Reassociation of Microvillar Core Proteins: Making a Microvillar Core In Vitro

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Abstract. Intestinal epithelia have a brush border membrane of numerous microvilli each comprised of a cross-linked core bundle of 15–20 actin filaments attached to the surrounding membrane by lateral cross-bridges; the cross-bridges are tilted with respect to the core bundle. Isolated microvillar cores contain actin (42 kD) and three other major proteins: fimbrin (68 kD), villin (95 kD), and the 110K-calmodulin complex. The addition of ATP to detergent-treated isolated microvillar cores has previously been shown to result in loss of the lateral cross-bridges and a corresponding decrease in the amount of the 110-kD polypeptide and calmodulin associated with the core bundle. This provided the first evidence to suggest that these lateral cross-bridges to the membrane are comprised as least in part by a 110-kD polypeptide complexed with calmodulin. We now demonstrate that purified 110K-calmodulin complex can be readded to ATP-treated, stripped microvillar cores. The resulting bundles display the same helical and periodic arrangement of lateral bridges as is found in vivo. In reconstitution experiments, actin filaments incubated in EGTA with purified fimbrin and villin form smooth-sided bundles containing an apparently random number of filaments. Upon addition of 110K-calmodulin complex, the bundles, as viewed by electron microscopy of negatively stained images, display along their entire length helically arranged projections with the same 33-nm repeat of the lateral cross-bridges found on microvilli in vivo; these bridges likewise tilt relative to the bundle. Thus, reconstitution of actin filaments with fimbrin, villin, and the 110K-calmodulin complex results in structures remarkably similar to native microvillar cores. These data provide direct proof that the 110K-calmodulin is the cross-bridge protein and indicate that actin filaments bundled by fimbrin and villin are of uniform polarity and lie in register. The arrangement of the cross-bridge arms on the bundle is determined by the structure of the core filaments as fixed by fimbrin and villin; a contribution from the membrane is not required.

SINCE the intestinal brush border was first recognized by Tilney as a simple model system for the study of the cytoskeleton and its association with the membrane (reviewed, for example, in Tilney, 1983), it has been the focus of many investigations aimed at isolating and characterizing the proteins involved and their interactions.

The brush border of the intestinal epithelium is comprised of numerous surface microvilli each having, as seen by electron microscopy, a core of ~20 bundled actin filaments attached to the plasma membrane by a helical array of lateral cross-bridges that repeats every 33 nm along the length (Mukherjee and Staehlein, 1971; Tilney and Mooseker, 1971; Mooseker and Tilney, 1975). Brush borders (Miller and Crane, 1961; Tilney and Mooseker, 1971) and, subsequently, pure microvilli (Bretscher and Weber, 1978; Glenney and Weber, 1980) can be isolated in great number. The isolated microvillar cytoskeleton contains actin and four other major proteins (Bretscher and Weber, 1978, 1980b; Matsudaira and Burgess, 1979; Howe et al., 1980). Two of the major proteins, fimbrin (68 kD) and villin (95 kD), were found to bundle the actin filaments within the core (Bretscher and Weber,

1980a,b; Mooseker et al., 1980; Bretscher, 1981; Glenney et al., 1981). Concurrently, Matsudaira and Burgess (1979) demonstrated that microvillar cores treated with ATP lacked the lateral cross-bridge arms; a corresponding loss in the amount of 110-kD and 16.5-kD polypeptides was observed. The smaller polypeptide was soon identified as calmodulin (Glenney et al., 1980; Howe et al., 1980). This was the first evidence to suggest that the lateral cross-bridges are at least in part comprised of the 110-kD polypeptide in association with calmodulin molecules. The definitive evidence, however, resides in the ability to restore the lateral arms removed from the ATP-stripped cores with the addition of purified 110K-calmodulin complex.

The 110-kD polypeptide exists in solution with three to four calmodulin molecules (Coluccio and Bretscher, 1987) and has several properties characteristic of a myosin-like molecule. Like myosin, the 110K-calmodulin complex binds actin in a calcium- and ATP-sensitive manner (Howe and Mooseker, 1983; Collins and Borysenko, 1984; Verner and Bretscher, 1985; Coluccio and Bretscher, 1987). Its ATPase activity is similar to myosin's (Collins and Borysenko, 1984)

and is modestly activated by F-actin in the presence of magnesium (Conzelman and Mooseker, 1987; Krizek et al., 1987) and inhibited in K+-EDTA (Krizek et al., 1987). The ATPase activity resides on the 110-kD polypeptide and is independent of the calmodulin light chains (Coluccio and Bretscher, 1988). Moreover, the complex will decorate actin filaments with an arrowhead decoration similar to that found after incubation of F-actin with the myosin fragments, heavy meromyosin, and S₁ (Coluccio and Bretscher, 1987). At this time, however, no evidence of motility of the 110K-calmodulin complex has been identified.

