

Partial Reconstruction of the Microvillus Core Bundle: Characterization of Villin as a Ca^{++} -dependent, Actin-bundling/depolymerizing Protein

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ABSTRACT The brush border, isolated from chicken intestine epithelial cells, contains the 95,000 relative molecular mass (M_r) polypeptide, villin. This report describes the purification and characterization of villin as a Ca^{++} -dependent, actin bundling/depolymerizing protein. The 100,000 g supernatant from a Ca^{++} extract of isolated brush borders is composed of three polypeptides of 95,000 (villin), 68,000 (fimbrin), and 42,000 M_r (actin). Villin, following purification from this extract by differential ammonium sulfate precipitation and ion-exchange chromatography, was mixed with skeletal muscle F-actin. Electron microscopy of negatively stained preparations of these villin-actin mixtures showed that filament bundles were present. The viscosity, sedimentability, and ultrastructural morphology of filament bundles are dependent on the villin:actin molar ratio, the pH, and the free Ca^{++} concentration in solution. At low free Ca^{++} ($<10^{-6}$ M), the amount of protein in bundles, when measured by sedimentation, increased as the villin: actin molar ratio increased and reached a plateau at approximately a 4:10 ratio. This behavior correlates with the conversion of single actin filaments into filament bundles as detected in the electron microscope. At high free Ca^{++} ($>10^{-6}$ M), there was a decrease in the apparent viscosity in the villin-actin mixtures to a level measured for the buffer. Furthermore, these Ca^{++} effects were correlated with the loss of protein sedimented, the disappearance of filament bundles, and the appearance of short fragments of filaments. Bundle formation is also pH-sensitive, being favored at mildly acidic pH. A decrease in the pH from 7.6 to 6.6 results in an increase in sedimentable protein and also a transformation of loosely associated actin filaments into compact actin bundles. These results are consistent with the suggestions that villin is a bundling protein in the microvillus and is responsible for the Ca^{++} -sensitive disassembly of the microvillar cytoskeleton. Thus villin may function in the cytoplasm as a major cytoskeletal element regulating microvillar shape.

The brush border microvillus is a model biological system for investigations aimed at understanding how a cell maintains its shape. Ultrastructural studies have shown that the microvillus contains a core of bundled microfilaments which extend into the underlying terminal web (44, 45). This core bundle is associated with the overlying plasma membrane by two types of attachments. The first type laterally connects the membrane along the length of the bundle by means of cross filaments (35, 39, 44, 45). In the second type of membrane attachment, the microfilaments insert end-on into the electron-dense cap at the tips of the microvilli. Earlier studies had suggested that both

types of attachments as well as the bundling protein in the microvillus core were composed of alpha-actinin (3, 44, 48). However, recent studies have shown instead that alpha-actinin is not a structural component of the microvillus but rather is a component of the terminal web (7, 16, 21, 43).

Biochemical studies have shown that demembrated microvillar cytoskeletons contain polypeptides with relative molecular masses, M_r , of 110,000, 95,000 (villin), 68,000 (fimbrin), 42,000 (actin), and 17,000 (calmodulin) (9, 15, 36, 44). The proposed structural roles of these proteins in the cytoskeleton were determined by experiments which selectively released

different cytoskeletal components from the microvillus. Changes in the ultrastructure of the microvillar cytoskeleton were correlated with changes in the polypeptide composition and led to the suggestion that the 110,000- M_r polypeptide is a component of the cross filaments, while villin and/or fimbrin were bundling components in the core bundle (35). This was in contrast to the evidence reported by Bretscher and Weber (8) that the cross-filaments were composed of villin. Recently, the demembrated cytoskeleton was shown to be extremely sensitive to Ca^{++} . Greater than micromolar levels of free Ca^{++} in solution caused disassembly of the bundle filaments (22, 28). However, the Ca^{++} -sensitive agent in microvilli was not identified.

To identify both the actin bundling protein and the Ca^{++} -sensitive factor in the microvillus, villin was isolated from preparations of chicken intestine brush borders and examined for its actin binding properties in the presence and absence of Ca^{++} . During the course of these studies, several labs independently reported the isolation and characterization of villin as a Ca^{++} -dependent, actin binding protein (10, 17, 22, 42). While the present results are in general agreement with their findings, we describe here the characteristics of this Ca^{++} -dependent, actin-bundling/depolymerizing phenomenon and identify several of the important factors which affect it. A preliminary report of this work has been published previously (37).

MATERIALS AND METHODS

Isolation of Brush Borders

Epithelial cells were collected from chicken intestines as described earlier (35). Brush borders were isolated from these cells using the protocol outlined by Mooseker et al. (41), with the modification that the pH of the solutions was adjusted to 6.9. The proteolytic inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to all preparations at a final concentration of 0.1 mM.

Isolation of Villin

The brush borders from three hens were resuspended in 20 ml of a solution containing 75 mM KCl, 1 mM $Ca(OH)_2$, and 10 mM TrisHCl, pH 7.5, at room temperature for 30 min. The Ca^{++} -extracted brush borders were sedimented at 100,000 g for 15 min in a type 65 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 4°C. The supernatant of this extract was sequentially fractionated by 0/20%, 20%/50%, and 50%/70% ammonium sulfate cuts with a saturated solution of ammonium sulfate (Schwarz-Mann, Orangeburg, NY). After 30 min on ice, each fraction was collected by centrifugation at 10,000 g for 10 min. The 20%/50% fraction was resuspended in Ca^{++} buffer (0.1 mM $Ca(OH)_2$ and 10 mM TrisHCl pH 7.5) and desalted through Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). The void volume was collected and located on a DEAE-Sephacel (Pharmacia Fine Chemicals) column (0.9 cm \times 5 cm) which was previously equilibrated with the Ca^{++} buffer. After the A_{280} of the column buffer returned to baseline, the column was eluted with 200 ml of a 0.0–0.3 M NaCl linear gradient containing 0.1 mM EGTA and 10 mM TrisHCl, pH 7.5. The salt concentration was monitored by measuring the conductivity of the fractions. The fractions containing villin were detected by SDS gel electrophoresis on a microslab gel apparatus (34). The fractions comprising the villin peak were simultaneously dialyzed and concentrated to ~0.3 ml against appropriate solutions by vacuum dialysis (Micro-Pro Di Con model 315; Bio-Molecular Dynamics, Beaverton, OR). Villin was used without further purification and stored at 4°C for as long as 1 wk without loss of bundling activity. Brush borders from three chickens yield ~1.5 mg of villin in this 2-d procedure.

