Calcium-regulated Cooperative Binding of the Microvillar 110K-Calmodulin Complex to F-Actin: Formation of Decorated Filaments

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Abstract. The 110K-calmodulin complex of intestinal microvilli is believed to be the link between the actin filaments comprising the core bundle and the surrounding cell membrane. Although not the first study describing a purification scheme for the 110K-calmodulin complex, a procedure for the isolation of stable 110K-calmodulin complex both pure and in high yield is presented; moreover, isolation is without loss of the associated calmodulin molecules since a previously determined ratio in isolated microvillar cytoskeletons of calmodulin to 110-kD polypeptide of 3.3:1 is preserved. We have found that removal of calmodulin from the complex by the calmodulin antagonists W7 or W13 results in precipitation of the 110-kD polypeptide with calmodulin remaining in solution. The interaction of 110K-calmodulin with beef skeletal muscle F-actin has been examined. Cosedimentation assays of 110K-calmodulin samples incubated with F-actin show the amount of 110K-calmodulin associating with F-actin to be ATP, calcium, and protein concentration dependent; however, relatively salt independent. In calcium, ~30% of the calmodulin remains in the supernatant rather than cosedimenting with the 110-kD polypeptide and actin. Electron microscopy of actin filaments after incubation with 110K-calmodulin in either calcium- or EGTA-containing buffers show polarized filaments often laterally associated. Each individual actin filament is seen to exhibit an arrowhead appearance characteristic of actin filaments after their incubation with myosin fragments, heavy meromyosin and subfragment 1. In some cases projections having a 33-nm periodicity are observed. This formation of periodically spaced projections on actin filaments provides further compelling evidence that the 110K-calmodulin complex is the bridge between actin and the microvillar membrane.

Actin filaments are generally considered to play an important role in giving cells their shape and structure. To achieve this, the cytoskeleton must be attached at discrete places to the cell membrane because many cellular functions, e.g., phagocytosis, cytokinesis, secretion, and the elongation of projections, are activities in which microfilaments are intimately associated with the membrane. Within the cell this interaction between microfilaments and the surrounding membrane is almost certainly under precise physiological control.

Intestinal microvilli provide a simple model system for the study of actin structure and how actin filaments can be attached to the membrane. In cross section, microvilli are found to consist of a bundle of ~20 microfilaments (31, 34). The isolated cytoskeleton contains actin and four major associated polypeptides (110 kD, a 95-kD protein known as villin, fimbrin of 68 kD, and a 17-kD protein identified as calmodulin) along with a number of other proteins found in lesser amounts (6, 26). The two actin-binding proteins, villin and fimbrin, serve to bundle the actin filaments within the core (2-4, 7, 8, 15, 27, 30). The filaments are attached to the surrounding cell membrane at the tip and by lateral arms at a 33-nm periodicity by an as yet unidentified substance thought to be comprised of a calmodulin-binding protein of 110 kD (14, 26, 28).

Evidence for the existence of the 110- and 17-kD polypeptides as part of the intestinal microvilli was originally presented by Matsudaira and Burgess (26). They found that treatment of microvillar cores with ATP and magnesium resulted in dissociation of the lateral arms and loss of the 110-kD polypeptide from the cytoskeletons; a simultaneous reduction in the amount of the 17-kD polypeptide was observed. Subsequently, the 17-kD polypeptide in the brush border was identified as calmodulin (12, 20). Direct evidence for the interaction of the 110-kD polypeptide with calmodulin came from gel overlay studies with 125I-calmodulin (12). The 110-kD polypeptide was the only protein in the microvillar core found to bind calmodulin; the interaction was determined to be at least partially Ca2+-independent. Additional evidence to suggest that the 110-kD polypeptide was a component of the link between the filament bundle and the membrane also came from Matsudaira and Burgess (26) who found that treatment of cores with deoxycholate solubilized all the proteins except the 110-kD polypeptide and some actin. By
electron microscopy the actin filaments were shown still to possess lateral arms. In a study using antibodies directed against the 110-kD polypeptide, Glenney and colleagues (14) localized the 110-kD polypeptide by immunoelectron microscopy to the microvillar cores in brush border cytoskeletons, providing further evidence that the 110-kD polypeptide is in a position where it could link the actin filaments to the membrane. The available evidence is convincing although not definitive that the 110K-calmodulin complex is a component of the lateral arms linking the core bundle to the brush border membrane.