We have added purified 110K-calmodulin complex to ATP-treated microvillar cores. The resulting cores have an organized arrangement of lateral projections indistinguishable from untreated microvillar cores as viewed by electron microscopy of both negatively stained preparations and in thin sections. We also describe reconstitution experiments involving all the major proteins in the microvillar core. Structures assembled by addition of fimbrin and villin to F-actin are smooth-sided bundles. Incubation of these bundles with the 110K-calmodulin complex results in the appearance of helically arranged projections regularly spaced at 33 nm along the entire length of the bundles. These reconstituted structures are remarkably similar to native microvillar cores.

Materials and Methods

Purification of Brush Borders, Microvilli, Cores, and Stripped Cores

Brush borders were isolated from chicken intestinal epithelial cells essentially according to the method of Bretscher and Weber (1978) with a second sucrose gradient as described by Glenney and Weber (1980). Microvillar cytoskeletons, cores, were prepared by treating the microvilli with 1% Triton X-100 for 10 min at room temperature in Solution I (10 mM imidazole, pH 7.3, 75 mM KCl, 0.1 mM MgCl₂, 1 mM EGTA) (Verner and Bretscher, 1983). Cores were stripped of their lateral cross-bridge proteins by incubation in Solution I containing 1 mM MgCl₂ and 5 mM ATP (after Matsudaira and Burgess, 1979) then suspended in Solution I. Brush borders were used either the same afternoon or the following morning. Microvilli, cores, and stripped cores were used the same day as prepared.

Purification of Proteins

Chicken intestinal villin was purified according to Bretscher and Weber (1980a). Fimbrin was also purified as previously described (Bretscher, 1981). 110K-calmodulin was isolated as recently described (Coluccio and Bretscher, 1987). Rabbit skeletal muscle actin was prepared according to Spudich and Watt (1971) and further purified by gel filtration as described by MacLean-Fletcher and Pollard (1980). All the purified proteins were used within the first few days after their isolation when little or no evidence of proteolysis was detectable.

Electron Microscopy

For negatively stained images, samples were applied to collodion-coated copper grids stabilized by a thin film of carbon and rendered hydrophilic immediately before use by glow discharge. After withdrawing the excess sample, the grids were washed for ∿5 s on drops of water, stained with 1% aqueous uranyl acetate for 30 s, then allowed to air dry. Tissues were prepared for thin-section electron microscopy according to Begg et al. (1973). Thin sections were stained with uranyl acetate and lead citrate. Negatively stained samples and thin sections were examined with an electron microscope (Model 301; Philips Electronics, Inc., Mahwah, NJ) at an accelerating voltage of 80 kV.

Binding Assays

Association of purified 110K-calmodulin complex to ATP-treated microvillar cores was done in buffer containing 10 mM imidazole, 75 mM KCl, 0.1
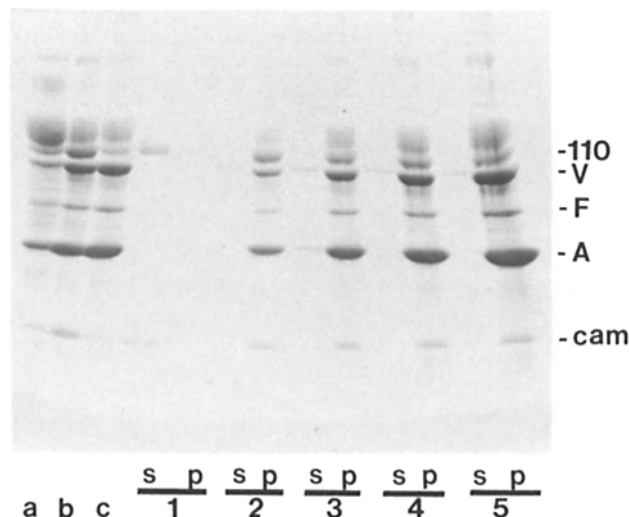

Figure 1. Purified 110K-calmodulin associates with ATP-treated microvillar cores. 110K-calmodulin was incubated with ATP-treated microvillar cores. After centrifugation, supernatants (s) and pellets (p) were analyzed by SDS-PAGE. Equivalent amounts of microvilli (lane a), microvillar cores after detergent treatment (lane b), and ATP-treated microvillar cores showing a loss of the 110-kD polypeptide and calmodulin (lane c) were run. Purified 110K-calmodulin at 0.06 mg/ml is shown in lanes 1s and 1p. The remaining lanes contain 110K-calmodulin at 0.06 mg/ml added to increasing concentrations of stripped microvillar cores: sample 2, 0.25 mg/ml; sample 3, 0.75 mg/ml; sample 4, 1.25 mg/ml; sample 5, 2.0 mg/ml. Migration of the 110-kD polypeptide (110), villin (V), fimbrin (F), actin (A), and calmodulin (cam) on a 7.5%/15% split minigel is indicated.

