Role of Myosin in Terminal Web Contraction in Isolated Intestinal Epithelial Brush Borders

THOMAS C. S. KELLER III, KAREN A. CONZELMAN, REBECCA CHASAN, and MARK S. MOOSEKER

Department of Biology, Yale University, New Haven, Connecticut 06511. Dr. Keller's present address is Department of Biology, Wesleyan University, Middletown, Connecticut 06457.

ABSTRACT We have investigated the role of myosin in contraction of the terminal web in brush borders isolated from intestinal epithelium. At 37°C under conditions that stimulate terminal web contraction (1 μM Ca^{++} and ATP), most (60–70%) of the myosin is released from the brush border. Approximately 80% of the myosin is also released by ATP at 0°C, in the absence of contraction. Preextraction of this 80% of the myosin from brush borders with ATP has no effect on either the time course or extent of subsequently stimulated contraction. However, contraction is inhibited by removal of all of the myosin with 0.6 M KCl and ATP. Contraction is also inhibited by an antibody to brush border myosin, which inhibits both the ATPase activity of brush border myosin and its ability to form stable bipolar polymers. These results indicate that although functional myosin is absolutely required for terminal web contraction only ~20% of the brush border myosin is actually necessary. This raises the possibility that there are at least two different subsets of myosin in the terminal web.

Myosin is a major component of the cytoskeletal apparatus associated with the brush border of intestinal epithelial cells (for recent reviews of brush border structure see references 2 and 30). In brush borders, myosin is localized in the terminal web (3, 9, 19, 22, 33). Although its function in vivo remains unknown, this myosin has been implicated as the force producer (5, 26) in ATP-dependent contraction of the terminal web first observed in isolated brush borders by Rodewald et al. (41). Further support for this possibility has come from the demonstration that contraction of the terminal web correlates directly with Ca^{++}-calmodulin-dependent phosphorylation of the regulatory light chain of brush border myosin (26). A similar type of contraction has also been stimulated in glycerinated cells from intestinal epithelia (5, 21) and retinal epithelia (35).

Based on the morphologic changes that occur during contraction, it appears that at least some of the force for this contraction is produced within a ring of filaments that circumscribes the apical ends of both intestinal (24) and retinal epithelial cells (35) at the level of their zonula adherens junctions. This circumferential ring has been isolated from retina and stimulated to contract in vitro (36). In intestinal brush borders, this ring of filaments contains closely apposed, antiparallel actin filaments (21, 22) and myosin (21, 22). Presumably, the terminal web contraction that is observed could result from an interaction between actin and myosin within this ring that constricts it in a "purse-string" fashion. However, in our original studies of contraction in isolated brush borders (26) and in glycerinated intestinal epithelial cells (21), we observed that most of the myosin was dissociated from the cytoskeleton during contraction. This observation made us question the role of myosin as a producer of force in terminal web contraction. In this and a companion study (31), we have investigated the nature of the association of myosin with the brush border cytoskeleton and the role of myosin in terminal web contraction.

Here, we present evidence that in the presence of physiologic concentrations of salt, a large percentage (70–80%) of the total brush border myosin is dissociated from the brush border by ATP, and that solubilization of this myosin by ATP does not require phosphorylation of its regulatory light chain. More importantly, we also demonstrate that ATP extraction of 80% of the myosin does not inhibit Ca^{++}- and ATP-dependent contraction of the brush border terminal web. On the other hand, incubation of brush borders with an affinity-purified antibody to brush border myosin completely inhibits contraction, as does removal of all of the myosin from the brush border structure with a combination of a high concentration of salt (0.6 M KCl) and ATP. Our results demonstrate that functional myosin is absolutely essential for brush border

contraction. However, the fact that only a fraction of the total myosin is required suggests that myosin may play more than one role in brush border function.

Preliminary reports of this work have been presented previously (25, 31).