Isolation of Actin

Actin was isolated from an acetone powder of chicken breast muscle by the method of Spudich and Watt (51) and stored at -70°C as G-actin.

Characterization of Bundling

All experiments were carried out at 2°C in 1.5-ml microfuge tubes. The

bundling solution consisted of 75 mM KCl, 5 mM $MgCl_2$, 0.1 mM ATP, and 10 mM imidazole, at pH 6.9. Actin and villin (expressed in this report as their actual molar ratios in micromoles of actin to micromoles of villin) were incubated at 28°C for at least 12 h, after which 150- μ l samples were withdrawn for various analyses. The apparent viscosity of 0.6-ml samples was measured in Cannon-Manning semi-micro viscometers (100) (Cannon Instrument Co., State College, PA) which were calibrated previously with sucrose solutions of known viscosities (ISCO Tables; Instrument Specialties Co., Lincoln, NE). For electron microscopy, 5- μ l samples were placed on 300-mesh copper grids covered with thin carbon films and negatively stained with 0.5% uranyl acetate (pH 4.1). For the sedimentation assays, samples were either centrifuged for 5 min at 10,000 g in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 8°C or centrifuged for 20 min at 30 psi (~180,000 g) in an airfuge (Beckman Instruments, Inc.) at 2°C. The supernatants were carefully withdrawn and the pellets resuspended to 150 μ l. The amount of protein in both the supernatants and the pellets was assayed by the Schacterle and Pollack (46) modification of the Lowry method (33).

pH Dependence

To minimize the pH contribution of the dialysis buffer in the following experiments, villin and actin were dialyzed against the bundling solution containing 1 mM of Imidazole at pH 7.0 and then reconstituted at a 1:2 molar ratio. The mixture was then diluted with the standard bundling solution whose pH was adjusted with concentrated HCl to 6.6, 6.8, 7.0, 7.2, 7.4, or 7.6. The final pH of the dilution solutions did not deviate from its preadjusted levels. In different experiments, the final concentration of actin was varied from 2.5 μ M to 10 μ M.

Ca⁺⁺ Dependence

Villin and actin, previously dialyzed against bundling buffer, were mixed in a 5:10 molar ratio. The Ca:EGTA ratio was varied by addition of $Ca(OH)_2$ from a 8.713-mM stock solution, pH 7.0, whose Ca^{++} concentration was measured by atomic absorption. Ca^{++} was added to 0.2, 0.15, 0.1, 0.08, 0.05, 0.04, 0.02, and 0.003 mM to bundling buffer with 0.1 mM EGTA to yield the pCa^{++} shown in Fig. 11. The free Ca^{++} in solution was calculated using a program written by Dr. Peter Chantler (Brandeis University) and expressed as pCa . This program takes into consideration the temperature, pH, Mg^{++} , EGTA, and ATP which all affect the level of free Ca^{++} in solution.

Villin:Actin Molar Dependence

Villin and actin were dialyzed against bundling buffer and mixed to give a final molar ratio ranging from 0:10–10:10 μ M villin: μ M actin.

SDS Gel Electrophoresis

Samples were analyzed on microslab gels poured with a 5–20% gradient of acrylamide and a 3–6 M gradient of urea (13) following the method described by Matsudaira and Burgess (34).

Electron Microscopy

Samples were processed for thin sectioning as described earlier (35). Silver sections were cut with a diamond knife on a Sorvall MT-5000 ultramicrotome (DuPont Instruments–Sorvall Biomedical Div., DuPont Co., Newtown, CT) and examined on a JEOL 100CX operated at 80 kV.

RESULTS

Purification of Villin

Extraction of isolated brush borders (Fig. 1a) with solutions containing 1 mM $Ca(OH)_2$ results in an apparent loss in the organization of the cytoskeleton and a uniform shortening of the microvilli (Fig. 1b and c). In the distal portions of the microvilli where underlying filaments are absent, the membrane appears vesiculated while in the basal portion of the same microvilli the core bundles remain relatively intact. In the terminal web the rootlets of the microvilli appear disrupted. After these extracts of brush borders are sedimented, three polypeptides remain in the supernatant: villin, fimbrin, and actin (Fig. 2, slot 2). The villin and actin coprecipitate in the 20%/50% ammonium sulfate fraction (Fig. 2, slot 3) and are