mM MgCl₂, and 1 mM EGTA, pH 7.3. Various amounts of stripped cores were incubated with 0.06 mg/ml 110K-calmodulin in a total volume of 100 µl at room temperature for 30 min. A 10-µl aliquot from each sample was negatively stained for electron microscopy. The remaining sample was centrifuged in a Beckman airfuge at 30 psi for 20 min. After centrifugation, the pellets were resuspended in 25 µl of 1 M Tris, pH 8.7, then prepared for SDS-PAGE by boiling in 25 µl of 2× sample buffer containing 80 mM Tris, pH 6.8, 1% β-mercaptoethanol, 5 mM EGTA, and 10% glycerol; proteins in the supernatants were first precipitated with 1% trichloroacetic acid. Pellets from an identical second set were embedded in Epon 812 for electron microscopy; the samples for this set were centrifuged at only 20 psi for 15 min.

For binding experiments, F-actin was allowed to assemble in buffer containing 5 mM Tris, pH 8.0, 0.5 mM DTT, 0.2 mM CaCl₂, 0.5 mM ATP, 100 mM KCl, and 1 mM MgCl₂, then dialyzed extensively into buffer containing 5 mM KH₂PO₄, pH 7.0, 1 mM EGTA, 1 mM DTT, 40 mM KCl, and 1 mM MgCl₂. Fimbrin, villin, and 110K-calmodulin were similarly dialyzed into the phosphate-EGTA buffer. F-actin was allowed to incubate overnight with fimbrin and villin at the stated concentrations. After this incubation at 4°C, 110K-calmodulin was added to some samples at the appropriate concentration for 1.5 h. Control samples containing the individual proteins alone and in combination were always run so that aggregation with subsequent precipitation could not be mistaken as association with F-actin in the pelleting assays. Aliquots of 10 µl were negatively stained for electron microscopy and the remaining 90-µl samples were centrifuged and assayed by gel electrophoresis as described above. Duplicate incubations were prepared for thin-section electron microscopy; however, centrifugation was at 20 psi for 15 min.

Other

Electrophoresis of proteins was done using 7.5%/15% acrylamide split minigels according to Laemmli (1970). Gels stained with Coomassie Blue were scanned using a quantitative densitometer (Quick-Scan R+D; Helena Laboratories, Beaumont, TX). The relative areas under the peaks were determined by excising and weighing with a Mettler analytical balance. Protein concentrations were determined by the method of Bradford (1976).
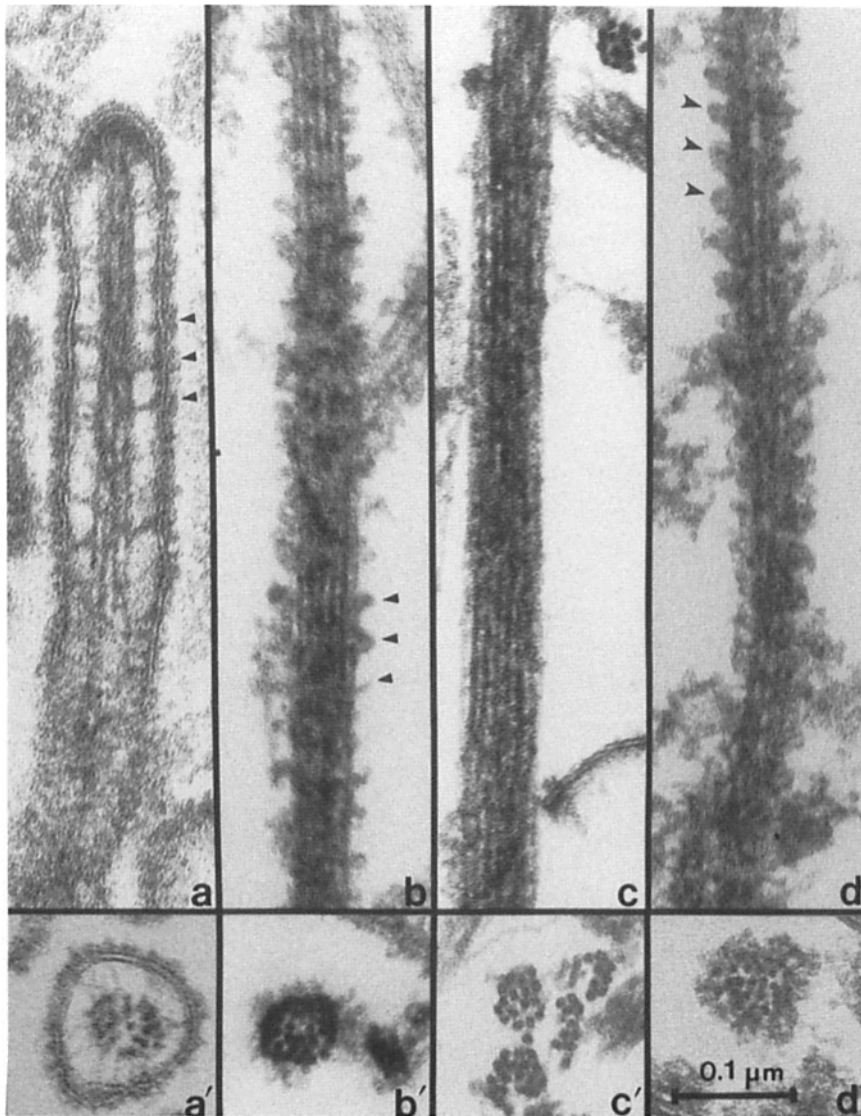
Figure 2. Appearance of ATP-treated cores after addition of 110K-calmodulin. Microvilli contain bundles of actin filaments attached to the surrounding cell membrane by lateral cross arms (*a*). Arms reaching to the membrane from the bundles are visible in cross section (*a'*). These helically arranged lateral arms (*arrowheads*) are particularly obvious on microvilli after removal of the membrane (*b*; cross section, *b'*). After addition of ATP the lateral links are absent from the bundles (*c*). No arms can be seen in cross section (*c'*). Incubation of the stripped cores with exogenous 110K-calmodulin resulted in the reassociation of arms (*d*), helically arranged at 35-nm intervals around the bundles and visible in cross section (*d'*).

