Characterization of the 110-kdalton Actin-Calmodulin-, and Membrane-binding Protein from Microvilli of Intestinal Epithelial Cells

CHRISTINE L. HOWE and MARK S. MOOSEKER
Department of Biology, Yale University, New Haven, Connecticut 06511

ABSTRACT One of the major proteins of the chicken intestinal microvillus is a calmodulin-binding protein of 105–110 kdaltons which has been tentatively identified as the bridge linking the microvillar filament bundle laterally to the membrane. We have treated isolated, membrane-intact brush borders with ATP and obtained solubilization of the 110-kdalton protein, calmodulin (CM), myosin, and lesser amounts of several other cytoskeletal proteins. Electron micrographs of ATP-extracted brush borders showed loss of the linkers between the actin filament bundle and the microvillar membrane, with "ballooning" of the membrane away from the filament bundle, particularly at the tip end. In brush borders treated with calcium and trifluoperazine to solubilize CM, precise arrangement and morphology of lateral bridges was unperturbed, but ATP treatment would no longer solubilize the 110-kdalton protein. This result suggests that associated CM is necessary for the ATP-induced solubilization of the 110-kdalton protein. A 110-kdalton protein-CM complex, with 110-kdalton protein:CM ratios of 1:1–2, was partially purified from ATP-extracts of brush borders by a combination of gel filtration and hydroxylapatite chromatography. The 110-kdalton protein-CM complex is an irregular, elongated molecule that ranged in size from 5 × 8 nm to 8 × 14 nm, with a Stokes' radius of 6.1 nm. This 110-kdalton protein-CM complex exhibited no Mg^{++}-ATPase activity and no detectable myosin light chain kinase activity. In co-sedimentation assays, the 110-kdalton protein-CM bound to F-actin in the absence but not the presence of ATP. Both the interaction of the complex with actin and the binding of CM to the 110-kdalton protein were calcium-independent. Negative stains of F-actin and 110-kdalton protein-CM in the absence of ATP showed loosely organized aggregates of actin with the 110-kdalton protein-CM complex coating the surface of the filaments. On the basis of our data, and in agreement with previous calculations (Matsudaira, P. T., and D. R. Burgess, 1979, *J. Cell Biol.* 83:667–673), we suggest that the lateral bridge of the microvillus is composed of a dimer of the 110-kdalton protein with four associated calmodulins.

Interactions between actin and membranes are an almost universal organizational feature of cortical cytoplasm in eucaryotic cells. Such interactions can be involved in establishment or maintenance of cell shape as well as in movements of the cell. Ultrastructural studies have shown that actin is localized near the plasma membrane in many cell types, with apparent attachments between actin filaments and the membrane. (For a review of membrane-cytoskeletal interactions, see references 52.) An excellent model for investigating how actin filaments interact with membranes is the brush border of intestinal epithelial cells. Each microvillus of the brush border contains an actin filament bundle connected both at its tip and along its length to the surrounding membrane. (For a review of brush border structure, see reference 34.) At the tip of the microvillus, where end-on connection of actin filaments with the membrane occurs, an electron dense "cap" is present. Significantly, this morphological "cap" does not prevent the polymerization of actin monomers onto the existing filament bundle at this site in vitro (36), although it can apparently maintain a connection between the growing actin filaments and the membrane in such a situation.

At the present time, more is known about the second type

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 OCTOBER 1983 974–985
© The Rockefeller University Press · 0021-9525/83/10/0974/12 $1.00

of filament-membrane connection in the microvillus, the lateral bridges. Structurally, "cross-filaments" in the microvillus were first detected by Millington and Finean (30). More convincing evidence for the existence of lateral bridges was presented in freeze-etch preparations of intestinal epithelium (38) and brush borders (32) and in brush borders treated with high Mg^{++} concentrations (32, 47). The bridges are attached to the actin filament bundle at a periodic interval of 33 nm along its length (27, 47) and appear to be arranged as twin spirals around the bundle (28, 29). The dimensions of bridges in membrane-intact brush borders range from 15 to 30 nm in length and 2 to 7 nm in width (32) but tend to be longer and thinner compared with bridges in demembranated microvilli, where their size has been measured as 7 × 20 nm (32) or their length as 12–16 nm (27). Membrane particles do not seem to associate directly with the lateral bridges (47). Even though lateral bridges remain attached to the actin filament bundle after demembranation of brush borders or microvilli, the bridges can be dissociated from the core filaments by treatment with ATP (2, 27). More specific biochemical information about the composition of the lateral bridges is still indirect. Morphological loss of bridges after ATP-treatment of demembranated microvilli corresponds to loss of two proteins, 110-kdaltons and 17-kdaltons, from the pellet fraction (27). Although the soluble 110-kdalton protein in the supernate was not demonstrated, it may have been proteolyzed since the 110-kdalton protein is extremely susceptible to proteolysis (33). On the basis of these data, it was suggested that the 110- and 17-kdalton proteins were components of the lateral bridges (27). The 17-kdalton protein was later identified as calmodulin (CM[1]; [19]), but the 110-kdalton protein has not yet been isolated and characterized.

The 110-kdalton protein is dissociated from demembranated (27) and membrane-intact (2) microvilli by ATP treatment; some CM also dissociates with the 110-kdalton protein under these conditions. The association between the 110-kdalton protein and CM implied by this result is further supported by in vitro gel overlay studies in which calmodulin was shown to bind to the 110-kdalton protein in a Ca^{++}-independent manner (13, 20). However, Glenney et al. (14) reported that loss of CM neither affects the binding of the 110-kdalton protein to the microvillus actin-filament bundle nor changes the morphology of the bundle or its lateral bridges, although ultrastructural evidence was not presented. Immunoelectron microscopy of the 110-kdalton protein by Glenney et al. (16) indicates that this protein is present on actin filaments in the microvillus core but not in the rootlet portion of the microvillus, an arrangement that corresponds to the location of the lateral bridges. In contrast, Coudrier et al. (6–8) and Reggio et al. (43), using a different antibody against the 110-kdalton protein, have presented evidence that localize this protein to the microvillar rootlet in the terminal web, to basal and lateral brush border membranes, and also to the sarcoplasmic reticulum of muscle in addition to the microvillus core. On the basis of 2-dimensional maps of total tryptic peptides (33), the 110-kdalton protein is similar in composition to the 95-kdalton protein of the microvillus actin bundle, villin. Functionally, however, the two proteins are quite different. Villin has been shown to bundle actin in the absence of Ca^{++}, and to "cut" actin filaments in the presence of Ca^{++} (2, 9, 28, 37, 51). These "solation" effects on the microvillus core do not involve any action of the 110-kdalton protein. The function of the 110-kdalton protein is apparently to bind calmodulin to the microvillus core and to attach the core filaments to the overlying microvillar membrane. The membrane attachment may be direct, or may be mediated by another, integral membrane protein, such as the 130-kdalton membrane protein as suggested by Coudrier et al. (6–8) and Reggio et al. (43).

All of these studies certainly implicate the 110-kdalton protein as a major component of the lateral bridge structure, and the data led us to explore further the structural and biochemical relationships between the lateral bridges, the 110-kdalton protein, and calmodulin in the brush border. We have been able to solubilize the 110-kdalton protein together with associated CM and keep it in solution with greatly reduced proteolysis by elimination of the demembranation procedure. We here report our initial experiments and characterize this major protein complex of the microvillus filament bundle.

[1] *Abbreviations used in this paper:* ATP-γ-S, adenosine-5'-*O*-(3-thiophosphate); CM, calmodulin; TFP, trifluoperazine.

MATERIALS AND METHODS

Isolation of Brush Borders: Brush borders were isolated from the small intestines of chickens according to the method described by Mooseker et al. (35), and modified by Keller and Mooseker (21). To help control proteolysis, 10–20 trypsin inhibitor units of aprotinin (Sigma Chemical Co., St. Louis, MO) per liter and 0.1 mM phenylmethysulfonylfluoride were added to all solutions.

Partial Purification of the Brush Border 110-kdalton Protein: Except where noted, all chemicals were obtained from Sigma Chemical Co. Pellets of isolated, membrane-intact brush borders were washed twice in solution A (75 mM KCl, 5 mM $MgSO_4$, 1 mM EGTA, 10 mM imidazole pH 7.2, 4 mM NaN_3, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride [Eastman Kodak Co., Rochester, NY]) and suspended in ~3 vol of solution A with 10 μg/ml phalloidin (Boehringer Mannheim Biochemicals, Indianapolis, IN) included to help stabilize actin filaments. After incubation on ice for 10 min, the suspension was extracted by addition of ~20 vol of solution A with 4 mM ATP and 0.2 M KCl. To test the nucelotide specificity of extraction, ATP-γ-S (adenosine-5'-O-(3-thiophosphate)), adenylyl-imidodiphosphate, (both obtained from Boehringer Mannheim Biochemicals), GTP, or sodium pyrophosphate was also used instead of ATP in the extraction step. After incubation on ice for another 10 min, the suspension was centrifuged for 10 min at 31,000 *g*. The supernate was made 60% in $(NH_4)_2SO_4$ by addition of solid salt, and the precipitated protein spun down at 31,000 *g* for 10 min. This pellet was suspended in a small volume of solution A with 1 mM ATP and 0.2 M KCl (buffer C), applied to a Sephacryl S-500 (Pharmacia Inc., Piscataway, NJ) gel filtration column, and eluted with this same buffer. Fractions containing the 110-kdalton protein were pooled, made 5 mM in potassium phosphate, and loaded onto a hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) column equilibrated with buffer C and 5 mM phosphate. The column was eluted with 100 mM phosphate in buffer C, then with 125 mM phosphate in buffer C. Fractions containing the 110-kdalton protein were used after dialysis into appropriate buffers.