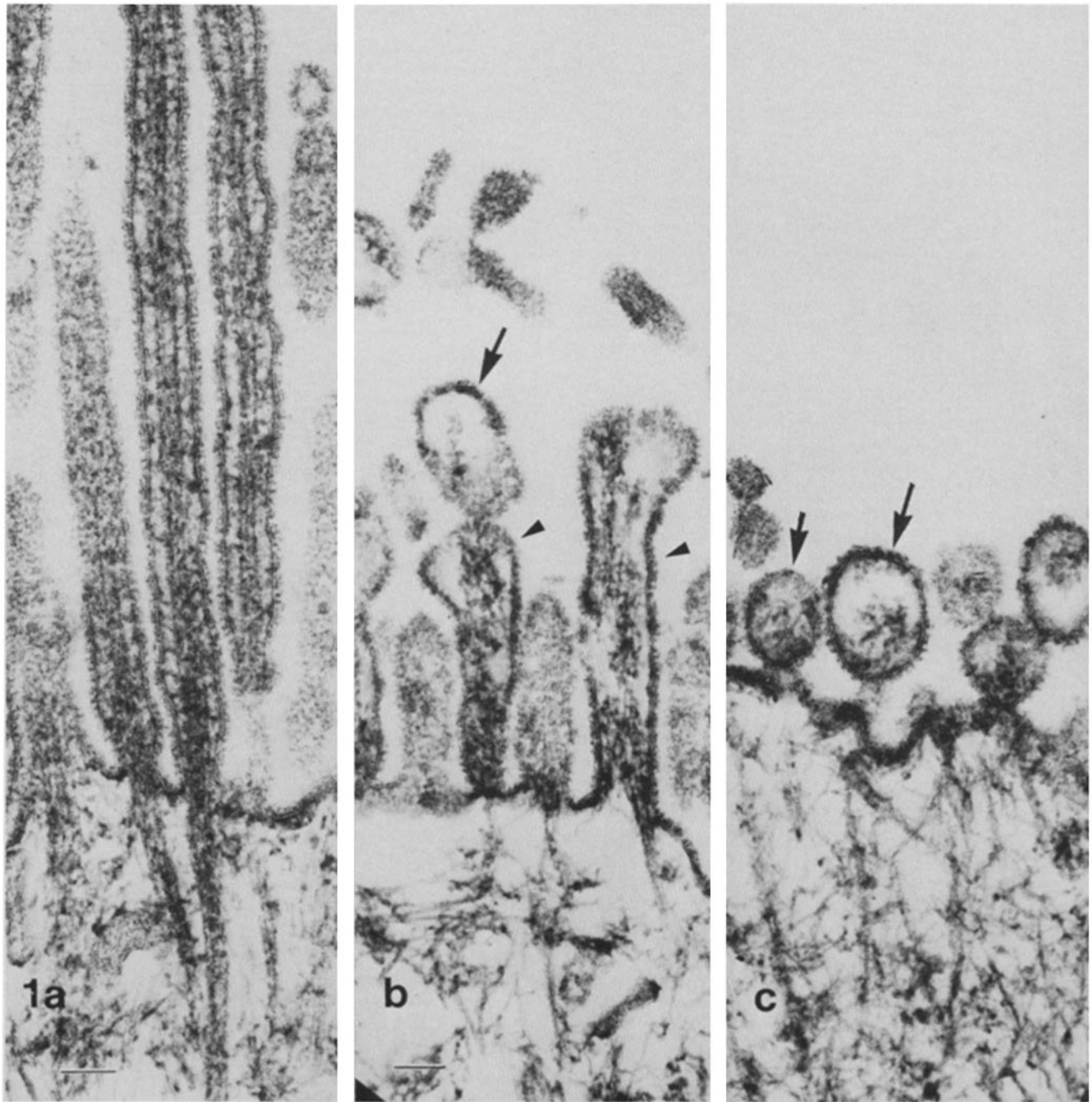


FIGURE 1 The effect of Ca^{++} on the integrity of the isolated brush border cytoskeleton. (a) The microfilaments remain bundled within the long microvilli (the tips are not shown) and extend into the underlying terminal web. (b and c). When incubated in 1 mM Ca^{++} for $\frac{1}{2}$ h at room temperature, there are drastic alterations in the morphology of the brush borders. The microvilli are shorter, with vesicle-like membrane blebs at the distal tips (arrows). The cytoskeleton within these blebs is disordered when compared to the more basal portions of the same microvilli (arrowheads). Bar, 100 nm. (a) $\times 90,000$. (b) $\times 84,000$. (c) $\times 84,000$.

free from contamination by fimbrin which is collected in the 50%/70% fraction (Fig. 2, slot 4). When the resulting villin-actin mixture is chromatographed as DEAE-Sephacel in the presence of 0.1 mM EGTA, villin elutes as a sharp peak at 75 mM NaCl while the actin elutes at 150 mM NaCl (Fig. 3). Gel electrophoresis of these fractions shows that the villin and the actin are separated without any detectable cross-contamination (Fig. 2, slots 5 and 6). No cross-contamination is evident even on overloaded gels. Approximately 0.5 mg of villin is isolated from the small intestine of each chicken. The villin isolated by this 2-d procedure was $>95\%$ pure as estimated from SDS gels.

Dependence of Bundle Formation on the Villin: Actin Ratio

Previous studies of fascin, the bundling protein isolated from extracts of sea urchin eggs, had demonstrated that bundle formation was complete at a fascin:actin molar ratio of 1:4 and at this ratio the actin binding sites for fascin were saturated (11). With this in mind, villin was tested for its ability to bundle actin by varying the villin:actin molar ratio. When the Ca^{++} concentration in solution is $<10^{-6}$ M the extent of bundle formation is strictly dependent on the villin:actin molar ratio.

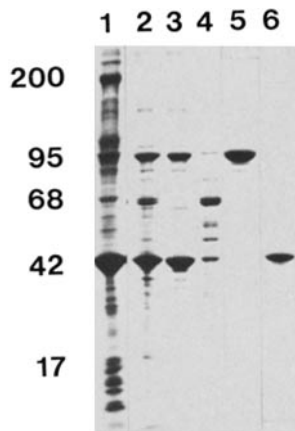


FIGURE 2 The polypeptide composition of the different extracts during villin purification analyzed on SDS microslab gels (5–20% gradient of polyacrylamide and a 3–6 M gradient of urea). (Lane 1). Triton X-100-demembrated brush borders with the M_r of myosin (200,000), villin (95,000), fimbrin (68,000), actin (42,000), and calmodulin (17,000) indicated for reference. The numbers on the figure are $\times 10^3$. Supernates from the extracts of Ca^{++} -treated brush borders (lane 2) are composed of villin, fimbrin, and actin. Villin and actin coprecipitate in the 20%/50% ammonium sulfate fraction (lane 3) while fimbrin is highly enriched in the 50%/70% ammonium sulfate fraction (lane 4). Villin elutes in the 75 mM salt peak (lane 5) from DEAE-Sephacel while actin elutes in the 150-mM salt peak (lane 6) from the same column.