Results

Removal and Reassociation of Lateral Arms to Microvillar Cores

As has been previously described, microvillar cores contain five main polypeptides; the 110-kD polypeptide, villin (95 kD), fimbrin (68 kD), actin (43 kD), and calmodulin (17 kD) (Fig. 1, lane *b*). The polypeptide migrating at 140 kD correlates with the presence of vesicles and is not detected in extensively washed microvillar core preparations. Addition of ATP resulted in a 90% reduction in the amount of the 110-kD polypeptide and a corresponding loss of calmodulin associated with the microvillar cores as determined by densitometry (Fig. 1, lane *c*). No loss of any of the other microvillar proteins could be detected. Incubation of purified 110K-calmodulin (Fig. 1, lane *1s*) with these stripped microvillar cores in the absence of ATP resulted in reassociation of 110K-calmodulin (Fig. 1, samples *2-5*).

In thin sections, microvilli treated with Triton X-100 appeared as bundles of actin filaments with lateral projections at 33-nm intervals (Fig. 2, *b* and *b'*). These projections correspond to the lateral bridges seen in intact microvilli to connect the core bundle of actin filaments to the membrane (Fig. 2, *a* and *a'*). When treated with ATP in the presence of magnesium, there was a characteristic loss of the lateral projections (Fig. 2, *c* and *c'*) as has been noted earlier (Matsudaira and Burgess, 1979; Verner and Bretscher, 1985). Although a small fraction of 110K-calmodulin pelleted with the microvillar cores after ATP treatment (Fig. 1, lane *c*), no cores with arms were observed. When 110K-calmodulin was added to the ATP-treated core samples after removal of the ATP, a dramatic reappearance of projections at 33-nm intervals along the length of the actin bundles was seen in thin sections of the pelleted material (Fig. 2 *d*); cross sections of these bundles showed an obvious association of arms (Fig. 2, compare *c'* with *d'*). The reassociated arms were angled as found in controls and their arrangement on the microvillar bundle appeared helical.