Iodination of Calmodulin and ^{125}I-Labeled Calmodulin Overlay Technique/ATPase Experiments: CM was purified, iodinated, and used in the gel overlay technique exactly as described by Howe et al. (20). ATPase activity was measured by incubating fractions in a reaction mixture containing final concentrations of 160 mM KCl, 5 mM $MgSO_4$, 1 mM EGTA, 0.1 mM dithiothreitol, 10 mM imidazole pH 7.2, and 2 mM ATP. For experiments with Ca^{++}, the mixture also contained 1.2 mM $CaCl_2$. Inorganic phosphate was measured using Pollard and Korn's (42) modification of the method of Martin and Doty (25) in which [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, IL) was added to the reaction mixture. The inorganic phosphate generated after 0 or 20 min at 20°C was quantitated by scintillation counting.

Myosin Light Chain Kinase Assay: Samples of the 110-kdalton protein or other fractions from the S-500 column were tested for myosin light chain kinase activity by mixing equal volumes (50 λ) of sample and either buffer C or partially purified brush border myosin (final concentration, ~0.03 μg/ml). Fractions obtained by direct hydroxylapatite chromatography of the brush border ATP-extract (without using the first gel filtration column) were also tested for myosin light chain kinase activity. The final assay mixture also contained 2 μg/ml CM, 1 mM $CaCl_2$, and 2.5 mM [γ-^{32}P]ATP (0.02 Ci/mmol). The mixtures were incubated for 5 min at 37°C, and the reaction was stopped

by adding 0.011 ml of 10 × Laemmli sample buffer (22) and boiling for 2 min. Samples were electrophoresed on 5–15% gradient SDS polyacrylamide gels, and the gels were dried and autoradiographed.

Analysis of the 110-kdalton Protein Phosphorylation: Phosphorylation of brush border 110-kdalton protein was assayed in solution A with 0.2 M KCl and either 1 mM EGTA or a calcium buffer (1 mM EGTA, 1 mM $CaCl_2$) which gave a free calcium ion concentration of ~1–2 μM at pH 7.0. Membrane-intact brush borders were suspended in the appropriate solution, 2 mM [γ-^{32}P]ATP (0.04 Ci/mmol) was added, and the suspension incubated at 37°C for 3 min. Gel samples were made of the whole brush border suspension, or of the supernate and pellet fractions obtained by centrifuging the suspension for 2 min in a minifuge (Eppendorf model 5412, Brinkmann Instruments, Inc., Westbury, NY). The samples were electrophoresed on SDS polyacrylamide gels (linear 4–10% gradient), and the gels were stained and dried for autoradiography.

Actin-binding Experiments: Actin was purified from chicken breast muscle according to the procedure of Spudich and Watt (45). F-actin, polymerized by addition of 75 mM KCl and 1 mM $MgSO_4$, was added to hydroxylapatite column fractions of the 110-kdalton protein which had been dialyzed into solution A with 0.2 M KCl. The final KCl concentration was 150 mM. ATP was added where appropriate to a final concentration of 4 mM. For experiments in which the Ca^{++} was a variable, solution A with 0.05 mM EGTA was used in the dialysis, and either EGTA or $CaCl_2$ was added to the experimental mixtures to final concentrations of 1 mM or 0.2 mM, respectively. The mixtures of F-actin and the 110-kdalton protein were incubated for 20 min at room temperature, samples taken for negative staining, and the mixtures centrifuged for 1 h at 100,000 *g* at 15°C. Gel samples were made of the resulting supernates and pellets. Control samples of either F-actin or the 110-kdalton protein-CM alone were treated in the same way.

Extraction of Demembranated Brush Borders: Gradient-purified brush borders or microvillus cores prepared in solution A with only 0.1 mM $MgSO_4$ (solution I; [17]) were stabilized in an equal volume of 250 μg/ml phalloidin in solution I for 10 min on ice. An equal volume of 2% Nonidet P-40 (Particle Data Laboratories, Ltd., Elmhurst, IL) in solution I was added to this suspension and allowed to incubate for 10 min on ice. Finally, an excess of 1% Nonidet P-40 in solution I was added and after 10 min the brush borders were pelleted. They were washed once in solution I and aliquotted to Eppendorf tubes. These stabilized, demembranated brush borders were extracted in various buffers containing solution I with 0.2 M KCl and various combinations of 1 mM ATP, 1.2 mM $CaCl_2$, and/or 0.25 mM trifluoperazine dihydrochloride (Smith Kline and French Laboratories, Philadelphia, PA). After extraction on ice for 10 min, the brush borders were centrifuged for 3 min in an Eppendorf minifuge. Gel samples of supernates and pellets were made, and second pellets from identical extractions were also fixed and embedded for thin-section electron microscopy. The same experiments were performed on demembranated brush borders and microvillus cores without the phalloidin treatment but, instead of 1.2 mM $CaCl_2$, a concentration of 0.9 mM $CaCl_2$ (in conjunction with the 1 mM EGTA in solution I) was used.

Electron Microscopy: Samples were prepared for thin-section electron microscopy according to the method of Begg et al. (1). Pellets were fixed on ice in 2% glutaraldehyde, 2 mg/ml tannic acid in 0.01 M sodium phosphate buffer pH 7, washed in buffer pH 7 and postfixed in 1% OsO_4 in phosphate buffer pH 6. After washing in cold distilled water, the pellets were en bloc stained with 1% uranyl acetate, dehydrated in an acetone series and propylene oxide, and embedded in EPON.

The negative stain technique involved diluting the sample with the appropriate buffer solution immediately before staining and placing a small drop onto a glow-discharged, parlodion and carbon-coated grid. After 30–60 s, excess sample was wicked away before a drop of 1% uranyl acetate was put onto the grid for about the same amount of time. The uranyl acetate was then wicked away and the grid allowed to dry.

Other Methods: Protein concentrations were determined using the method of Lowry et al. (24) using BSA as a standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS PAGE) was performed using the methods of Laemmli (22) and Matsudaira and Burgess (26). Mini-gels were stained using the method of Weber and Osborn (50), and other gels stained by the method of Fairbanks et al. (12). Gel scans were performed at 643 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Protein peaks were quantitated by cutting and weighing.

RESULTS

ATP-extraction of Membrane-intact Brush Borders

Treatment of isolated, membrane-intact brush borders with ATP in a buffer (solution A; see Materials and Methods) which otherwise serves to stabilize brush border structure resulted in solubilization of the 110-kdalton protein and several other cytoskeletal proteins including calmodulin, myosin, and lesser relative amounts of actin, villin, fimbrin, and the spectrin-like 260/240-kdalton subunits (Fig. 1). The extraction of the 110-kdalton protein was ATP-dependent. Although use of ATP without increase in the KCl concentration did solubilize some 110-kdalton protein, best extraction occurred when the salt concentration was raised to at least 0.2 M KCl. In the presence of 0.2 M KCl in solution A, concentrations of ATP as low as 10 μM will extract the 110-kdalton protein, although more of this protein was solubilized when ATP concentrations in the millimolar range were used. We have examined the nucleotide specificity of the 110-kdalton protein solubilization by conducting similar experiments using GTP, ATP-γ-S, adenylyl-imidodiphosphate, or sodium pyrophosphate. Both GTP and the analog ATP-γ-S solubilized the 110-kdalton protein but to a lesser extent than ATP at the same concentration. Neither adenylyl-imidodiphosphate nor pyrophosphate induced solubilization of the 110-kdalton protein. In contrast, myosin was solubilized only by ATP and GTP.

The morphology of isolated brush borders after ATP extraction was unaltered at the light-microscope level of resolution. However, examination of these preparations by electron microscopy revealed that several major structural changes did occur (Fig. 2). In almost all of the brush borders, the lateral bridges linking the microvillar actin filament bundle to the surrounding membrane were gone. The actin bundles themselves remained relatively intact, although "loosen-

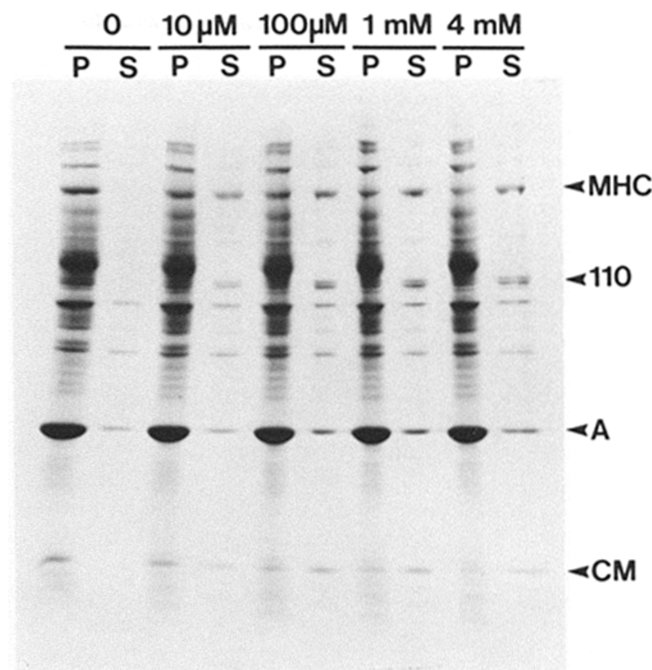


FIGURE 1 Extraction of isolated, membrane-intact brush borders with ATP. Brush borders were extracted on ice for 10 min with various concentrations of ATP (0, 10 μM, 100 μM, 1 mM, 4 mM) in solution A with 0.2 M KCl. Gel samples were made of the pellet (*P*) and supernate (*S*) fractions after a 31,000 *g* centrifugation of these suspensions and analyzed by SDS PAGE on 4–16% gradient gels. Proteins extracted by ATP concentrations as low as 10 μM include 10-kdalton protein (*110*), calmodulin (*CM*), myosin (*MHC*), and some actin (*A*).