Isolation of the 110-K calmodulin complex in pure and in native form with adequate recovery has been hampered, mostly due to solubility problems (19). The 110-kD polypeptide has been found to precipitate easily from solution, a property that has been attributed to loss of its associated calmodulin(s) (19). One report has claimed that the 110-kD polypeptide requires detergent for its solubilization and is therefore an integral membrane protein (11). This has been questioned by a subsequent study of Verner and Bretscher (35) who analyzed in detail the properties of the complex and found it to be water soluble.

Using gel-filtration and ion-exchange chromatography, we have devised an improved purification procedure for intact 110K-calmodulin complex which yields milligram quantities of the complex with relatively few contaminants; moreover, the complex is stable in solution for extended periods. We have studied the 110K-calmodulin complex and characterized the binding of the complex to filamentous actin. Association of the 110K-calmodulin complex with F-actin is calcium regulated and exhibits cooperativity. Visualization by electron microscopy of actin filaments incubated with the 110K-calmodulin complex shows some filaments having projections with a 33-nm spacing, the same periodicity determined in brush border for the lateral links connecting the microfilament core to the membrane (6, 27, 28, 31). The morphology of most individual decorated actin filaments resembles the arrowhead appearance characteristic of F-actin after incubation with the myosin fragments, heavy meromyosin or subfragment 1.

**Materials and Methods**

**Materials**

Fresh chicken intestines were obtained from Larson's Poultry House in Odessa, NY. N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W7), N-(4-aminobutyl)-5-chloro-2-naphthalene-sulfonamide (W13), and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Sepharose 4B, Fast Flow Q-Sepharose, and Fast Flow S-Sepharose were purchased from Pharmacia AB (Upsala, Sweden). The listed standards for Stokes radius and sedimentation coefficient determination were purchased from Pharmacia Fine Chemicals (Div. Pharmacia, Inc., Piscataway, NJ).

**Isolation of the 110K-Calmodulin Complex**

Brush borders were isolated from the intestinal epithelial cells of 20 chick-ens as previously described by Bretscher and Weber (6). To inhibit proteolysis, phenylmethylsulfonylfluoride (PMSF) and benzamidine were added to all solutions at 0.3 and 0.5 mM, respectively. The brush borders were preextracted in 10 mM imidazole, pH 7.3, 300 mM NaCl, 5 mM MgCl2, 75 mM KCl, 1 mM EGTA, and centrifuged at 4000 rpm for 10 min (Sorvall RC3B; 1 Abbreviations used in this paper: W7 and W13, calmodulin agonists N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide and N-(4-aminobutyl)-5-chloro-2-naphthalene-sulfonamide, respectively.

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Figure L Isolation of the 110K-calmodulin complex. Brush borders were extracted in ATP (lane a), precipitated with ammonium sulfate (lane b), resuspended and loaded (lane c) after clarification (lane d, pellet) onto a Sepharose 4B column. High molecular mass proteins (lanes e and f) eluted early in the separation. 110K-calmodulin-containing fractions (lanes g–j) were pooled (lane k) and chromatographed over a DNase-I-Sepharose column that removed actin (lane l). Flow-through was precipitated with ammonium sulfate (lane m) and chromatographed on Q-Sepharose. Flow-through fractions (lane n) were rich in villin. Fractions containing the 110K-calmodulin complex (lanes o–r) eluted with 0.4 M salt. These fractions were pooled (lanes s) and chromatographed on S-Sepharose. Purified 110K-calmodulin was eluted with a step gradient (lanes t–w). Fractions were analyzed by SDS-PAGE on 7.5/15% split mini gels. Arrows indicate the 110-kDa polypeptide and 17-kDa calmodulin.