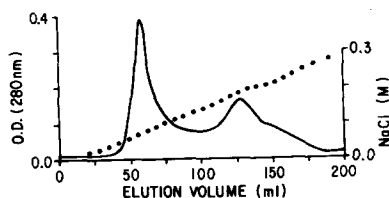


FIGURE 3 The elution profile (solid line) of the 20%/50% ammonium sulfate fraction from a DEAE-Sephacel column when developed with a 0–0.3 M NaCl salt gradient (dotted line). Two peaks appear at 75 mM NaCl and are composed of villin (Fig. 2, lane 5) and actin (Fig. 2, lane 6), respectively. The elution buffers contain 10 mM Imidazole, pH 7.0, and 0.1 mM EGTA.

In the absence of villin, actin filaments do not form bundles under the conditions tested (Fig. 4*a*), although there is some unexplained aggregation or lateral association. However, when purified villin is mixed with F-actin in the presence of 0.1 mM EGTA, large bundles of filaments are formed (Fig. 4). As the amount of villin added to 10 μM F-actin increases to a 2:10 ratio, large arrays of filaments become aligned laterally with one another (Fig. 4*b*). Incubation of villin with F-actin at ratios of 5:10 (Fig. 4*c*) and 10:10 (Fig. 4*d*) results in most of the filaments becoming closely aligned into bundles. Correlated with bundle formation is a decrease in the number of free filaments seen in negative stain (Fig. 4).

Attempts were made to establish the arrangement of villin with respect to actin in the bundles. In previous studies with fascin a distinct 11-nm periodic striation was seen in the fascin-actin bundles (11). In negatively stained samples of villin-actin preparations, distinct cross-links are occasionally seen spanning the distance between adjacent filaments (arrows, Fig. 5*a* and *b*). The distance between cross-links varies between 6 and 45 nm, with the majority measuring between 16 and 24 nm. However, a clear periodicity or a paracrystalline striation in

the bundles was not obvious.

Sedimentation was used as a quantitative measure for bundle formation. Sedimentation in a microfuge can be used as a measure of bundle formation because the microfuge produces a force sufficient to sediment actin bundles but not single actin filaments (J. Bryan, personal communication). This assay detected very little protein sedimented at low villin:actin ratios (<2:10). An increase in the villin:actin ratio to 4:10 results in an increase in sedimentable protein. At ratios >4:10 the amount of protein sedimented remained relatively constant (Fig. 6). When assayed by sedimentation in an airfuge, bundle formation displays very different results (Fig. 7). Addition of villin to 10 μM F-actin resulted in an initial increase and a leveling off in the amount of protein sedimented. The villin:actin ratio at which the protein in the pellet reached this plateau varies in different experiments from 3:10 to 5:10 in both the microfuge and airfuge assays. Beyond a 5:10 ratio, addition of villin to actin results in a proportional increase in the amount of protein found in the supernatant for both assays. The plateau represents a quantitative measure of the extent of bundle formation and is in agreement with the electron microscopy evidence that bundle formation is maximal at approximately a 4:10 villin:actin role.

The viscosity of the villin:actin mixtures was used to monitor bundle formation. The viscosity of the villin-actin mixtures exhibited a very complex dependence on the villin:actin ratio (Fig. 8). Increasing the villin:actin ratio to 2:10 results in a 50% decrease in the apparent viscosity of the bundles when compared to the value measured for F-actin (Fig. 8). This decrease in apparent viscosity was not due to F-actin depolymerization since both electron microscopy and sedimentation studies showed the presence of filaments and sedimentable protein. Instead, the decrease in viscosity can be explained by polymer theory (53) as a result of cross-linking single actin filaments into actin filament bundles. Further increases in the ratio result in an increase in the apparent viscosity of the mixtures. At ratios >5:10 (Fig. 8) the viscosity remains constant. A possible explanation for this subsequent increase in viscosity could be explained by postulating that the bundles become rigid at villin:actin ratios >2:10 and therefore impede the flow of the mixture through the capillary portion of the viscometer. Electron microscopy of the bundles suggests that this situation may occur since samples after measurement in the viscometer are sheared to shorter lengths as monitored by negatively stained samples.

Ca^{++} Dependence of Bundling

Raising the calcium level in the solution to $>10^{-6}$ M causes depolymerization of actin filaments and disassembly of actin bundles within 2 min after addition of Ca^{++} . In the electron microscope, short fragments of filaments were observed at villin:actin ratios of 2:10 (Fig. 9*a*) and 0.5:10 (Fig. 9*b*) instead of actin filaments or filament bundles. The fragments formed from a 2:10 ratio appeared shorter than those formed from a 0.5:10 ratio. These observations were correlated with viscometric studies which measured a decrease in the apparent viscosities of the villin-actin mixtures to a level measured for the buffer alone (Fig. 8). Others (9, 42) have reported that this disassembly of the bundles is reversed by removal of Ca^{++} and their results suggested that the Ca^{++} -induced disassembly of the bundles is not caused by Ca^{++} -activated proteases.

The Ca^{++} sensitivity of the villin-actin bundles was compared to the reported values for the Ca^{++} -induced disassembly