MATERIALS AND METHODS

Contraction and Extraction of Isolated Brush Borders

BRUSH BORDER ISOLATION: Sheets of brush borders attached to their neighbors by their normal intercellular junctions were isolated from the intestinal epithelia of chickens by the method of Mooseker (29), as modified by Keller and Mooseker (26). The sheets of brush borders, once purified, were transferred into a brush border stabilization buffer (BBSB)[1] composed of 75 mM KCl, 5 mM $MgSO_4$, 1 mM EGTA, 0.2 mM dithiothreitol, 10 mM imidazole, pH 7.2, with 0.2 mM phenylmethylsulfonyl fluoride, and 20 trypsin inhibitor units/liter of aprotinin (Sigma Chemical Co., St. Louis, MO.).

BRUSH BORDER CONTRACTION: Brush borders were stimulated to contract by resuspending the sheets (at a 5% suspension) in a contraction solution composed of 50 mM KCl, 5 mM $MgSO_4$, 1 mM EGTA, 0.9 mM $CaCl_2$, 0.2 mM dithiothreitol, 25 mM PIPES-KOH, pH 6.9 (CS) that contained 4 mM ATP (26), or CS that contained 150 mM KCl (CS-150) and ATP, and warming them to 37°C. Contraction was monitored routinely with light microscopy as described in Keller and Mooseker (26).

To determine the effect of preextraction of the brush borders with ATP (see below) on contraction, aliquots of contracting brush borders were fixed with 1% glutaraldehyde at 1, 3, 5, 10, and 12 min after warming to 37°C. The fixed brush borders were mounted on slides and multiple fields from each time point were recorded with videomicroscopy. The video images were then analyzed semiquatitatively by an investigator who was unaware of the specific experimental condition being scored. In each field, the number of sheets of brush borders that were (*a*) extensively contracted, (*b*) not extensively contracted, and (*c*) too ambiguous to score conclusively were recorded. At least 100 sheets of brush borders were scored for each condition. The number of sheets too ambiguous to score did not exceed 5% for any of the conditions. For these assays, preparations of brush borders were used in which >90% of the brush borders were in sheets and in which >90% of the sheets of control brush borders contracted. Only sheets of brush borders were scored, because of the difficulty in unambiguously scoring contraction in individual brush borders.

EXTRACTION METHODS: Brush borders were incubated in various extraction buffers for 15 min at 0°C. The extraction buffers included CS-150, CS that contained 0.6 M KCl, and BBSB that contained 1 mM CaCl and 0.15 M KCl, as well as all of these buffers containing 4 mM ATP. The brush borders were then centrifuged at 12,000 *g* for 4 min and the supernatant was removed. Samples of the pellets were resuspended in CS-150 with ATP and warmed to stimulate contraction, after which they were repelleted.

Preparation and Characterization of Anti–Brush Border Myosin

BRUSH BORDER MYOSIN PURIFICATION: Myosin was extracted from brush borders by incubating them in BBSB containing 0.15 M KCl and 4 mM ATP on ice for 20 min. The brush borders were then centrifuged for 30 min at 30,000 *g*, and the supernatant loaded directly onto a column of hydroxylapatite equilibrated with BBSB containing 1 mM ATP and 0.3 M KCl. After loading, the column was eluted with a linear gradient of 50–200 mM potassium phosphate made in BBSB-0.3 M KCl to remove contaminants including TW 260/240, the brush border-specific form of spectrin (15, 16, 20, 37). Pure myosin was then eluted with 1 M potassium phosphate. Under these conditions, some of the myosin eluted at ~200–250 mM potassium phosphate, but efficient elution of all of the myosin required higher concentrations of phosphate. The pure myosin was pooled and exhaustively dialyzed into myosin storage buffer (MSB) consisting of 150 mM KCl, 1 mM $MgCl_2$, 10 mM imidazole-Cl, pH 7.0, and 0.2 mM DTT, in which it assembled into bipolar filaments. The bipolar filaments were then pelleted at 20,000 *g* for 30 min and either resuspended as a concentrated suspension in MSB or dissolved in MSB containing 0.6 M KCl before use. >25% of the brush border myosin was recovered at a purity of >95%.