catalase (11.3 s), aldolase (7.3 s), and BSA (4.3 s). The Stokes radius was estimated on a 250-ml Sephacryl S400 column using the following internal standards: thyroglobulin (85 Å), apoferritin (61 Å), catalase (52 Å), and aldolase (48 Å). In both cases fractions were analyzed by SDS-PAGE.

Electron Microscopy
Samples were negatively stained with 1% aqueous uranyl acetate onto collodion-coated copper grids stabilized by a thin film of carbon. Grids were examined with a Philips 301 electron microscope at an accelerating voltage of 100 kV.

SDS-PAGE
PAGE in the presence of SDS was performed essentially according to Laemmli (22). Split mini-gels of 7.5/15% were used to resolve both the 110-kDa polypeptide and 17-kDa calmodulin. Sample buffer contained final concentrations of 80 mM Tris, pH 6.8, 1% mercaptoethanol, 5 mM EGTA, and 10% glycerol.

Other
Bovine brain calmodulin was prepared according to the method of Lin et al. (23). Column fractions were examined for the presence or absence of protein by dotting 3 μl of each fraction onto filter paper, then air drying before precipitating any protein present with 10% TCA for 1–2 min. Protein was visualized by staining in 0.025% Coomassie Blue in 15% methanol, 10% acetic acid for 1 min followed by destaining in 15% methanol, 10% acetic acid. The ratio of calmodulin to the 110-kDa polypeptide was determined by quantitation of Coomassie Blue-stained proteins after SDS-PAGE (16). BSA and bovine brain calmodulin were used as standards. Gel bands were sliced out of the gel, macerated, and the Coomassie dye eluted by overnight incubation in 25% pyridine in water. The amount of protein present was quantitated by the absorbance of the eluted dye at 605 nm. Alternatively, gel bands stained with Coomassie Blue were scanned using a quantitative densitometer (Quick-Scan R+D; Helena Laboratories, Beaumont, TX).

Results

Purification of the 110K-Calmodulin Complex by Gel Filtration and Ion Exchange Chromatography
110K-calmodulin complex was isolated by a series of steps including gel filtration, anion, and cation chromatography. In particular, gel filtration served to separate the 110K-calmodulin complex from high molecular mass contaminants including myosin heavy chain (200 kD; Fig. 1, lanes e and f). The remaining major contaminating bands at this step included villin (95 kD) and actin (43 kD) (Fig. 1, lanes g–j).

Actin was selectively removed from the extract by chromatography over Sepharose to which DNase I had been immobilized (Fig. 1, lane l). Anionic exchange chromatography served to separate villin from the 110K-calmodulin complex (Fig. 1, lane n). Further purification was performed by cation exchange using a step gradient containing 0.7 M NaCl (Fig. 1, lanes t–w). Use of a linear salt gradient was unsuccessful, resulting in the elution of the 110K-calmodulin complex along the entire gradient. Yield was usually 1–2 mg. The 110K-calmodulin was found to be stable at 4°C over several days; after 14 d some proteolytic breakdown products became evident by gel electrophoresis.