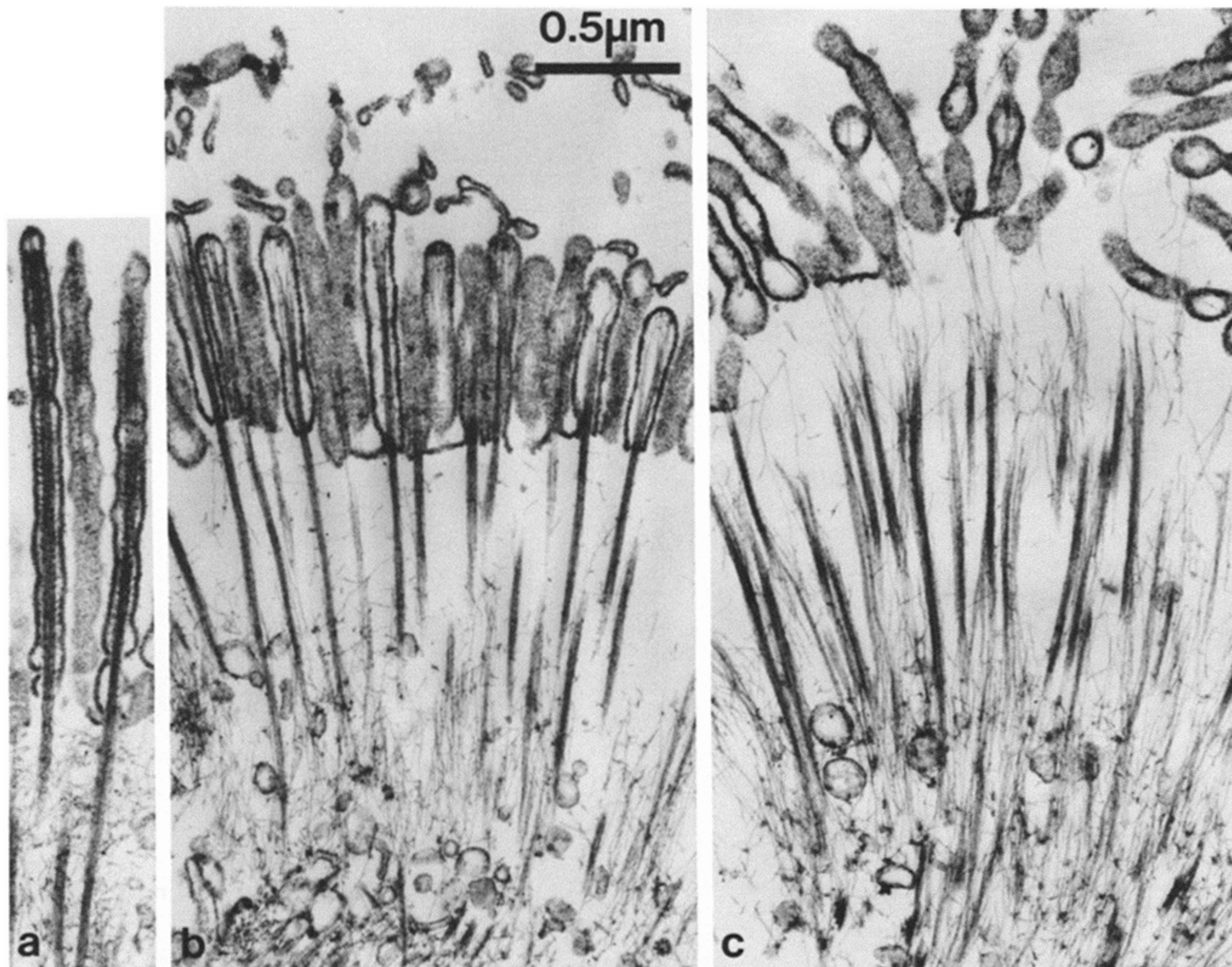


FIGURE 2 Thin-section electron microscopy of ATP-extracted brush borders. Membrane-intact brush borders were extracted in solution A with 0.2 M KCl in the absence (*a*) or presence (*b* and *c*) of 4 mM ATP. Normal morphology, including the presence of lateral bridges, is observed in controls (a); after ATP-extraction, (*b* and *c*) lateral bridges are absent, and the microvillar membrane appears to "lift" from the actin bundles and away from the terminal web region. Bar, 0.5 µm. × 40,000.

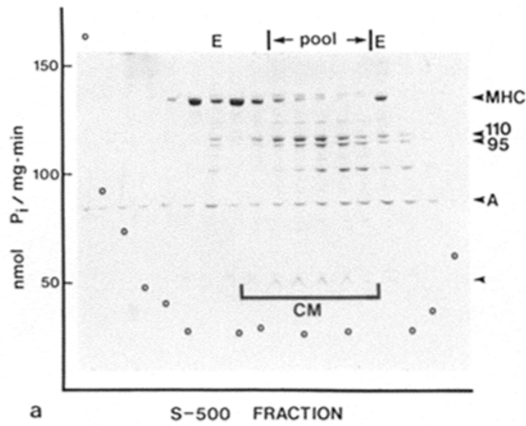
ing" of the bundle may occur at the distal end. The terminal web region was much less dense, consistent with the loss of myosin and known terminal web cross-linking proteins such as the terminal web 260/240 (15). A very dramatic change in the structural position of the microvillus membrane was evident in most brush borders (Fig. 2*b*). The membrane appeared to be "lifted" from the actin bundles, vesiculating at the tip as it left the bundle behind, intact but without lateral bridges. In some cases, the membrane seemed to be almost completely removed from the brush border cytoskeleton (Fig. 2*c*).

Partial Purification of a 110-kdalton Protein-Calmodulin Complex

It was possible to partially purify the 110-kdalton protein from the ATP extract of brush borders by a combination of gel filtration and hydroxylapatite chromatography (Fig. 3). In both columns, CM co-eluted with the 110-kdalton protein. The molar ratio of the 110-kdalton protein to CM in the gel filtration fractions as determined by scans of Coomassie-stained gels was 1:2 and ranged from 1:1 to 1:2 in the hydroxylapatite fractions. These ratios can be regarded as only rough estimates, however, because the relative dye binding by the 110-kdalton protein and CM has not been determined. Preparations of the 110-kdalton protein-CM obtained by these methods contained variable amounts of minor contaminating proteins with subunit molecular weights of about 130-, 80-, 68-, and 32-kdaltons. The 130-kdalton subunit was the most consistent contaminant, and we have tentatively identified it as a proteolytic fragment of the 240-kdalton subunit of the spectrin-like protein TW 260/240 (12). Identification was made using the one-dimensional peptide mapping technique of Cleveland et al. (5; [K. Conzelman and M. S. Mooseker, unpublished observations]). The other contaminating subunits, with the possible exception of the 68-kdalton subunit, which may be fimbrin, are probably proteolytic fragments of the 110-kdalton protein because their amounts increased with storage of the 110-kdalton protein fractions.

Characterization of the 110-kdalton Protein-Calmodulin Complex

Because CM was associated with the 110-kdalton protein throughout our purification scheme, we assumed that the 110-kdalton protein was in fact the same CM-binding protein as that identified by a ^{125}I-calmodulin gel overlay technique (13, 20). To verify that assumption, we used the technique to



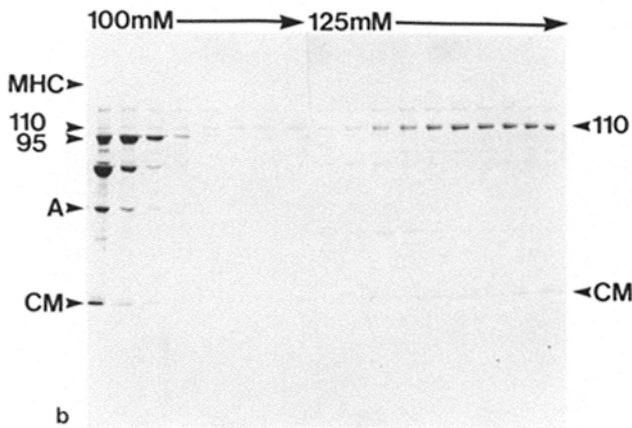


FIGURE 3 Purification of 110-kdalton protein-CM from an ATP-extract of brush borders and enzyme activity of fractions. (*a*) The ATP-extract (*E*) was applied to a S-500 gel filtration column equilibrated in buffer C. Calmodulin (*CM*) co-elutes with 110-kdalton protein (*110*) in molar rations of ~2:1 as shown by SDS PAGE. The axes alongside the gel indicate Mg^{++}-ATPase activity of fractions on the gel. The ATP-extract (*E*) had an Mg^{++}-ATPase activity of 100 nmol P_i/mg-min, but activity is decreased in fractions containing 110-kdalton protein-CM. (*b*) The 110-kdalton protein-CM fractions (*pool* as shown in *a*) were made 5 mM in phosphate and applied to a hydroxylapatite column. After a 100-mM phosphate step (left side of gel), 110-kdalton protein (*110*) and calmodulin (*CM*) are co-eluted with 125 mM phosphate (right side of gel).The 110-kdalton protein-CM fractions after this step have an Mg^{++}-ATPase activity of ~30 nmol P_i/mg-min. Contaminating proteins are discussed in the text. 4-16% SDS PAGE. *95*, villin, *A*, actin, *MHC*, heavy chain of myosin.

test samples of the initial ATP extract, gel filtration column fractions, and the hydroxylapatite-purified 110-kdalton protein-CM complex. The labeled CM bound to the 110-kdalton protein, and this binding occurred in the presence (Fig. 4) and absence (results not shown) of Ca^{++}.