ANTIBODY PRODUCTION: A polyclonal antibody was elicited in a female New Zealand White rabbit by a series of subcutaneous injections of purified brush border myosin emulsified with Freund's adjuvant. Specific antibodies were purified from whole serum by affinity chromatography using brush border myosin conjugated to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) The monospecific antibodies were eluted with 4.5 M $MgCl_2$. Where necessary, these antibodies were further concentrated by ammonium sulfate precipitation and desalted into an appropriate buffer, e.g., CS-150, by centrifuge chromatography (8) using Sephadex G-25 (Pharmacia Fine Chemicals).

ANTIBODY CHARACTERIZATION: These antibodies were characterized by autoradiography of one-dimensional immunoblots (7), using ^{125}I-Protein A as the secondary probe.

Antibody Inhibition Assays

MYOSIN BIPOLAR FILAMENT ASSAY: For the myosin bipolar filament assembly and disassembly assays, myosin in MSB containing 0.6 M KCl was centrifuged at 100,000 *g* for 2 h to remove a small number of stable bipolar filaments. In the assembly assay, the disassembled myosin (0.16 mg/ml) was incubated for 10 min in MSB containing 0.3 M KCl, either in the presence or absence of 2 mg/ml affinity-purified antibody to brush border myosin (at a molar ratio of approximately 20:1, antibody to brush border myosin, assuming a mol wt of 240,000 for the myosin). It was then diluted twofold to a final KCl concentration of 0.15 M and myosin concentration of 0.08 mg/ml. At specified time intervals, samples were removed, placed on carbon-parloidin-coated grids, and negatively stained with 1% uranyl acetate. In the bipolar filament disassembly assay, the myosin was first diluted to 0.11 mg/ml in MSB containing 0.15 M KCl and allowed to assemble for 10 min. Then the antibody or an appropriate buffer control solution was added so that the final concentrations of myosin, antibody, and KCl were equivalent to those in the assembly assay described above.

Mg^{++}-ATPase ASSAY: The Mg^{++}-ATPase activity of both phosphorylated and nonphosphorylated myosin was determined in the presence and absence of both actin and anti-brush border myosin antibody. The assay buffer contained 0.15 M KCl, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM imidazole, pH 7.0, and where present, the final concentration of myosin was 0.12 mg/ml, chicken breast skeletal muscle actin was 0.5 mg/ml, chicken gizzard myosin light chain kinase (isolated by a modification of the method of Guerriero et al. [17]) was 0.065 mg/ml, bovine brain calmodulin was 0.02 mg/ml, affinity-purified antibody to brush border myosin was 0.24 mg/ml (approximately a 3.5:1 molar ratio of antibody to myosin), and rat IgG, when used as a control, was 0.25 mg/ml. Purified brush border myosin was incubated with gizzard myosin light chain kinase, calmodulin, and 0.1 mM ATP at 37°C for 10 min immediately before starting the ATPase assay. Under these conditions, all of the myosin's regulatory light chain contained at least one phosphate group, as indicated by urea-glycerol PAGE (see below). In all cases, the ATPase assay was initiated by adding [γ-^{32}P]ATP to a final concentration of 2 mM (0.05 Ci/mmol) and warming to 37°C. After specified times of incubation, aliquots of the reaction mixture were removed and the inorganic phosphate present was determined by a modification of the method of Pollard and Korn (40), in which the amount of $^{32}P_i$ partitioned into the organic phase of the stop solution was determined by scintillation counting.

K^+-EDTA-ATPase ASSAY: The K^+-EDTA-ATPase activity of the brush border myosin (0.09 mg/ml) was determined in an assay solution containing 0.5 M KCl, 10 mM EDTA, 10 mM imidazole, pH 7.0, and various concentrations (from 0 to 1.7 mg/ml) of affinity-purified antibody to brush border myosin.

