoretic mobilities. Slot F, chicken gizzard α-actinin, molecular weight marker. Slot G, 30 μg of chicken gizzard α-actinin to show the purity of the preparation used both as an antigen and to make the affinity column for the preparation of the α-actinin monospecific antibodies. Origin of the gels is at the top of the figure.

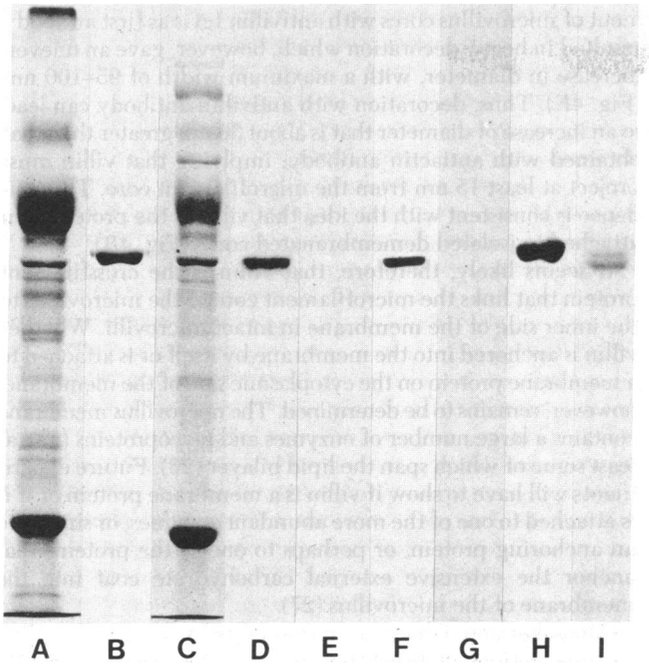

FIG. 2. Immune replicas showing the reaction between proteins separated by $NaDodSO_4$ gel electrophoresis and immune sera. Proteins were separated on a 7.5% $NaDodSO_4$/polyacrylamide gel and the gel was laid on agarose containing the appropriate serum. After incubation to allow the separated proteins to diffuse into the agarose, the agarose gel was washed and the precipitated immune complexes were labeled with ^{125}I-labeled protein A and detected by autoradiography of the dried gel (for details see ref. 17). Slots A–C, Coomassie blue-stained gel of separated microvillus proteins (A), chicken gizzard α-actinin (B), and purified brush borders (C). Identical gels were used to test the immunological reaction against antivillin serum (slots D–F) and anti-α-actinin serum (slots G–I). The autoradiographs (D–I) show that the antivillin serum reacts specifically with villin (D and F) but not α-actinin (E), whereas the anti-α-actinin serum does not react with any microvillus proteins (G) but does react with α-actinin (H) and minor proteins present in brush borders (I).

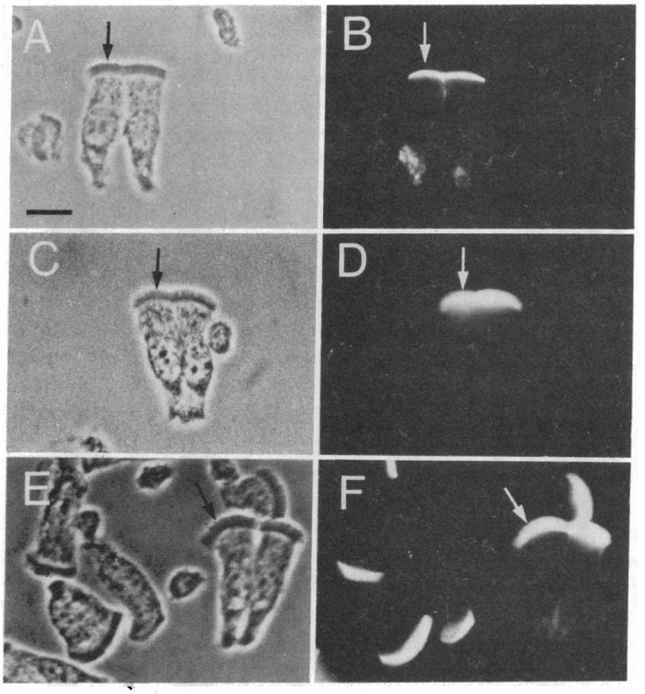

FIG. 3. Immunofluorescence microscopy on intestinal epithelial cells. Corresponding phase (*A*, *C*, and *E*) and fluorescence (*B*, *D*, and *F*) micrographs of cells stained with antibodies to α-actinin (*A* and *B*), villin (*C* and *D*), and actin (*E* and *F*). Arrows indicate the positions of the ends of microvilli in corresponding micrographs. All micrographs are at the same magnification; bar in *A* is 10 μm.