Properties of the Purified 110K-Calmodulin Complex
The amino acid composition of the 110K-calmodulin complex showed no particularly noticeable characteristics (Table I). Several physical properties of the complex were determined and are listed in Table II. BSA and bovine brain calmodulin were prepared according to the method of Lin et al. (23). Column fractions were examined for the presence or absence of protein by dotting 3 μl of each fraction onto filter paper, then air drying before precipitating any protein present with 10% TCA for 1–2 min. Protein was visualized by staining in 0.025% Coomassie Blue in 15% methanol, 10% acetic acid for 1 min followed by destaining in 15% methanol, 10% acetic acid. The ratio of calmodulin to the 110-kDa polypeptide was determined by quantitation of Coomassie Blue-stained proteins after SDS-PAGE (16). BSA and bovine brain calmodulin were used as standards. Gel bands were sliced out of the gel, macerated, and the Coomassie dye eluted by overnight incubation in 25% pyridine in water. The amount of protein present was quantitated by the absorbance of the eluted dye at 605 nm. Alternatively, gel bands stained with Coomassie Blue were scanned using a quantitative densitometer (Quick-Scan R+D; Helena Laboratories, Beaumont, TX).

Table I. Amino Acid Composition of the 110K-Calmodulin Complex

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mole (M)</th>
<th>%</th>
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<tbody>
<tr>
<td>Aspartic acid</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.8</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
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<td></td>
</tr>
<tr>
<td>Glycine</td>
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<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Arginine</td>
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<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
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<td></td>
</tr>
<tr>
<td>Methionine</td>
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<td></td>
</tr>
<tr>
<td>Cysteine</td>
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</tr>
<tr>
<td>Isoleucine</td>
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</tr>
<tr>
<td>Leucine</td>
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<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
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<tr>
<td>Lysine</td>
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modulin were used as standards to determine the ratio of calmodulin to the 110-kD polypeptide. Assuming that the 110-kD polypeptide and BSA bind dye equivalently on a mass basis, quantitation by absorbance of the eluted dye from stained gel bands gave a ratio of 3.3 calmodulin molecules per one 110-kD molecule.

Incubation of the 110K-calmodulin complex with the calmodulin antagonist W13 (18) affected the solubility of the 110-kD polypeptide. A substantial amount of 110-kD polypeptide pelleted at concentrations of W13 above 100 µM; the majority of the calmodulin, however, remained in the supernatant (Fig. 2). Nearly identical results were found with the antagonist W7. Neither WI3 nor W7 had any detectable effect in the absence of calcium (data not shown).

Interaction of the 110K-Calmodulin Complex with F-Actin Is Calcium Regulated and Concentration Dependent

In buffer containing calcium but no ATP, at 110K-calmodulin concentrations ranging from 0.045 to 0.45 mg/ml, 100% of the 110-kD polypeptide was found to bind to and cosediment with the actin filaments (Fig. 3 A). In these assays, ~90% of the 0.3 mg/ml actin used pelleted. Interestingly, under these conditions about one-third of the calmodulin dissociated from the complex and remained in the supernatant, whereas the 110-kD polypeptide and remaining calmodulin sedimented with the F-actin. In the presence of 5 mM ATP, however, regardless of the amount of 110K-calmodulin present, >72% of the 110-kD polypeptide stayed in the supernatant (Fig. 3 B). A portion of the small fraction of 110-kD polypeptide that pelleted could be attributed to that which pelleted on its own (~14%), plus a fraction that either apparently associated with the F-actin or became trapped with the F-actin during sedimentation.

Similar experiments were done in 1 mM EGTA to reduce the calcium concentration. In the absence of ATP at 110K-calmodulin concentrations below 0.18 mg/ml, 75% of the 110-kD polypeptide did not cosediment with F-actin but remained in the supernatant (Fig. 3 C). As the amount of 110K-calmodulin used in the assay was increased, a larger fraction of it bound to and sedimented with the F-actin. In contrast to that found in the presence of calcium, all the calmodulin stayed associated with the 110-kD polypeptide. Results in the presence of 1 mM EGTA and 5 mM ATP were similar to those found for ATP when calcium was present: most (>80%) of the 110K-calmodulin was found in the supernatant (compare Fig. 3, B and D).