All the cores in Fig. 1, sample *2*, displayed reattached arms as seen in thin sections by electron microscopy (Fig. 3 *a*). The molar ratio in that sample of actin/fimbrin/villin/110K-calmodulin as determined by densitometry was 10:0.21:1.1:
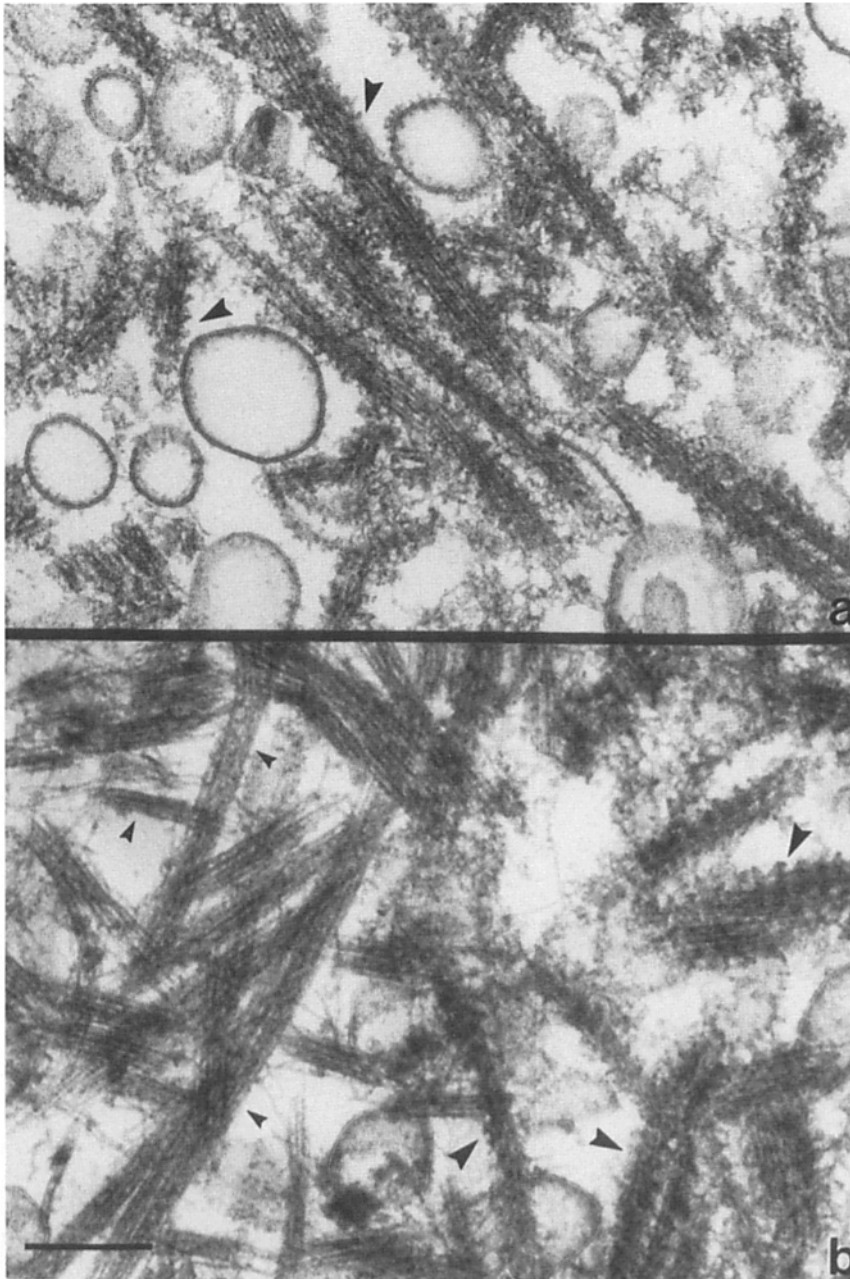
Figure 3. Decoration of ATP-treated microvillar cores by the addition of purified 110K-calmodulin. Addition of purified 110K-calmodulin to ATP-treated cores resulted in the appearance of lateral links along the length of all the bundles when the amount of 110K-calmodulin added (0.06 mg/ml) was not limiting. *a* shows a micrograph of a thin section of the pelleting material found in Fig. 1, sample 2 (core concentration, 0.25 mg/ml). All the bundles display arms. At higher concentrations of cores such as that found in Fig. 1, sample 5 (2.0 mg/ml), structures showing reconstituted arms were among bundles completely devoid of arms (*b*). Bar, 0.2 μm.

0.8. This ratio approaches the ratio of 10:0.65:1.3:0.8 previously determined for isolated microvilli (Bretscher, 1983). However, in samples where the amount of added 110K-calmodulin was limiting (Fig. 1, sample 5), stripped cores were found among the cores showing reconstituted arms along the entire length (Fig. 3 *b*).

Reconstitution of Synthetic Cores from Purified Components

Assembly of structures from purified actin, fimbrin, villin, and the 110K-calmodulin complex (Fig. 4) was done by incubating actin filaments with fimbrin and villin overnight and then adding the 110K-calmodulin complex for 1.5 h. Buffer conditions such as salt, pH, and presence of EGTA

were selected to optimize binding of the isolated proteins to F-actin (Bretscher, 1981; Glenney et al., 1981; Matsudaira and Burgess, 1982b; Coluccio and Bretscher, 1987).