Although its ATP-induced dissociation from both the actin bundle and membrane suggested that the 110-kdalton protein-CM may have an ATP binding site(s), we have been unable to demonstrate significant Mg^{++}-ATPase activity of the 110-kdalton protein-CM in the presence or absence of Ca^{++} or added CM purified from bovine brain. The ATP-extract of membrane-intact brush borders has an Mg^{++}-ATPase activity of ~100 nmol P_i/mg-min, but when the extract is fractionated by gel filtration this ATPase activity is enriched to ~150 nmol P_i/mg-min in fractions eluting ahead of the 110-kdalton protein-CM and is decreased in the fractions containing the 110-kdalton protein-CM to ~25–50 nmol P_i/mg-min (see Fig. 3*a*). The 110-kdalton protein-CM fractions further purified by hydroxylapatite chromatography exhibit a Mg^{++}-ATPase activity of ~30 nmol P_i/mg-min.

Given that the molecular weight of the 110-kdalton protein-CM is similar to that of myosin light chain kinase, we have asayed the 110-kdalton protein-CM for kinase activity in the presence or absence of calcium and added CM. Our 110-kdalton protein-CM-fractions had no detectable myosin light chain kinase activity, but such light chain kinase activity was present in isolated brush borders, in the ATP-extract of brush borders, and in fractions eluting ahead of the 110-kdalton protein-CM on the S-500 column and in the 100 mM phosphate step on the hydroxylapatite column.

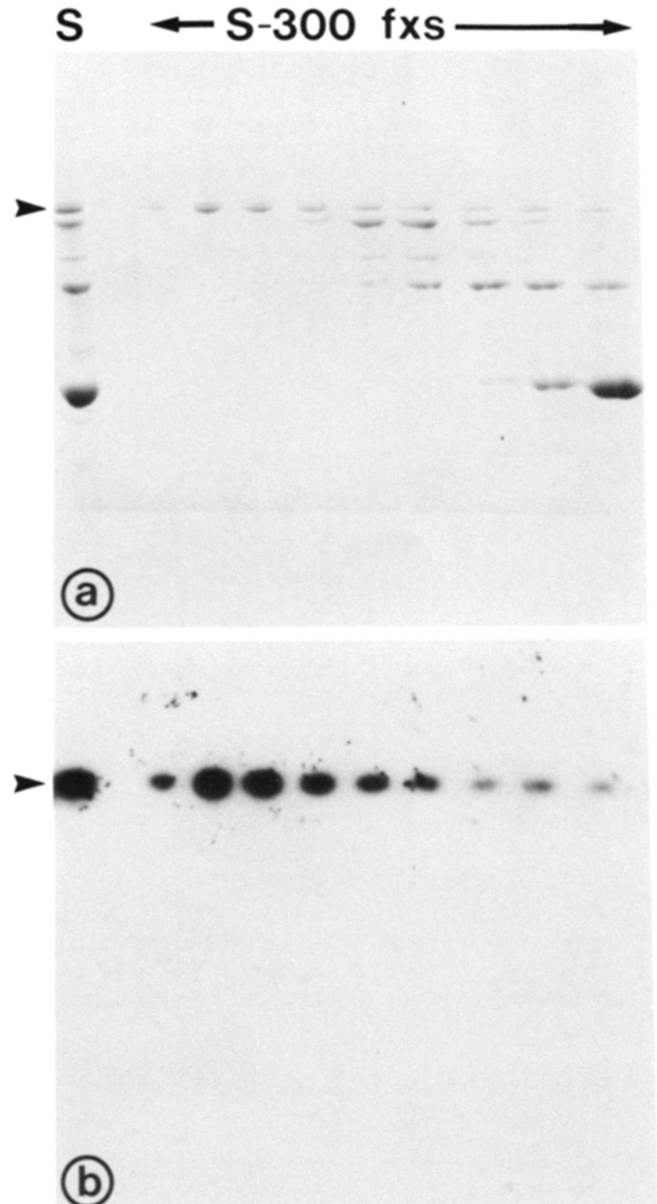


FIGURE 4 ^{125}I-calmodulin gel overlay of ATP extract and gel filtration column fractions. An SDS polyacrylamide gel of an ATP-extract (*S*) and fractions from a gel filtration column (*S-300 fxs*) was incubated with ^{125}I-calmodulin as described by Howe et al. (20). A Coomassie Blue-stained gel (*a*) is shown above its corresponding autoradiogram (*b*). The 110-kdalton protein (arrow) binds CM in the presence (or absence, results not shown) of calcium.

There is preliminary evidence to indicate that the 110-kdalton protein-subunit can be phosphorylated by a membrane-associated kinase. In experiments where 2 mM [γ-^{32}P]ATP was added to isolated, membrane-intact brush borders, a 105–110-kdalton-subunit was one of the major phosphorylated bands (results not shown, but see Fig. 6*a* in Keller and Mooseker [21]). That this band is the 110-kdalton protein is suggested by the fact that when the ATP-treated brush borders were centrifuged and gel samples made of the resulting ATP-extract and the extracted pellet fractions, this phospho-

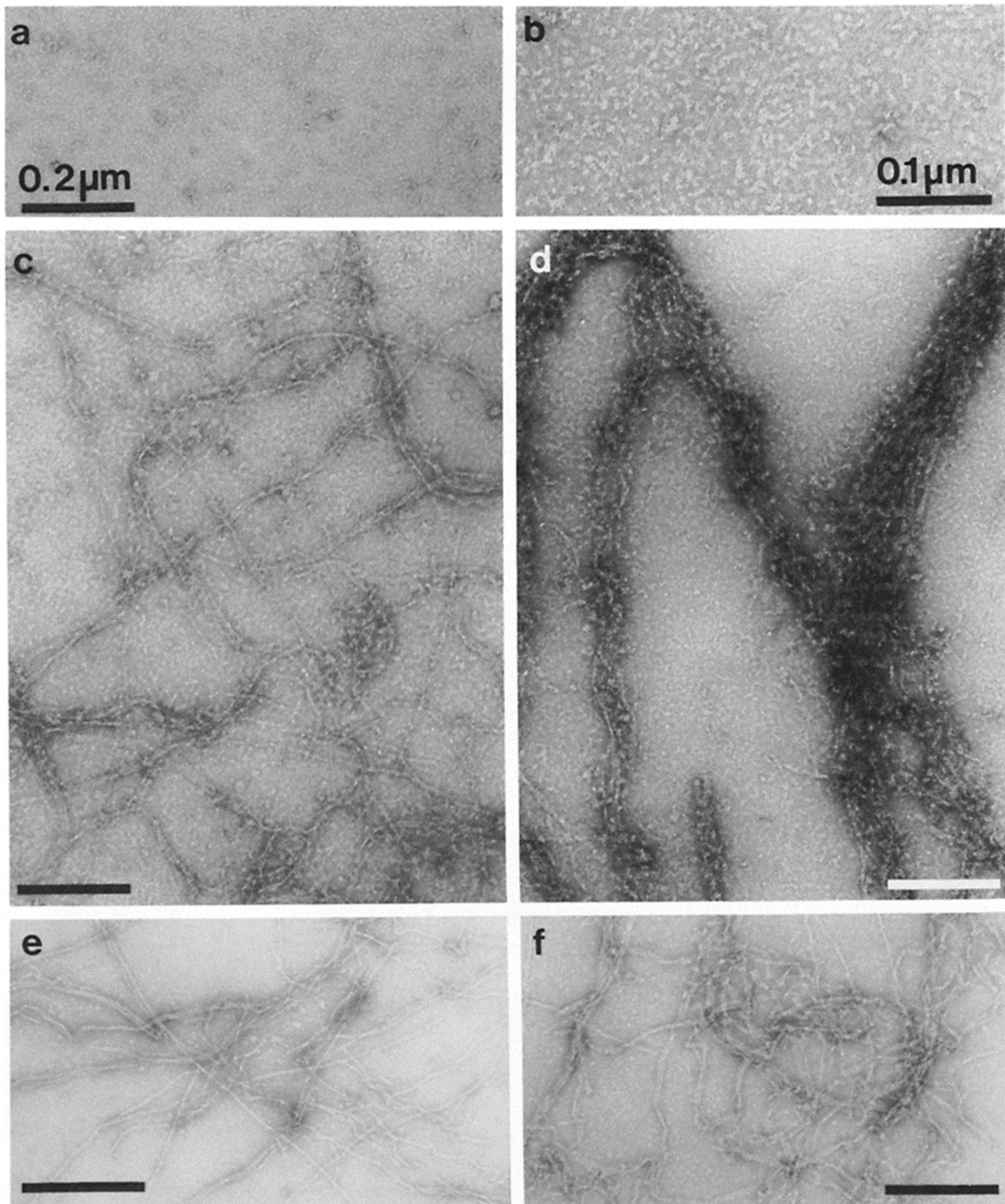


FIGURE 5 Electron microscopy of 110-kdalton protein-CM and actin. Electron micrographs of negatively stained (*a* and *b*) preparations of 110-kdalton protein-CM show irregularly shaped, elongated molecules. When 110-kdalton protein-CM (0.02 mg/ml) is added to F-actin (0.25 mg/ml) in the presence of ATP (*c*), normal actin filaments are seen on a uniform background of 110-kdalton protein-CM. In the absence of ATP (*d*), 110-kdalton protein-CM coats the actin filaments, which are collected into loose aggregates. See text for details. Actin controls in the presence (*e*) and absence (*f*) of ATP are also shown. Bar, 0.2 μm (*a* and *c–f*); Bar, 0.1 μm (*b*). × 90,000 (*a* and *c–f*); 190,000 (*b*).

rylated 105–110-kdalton-band was present in the ATP-extract, as was the 110-kdalton protein (results not shown). Moreover, phosphorylation of the 110-kdalton protein is not observed if demembranated (Triton-treated) brush borders are used rather than membrane-intact brush borders (see Fig. 6*b* in Keller and Mooseker [21]), indicating that the kinase responsible for phosphorylating the 110-kdalton protein was solubilized or inactivated by detergent treatment.