Table II. Properties of the 110K-Calmodulin Complex

<table>
<thead>
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<th>Property</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Stokes radius</td>
<td>63 Å</td>
</tr>
<tr>
<td>Sedimentation coefficient ±ATP</td>
<td>6.7 s</td>
</tr>
<tr>
<td>Ratio of calmodulin to 110-kD polypeptide</td>
<td>3.3:1</td>
</tr>
<tr>
<td>Binds to anionic resin, pH 7.4</td>
<td></td>
</tr>
<tr>
<td>Binds to cationic resin, pH 8.0</td>
<td></td>
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</table>

Figure 2. Incubation of 110K-calmodulin with the calmodulin antagonist, W13. Aliquots of 110K-calmodulin at 0.09 mg/ml (0.7 µM) were incubated with W13 at 0–250 µM in buffer containing 0.2 mM CaCl₂, then centrifuged. Plotted are the percent of 110-kD polypeptide (●) and calmodulin (○) pelleting as a function of W13 concentration.

Figure 3. Cosedimentation of 110K-calmodulin with F-actin. F-actin at 0.3 mg/ml was incubated with increasing amounts of 110K-calmodulin in 0.2 mM CaCl₂ without ATP (A), or with 5 mM ATP (B), or in 1 mM EGTA without ATP (C), or with 5 mM ATP (D). Percentage of 110-kD polypeptide (●) and calmodulin (○) cosedimenting with F-actin is plotted vs. 110K-calmodulin concentration.
The association of 110K-calmodulin with F-actin in the absence of ATP was readily reversible in either calcium or 1 mM EGTA by the addition of ATP to a final concentration of 5 mM (data not shown).

An actin-concentration dependence on the binding of 110K-calmodulin was observed by varying the amount of actin used in the assays while keeping the 110K-calmodulin concentration constant (Fig. 4, A and C). As the actin concentration decreased from 0.3 to 0.07 mg/ml, the amount of 110-kD polypeptide cosedimenting increased, clearly demonstrating that 110K-calmodulin binds cooperatively to F-actin. When the actin concentration was reduced below 0.07 mg/ml, the amount of 110K-calmodulin associating with F-actin declined, possibly indicating that the F-actin was saturated with the complex. This series of assays demonstrates that 110K-calmodulin binds cooperatively to F-actin in a saturable manner in 1 mM EGTA.

However, different results were observed under similar conditions in the presence of calcium (Fig. 4, B and D). At high actin concentrations, essentially all the 110-kD polypeptide and two-thirds of the calmodulin cosedimented with the F-actin. As the actin concentration decreased, an apparently simple saturation curve was obtained, but with the ratio of 110K-calmodulin/actin at saturation being about half that found in the presence of 1 mM EGTA (Fig. 4 D). The reason for this difference is not yet clear. In both calcium and EGTA, the amount of actin monomer remaining in the supernatant was noticeably reduced at high ratios of 110K-calmodulin/actin (Fig. 4, A and B).

In the range of 10^-4–10^-6 M free calcium (8, 17) the amount of 110K-calmodulin cosedimenting with F-actin remained unchanged (Fig. 5) whereas in 1 mM EGTA, 75% of the 110-kD polypeptide along with 75% of the calmodulin remained in the supernatant (Fig. 3 C). These results indicate that a change in the interaction of the complex with F-actin occurs in a range of free calcium typical of physiological levels.

**Ionic Strength Has Little Effect on 110K-Calmodulin Binding to F-Actin**

In 0.2 mM CaCl₂, the amount of 110K-calmodulin associating with F-actin was unaffected by KCl concentration in the