Electrophoretic Methods

SDS PAGE: SDS PAGE was performed as described previously (26). Because we have been criticized in the literature for using one-dimensional SDS PAGE for identifying the regulatory light chain of brush border myosin (see reference 4), it is necessary to reemphasize that including EGTA in the sample buffer causes the calmodulin to migrate with an apparent mol wt of ~21,000 (6). This is both slower than and easily resolvable from the migration of the regulatory light chain of brush border myosin, allowing a positive identification of the regulatory light chain. The estimates of the relative amounts of myosin in samples of pellets and supernatants were determined by scanning densitometry of Coomassie Blue-stained (11) tube gels.

UREA-GLYCEROL-PAGE: The phosphorylation state of the regulatory light chain of brush border myosin in both ATP-extracted supernatants of brush border and in the Mg^{++}ATPase assay was determined by urea-glycerol PAGE (38). Samples were processed by adding ice-cold trichloroacetic acid to a final concentration of 3% (42), which immediately precipitates the protein and completely inhibits further kinase or phosphatase activity (which is a significant problem especially in brush border samples). The trichloroacetic acid pellet was then washed with 95% acetone and resuspended in urea sample buffer. Typically, the extracted protein in 2 ml of the supernatant from a 5% suspension of brush borders was loaded on each lane of the gel. Because PIPES

[1] *Abbreviations used in this paper:* BBSB, brush border stabilization buffer; CS, contraction solution; MSB, myosin storage buffer.

buffer precipitates with the trichloroacetic acid and interferes with the subsequent electrophoresis, BBSB containing 0.15 M KCl/ATP and Ca^{++} was used for all of the extractions in this procedure. After autoradiography, the regulatory light chain bands were excised from the dried gels, dissolved in H_2O_2, and mixed with Aquasol (New England Nuclear, Boston, MA) for scintillation counting.

RESULTS

ATP-dependent Solubilization of Myosin from Brush Borders

When isolated brush borders were stimulated to contract, most (60–70%) of the myosin appeared to be solubilized from the brush border structure (Fig. 1 *a*, gel S_2). This loss of myosin during contraction was confirmed (Fig. 1 *a*, autoradiogram S_2) by immunoblotting SDS gels with an affinity-purified antibody to brush border myosin (characterized below). Contraction, which was stimulated by incubating brush borders at 37°C in the presence of Ca^{++} (1 μM), 0.15 M KCl, and 4 mM ATP, also resulted in the solubilization of some of the TW 260/240, actin, villin, fimbrin, and 110,000-mol wt protein-calmodulin complex. To determine the basis for the release of these proteins, especially myosin, from the brush border structure and to better define the role of myosin in brush border contraction, we have examined the effects of