We have examined negatively stained (Fig. 5, *a* and *b*) preparations of the 110-kdalton protein-CM complex and saw irregularly-shaped elongated molecules ranging in size from 5 × 8 to 8 × 14 nm. The Stokes' radius of the complex measured by gel filtration was ~6.1 nm. Thus far we have been unsuccessful in attempts to determine the pI of the 110-kdalton protein using isoelectric focusing gel techniques. The 110-kdalton protein will not electrophorese or focus with standard procedures (39), nonequilibrium electrophoresis (40), or inclusion of 0.1% SDS in the sample lysis buffer (40).

Binding of the 110-kdalton Protein-Calmodulin Complex to Actin

Interaction of the 110-kdalton protein with actin is of obvious importance because of the suggestions that 110-kdalton protein is a component of the lateral bridge. Moreover, the binding of CM to the microvillus core must be mediated by 110-kdalton protein because we have shown previously (19) that CM does not bind directly to actin. In co-sedimentation assays, the partially purified 110-kdalton protein-CM complex bound to actin in an ATP-dependent, Ca^{++}-independent manner (Fig. 6). Binding occurred in the absence but not the presence of ATP. The fact that calmodulin co-sedimented with actin and the 110-kdalton protein in both the presence and absence of calcium indicates that the binding of calmodulin by "native" 110-kdalton protein is Ca^{++}-independent and confirms this idea which was first shown using the gel overlay technique (13, 20). Negative stains of actin and 110-kdalton protein-CM in the presence of ATP show normal actin filaments present on a uniform, homogeneous background of 110-kdalton protein-CM (Fig. 5*b*). In the absence of ATP, however, the 110-kdalton protein-CM complex coated loosely organized aggregates or bundles of actin (Fig. 5*d*). The precise spiral arrangement of lateral bridges seen on microvillus cores was not observed in these actin aggregates. Moreover, because the actin aggregates are not highly organized, it was impossible to see whether 110-kdalton protein-CM was interacting with a given actin filament at periodic intervals along the length of the filament.

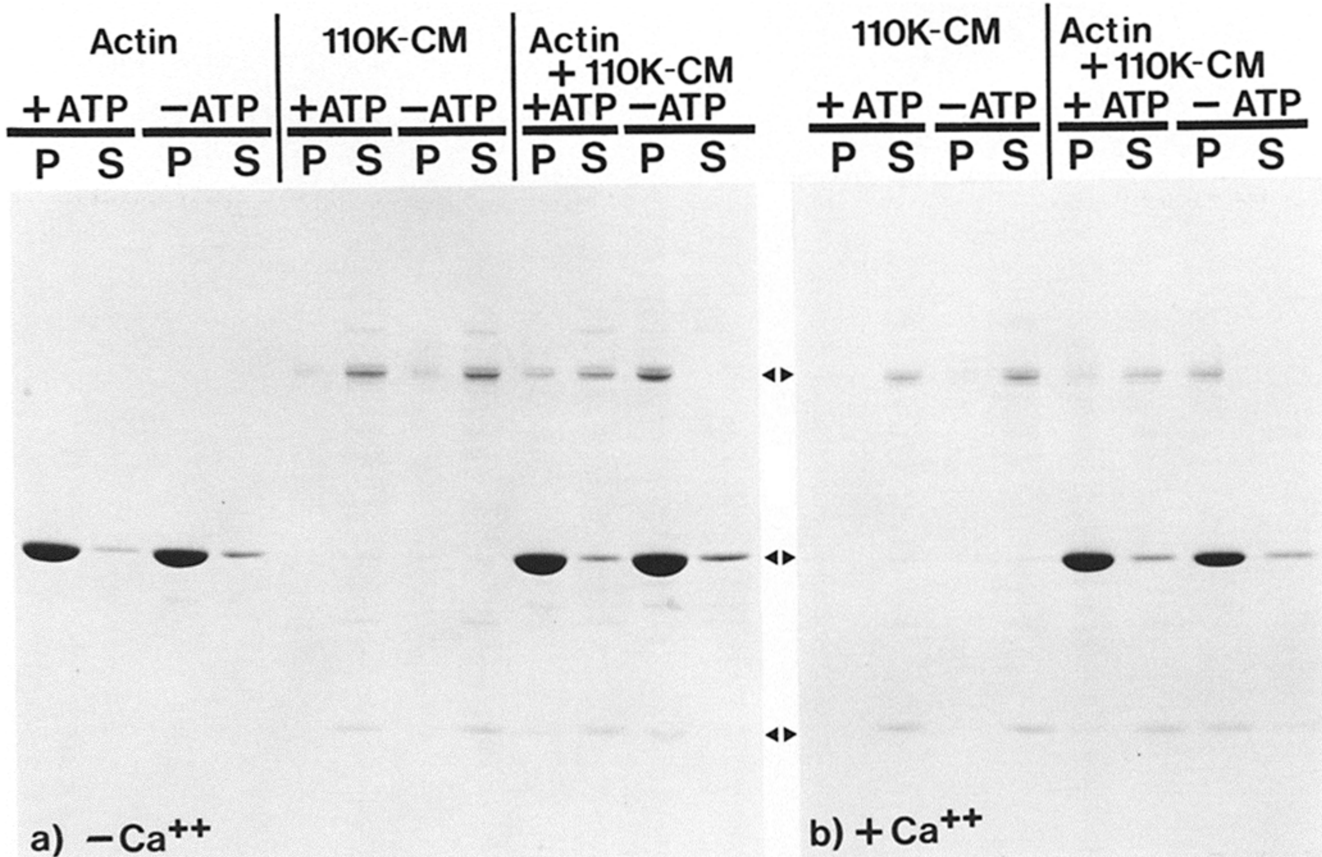


FIGURE 6 Co-sedimentation of 110-kdalton protein-CM with F-actin. (*a*) In samples containing 1 mM EGTA ($-Ca^{++}$) that were centrifuged for 1 h at 100,000 *g*, actin alone (*Actin*) in the presence (*+ATP*) or absence (*−ATP*) of ATP sediments into the pellet fraction (*P*). Hydroxylapatite-purified 110-kdalton protein-CM (*110K-CM*) alone remains in the supernate (*S*) in the presence or absence of ATP. When F-actin and the 110-kdalton protein-CM fraction are mixed (*Actin + 110K-CM*) and centrifuged, little interaction is seen in the presence of ATP. In the absence of ATP, however, all of the 110-kdalton protein and all of the calmodulin (*CM*) pellet with the actin. (*b*) In the presence of 0.2 mM calcium ($+Ca^{++}$), the same results are observed. Only in the absence of ATP does 110-kdalton protein-CM pellet with F-actin. The concentrations of actin and 110-kdalton protein-CM were ~0.25 mg/ml and 0.02 mg/ml, respectively. 4-16% SDS PAGE. Arrowheads indicate 110-kdalton protein, actin, and CM on each gel.

Effects of Calcium and Trifluoperazine on Lateral Bridge Structure and ATP-dependent Dissociation of 110-kdalton Protein-Calmodulin

Glenney et al. (14) demonstrated that treatment of microvillus cores with the drug trifluoperazine (TFP), both in the presence and in the absence of Ca^{++}, results in solubilization of CM without release of 110-kdalton protein from the microvillus core pellet. They report, but data are not shown, that this loss of CM has no effect on lateral bridge structure. We have repeated and extended this observation, conducting extensive ultrastructural studies, to further define the various associations of CM with the brush border cytoskeletal apparatus and the role that CM plays in the structure of the lateral bridge. For the experiments shown here (Fig. 7), demembranated brush borders were pretreated with phalloidin to prevent Ca^{++}-dependent solation of microvillus cores by villin (2, 9, 28, 37, 51). In addition, we also conducted identical experiments (results summarized in Table I) with brush borders without phalloidin by using free Ca^{++} concentrations (1–2 μM; Ca^{++}/EGTA ratio = 0.9) which are sufficiently high to saturate Ca^{++}-binding by CM (as assayed by Ca^{++}-activation of brush border myosin light chain kinase), but low enough that the "cutting" activity of villin is not activated (21).

As first demonstrated by Glenney et al. (14), treatment of microvillus cores, or in our case demembranated brush borders as well, with Ca^{++} and TFP results in solubilization of CM without concomitant solubilization of the 110-kdalton protein. Unlike Glenney et al., we were unable to extract CM with TFP in the absence of Ca^{++}, but examination of demembranated brush borders treated with Ca^{++} and TFP, or Ca^{++} or TFP alone, indicated that lateral bridge structure is unaltered (Fig. 7, *a* and *b*).

The ATP-dependent solubilization of 110-kdalton protein-CM, first reported by Matsudaira and Burgess (27), occurred equally well in the presence or absence of Ca^{++} over a wide range of Ca^{++} concentrations (10^{-8} to 10^{-4} M), as well as in the presence of TFP alone (no Ca^{++}) (Fig. 7, *c* and *d*). In most experiments, only 40–75% of 110-kdalton protein-CM was solubilized by ATP-treatment (Table I), presumably because of the detergent effects on 110-kdalton protein-CM solubility. Despite incomplete solubility of 110-kdalton protein-CM, the lateral bridges were absent from all of the microvillus cores after these ATP-treatments (Fig. 7, *c* and *d*).