on intact cells contain very little α-actinin because it is quite likely that a substantial proportion of the α-actinin is lost during brush border isolation. Very recently (21), an independent report also documented the existence of a 100,000-dalton protein in chicken intestinal epithelial cells which is recognized by antibodies directed against chicken gizzard α-actinin. The apparent absence from the microvilli of a protein that cross-reacts with anti-α-actinin serum does not exclude the possibility that our purified microvilli also contain a very small, presently undetectable, amount of α-actinin; rather, it demonstrates that the villin in the microvilli and the α-actinin detected in whole brush borders are immunologically different proteins. However, it is still conceivable, as has been suggested (22), that villin and α-actinin may share some homology at the amino acid sequence level.

Further evidence for the lack of immunological crossreactivity between α-actinin and villin is provided by the different staining patterns obtained with antibodies to villin and α-actinin in immunofluorescence microscopy. Purified specific IgGs to villin and α-actinin were prepared by affinity column chromatography on Sepharose 4B to which the corresponding purified antigen was covalently linked. The resulting purified immune IgGs were used in immunofluorescence microscopy on mouse intestinal epithelial cells (13). Antibodies against α-actinin decorated the structures of the terminal web of the brush border but did not show any staining of the microvilli (Fig. 3 *A* and *B*). By contrast, antibodies against villin stained the microvilli, giving rise to a very intense fluorescence pattern (Fig. 3 *C* and *D*), very similar to the decoration seen when the microfilaments of the microvilli are decorated with antiactin antibody (Fig. 3 *E* and *F*). In addition, the staining pattern of mouse 3T3 tissue culture cells by these antibodies also demonstrated that villin and α-actinin are immunologically different proteins. The antibody against α-actinin revealed the typical distribution of α-actinin along stress fibers and in the region of their attachment point to the membrane, as previously reported by others (10) and ourselves (23). The villin antibody, however, did not show any decoration of these structures.

Thus, since villin and α-actinin show no immunological crossreaction and antibodies to these proteins decorate different structures in immunofluorescence microscopy, the immunological data prove that villin and α-actinin are different proteins. Therefore, the inability of α-actinin-specific antibodies to stain microvilli is not due to the inaccessibility of the 95,000-dalton protein in the microvillus to the antibodies, but is clearly due to the fact that α-actinin and villin are different proteins. Furthermore, the different immunofluorescence staining patterns obtained by antibodies to villin and α-actinin in the brush border and tissue culture cells suggest that these proteins may perform rather different skeletal functions.

Is Villin the Crossfilament Protein of Intestinal Microvillus? The crossfilaments seen in thin sections of intact microvilli linking the microfilament core to the membrane (Fig. 4*A*) can clearly be seen to remain attached and projecting from the isolated demembranated microvillus core (Fig. 4*B*). These crossfilaments are regularly spaced longitudinally about 33 nm apart down the core (refs. 3 and 12; Fig. 4 *A* and *B*).

Two lines of evidence suggest that villin may be the crossfilament protein seen attached to the demembranated microvillus core. The first is that villin, the major protein associated with actin in the microvillus core (see Fig. 1 slot B), is present in