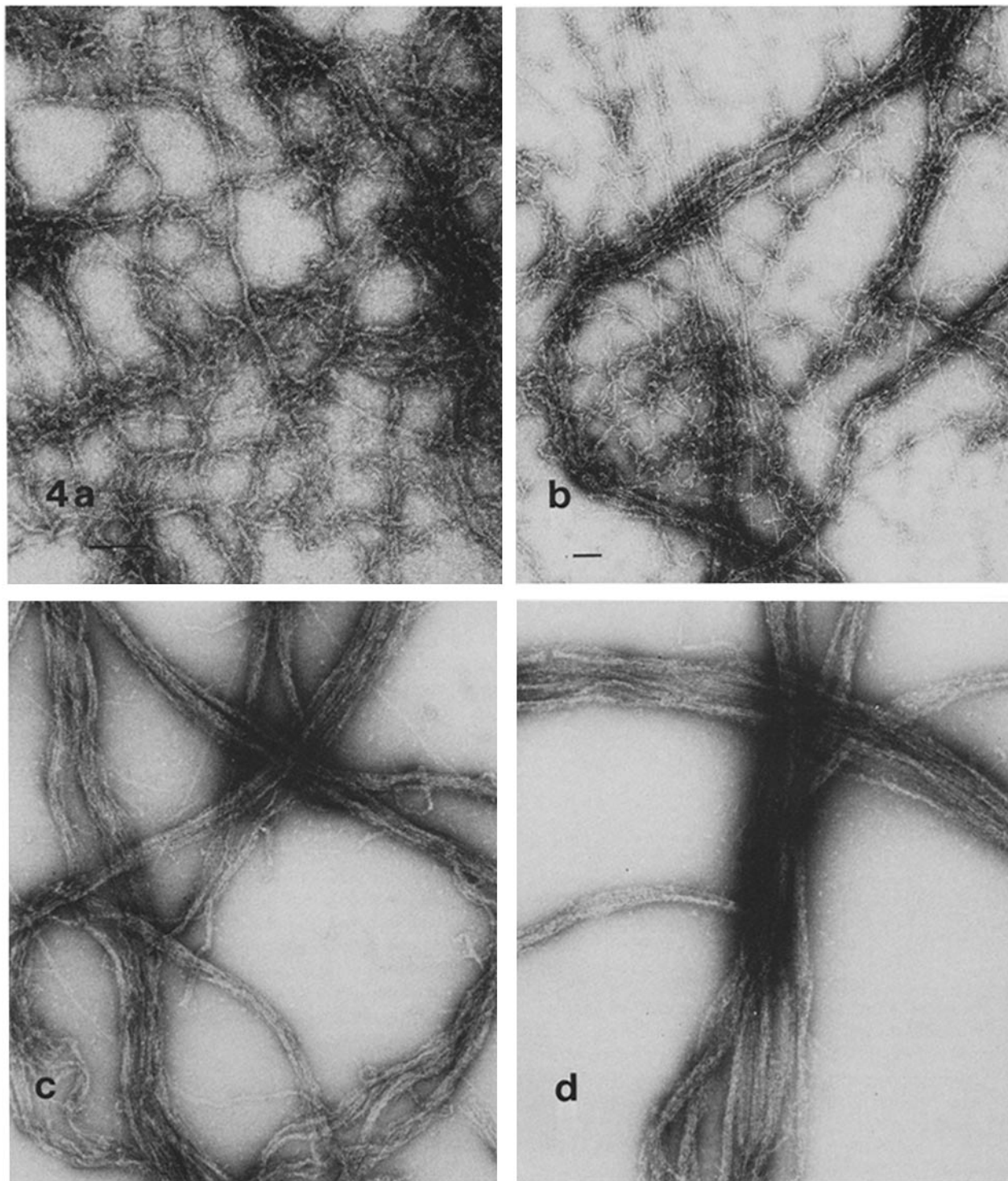


FIGURE 4 The dependence of bundle formation on the villin:actin ratio. At a 0:10 ratio, F-actin alone (Fig. 4 *a*) is loosely arranged on the grid without formation of distinct bundles. As the villin:actin ratio increases to 2:10 (Fig. 4 *b*), a few bundles of filaments appear. These bundles appear composed of loosely associated filaments. At saturating conditions, villin:actin = 5:10 (*c*), 10:10 (*d*), the lack of any single actin filaments suggests that most of the filaments are recruited into bundles. The bundles are very straight and ordered in appearance. Actin was present at $10\ \mu\text{M}$ in all these preparations. Bar, 100 nm, (*a*) $\times 97,500$, (*b*, *c*, and *d*) $\times 51,000$.

of the microvillar cytoskeleton (22, 28). Bundle formation by villin-actin mixtures exhibits a sigmoidal dependence on the free Ca^{++} concentration in solution when measured by viscometry or sedimentation. Bundles disassembled and filaments depolymerized at Ca^{++} concentrations $>10^{-6}$ M. At free Ca^{++} concentrations $<10^{-6}$ M, actin filaments were organized as

bundles. The $\text{max}_{1/2}$ for this Ca^{++} effect was 1.12×10^{-6} at pH 6.9 (Fig. 10).

pH Dependence of Bundling

The ultrastructural morphology of the bundles appears de-

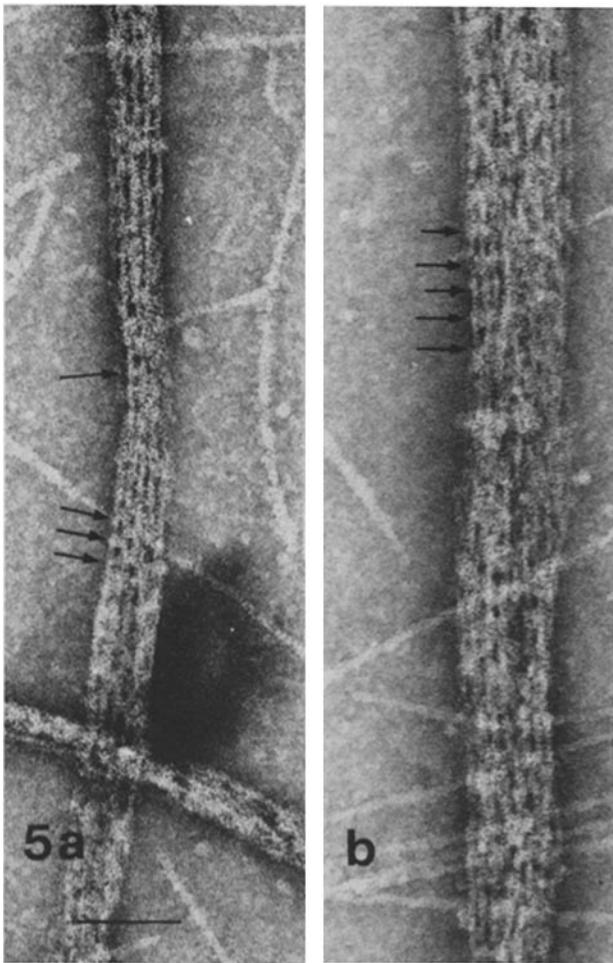


FIGURE 5 At higher magnifications, distinct cross-links are seen between adjacent filaments within a bundle. The bundles were reconstituted from a 2.5:5 mixture of villin and actin. Bar, 100 nm. $\times 135,300$.