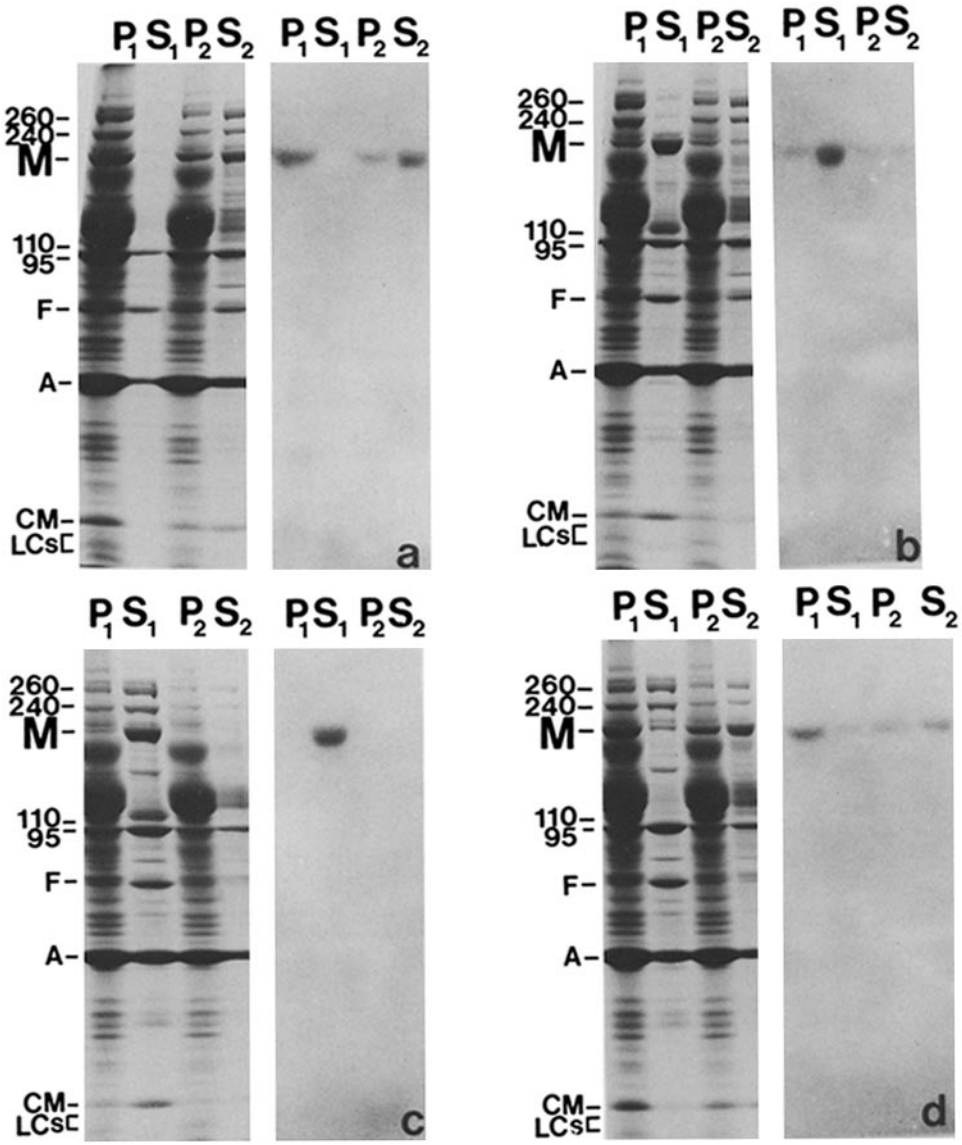

FIGURE 1 SDS PAGE and immunoblot analysis of extraction and contraction of isolated brush borders. Isolated brush borders that were either contracted or extracted with 0.15 M KCl/ATP, 0.6 M KCl, or 0.6 M KCl/ATP and then contracted were analyzed by SDS PAGE and by immunoblotting with affinity-purified antibody to brush border myosin. For extraction, the brush borders were incubated on ice for 15 min in (*a*) CS-150 containing no ATP, (*b*) CS-150 with 4 mM ATP, (*c*) CS-0.6 M KCl with 4 mM ATP, and (*d*) CS-0.6 M KCl containing no ATP. They were then pelleted at 12,000 *g* for 4 min (P_1). All of the pelleted brush borders were resuspended in CS-150 containing 4 mM ATP, warmed to 37°C for 15 min, and repelleted (P_2) and the supernatant was removed (S_1). All of the pelleted brush borders were resuspended in CS-150 containing 4 mM ATP, warmed to 37°C for 15 min, and repelleted (P_2) and the supernatant was removed (S_2). Proportional amounts of the resulting pellets and supernatants were analyzed by SDS PAGE. An equivalent gel was transferred to nitrocellulose and probed with an affinity-purified antibody to brush border myosin. The Coomassie Blue-stained SDS gel is shown on the left and the autoradiograph of the immunoblot is on the right in each panel. Of the numerous proteins present in brush borders the antibody reacts with only one, the myosin heavy chain (*M*). The migration positions of TW 260/240 (*260* and *240*), the 110,000-mol wt microvillus protein (*110*), villin (*95*), fimbrin (*F*), actin (*A*), calmodulin (*CM*), and the myosin light chains (*LCs*) are indicated.

ATP, temperature, and salt concentration on the structure and contraction of isolated brush borders.