Incubation of 0.1 mg/ml F-actin with 0.1 mg/ml fimbrin in buffer containing 5 mM KH_2PO_4, 1 mM DTT, 1 mM EGTA, 40 mM KCl, 1 mM $MgCl_2$ at pH 7.0 resulted in 13% of the fimbrin associating with the F-actin (Fig. 5, sample 2p). Incubation of 0.1 mg/ml F-actin with 0.15 mg/ml villin resulted in association of 46% of the villin with the filaments under these conditions (Fig. 5, sample 3p). When actin filaments were incubated with both fimbrin and villin at an actin/fimbrin/villin molar ratio approximating 10:7:7, 16% of the fimbrin and 43% of the villin cosedimented (Fig. 5, sample 5). In samples negatively stained for electron microscopy, the actin filaments bundled in either the presence of fimbrin
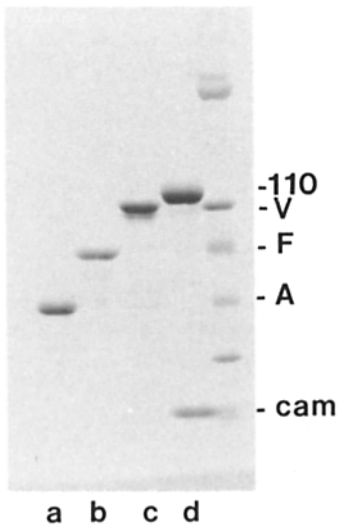
Figure 4. SDS gel electrophoresis of the five major components of the microvillar core. Samples of (*a*) actin, 43 kD; (*b*) fimbrin, 68 kD; (*c*) villin, 95 kD; and (*d*) the 110K-calmodulin complex (110 kD, 17 kD) are shown on 7.5%/15% split minigel.

Figure 5. Incubation of the isolated microvillar core proteins. Fimbrin at 0.10 mg/ml and villin at 0.15 mg/ml were incubated overnight at 4°C with F-actin at 0.1 mg/ml in 5 mM NaH$_2$PO$_4$, 1 mM DTT, 1 mM EGTA, 40 mM KCl, 1 mM MgCl$_2$, pH 7.0. 110K-calmodulin at 0.08 mg/ml was added to samples *1*, *4*, and *6* for 1.5 h before centrifugation. Supernatants (*s*) and pellets (*p*) were analyzed by gel electrophoresis on 7.5%/15% split minigels. Sample *1*, fimbrin (*F*), villin (*V*), and 110K-calmodulin (*110* and *cam*) in the absence of F-actin (*A*); sample *2*, association of fimbrin with F-actin; sample *3*, association of villin with F-actin; sample *4*, association of 110K-calmodulin with F-actin; sample *5*, incubation of fimbrin and villin with F-actin; sample *6*, association of F-actin–fimbrin–villin bundles with 110K-calmodulin.

or villin (Fig. 6, *a* and *b*) or both fimbrin and villin (Fig. 6 *c*) appeared smooth sided.

When 110K-calmodulin was added to F-actin at an actin/110K-calmodulin molar ratio of 10:2 (Fig. 5, sample *4*) 90 min before sedimentation, all the 110K-calmodulin associated with the F-actin (Fig. 5, sample *4*). Virtually no 110K-calmodulin pelleted in the absence of F-actin (Fig. 5, sample *1p*). In the presence of 110K-calmodulin, most of the actin filaments were single; however, some filament association as displayed in Fig. 6 *d* was evident. When 110K-calmodulin was added to actin–fimbrin–villin bundles, no change in the amount of fimbrin or villin pelleting with the F-actin could be observed. The final ratio of actin/fimbrin/villin/110K-calmodulin found in the resulting pellet was 10:1:3:2. Negative stain revealed bundles with an interfilament spacing comparable to that seen in intact microvilli (see Fig. 2). Lateral projections regularly spaced at 33-nm intervals are visible down the entire length of the bundles in both negatively stained (Fig. 7 *a*) and thin-sectioned materials (Fig. 7 *b*). These projections appear helically arranged and are uniformly tilted revealing the polarity of the bundles. The number of filaments within each bundle varied as seen in cross sections (Fig. 7 *b, inset*).