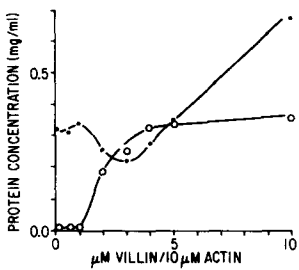


FIGURE 6 The dependence of protein sedimented in the microfuge on the villin:actin ratio. The amount of protein found in the pellet (circles) follows a sigmoidal curve and reaches a plateau at approximately a 4:10 ratio. The excess protein in the supernatants (dots) increases linearly from a 3:10 ratio after an initial decrease.

pendent on the pH of the solution. Bundles assembled at pH 6.6 (Fig. 11 a) or 6.9 (Figs. 4 and 5) appear composed of many tightly packed filaments. There are few free filaments, indicating that most of the filaments are recruited into bundles. In comparison, the bundles assembled at pH 7.6 (Fig. 11 b) generally have fewer filaments parallel with one another, and filaments are not so closely spaced within a bundle as those formed at the acidic pHs. Correlated with the ultrastructural observations is a dependence of the amount of protein sedimented on the pH at which the bundles are formed. When assayed with the microfuge or the airfuge, the amount of sedimentable protein characteristically decreases as the pH increases (Fig. 12) and approaches the quantity sedimented in actin controls (dashed line). In the control experiments, there

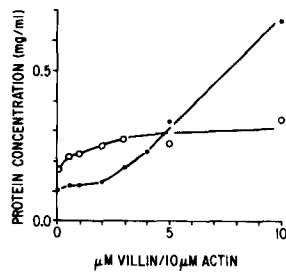


FIGURE 7 The dependence of protein sedimented in the airfuge on the villin:actin ratio. Under these conditions, F-actin filaments are pelleted and, as the villin:actin ratio increases, there is a linear increase in the protein in the pellet (circles) which reaches a plateau at a 3:10 ratio. The amount of protein in the supernatant (dots) remains initially constant, then increases linearly at ratios $>2:10$.

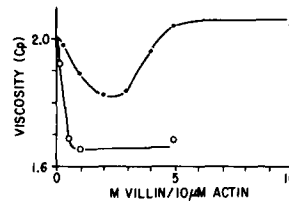


FIGURE 8 The influence of both the villin:actin ratio and the free Ca^{2+} in on the apparent viscosity. At free Ca^{2+} ($<10^{-6} M$) (dots), there is a reproducible drop in the apparent viscosity of the mixtures to a level midway between the viscosity of the F actin (2.0 centipoise [Cp]) and the buffer (1.65 Cp) as the villin:actin ratio increases from 0:10 to 2:10. With a subsequent increase in the villin:actin ratio, the apparent viscosity increases to a value slightly higher than for F-actin and then remains constant. When the free Ca^{2+} in solution is $>10^{-6} M$ (circles), there is a sharp and dramatic decrease in the apparent viscosity as the ratio increases to 0.5:10. The $max_{1/2}$ of this drop is approximately at 0.25:10. At ratios $>0.5:10$, the viscosity remains constant at levels measured for the buffer alone.

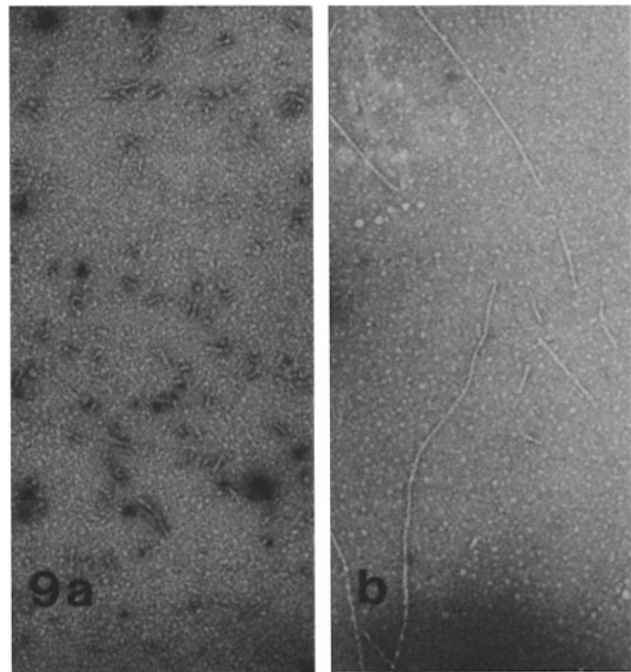


FIGURE 9 If the villin-actin mixtures are incubated in the presence of $Ca^{2+} >10^{-6} M$, only fragments of filaments are now detected. Compare to Figs. 4 and 5. The length of the fragments appears qualitatively to be affected by the villin:actin ratio (Fig. 10 a = 2:10, Fig. 10 b = 0.5:10) as seen in negative stain. Bar, 100 nm. $\times 73,100$.

is a slight decrease in F-actin sedimented in the airfuge (dashed line, Fig. 12), indicating that F-actin polymerization is not affected greatly over the same pH range.

DISCUSSION

Our results complement and extend the recently reported ob-

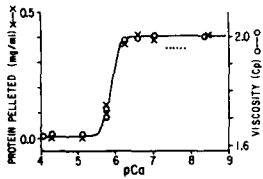


FIGURE 10 Bundling as measured by sedimentation in the microfuge (X) or the apparent viscosity (O-O) shows a sigmoidal dependence on the free Ca^{++} in solution. At pH 6.9, the $\text{max}_{1/2}$ is at pCa 5.96 or 1.12 μM . At $\text{Ca}^{++} < 10^{-6}$ M, the apparent viscosity is slightly greater than that measured for F-actin (dotted line). At $\text{Ca}^{++} > 10^{-6}$ M, the viscosity drops to approximately buffer levels and very little protein is pelleted. The villin:actin ratio was held at 5:10.