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**Figure 4.** Cosedimentation of 0.09 mg/ml 110K-calmodulin with F-actin ranging from 0 to 0.3 mg/ml in buffer containing either 1 mM EGTA (A) or 0.2 mM CaCl₂ (B). After incubation samples were centrifuged and the supernatants (s) and pellets (p) analyzed by SDS-PAGE on 7.5/15% split gels. The percentage of 110-kD polypeptide (○) and calmodulin (○) sedimenting with F-actin in buffer containing 1 mM EGTA (C) or 0.2 mM CaCl₂ (D) is plotted vs. the actin concentration.
range of 0.5 M to 75 mM; at 50 mM some of the 110-kD polypeptide was left in the supernatant (Fig. 6 A). In EGTA, under the chosen conditions, approximately half of the 110K-calmodulin remained in the supernatant after centrifugation of the F-actin. This was unaffected by KCl concentration in a range from 0.1 to 0.5 M; a somewhat smaller fraction of the 110K-calmodulin cosedimented with F-actin at KCl concentrations of 75 and 50 mM (Fig. 6 B).

Decoration of F-Actin after Incubation with 110K-Calmodulin

The interaction of 110K-calmodulin with F-actin was examined by electron microscopy of negatively stained preparations. Control grids of F-actin only showed individual actin filaments in either the presence or absence of calcium (Fig. 7 A). Incubation with 110K-calmodulin resulted in some lateral association of the actin filaments. In either 0.2 mM CaCl₂ (Fig. 7, B and C) or 1 mM EGTA (Fig. 7 D), a change in the morphology of most of the F-actin was particularly obvious at an actin concentration of 0.30 mg/ml with 0.09 mg/ml 110K-calmodulin. On individual filaments presumably decorated with 110K-calmodulin, a polarity of the filaments was observed with directionality as illustrated by the arrow in Fig. 7 C. The appearance of these single filaments resembled the characteristic arrowhead formation found on actin filaments after their incubation with the myosin fragments, heavy meromyosin and subfragment 1. In either calcium or EGTA, filaments with apparently no associated 110K-calmodulin were also observed (Fig. 7, B and C, arrowheads). On some filaments projections 35-nm long at 33-nm intervals were visible, giving the filaments the appearance of a ladder (Fig. 7 D).

Discussion

Although earlier studies have outlined methods for the purification of the 110-kD polypeptide or the 110K-calmodulin complex, until now a procedure for the isolation of native 110K-calmodulin both pure and in high yield has not been described. One problem initially encountered was the dissociation under certain isolation conditions of some of the calmodulin to give complexes containing variable ratios of calmodulin to the 110-kD polypeptide. Under the worst of conditions, removal of calmodulin from the complex resulted in its precipitation and subsequent loss during isolation (19). Other earlier methods were also hampered by what appeared as insolubility of the complex since at low levels of ATP with the slightest trace of F-actin present, the 110K-calmodulin and actin pelleted (35). This is likely explained by the present finding of cooperative binding of 110K-calmodulin to F-actin with an apparent reduction in actin's critical concentration.

The method described here takes 4 d and yields milligram quantities of the complex which is stable at 4°C for at least a week. A number of properties indicate that the complex has been isolated in an intact native form. The molar ratio of the 110-kD polypeptide to calmodulin in the purified complex is 1:3.3, a value identical to that found in isolated microvillar cytoskeletons (13, 20). This suggests that most, if not all, of the calmodulin in the microvillar core is associated with the 110-kD polypeptide and remains associated as a complex during purification. Secondly, the 110-kD polypeptide binds to F-actin in the absence but not presence of ATP, and this binding in either calcium or EGTA is reversible since addition of ATP to 110K-calmodulin-actin filaments results in dissociation of the 110K-calmodulin complex from the F-actin. Preliminary experiments in this laboratory have confirmed the Mg²⁺ and K⁺-EDTA ATPase activity of the 110K-
Figure 7. Electron microscopy of negatively stained images of F-actin after incubation with 110K-calmodulin. 110K-calmodulin (0.09 mg/ml) was allowed to incubate with 0.3 mg/ml F-actin in buffer containing either 0.2 mM CaCl2 or 1 mM EGTA. Control grids of F-actin only in either buffer showed individual filaments (A). F-actin incubated with 110K-calmodulin in either calcium (B and C) or EGTA (D) showed polarized filaments often laterally associated. Some filaments apparently free of 110K-calmodulin are seen in the background (B and C, arrowheads). Individual filaments presumably bound with 110K-calmodulin show a prominent polarity with directionality indicated by the arrow (C). Periodic projections linking neighboring filaments are indicated (D). Bars, 0.5 μm.
calmodulin complex (9), but differ in that the Mg²⁺ ATPase is stimulated by F-actin.