Discussion

Previous work has demonstrated that actin bundles of indefinite length but containing ratios of actin/fimbrin/villin resembling that found in isolated microvillar cores could be generated in vitro from purified components (Bretscher, 1981; Glenney et al., 1981; Matsudaira and Burgess, 1982*b*); these bundles, however, lacked the characteristic arms observed on microvilli in vitro. Since Matsudaira and Burgess (1979) first demonstrated that the lateral arms could be removed from microvillar cores by the addition of ATP with a concomitant loss of the 110-kD polypeptide, it has been suspected that the 110-kD polypeptide might be the major component of these projections.
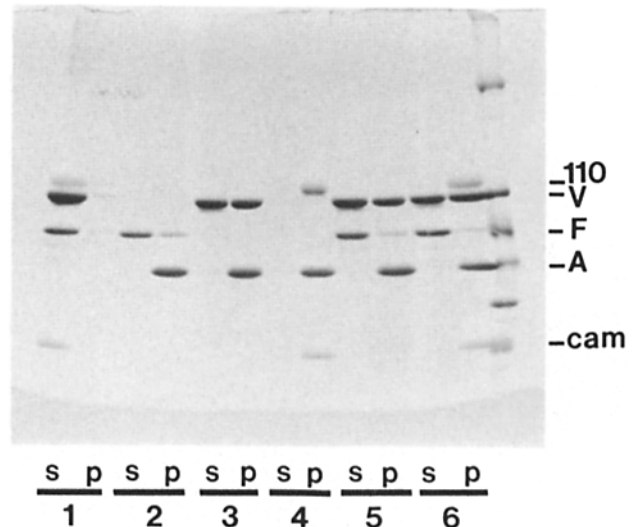
We now show that isolated 110K-calmodulin complex reassociates with stripped microvillar cores to restore the lateral arms. All structures in samples where the amount of 110K-calmodulin added mimicked that found in vivo, have a periodic arrangement of lateral arms seemingly identical to that found in the untreated microvillar cores (Matsudaira and Burgess, 1982*a*). When the amount of 110K-calmodulin added to stripped cores was limiting, structures were found by electron microscopy either to be decorated completely with projections or devoid of arms. These results may suggest that binding of 110K-calmodulin to stripped cores is cooperative as previously found for binding of 110K-calmodulin to single actin filaments (Coluccio and Bretscher, 1987). We interpret these results as definitive evidence that the 110K-calmodulin complex is the major component of the cross-bridge arms linking the actin bundles to the membrane. The ability to add 110K-calmodulin back to the stripped cores to give rise to a helical array indicates that the regular helicity and periodicity of the arms are a consequence of the geometry of the actin bundle and are not dictated by the membrane. Whether an additional anchor protein at the membrane is involved is not known. A previous study indicates that the 110K-calmodulin complex may be associated with an integral membrane polypeptide of 140 kD (Coudrier et al., 1983).

Bundles reconstituted in vitro from pure actin, fimbrin, villin and the 110K-calmodulin complex are remarkably similar to microvillar cores. The ratio of actin/fimbrin/villin/110K-calmodulin in the reconstituted bundle (10:1:3:2) did not differ dramatically from that determined for microvilli

Figure 6. Bundle formation by the microvillar proteins. Electron micrographic images of negatively stained preparations containing (*a*) F-actin and fimbrin, (*b*) F-actin and villin, (*c*) F-actin plus fimbrin and villin, and (*d*) bundling of F-actin in the presence of 110K-calmodulin only. Concentrations and conditions are those described in the legend to Fig. 5. Bar, 0.1 μm.

in vitro (10:0.65:1.3:0.8). It now seems fairly clear that no 110K-calmodulin resides within the structure, but rather lies around the outer surface of the cores. The seemingly increased amount of 110K-calmodulin associating with the reconstituted bundles may simply be a consequence of too low an estimate in the native cores due to proteolysis. The interfilament distance in the reconstituted bundles of ∼12 nm (although not rigorously derived) compares with that observed in native microvilli (Matsudaira et al., 1983). Moreover, both the reassociated arms on previously stripped cores and the arms on our reconstituted bundles, like the lateral bridges found on native microvilli (Tilney and Mooseker, 1971), bind at an angle thereby revealing the uniform polarity of the actin filaments within the bundle. These results complement our previous finding that the 110K-calmodulin complex can decorate actin filaments in a polar fashion similar to the arrowhead decoration of F-actin after incubation with the myosin fragments, heavy meromyosin, and S₁ (Coluccio and Bretscher, 1987) and further demonstrate that the 110K-calmodulin as isolated is in a native state. Moreover, the addition of 110K-calmodulin to actin bundles containing fimbrin and villin results in the appearance of projections which, as viewed by electron microscopy and using the same criteria

previously established for predicting helicity of the lateral arms on native microvilli (Matsudaira and Burgess, 1982*a*), appear helically arranged around the bundle. Likewise, using the same argument presented for microvillar cores (Matsudaira and Burgess, 1982*a*), we propose that the actin filaments within the reconstituted bundles are in register. It is now clear that all the structural information required to make an actin bundle with regularly spaced, helically arranged, lateral arms is dictated by the four proteins: actin, fimbrin, villin, and the 110K-calmodulin complex.

There are two major differences between the reconstituted bundles and microvilli as found in vivo. Unlike microvillar cores, the reconstituted structures are of indeterminate length indicating that the cell possesses the ability to regulate filament length. This may be under the control of villin, which is known to sever actin filaments in the presence of micromolar concentrations of calcium. Also, the number of filaments comprising a reconstituted bundle in general was fewer than found in vivo. One possibility is that in vivo nucleation of actin filaments which will form microvilli is regulated by another protein, such as the microvillar tip component.