Finally, when demembranated brush borders were pretreated with Ca^{++} and TFP to extract CM, and then treated with ATP, no solubilization of the 110-kdalton protein occurred. A similar result was observed if demembranated brush borders were treated simultaneously with ATP, Ca^{++}, and TFP. However, even though the 110-kdalton protein remained in the insoluble pellet fraction, the lateral bridges were not present on the microvillus cores. Rather, the cores were coated with aggregates of dense staining material (Fig. 7, *e* and *f*).

DISCUSSION

Isolation of 110-kdalton Protein-Calmodulin

Our initial attempts to isolate and purify the microvillar 110-kdalton protein involved extraction of partially or extensively demembranated brush borders and microvilli. Although we obtained the same qualitative nucleotide-dependent release of 110-kdalton protein in these cases as we report from membrane-intact brush borders, above, the amount of 110-kdalton protein and CM that was solubilized from detergent-treated organelles was much greater than that from membrane-intact brush borders. However, the 110-kdalton protein in ATP-extracts from detergent-treated preparations did not remain soluble in the succeeding purification steps and in fact could be pelleted out of the ATP-extract, along with some of the other extracted proteins, by high speed centrifugation. Because of these solubility problems, we used the ATP-extract from membrane-intact brush borders for further purification even though we had lower quantitative yields of 110-kdalton protein from such preparations.

Other schemes besides the one listed in Materials and Methods for purification of 110-kdalton protein that were tried included various combinations of gel filtration (using agarose, dextran, or polyacrylamide beads), ion exchange (DEAE- or CM-Sephadex beads), affinity (CM coupled to Sepharose), and hydroxylapatite chromatography. Both step and gradient elutions from ion exchange and hydroxylapatite columns were used, and fractions were concentrated when needed by ammonium sulfate precipitation. We used buffers ranging from 25 mM to 0.6 M salt, with or without ATP and/or calcium, at pH's from 6.5 to 9, to try to find conditions in which the insolubility of 110-kdalton protein (even from nondetergent-treated brush borders) was minimized. The Sephacryl-500 gel filtration and a hydroxylapatite column gave us the best results in terms of both relative purity and retention of solubility. Other labs have noted the insolubility of the 110-kdalton protein (8, 51), and, although we have been able to overcome this difficulty to some degree, we do have trouble keeping it in solution if lengthy purification procedures are used. Specifically, we have noted that precipitation of 110-kdalton protein is accompanied by a partial or total loss of associated CM. Whether this relationship between 110-kdalton protein insolubility and CM loss is due to a turnover of CM that may cause 110-kdalton protein to alter its conformation and precipitate, or a destructive change in the structure of the 110-kdalton protein-molecule such that it can no longer bind CM, is not clear. Addition of exogenous brain CM had no effect on the retention of solubility by the crude or partially purified 110-kdalton protein-CM complex.

Morphology of ATP-extracted Brush Borders

The morphological changes that we have seen in ATP-extracted brush borders were consistent with the ideas that 110-kdalton protein and CM are components of the lateral bridges of the microvillus, and that these bridges do structurally link and physically tether the microvillar membrane to the underlying cytoskeletal framework. However, as pointed out by Bretscher (2), ATP-induced release of 110-kdalton protein and CM from the membrane-intact structure means that not only the actin-binding site but also the membrane-binding site for this protein complex is ATP-sensitive in vitro. This fact poses a problem in vivo if the 110-kdalton protein-CM complex composes the lateral bridge and is involved in structural attachment of the actin bundle with the membrane. Perhaps not all of the attachments are broken at the same time in the presence of ATP, or perhaps there are other controlling factors for attachment in vivo that are lost in vitro. The dense tip of the microvillus, at the site of end-on filament interaction with the membrane, also seems to link actin with the membrane, since in extracted brush borders there are some actin filaments associated with the tip structure that

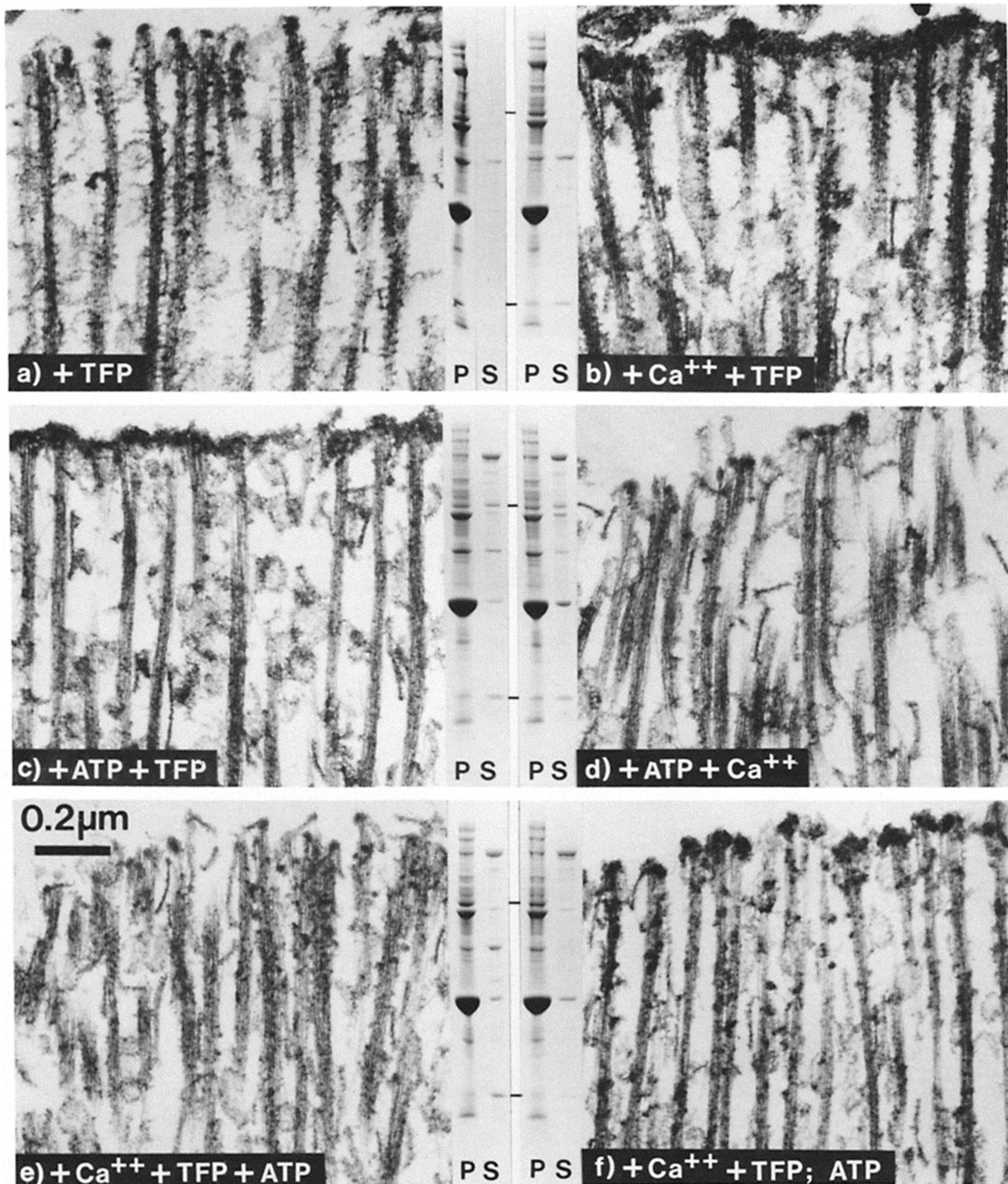


FIGURE 7 Thin-section electron microscopy of phalloidin-stabilized, demembranated brush borders after extraction. Results of corresponding SDS PAGE of pellets (*P*) and supernates (*S*) are also shown. Brush borders extracted in solution I (see text) with 0.25 mM trifluoperazine (*a*; *+ATP*) or with 0.2 mM calcium and TFP (*b*; *+*Ca^{++} *+ TFP*) retain normal lateral bridges, although CM is solubilized in *b* (bar). Lateral bridges are lost and 110-kdalton protein-CM is released into the supernate (bars) after extraction with 1 mM ATP and TFP (*c*; *+ATP + TFP*) or with ATP and 0.2 mM calcium (*d*; *+ATP +* Ca^{++}). When CM is solubilized by calcium and TFP (bar), neither concomitant (*e*; + Ca^{++} *+TFP + ATP*) nor subsequent (*f*; *+*Ca^{++} *+ TFP; ATP*) ATP treatment will solubilize 110-kdalton protein (bar), and amorphous aggregates are present on the actin filament bundles. Bar, 0.2 μm. × 65,000.

seem to be "pulled" out of the bundle and along with the membrane when it is "lifted" away. Unlike the lateral attachments, therefore, the end-on connection of actin with the membrane does not appear to be ATP-sensitive, and is persistent even in increased salt concentrations. Incidentally, the ATP-dependent lifting up of the brush border membrane, together with the "solation" of the microvillus core, is the reason why one of us was "tricked" into thinking that Ca^{++} and ATP caused a retraction of microvillus cores into the terminal web (e.g., see Fig. 7 in reference 31).

Table I

Effects of ATP, Ca^{++}, and TFP on Solubilization of the 110-kdalton Protein and Calmodulin and the Presence of Lateral Bridges

Extraction condition	110-kdalton Protein in sup	Calmodulin in sup	Bridges
	%	%	
Control	0	0	+
ATP[*]	40–75	40–60	–
Ca^{++}[‡]	0–10	0-tr	+
TFP[§]	0	0-tr	+
Ca^{++} + TFP	0	20–90	+
Ca^{++} + ATP	10–75	50–75	–
ATP + TFP	60	60	–
Ca^{++} + TFP + ATP	0	70–90	–[‖]
Ca^{++} + TFP; then ATP	0–tr	0-tr	–[‖]
ATP; then Ca^{++}	0[¶]	0[¶]	–
Ca^{++}; then ATP	60–80	60–80	–

Demembranated microvilli ("microvillar cores") and brush borders, some of which were pretreated with phalloidin to stabilize actin filaments, were extracted with different solutions (see Materials and Methods), centrifuged, and the amounts of 110-kdalton protein and calmodulin in the supernate fraction assayed by SDS PAGE. Thin-section electron microscopy of extracted pellets was used for ultrastructural analysis of lateral bridges. This table summarizes results of seven separate experiments. *sup*, supernate; *tr*, trace amounts in the supernate.