We have found that the complex can be stripped of some of its calmodulin by the calmodulin antagonists W7 and W13 in the presence of calcium and that this renders the 110-kD polypeptide insoluble. This finding might be used to help explain some earlier results. In particular, Howe and Mooseker (19) found that the 110-kD polypeptide could not be solubilized from microvillar cores after the calmodulin had been removed by trifluoperazine and calcium; this is probably a direct result of rendering the 110-kD polypeptide insoluble. Howe and Mooseker also reported that their isolated complex had 1:2 calmodulins associated with each 110-kD polypeptide and that it tended to precipitate out of solution. Contrast, our preparation with presumably the native number of associated calmodulins remains soluble for extended periods. Finally, Glenney and Glenney (11) reported that the 110-kD polypeptide was an integral membrane protein because it required a detergent to remain in solution. Since it has previously been demonstrated that the 110K-calmodulin complex is freely soluble in aqueous buffer in the absence of detergent (35), we can offer a simple explanation for their results. In their experiments, before extraction of the 110-kD polypeptide in 0.25 % Triton X-100, 0.05 % SDS, and pyrophosphate, the cytoskeletons were pretreated with detergent only; this pretreatment removed the calmodulin from the complex. The 110-kD polypeptide was then found to remain soluble only in the presence of an ionic detergent. Consequently they used this and other evidence to conclude that the 110-kD polypeptide is an integral membrane protein. Although it is now clear that the 110-kD-calmodulin complex is not an integral membrane protein since detergent is not required for its release, we are in agreement with Glenney and Glenney (11) that the 110-kD polypeptide, once stripped of its calmodulin, is not freely soluble in aqueous buffer. At the present time there is no direct evidence for an association between the 110-kD-calmodulin complex and the microvillar membrane.

We were able by adding exogenous brain calmodulin to resolubilize only a fraction of the 110-kD polypeptide precipitated with removal of the calmodulin with W13. These results can be compared to Howe and Moosiker's (19) vain attempt to aid isolation of the 110K-calmodulin complex by addition of exogenous calmodulin in the hope of preventing precipitation of the 110-kD polypeptide. Apparently conditions appropriate for the quantitative reconstitution of the complex have not yet been identified. Calmodulin removal from the 110K-calmodulin complex by the antagonists occurred only in the presence of calcium. This suggests that the calmodulin molecules in the complex bind calcium to undergo a conformational change making them susceptible to antagonist release.

In the absence of ATP the association of 110K-calmodulin with actin differs in 1 mM EGTA as compared with free calcium concentrations above 10⁻⁶ M. In EGTA, at protein concentrations that represent a 110-kD-calmodulin to actin mass ratio of 1:1, only a fraction of the 110K-calmodulin pellets with the F-actin, whereas at concentrations representing high mass ratios up to 1:5:1, all the 110K-calmodulin pellets with the actin. Above this value, no additional 110K-calmodulin is sedimented so the F-actin is presumably saturated. The binding of 110K-calmodulin to F-actin therefore clearly demonstrates cooperativity as the 110K-calmodulin concentration increases up to saturation. In addition, the 110-kD polypeptide and calmodulin bind as a complex, i.e., the calmodulin distribution between the pellet and the supernatant follows that of the 110-kD polypeptide. The critical concentration of actin (i.e., the amount of actin remaining in the supernatant) appears to decrease in the presence of increasing amounts of 110k-calmodulin. It is likely that the 110K-calmodulin acts to stabilize the actin filaments thus reducing the likelihood for dissociation of actin monomers, the phenomenon responsible for the critical concentration.