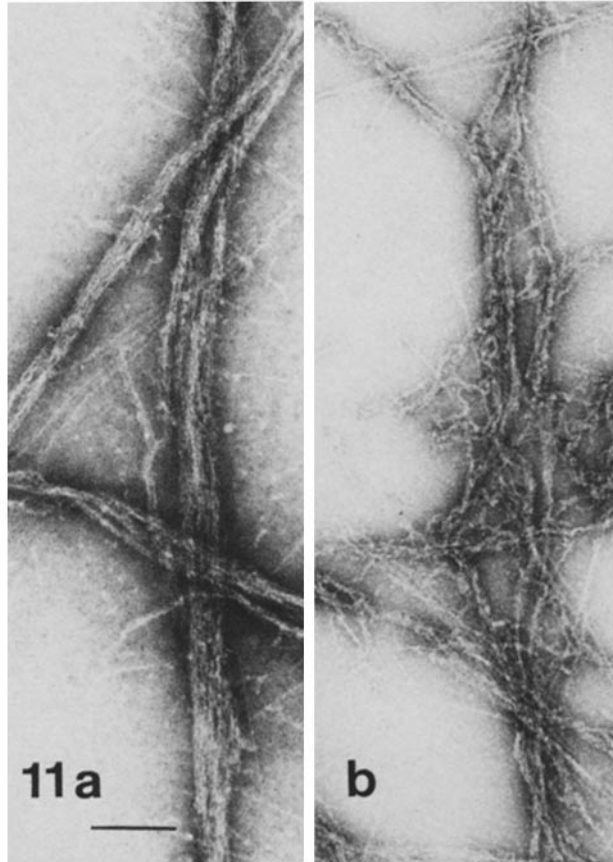


FIGURE 11 The morphology of the bundles is dependent on the pH at which they are assembled. At pH 6.6 (a), most of the filaments are organized into bundles. In comparison, at pH 7.6 (b), the filaments are loosely associated as bundles with many free filaments visible. The villin:actin ratio is 5:10. Bar, 200 nm. $\times 56,000$.

servations of other labs on villin-actin interactions (10, 17, 22, 42). Here we identified the factors which affect villin-actin interactions in order to establish the optimal conditions for studying the formation of villin-actin bundles. This report, as well as others (10, 22, 42), demonstrates that the level of Ca^{++} in solution must be carefully controlled. If the free Ca^{++} in solution is $> 10^{-6}$ M, then villin acts as an actin depolymerizing protein rather than an actin filament bundling protein. This Ca^{++} effect is possibly a reason why Craig and Powell (17) were unable to detect bundles of filaments in their preparations since their solutions were not buffered for Ca^{++} . The pH also affects bundle formation. If the pH is < 7.0 , then the villin-actin preparations form well ordered bundles compared to the bundles formed at the higher pHs. In other studies (9, 42) bundle formation was studied at pHs ranging from 7.3 to 7.8. Whether this pH difference with respect to the more optimal low pH conditions (6.8–7.0) would alter the results of kinetic

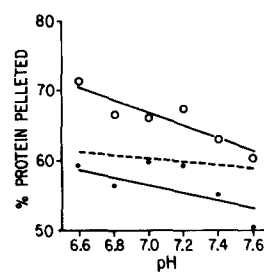


FIGURE 12 The amount of protein found in the pellet is dependent on the pH of the solution. As the pH increases from 6.6 to 7.6 the amount of protein sedimented in the microfuge (dots) or the airfuge (circles) decreases. At pH 7.6, the amount of sedimented protein approaches the level measured for F-actin alone (dashed line). The villin:actin ratio is 5:10.

studies (42) should be reinvestigated. We also demonstrate here that the amount of villin in relation to actin also influences the formation of bundles. Although other labs have studied villin-actin interactions at villin:actin ratios $< 1:30$ (17, 42), bundle formation should be studied under saturating conditions (villin:actin = 5:10), otherwise subtle variation in the villin:actin ratio could have a profound effect on the rate or extent of bundle formation. Finally, we point out here the problems associated with the use of viscometry to measure bundle formation. As pointed out by the results, the viscosity of villin-actin mixtures formed in the absence of Ca^{++} behaves anomalously at villin:actin ratios $> 2:10$. This variation in viscosity places doubt on the usefulness of viscometry to quantify bundle formation with at least an Ostwald-type viscometer.

These results demonstrate that the level of free Ca^{++} in solution modulates the integrity of an actin bundle formed with villin. Raising the free Ca^{++} to $> 10^{-6}$ M induces actin to depolymerize into short fragments (Figs. 8 and 9; see also references 17, 42). The size of the filaments seems to be dependent on the villin:actin ratio (Fig. 9; see also references 17, 42). The DNase I studies of Craig and Powell (17) and the gel filtration studies of Mooseker and colleagues (42) suggest that the Ca^{++} -induced shortening of filaments may involve breakage of the filaments into short pieces rather than depolymerization by loss of subunits from the ends of filaments. These results are sufficient to explain the Ca^{++} -sensitive disassembly of the isolated microvillus cytoskeleton first demonstrated by Howe et al. (28) and clearly suggests that villin may play a major role in regulating the integrity of the actin core structure in intact microvilli. While calmodulin is the other Ca^{++} -binding protein in the microvillus (23, 28), its *in vivo* or *in vitro* function is unknown since both Howe et al. (28) and Glennly et al. (22) have demonstrated clearly that calmodulin's presence is not required for the Ca^{++} -induced disassembly of the microvillar cytoskeleton. Glennly and Weber (23) have proposed that calmodulin may serve as a Ca^{++} buffer during Ca^{++} transport across the brush border. Their suggestion is based on the large amount of calmodulin in the microvillus (1 mol calmodulin/2 mol of actin) discovered by Howe et al. (28). However, in the microvillus, a more suitable calcium buffer may be the 21,500 *M*, water-soluble calcium binding protein (5). One clue to calmodulin's function may come from the recent studies of Glennly and Weber (23) who demonstrated that calmodulin is associated with the 110,000-*M*, polypeptide and possibly may function in regulating membrane-microfilament connections or membrane-associated activity.