[*] ATP concentrations used were 1-4 mM.

[‡] The calcium concentration used for phalloidin-stabilized organelles was ~1–2 μM (Ca^{++}/EGTA ratio = 1.2), and for nonphalloidin-treated organelles it was ~0.5 μM (Ca^{++}/EGTA ratio = 0.9).

[§] The trifluoperazine concentration used was 0.25 mM.

[‖] For these extraction conditions, normal bridges were absent, but densely-staining aggregates remained with the pellet fraction; see Discussion for details.

[¶] Both the 110-kdalton protein and calmodulin were solubilized by the first ATP treatment (see condition 2), and subsequent Ca^{++} extraction did not induce further solubilization.

Phosphorylation of the 110-kdalton Protein

The 110-kdalton protein appears to be phosphorylated by an endogenous brush border kinase. This phosphorylation reaction is calcium- and calmodulin-independent (see Fig. 6 *a*, Keller and Mooseker [27]) but occurs to a very much reduced extent, if at all, in demembranated brush borders (see Fig. 6 *b*, Keller and Mooseker [21]). This data, combined with the fact that 110-kdalton protein in an ATP-extract of brush borders was not labeled by subsequent addition of [γ-^{32}P]ATP (data not shown), suggest that the kinase may be present in the brush border membrane fraction. It is important to note that ATP-treatment results in solubilization of 110-kdalton protein-CM from both membrane-intact and demembranated brush borders, suggesting that phosphorylation of the 110-kdalton protein may not be responsible for its dissociation. Further experiments need to be done on the 110-kdalton protein phosphorylation reaction, for example to determine the location of the kinase and to see whether 110-kdalton protein solubilized by ATP-treatment is in a phosphorylated state.

110-kdalton Protein-Calmodulin-binding to Actin Filaments

Despite numerous attempts, we have not yet been able to restore bridges by adding 110-kdalton protein-CM to demembranated microvillus cores from which the lateral bridges and endogenous 110-kdalton protein-CM have been removed by ATP-treatment. We think that residual detergent on these demembranated microvilli, even after extensive washing of the cores, may be interfering with binding and/or solubility of the 110-kdalton protein-CM. However, we have been successful in adding 110-kdalton protein-CM to actin isolated from muscle, and this binding of 110-kdalton protein-CM to pure actin filaments is identical in nature to the binding of the lateral bridge to the microvillus core in that it occurs only in the absence of ATP and is Ca^{++}-independent (Fig. 6).

The apparent cross-linking of actin filaments by 110-kdalton protein-CM is disturbing since, if this complex is the lateral bridge, one would expect that it could bind to only one actin filament. We do not consider 110-kdalton protein-CM to be an actin "bundling" complex, as are villin and fimbrin, since the negative stains show aggregates, not bundles, of actin filaments. The slight amounts of contaminating proteins in the 110-kdalton protein-CM preparation (e.g., the 130-kdalton fragment of TW 240, and fimbrin) may contribute to this aggregation; however, we think that this is unlikely. Neither the TW 240 fragment nor fimbrin were present in significant amounts, and actin bundled by either fimbrin (17) or intact TW 260/240 (M. S. Mooseker and D. Fishkind, unpublished observations) has a different morphology than the actin aggregates we observed. Moreover, TW 260/240 does not bind significantly to actin under the salt conditions used in the 110-kdalton protein-CM-actin binding experiments (41). Most importantly, the cross-linking of actin by either fimbrin or intact TW 260/240 is not ATP-dependent, while the actin aggregates we observed (Fig. 5) and the 110-kdalton protein-CM binding to actin seen on gels (Fig. 6) occurred only in the absence of ATP.

Thus, the aggregation of actin filaments by 110-kdalton protein-CM in the absence of ATP could be an artifact of nonspecific binding. For example, the 110-kdalton protein-CM could have a "sticky" or hydrophobic domain at its membrane binding site. Consequently, actin would be cross-linked by 110-kdalton protein-CM either by nonspecific association of its membrane binding site with actin or by a head-to-head interaction between 110-kdalton protein-CM complexes bound to two different actin filaments. In support of this notion, we have observed that, in negatively stained preparations of isolated, demembranated microvilli with intact lateral bridges, clumps consisting of cores closely associated along their lengths often occurred.

The actin filaments in these aggregates are not analogous to microvillar actin filaments, because they are most likely of non-uniform polarity and are not packed in a precise array by bundling proteins (see references 10, 11, 46). The uniform arrangement of lateral bridges in a microvillus might be specified by the uniform filament polarity and packing, by other proteins in the actin filament bundle e.g., 95-kdalton (villin) or 68-kdalton (fimbrin), by structural units in the microvillus membrane, or by a combination of these or other factors.

Relationships of Calmodulin to the Lateral Bridge and the ATP-dependent Dissociation of 110-kdalton Protein-Calmodulin

On the basis of our electron micrographs of CM-depleted brush borders (Fig. 7), we agree with Glenney et al. (14) that the lateral bridges have a normal morphology even when more than half of the calmodulin is absent from the core. Unlike them, we have not been able to obtain complete CM

release in the presence of only calcium and trifluoperazine (Table I and Fig. 7). The material that remained in the pellet after extraction with calcium and TFP and that co-migrated with the calmodulin band in the supernate fraction was assumed to be CM, based on two observations. In some gels in which EGTA was not added to the samples, this band exhibited the mobility shift (usually seen as a "smile" unique to that band; see Fig. 3*a*) characteristic of and unique to CM (4). Also, no protein visible on Coomassie-stained gels was present in the brush border in amounts comparable to CM at that specific location directly above the myosin light chain (19). However, this difference in our results may be due to some factor in their preparation that also caused the release of ~50% of the CM by TFP alone (14), an effect we do not see and an unexpected result if the usual mechanism of TFP interaction with CM is applicable (23). Perhaps they are also losing CM from the extracted preparation by some nonspecific means, for the amount of CM in the supernate fraction after TFP extraction does not equal the amount of CM present in the nonextracted pellet (see Fig. 1, lanes *E, F,* and *G*) in Glenney et al. [14]).

When CM-depleted brush borders were treated with ATP, the 110-kdalton protein was not solubilized, but normal lateral bridges were absent. We believe that under these conditions 110-kdalton protein may be released from its usual association with actin, but remains in the pellet fraction because of insolubility somehow associated with the absence of bound CM. In support of this interpretation, we observe that the isolated 110-kdalton protein-CM complex dissociated over time into a soluble CM fraction and an insoluble 110-kdalton protein fraction.

Is the 110-kdalton Protein-Calmodulin Complex the Lateral Bridge?

All previous evidence (14, 20, 27) and the results presented here strongly indicate that 110-kdalton protein-CM composes the lateral bridge. Definite proof of this likely possibility will require reconstitution of the lateral bridges by addition of purified 110-kdalton protein-CM. The elongated shape and size of the 110-kdalton protein-CM complex, its presumed stoichiometry (110-kdalton protein:CM = 1:2), and its Stokes' radius are consistent with the idea that one complex is composed of two 110-kdalton protein-molecules with four associated CM. These data agree with the figures of Bretscher and Weber (3) and Matsudaira and Burgess (27) who, using calculations on the number of lateral bridges per actin in a microvillus and the molar ratio of 110-kdalton protein to actin, suggested that the lateral bridge should be composed of at least a dimer of 110-kdalton protein.

Many questions about the function of 110-kdalton protein-CM in the brush border remain unanswered. We do not know the significance of its ATP-induced release or how it interacts with the membrane. The functions of CM so tightly associated with 110-kdalton protein likewise must be defined. For example, could the 110-kdalton protein-CM complex be involved in regulation of enzymes in the microvillus membrane? Finally, nothing is known about linker proteins to which 110-kdalton protein may be related (18, 48), such as the lateral connections between the actin filament bundle and the membrane in stereocilia of the chick ear hair cell. These connections have dimensions very similar to those of the lateral bridge but are labile towards fixation and/or dehydration and have only been observed in quick frozen and deeply etched samples (18). Thus, there may be a family of molecules similar to the microvillar 110-kdalton protein-CM, possessing both actin- and membrane-binding sites and found in different cell types, which may be functionally related to 110-kdalton protein and with which microvillar 110-kdalton protein-CM[2] may or may not cross-react immunologically. In this regard, it is interesting to note the recent findings of Siliciano and Craig (44) on the identification of a vinculin-like protein (vinculin has been found in many cell types at sites of actin-filament-membrane interaction; see reference 9) with solubility properties characteristic of an integral membrane protein.

During the course of this work we had helpful discussions and assistance from Hector Alicea, Ed Bonder, Becky Chasen, Ann Chien, Karen Conzelman, Doug Fishkind, Tom Keller, and Mirilee Pearl. Joel Rosenbaum generously let us use his electron microscope facility. We thank Tom Keller for performing the myosin light chain kinase assays, Karen Conzelman for conducting Cleveland digests on the 130-kdalton fragment, and Doug Fishkind for extremely capable technical assistance at all stages of the work; they also provided cheerful help during numerous early morning chicken preps. Thanks are due Doug Keene for excellent thin sections. We also thank Lorraine Klump for help in preparing the manuscript.