The situation in free calcium >10⁻⁶ M is different. At all concentrations used up to saturation, essentially all the 110-kD polypeptide binds to F-actin. However, a portion of the calmodulin remains soluble. This suggests that in the presence of calcium and F-actin, some calmodulin is dissociated from the complex and the partially depleted complex binds better to F-actin than the intact complex in the absence of calcium. In calcium, the critical concentration of actin is also seen to decrease with increasing 110K-calmodulin complex. From our biochemical data alone, we have not demonstrated that binding of 110K-calmodulin to F-actin in the presence of calcium is also cooperative. It is possible that cooperativity exists in calcium; however, under the conditions used, it was not observed.

We have found that the interaction of 110K-calmodulin and F-actin is remarkably independent of salt concentration. As a means of comparison, the cooperative interaction of tropomyosin with F-actin is tighter at intermediate salt, although it is abolished in high salt (10).

Examination by electron microscopy of 110K-calmodulin bound to F-actin revealed filaments apparently saturated with bound complex together with a smaller number of undecorated actin filaments. No partially decorated filaments were observed. This result, found either in the presence or absence of calcium, provides ultrastructural evidence for the cooperative binding seen biochemically in the presence of EGTA. At near saturation, decorated filaments were often seen to associate laterally. It is not clear what significance this association, if any, has. Closer ultrastructural examination of 110K-calmodulin bound to F-actin reveals regular structures projecting at an angle from the filament. This leads to an arrowhead-like appearance of the 110K-calmodulin-decorated actin filaments similar to that seen when actin filaments are incubated with the myosin fragments heavy meromyosin and subfragment 1 (21). As of yet, we have been unable to determine the directionality of the complex's attachment but presume that like actin filaments decorated with myosin fragments, the preferred end for monomer addition to the actin filament will be the barbed end. This is already implicated by electron microscopy of the cross-links in intestine; the cross-bridges have been shown to be angled away from the microvillar tip (26), where actin assembly is presumed to occur (29). Besides the ultrastructural similarities with skeletal muscle myosin, there is a striking similarity between actin filaments decorated with 110K-calmodulin and those observed in the presence of Acanthamoeba myosin-1 (24, 32). In both cases, the thickened actin filaments become laterally associated and in some regions regularly spaced (30-35 nm) cross-links are observed (24). This myosin-like decoration of F-actin is not the only similarity between the 110K-calmodulin complex and myosin. Collins and Bory-
senko (9) have reported that the complex has K⁺-EDTA and Ca²⁺ ATPase activities characteristic of myosin and that the K⁺-EDTA activity is not stimulated by F-actin. By the use of proteolytic digest analysis and immunoblotting they found it to be distinct from brush border myosin and therefore not a myosin breakdown product. Whether or not the 110K-caldesmon complex is involved in any contractile activity is not yet known.

The result that the 110K-caldesmon complex binds to F-actin in a calcium-regulated manner and that with this association some of the caldesmon is released is particularly interesting. Thus, two out of the three major actin-associated proteins in the isolated microvillous core, i.e., villin and the 110K-caldesmon complex, show calcium regulation in the physiologically important range. Consistent with calcium-regulated events in other cytoskeletal systems, this finding adds support to the suggestion that microvillar structure may be modified by calcium fluxes during the normal functioning of the structure. Exactly what ultrastructural changes may be controlled by calcium remain to be determined.

The decorated actin filaments after incubation with the 110K-caldesmon complex revealed with electron microscopy that the 110K-caldesmon complex projects ~35 nm from the filament and repeats every 33 nm down the length of the filament. These numbers are the same as those measured for the cross-filaments that connect the microvillar core bundle to the brush border membrane. This lends substantial credibility to the proposed role of the 110K-caldesmon complex as the link between the actin filaments and the membrane.

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