These in vitro reconstitution experiments give insight into how the microvillar core may be assembled in vivo. It would
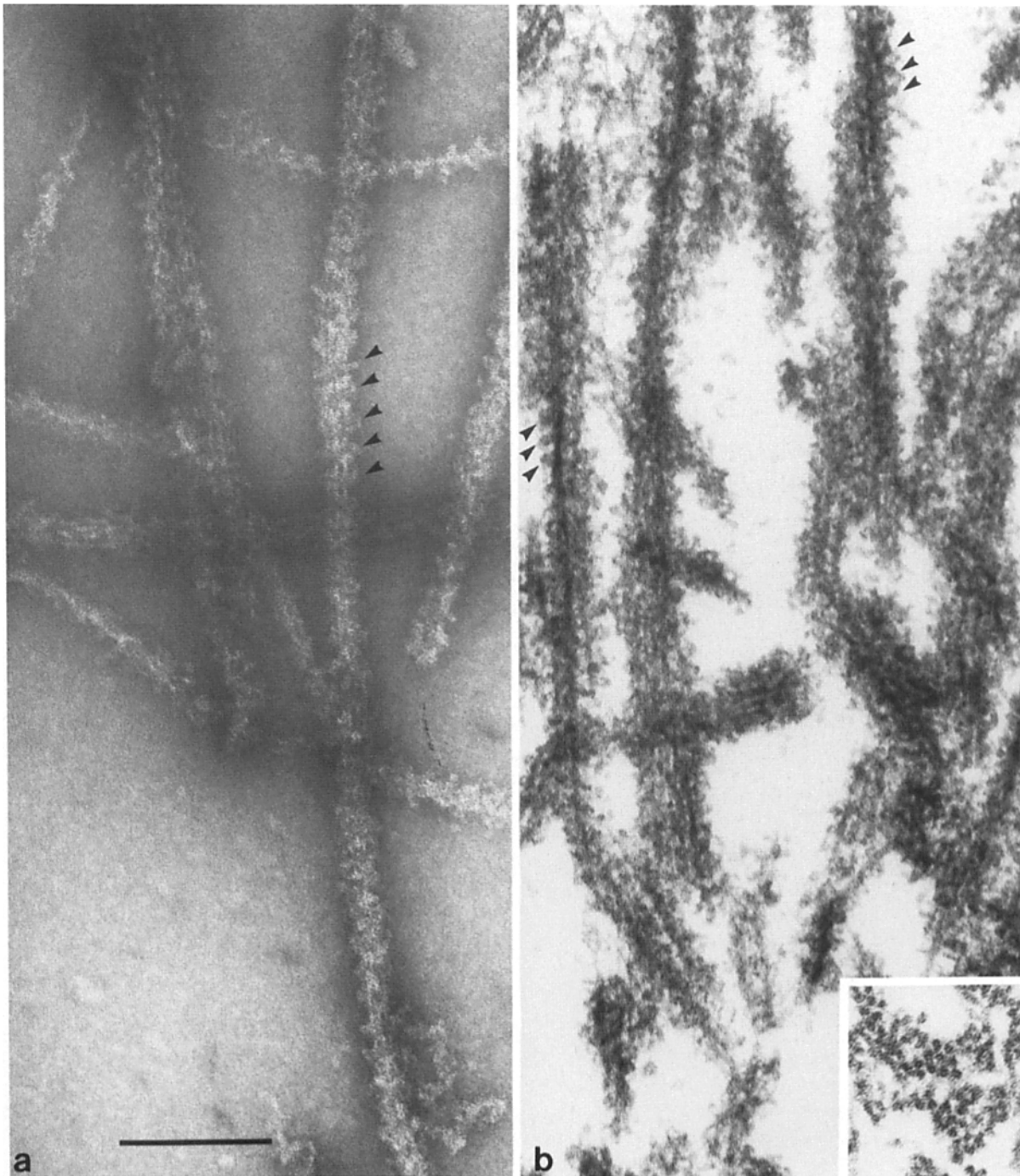
Figure 7. Reconstituted bundles as viewed in negatively stained (*a*) and thin-sectioned (*b*) samples. F-actin–fimbrin–villin bundles were incubated with 110K-calmodulin at concentrations described in the legend to Fig. 5. The final molar ratio of actin/fimbrin/villin/110K-calmodulin in the sedimenting structures was 10:1:3:2. Arrowheads indicate the presence of polarized projections helically arranged at 33-nm intervals down the length of the bundle. (Note that although all the arms on one bundle point in the same direction, not all the bundles are oriented in the same direction.) *Inset,* cross section. Bar, 0.1 μm.

appear that actin is first assembled from the membrane and then the accessory proteins, fimbrin and villin, bundle the filaments. Once the unipolar bundles are formed, the 110K-calmodulin, now firmly established as the major component of the lateral arms, could associate and thereby form the microvillus.

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