This work was supported by a National Institutes of Health Predoctoral Fellowship to C. L. Howe and Institute of Arthritis, Metabolism and Digestive Diseases grant AM-25387 and a Basil O'Connor Starter Research Grant from the March of Dimes to M. S. Mooseker.

Received for publication 29 December 1982, and in revised form 27 May 1983.

REFERENCES

1. Begg, D. A., R. Rodewald, and L. I. Rebhun. 1978. The visualization of actin filament polarity in thin sections. Evidence for the uniform polarity of membrane-associated filaments. *J. Cell Biol.* 79:846–852.
2. Bretscher, A. 1982. Characterization and ultrastructural role of the major components of the intestinal microvillus cytoskeleton. Cold Spring *Harbor Symp. Quant. Biol.* 46:871–879.
3. Bretscher, A., and K. Weber. 1979. Villin: the major microfilament-associated protein of the intestinal microvillus. *Proc. Natl. Acad. Sci. USA.* 76:2321–2325.
4. Burgess, W. H., D. K. Jemiolo, and R. H. Kretsinger. 1980. Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochim. Biophys. Acta.* 623:257–270.
5. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.
6. Coudrier, E., H. Reggio, and D. Louvard. 1981. Immunolocalization of the 110,000 molecular weight cytoskeletal protein of intestinal microvilli. *J. Mol. Biol.* 152:49–66.
7. Coudrier, E., H. Reggio, and D. Louvard. 1982. Characterization of an amphipathic glycoprotein associated with the microvillus cytoskeleton of intestinal brush border. *Biology of the Cell.* 45:253*a*. (Abstr.)
8. Coudrier, E., H. Reggio, and D. Louvard. 1982. The cytoskeleton of intestinal microvilli contains two polypeptides immunologically related to proteins of skeletal muscle. *Cold Spring Harbor Symp. Quant. Biol.* 46:881–892.
9. Craig, S. W., and T. D. Pollard. 1982. Actin binding proteins. *Trends Biochem. Sci.* 7:88–91.
10. DeRosier, D., E. Mandelkow, A. Silliman, L. Tilney, and R. Kane. 1977. Structure of actin containing filaments from two types of non-muscle cells. *J. Mol. Biol.* 113:679–695.
11. DeRosier, D. J., L. G. Tilney, and E. Egelman. 1980. Actin in the inner ear: the remarkable structure of the stereocilium. *Nature (Lond.).* 287:291–296.
12. Fairbanks, G., T. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
13. Glenney, J. R., Jr., and K. Weber. 1980. Calmodulin-binding proteins of the microfilaments present in isolated brush borders and microvilli of intestinal epithelial cells. *J. Biol. Chem.* 255:10551–10554.
14. Glenney, J. R., Jr., A. Bretscher, and K. Weber. 1980. Calcium control of the intestinal microvillus cytoskeleton: its implications for the regulation of microfilament organizations. *Proc. Natl. Acad. Sci. USA.* 77:6458–6462.
15. Glenney, J. R., Jr., P. Glenney, and K. Weber. 1982. Erythroid spectrin, brain fodrin, and intestinal brush border proteins (TW-260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit. *Proc. Natl. Acad. Sci USA* 79:4002–4005.
16. Glenney, J. R., Jr., M. Osborn, and K. Weber. 1982. The intracellular localization of

[2] Although the functions of the microvillar 110-kdalton protein are now better defined, we believe that to name the protein at this time is premature, and we choose to wait so that the name finally given to the 110-kdalton protein will be biologically meaningful.

the microvillus 110K protein, a component considered to be involved in side-on membrane attachment of F-actin. *Exp. Cell Res.* 138:199–205.
17. Glenney, J. R., Jr., P. Kaulfus, P. Matsudaira, and K. Weber. 1981. F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of microvillus core filaments. *J. Biol. Chem.* 256:9283–9288.
18. Hirokawa, N., and L. G. Tilney. 1982. Interactions between actin filaments and membranes in quick frozen and deeply etched hair cells of the chick ear. *J. Cell Biol.* 95:249–261.
19. Howe, C. L., M. S. Mooseker, and T. A. Graves. 1980. Brush border calmodulin. A major component of the isolated microvillus core. *J. Cell Biol.* 85:916–923.
20. Howe, C. L., T. C. S. Keller III, M. S. Mooseker, and R. H. Wasserman. 1982. Analysis of cytoskeletal proteins and Ca^{++}-dependent regulation of structure in intestinal brush borders from rachitic chicks. *Proc. Natl. Acad. Sci. USA.* 79:1134–1138.
21. Keller, T. C. S. III, and M. S. Mooseker. 1982. Ca^{++}-Calmodulin-dependent phosphorylation of myosin, and its role in brush border contraction in vitro. *J. Cell Biol.* 95:943–959.
22. Laemmli, V. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature (Lond.).* 227:680–685.
23. Levin, R. M., and B. Weiss. 1977. Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *Mol. Pharmacol.* 13:690–697.
24. Lowry, O.H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
25. Martin, J. B., and D. M. Doty. 1949. Determination of inorganic phosphate. Modification of isobutyl alcohol procedure. *Anal. Chem.* 21:965–967.
26. Matsudaira, P., and D. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386–396.
27. Matsudaira, P. T., and D. R. Burgess. 1979. Identification and organization of the components in the isolated microvillus cytoskeleton. *J. Cell Biol.* 83:667–673.
28. Matsudaira, P. T., and D. R. Burgess. 1982. Organization of the cross-filaments in intestinal microvilli. *J. Cell Biol.* 92:657–664.
29. Matsudaira, P. T., and D. R. Burgess. 1982. Structure and function of the brush-border cytoskeleton. *Cold Spring Harbor Symp. Quant. Biol.* 46:845–854.
30. Millington, P. F., and J. B. Finean. 1962. Electron microscope studies of the structure of the microvilli on principal epithelial cells of rat jejunum after treatment in hypo- and hypertonic saline. *J. Cell Biol.* 14:125–139.
31. Mooseker, M. S. 1976. Brush border motility. Microvillar contraction in Triton-treated brush borders isolated from intestinal epithelium. *J. Cell Biol.* 71:417–433.
32. Mooseker, M. S., and L. G. Tilney. 1975. The organization of an actin filament-membrane complex: filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. *J. Cell Biol.* 67:725–743.
33. Mooseker, M. S., and R. E. Stephens. 1980. Brush border alpha-actinin? Comparison of two proteins of the microvillus core with alpha-actinin by two-dimensional peptide mapping. *J. Cell Biol.* 86:466–474.
34. Mooseker, M. S., and C. L. Howe. 1982. The brush border of intestinal epithelium: a model system for analysis of cell-surface architecture and motility. *In* Methods and Perspectives in Cell Biology. L. Wilson, editor. Academic Press, Inc., New York. 25:143–174.
35. Mooseker, M. S., T. D. Pollard, and K. Fujiwara. 1978. Characterization and localization of myosin in the brush border of intestinal epithelial cells. *J. Cell Biol.* 79:444–453.
36. Mooseker, M. S., T. D. Pollard, and K. A. Wharton. 1982. Nucleated polymerization of actin from the membrane-associated ends of microvillar filaments in the intestinal brush border. *J. Cell Biol.* 95:223–233.
37. Mooseker, M. S., E. M. Bonder, B. G. Grimwade, C. L. Howe, T. C. S. Keller III, R. H. Wasserman, and K. A. Wharton. 1982. Regulation of contractility, cytoskeletal structure, and filament assembly in the brush border of intestinal epithelial cells. *Cold Spring Harbor Symp. Quant. Biol.* 46:855–870.
38. Mukherjee, T. M., and L. A. Staehelin. 1971. The fine structural organization of the brush border of intestinal epithelial cells. *J. Cell Sci.* 8:573–599.
39. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
40. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133–1142.
41. Pearl, M. L., D. J. Fishkind, and M. S. Mooseker. 1982. High molecular weight actin crosslinker from intestinal brush border. *J. Cell Biol.* 95 (2, Pt. 2): 282*a*. (Abstr.)
42. Pollard, T. D., and E. D. Korn. 1973. Acanthamoeba myosin. I. Isolation from *Acanthamoeba castellanii* of an enzyme similar to muscle myosin. *J. Biol. Chem.* 248:4682–4690.
43. Reggio, H. E. Coudrier, and D. Louvard. 1982. The Ca^{++}-ATPase from the sarcoplasmic reticulum of striated muscle is immunologically related to a membrane glycoprotein associated with the cytoskeleton of intestinal microvilli. *Biology of the Cell.* 45:253*a*. (Abstr.)
44. Siliciano, J. D., and S. W. Craig. 1982. Meta-vinculin—a vinculin-related protein with solubility properties of a membrane protein. *Nature (Lond.).* 300:533–535.
45. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
46. Spudich, J. A., and L. A. Amos. 1979. Structure of actin filament bundles from microvilli of sea urchin eggs. *J. Mol. Biol.* 129:319–331.
47. Tilney, L. G., and M. S. Mooseker. 1976. Actin filament-membrane attachment: are membrane particles involved? *J. Cell Biol.* 71:402–416.
48. Tyler, J. M., and D. Branton. 1980. Rotary shadowing of extended molecules dried from glycerol. *J. Ultrastruct. Res.* 71:75–102.
49. Vial, J. D., and J. Garrido. 1976. Actin-like filaments and membrane rearrangement in oxyntic cells. *Proc. Natl. Acad. Sci. USA.* 73:4032–4036.
50. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecylsulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.
51. Weber, K., and J. R. Glenney, Jr. 1982. Calcium-modulated multifunctional proteins regulating F-actin organization. *Cold Spring Harbor Symp. Quant. Biol.* 46:541–552.
52. Weihing, R. R. 1979. The cytoskeleton and plasma membrane. *Meth. Achiev. Exp. Pathol.* 8:41–209.