The release of myosin from the brush border depended strictly on ATP. Myosin was not released from brush borders incubated in 0.15 M KCl and Ca^{++} in the absence of ATP, either at 0°C (Fig. 1*a*, S_1) or at 37°C (not shown). On the other hand, myosin was released in the presence of ATP, even at 0°C in the absence of any contraction, In fact, ~80% of the total brush border myosin was solubilized by ATP at 0°C in the presence (Fig. 1*b*, S_1) or absence of Ca^{++} (not shown), or even in the presence of EDTA (not shown). This ATP-dependent solubilization of 80% of the brush border myosin occurred consistently at 0°C in the presence of 0.15 M KCl. Extraction of a higher percentage of the myosin by ATP required concentrations of KCl of greater than ~0.45–0.5 M KCl; occasionally less myosin (sometimes as little as 60% of the total) was extracted by ATP in the presence of lower concentrations (0.05 M) of KCl. Therefore, all of the low salt extraction and contraction buffers used in this study contained 0.15 M KCl. Even so, during contraction in solutions containing 0.15 M KCl/ATP sometimes only ~70% of the myosin was extracted (as in Fig. 1*a*, S_2). The 20% of the myosin that always remained associated with the brush border in 0.15 M KCl/ATP at 0°C was not extracted even after numerous sequential incubations or after extended periods of dialysis against large volumes of the buffer (not shown).

In the presence of 0.6 M KCl, ATP extracted essentially all of the myosin from brush borders, including the residual 20% associated with brush borders that were previously extracted with 0.15 M KCl/ATP (Fig. 1*c*, P_1 and S_1). Although two polypeptides of ~200,000–215,000 mol wt remained associated with the brush border, immunoblot analysis (Fig. 1*c*, P_1) indicates that neither of these appears to be myosin. In the absence of ATP, 0.6 M KCl extracted only a small fraction of the myosin (Fig. 1*d*, S_1). However, at least some of the two 200,000–215,000 polypeptides were extracted under these conditions.

Of the proteins besides myosin that were solubilized during contraction, a significant amount of the actin, villin, and fimbrin was released in the absence of ATP at 0°C (Fig. 1*a*, S_1), and their release was enhanced by higher concentrations of KCl (Fig. 1*d*, S_1). High concentrations of KCl also enhanced the release of TW 260/240. In 0.15 M KCl, most of the TW 260/240 was released when the brush borders were warmed (Fig. 1*a*, S_2 and *b*, S_2), and was independent of the presence of ATP or extraction of myosin (not shown). In contrast, the release of the 110,000-mol wt calmodulin complex depended on ATP (compare Fig. 1*a*, S_1, with *b*, S_1), but was greatly enhanced by concentrations of KCl above 0.15 M KCl (Fig. 1*c*, S_1; see also reference 23).

Effects of Myosin Extraction on Brush Border Contraction

Surprisingly, preextraction of 80% of the total brush border myosin with 0.15 M KCl/ATP had no discernible effect at least at the light microscope level of resolution on either the morphology of the brush borders (Fig. 2*a*) or on the time course or extent of subsequently stimulated contraction (Fig. 2*b*). When large populations of the brush borders were assayed semiquantitatively (as outlined in Materials and Methods), essentially equivalent amounts of contraction were scored at