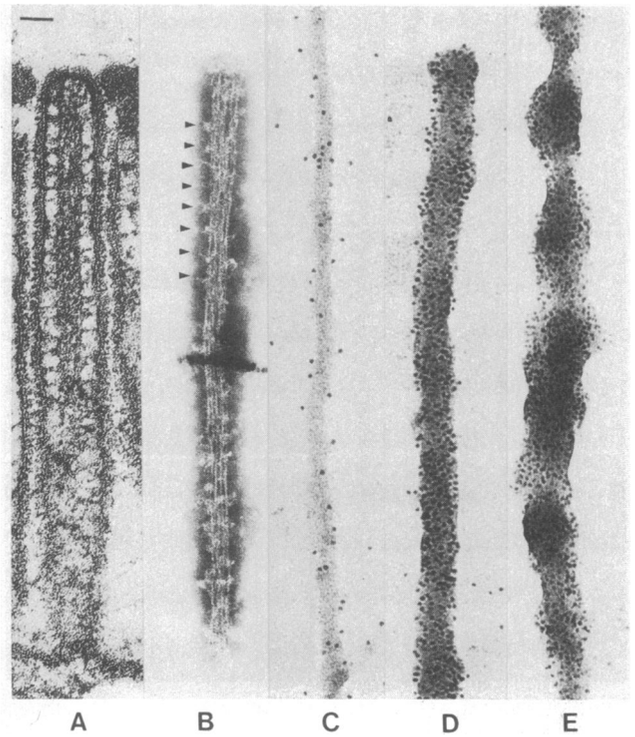

FIG. 4. (*A*) Thin section of an intestinal microvillus from an intact piece of chicken intestine. Crossfilaments are clearly visible. (*B*) Demembranated microvillus core negatively stained with 2% phosphotungstic acid. Note the regular crossfilaments (arrows). (*C–E*) Microvillus cores decorated with rabbit IgGs followed by ferritin-labeled sheep anti-rabbit IgGs; first antibody is nonimmune IgGs (*C*), actin IgGs (*D*), or villin IgGs (*E*). All micrographs are at the same magnification; bar is 50 nm.

approximately the abundance expected for the number of crossfilaments present. Since the crossfilaments are spaced every 33 nm, which is about every five actin dimers, along the microfilaments on the outside of the core bundles, one would expect the crossfilament protein to be present in a molar ratio of 1:10 with actin monomers along microfilaments that are linked to the membrane. The microvillus core contains about 20 hexagonally packed microfilaments (6), of which approximately two thirds are on the outside of the core and could be linked to the membrane. Therefore, the polypeptide molar ratio expected for the crossfilament protein to monomeric actin is in the region of 1:15 (if the crossfilament protein is a monomer) and 1:7.5 (if it is a dimer). The finding that the molar ratio of villin to actin in the microvillus core was about 1:10 (ref. 12; see also Fig. 1 slot B), shows that villin is present in approximately the right amount to be the crossfilament protein.

The second line of evidence stems from the decoration of microvillus cores seen after treatment with rabbit antibodies to either actin or villin, followed by ferritin-labeled sheep anti-rabbit IgG antibodies (Fig. 4 *D* and *E*). The electron micrographs show that this method is very specific because treatment of cores with nonspecific, instead of specific, IgGs gave rise to only a very low nonspecific background decoration (Fig. 4*C*). However, treatment with antiactin IgGs as first antibody led to substantial uniform decoration down the length of the core which increased the diameter from 33–47 nm to 63–75 nm (Fig. 4*D*). The 30-nm diameter increase in width by the two layers of antibodies on either side of the core is in agreement with that reported for the increase in diameter of a microtubule after addition of two layers of antibody around the circumference of the tubular structure (24). Similar treatment of microvillus cores with antivillin IgGs as first antibody resulted in heavy decoration which, however, gave an uneven increase in diameter, with a maximum width of 95–100 nm (Fig. 4*E*). Thus, decoration with antivillin antibody can lead to an increase of diameter that is about 30 nm greater than that obtained with antiactin antibody, implying that villin must project at least 15 nm from the microfilament core. This evidence is consistent with the idea that villin is the protein seen attached to isolated demembranated cores (Fig. 4*B*).

It seems likely, therefore, that villin is the crossfilament protein that links the microfilament core of the microvillus to the inner side of the membrane in intact microvilli. Whether villin is anchored into the membrane by itself or is attached to a membrane protein on the cytoplasmic side of the membrane, however, remains to be determined. The microvillus membrane contains a large number of enzymes and glycoproteins (25), at least some of which span the lipid bilayer (26). Future experiments will have to show if villin is a membrane protein or if it is attached to one of the more abundant enzymes, or simply to an anchoring protein, or perhaps to one of the proteins that anchor the extensive external carbohydrate coat into the membrane of the microvillus (27).

Mooseker and Tilney (3) have reported occasionally seeing in thin sections of brush borders a protein that appears to crosslink adjacent F-actin filaments. We have no data on whether these crosslinks are composed of villin or another, relatively minor, microfilament-associated protein of the microvillus.

Immunofluorescence microscopy revealed that villin is not found in the bundles of microfilaments typical for some tissue culture cells nor is it found located where microfilament fibers are attached to the membranes. Indeed, we know only that it is present in the microvillus of the brush border. Therefore it will be interesting to determine whether villin is also a constituent of the more transient microvilli found on tissue culture cells or whether it is a highly specialized protein restricted to the permanent microvilli of structures like the brush border of the intestinal epithelium.

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