Villin's Role in the Microvillus

Villin not only depolymerizes actin filament proteins but also gathers actin filaments into bundles. This demonstration of villin's bundling properties (10, 37, 42) is consistent with the suggested bundling role of villin in the microvillar cytoskeleton

(35). Bundled actin filaments are readily detected by negative staining at the lowest villin:actin ratios (Fig. 6b). The actin bundles formed are morphologically similar but not identical to the core bundle or microfilaments from the brush-border microvillus (35, Fig. 1d) which contain villin at an approximate ratio with actin of 1:10 (6, 35). The main difference between the in vivo and in vitro bundles is that the microvillar bundles appear more rigid, i.e., straighter than the bundles reconstituted with a 1:10–2:10 villin:actin ratio. This suggests that another protein, probably fimbrin, is also a bundling protein and is consistent with earlier studies (35) and with the immunological localization of fimbrin antibodies to microvilli of tissue culture cells (9).

The Ca^{++} -dependent depolymerizing properties of villin lead to the question of its role in the microvillus. While others have proposed that villin regulates the length of the microvillus (17) or facilitates microvillar movement (42), villin's function may be suggested from reports documenting the vesiculation of the microvillus membrane. The intact epithelial cell can be induced to shed its microvilli in a variety of ways. Nutritional stress (38, 39), anoxia (4), metabolic inhibitors (4), hydrostatic pressure (56), hormones (2), antibodies (31), and glutens (32) all have been reported to effect a disassembly of the microvillar core bundle, vesiculation of the membrane, and shortening of the microvillus. These same effects can be mimicked in vitro by incubating the isolated brush border in solutions containing Ca^{++} (Fig. 1b and c) or other reagents (4). Phalloidin, which stabilizes actin filaments, inhibits vesiculation (4), presumably by stabilizing the cytoskeleton (4, 28). These observations, then, suggest a simple mechanism for vesiculation. Binding of a membrane active agent or unfavorable energetic conditions could uncouple normal calcium transport and cause an increase in the cytoplasmic free Ca^{++} . This Ca^{++} flux would activate villin to depolymerize the actin core in the microvillus. The disassembly of the cytoskeleton would leave a finger of membrane which, due to thermodynamic considerations (54), would fragment into vesicles. This would rid the cell of pathological agents, reduce metabolic demands on the cell, and account for high membrane protein turnover rate in normal cells.

The characteristic of villin as a Ca^{++} -dependent actin depolymerizing protein also raises a paradox concerning the reported Ca^{++} ATP-dependent retraction of the microvillar cores into the terminal web (40). Since Ca^{++} alone causes total disassembly of the microvillar cytoskeletons (28) and a resulting shortening of the microvillus (Fig. 1b and c), then the time-lapse cinematographic and electron microscopy documentation of Ca^{++} -activated motility, i.e., retraction of the microvilli into the terminal web (40), can be alternatively reinterpreted as microvillar dissolution. This latter interpretation is consistent not only with the biochemical properties of villin but also with the observation that demembrated brush borders not previously incubated with glucose-hexokinase are sensitive to Ca^{++} alone (40). Resolution of this apparent inconsistency is important in determining the role and organization of the cytoskeletal components in the terminal web.

Villinlike Proteins in Nonmuscle Cells

Some of the actin depolymerizing proteins from other systems share some characteristics with villin. Proteins of the same general size, such as gelsolin (59–61) or actin depolymerizing factor, ADF, from pig serum (25) or a platelet polypeptide (58) have been reported to be Ca^{++} -sensitive depolymerizing pro-

teins but as yet have not shown an actin bundling activity. The Ca^{++} -sensitive properties of villin in the cytoplasm predict that, under normal situations (low free Ca^{++}), villin would act as an actin-bundling protein. However, when the intracellular Ca^{++} increases to $>10^{-6}$ M, villin would act as an actin-depolymerizing protein. Under these same conditions, Ca^{++} would affect also the activity of other proteins such as myosin which would form filaments as a result of Ca^{++} -dependent phosphorylation of the light chains (47, 52). Thus, actin filaments would depolymerize under the same conditions in which myosin filaments would be formed. It is not clear how an effective actomyosinlike interaction could occur in this situation unless there were microdomains of local Ca^{++} fluctuations.

Although villin is an actin bundling protein located in intestinal microvilli, villin may not be a bundling protein in the cytoplasm of other cells. Efforts to demonstrate villin's presence in other cells have proved unsuccessful (7). It may be that the microvillus cytoskeleton represents a unique organization of actin bundles. Studies on reconstituted actin bundles and other actin bundles such as those found in the actin paracrystal (24), stereocilia (18, 55), acrosome process (57), sea urchin filopodia (19, 30), or sea urchin microvilli (12, 50) have shown the presence of transverse striping patterns indicative of the organization of the helical cross-over points within the bundle. The lack of such a striping pattern in the microvilli (35) or in the reconstituted bundle (this report) suggests that the cross-links within the villin-actin bundles might be arranged in a different manner than found for the other actin bundles. Villin's ability to bundle actin is significantly influenced by the pH of the solution. Ultrastructural and sedimentation measurements suggest that bundle formation is favored at a pH slightly below 7.0, which agrees with the studies of others who have demonstrated a pH-dependence of gelation by the *Dictyostelium* 95,000-M, polypeptide (14, 27). Measurements of the pH in amoeba show that the cytoplasm is also slightly acidic (26) and justifies conducting these experiments at pH 6.9.

Changes in pH may possibly regulate the organization of the microvillar cytoskeleton by affecting villin's ability to bind actin. In cells, a pH change has been demonstrated to accompany changes in the organization of the cytoskeleton. For example, pH changes occur during sea urchin egg fertilization (the pH increases from 6.8 to 7.2) (29, 49) where there occurs, both in vivo and in vitro, a rapid assembly of actin filaments into the core bundles of the fertilized egg microvilli (1, 20). The studies on fertilization together with studies on amoeboid movement (26) and the acrosome reaction (57) should provide an understanding of how pH affects cytoskeletal organization.

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