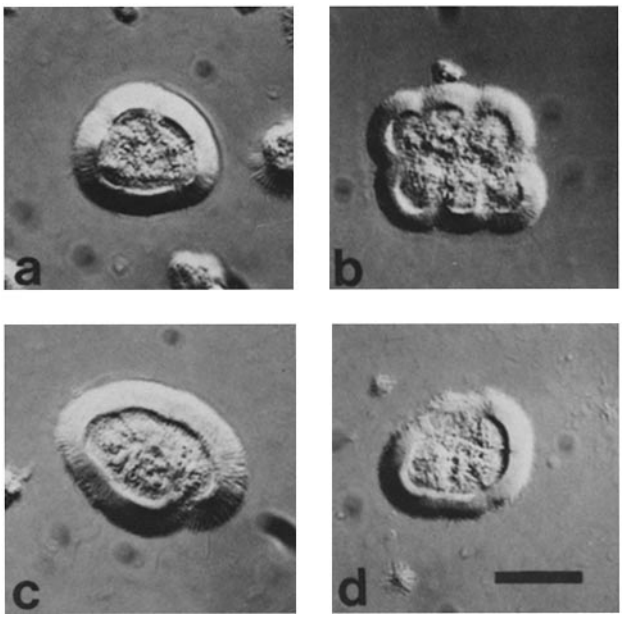

FIGURE 2 Light microscopy of contraction in preextracted brush borders. Sheets of 5–10 brush borders interconnected by their junctional complexes (from the same preparations analyzed in Fig. 1) were extracted by resuspension at 0°C in (*a*) CS-150 containing 4 mM ATP or in (*c*) CS with 0.6 M KCl and ATP. After pelleting, the extracted brush borders (*a* and *c*) were then resuspended in CS-150 with ATP and warmed to 37°C. Under these conditions the brush borders previously extracted with CS-150/ATP, which retain ~20% of their myosin contracted (*b*), whereas those extracted with CS-0.6 M/ATP, lacking all of their myosin, did not (*d*). Differential-interference optics. × 5,000; Bar, 10 μm.

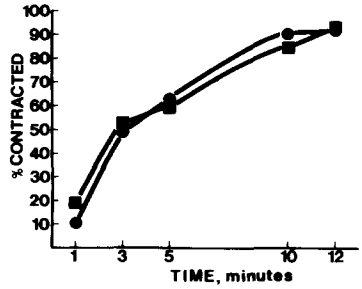

FIGURE 3 Comparison of contraction in ATP-extracted and nonextracted brush borders as a function of time. The percentage of brush border sheets that were scored as being extensively contracted is plotted as a function of time of incubation at 37°C in CS-150 with 4 mM ATP. Contraction of ATP-extracted brush borders (■) and untreated brush borders (●) occurred at approximately the same rate.

time intervals between 1 and 12 min after warming to 37°C (Fig. 3). After 12 min, >90% of both preextracted and control brush borders were extensively contracted. During contraction, there was little additional loss of myosin from previously extracted brush borders (Fig. 1*b*, S_2).

Extraction of virtually all of the brush border myosin with 0.6 M KCl/ATP, which also had little effect on the overall morphology of the brush border (Fig. 2*c*), completely inhibited contraction (Fig. 2*d*), even after long periods (>30 min) of incubation. In contrast, preincubation of brush borders with 0.6 M KCl alone which resulted in the loss of little

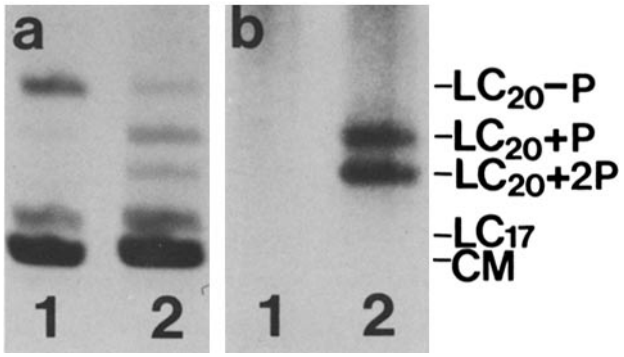

FIGURE 4 Urea-glycerol gel analysis of the phosphorylation state of ATP-extracted brush border myosin. Brush borders were incubated with BBSB containing 1 μM Ca^{++} (free) and 4 mM [γ-^{32}P]ATP for 15 min on ice and then pelleted at 12,000 *g* for 4 min. Half of the resulting supernatant was immediately processed for analysis (a), while the other half of the ATP extract was warmed to 37°C (2) for 15 min and then processed. Both samples were electrophoresed on a glycerol-polyacrylamide gel along with standards of chicken gizzard light chains (not shown). The migration positions of an unphosphorylated chicken gizzard regulatory light chain ($LC_{20}-P$), the same light chain phosphorylated with exogenously added chicken gizzard myosin light chain kinase ($LC_{20}+P$), and the 17,000-mol wt brush border light chain (LC_{17}) are indicated as is the position of the brush border regulatory light chain that has incorporated two phosphates ($LC_{20}+2P$). As extracted from brush borders by ATP, the regulatory light chain of the brush border myosin is predominantly unphosphorylated (*a1* and *b1*). However, when warmed at 37°C, most of the light chain becomes phosphorylated at least once and ~25% of it becomes phosphorylated twice (*a2* and *b2*), even in the absence of exogenously added myosin light chain kinase.

myosin (Fig. 1 *d*, S_1) had no effect on contraction (not shown). Moreover, the same amount of myosin was solubilized from 0.6 M KCl-extracted brush borders as from nonextracted brush borders during subsequently stimulated contraction (compare Fig. 1 *d*, S_2, with *a*, S_2).

Phosphorylation State of ATP-solubilized Brush Border Myosin

Consistently, most of the regulatory light chain of myosin extracted by 0.15 M KCl/ATP (actually [γ-^{32}P]ATP, 10 Ci/mole) at 0°C was unphosphorylated, as indicated by urea-glycerol gel electrophoresis (Fig. 4, *a1* and *b1*). However, when the ATP-extract was warmed to 37°C, most of the myosin regulatory light chain became phosphorylated (Fig. 4, *a2* and *b2*), as indicated both by the shift in its migration on the gel and by its incorporation of ^{32}P-labeled (fig. 4*b2*). In fact, some of the regulatory light chain appears to have become doubly phosphorylated, as indicated by the additional shift in migration of ~25% of the total light chain and evident increase in specific activity of ^{32}P-labeling. From the amount of radioactivity incorporated into the individual regulatory light chain bands, we estimate that there are 0.8–0.9 mol of phosphate/mol of light chain in the major regulatory light chain band in lanes *2* and *4*, and ~1.8 mol of phosphate/mol of light chain in the faster migrating species. Similar shifts in migration of the regulatory light chain and in ^{32}P-incorporation were observed in purified brush border myosin that was incubated with purified chicken gizzard myosin light chain kinase (Keller, T. C. S. III, K. A. Conzelman, and M. S. Mooseker, unpublished observations).

Although these results demonstrate that myosin light chain kinase is extracted from brush borders by 0.15 M KCl/ATP, at least some of the myosin light chain kinase activity remained associated with the brush border under these conditions, because the remaining myosin became phosphorylated during brush border contraction (results not shown).

Effects of Affinity-purified Antibody on Brush Border Myosin

The affinity-purified polyclonal antibody used in the immunoblot analysis (Fig. 1) to assess the extraction of myosin under various conditions reacted with only one polypeptide of the numerous proteins present in the brush border—the myosin heavy chain. There was no detectable reaction with either of the two myosin light chains. The antibody also apparently did not distinguish between the 0.15 M KCl/ATP-dissociable myosin and that which remained associated with the brush border structure under those conditions. Consistent with the specificity of the antibody for the brush border myosin heavy chain, this antibody both inhibited the formation of brush border myosin bipolar filaments and disrupted their structure once formed in vitro (Fig. 5, *a* and *b*).

In addition to affecting the filamentous structure of brush border myosin, the antibody also interfered with the enzymatic activity of brush border myosin in vitro. The Mg^{++}-ATPase of brush border myosin whose regulatory light chain had been fully phosphorylated (at least one phosphate group incorporated per light chain as indicated by urea-glycerol gels) by incubating it with exogenously added chicken gizzard, myosin light chain kinase was activated by actin (to 0.28 μmol P_i/mg per min) ~10-fold over the activity of brush border myosin alone (0.03 μmol P_i/mg per min). In the presence of the antibody at a molar ratio 5:1 (antibody to 240,000 mol wt of myosin), this actin-activated Mg^{++}-ATPase activity was inhibited by 73% (Table I). Nonimmune rat IgG at a similar concentration had essentially no effect on the actin-activated activity (Table I). Although the antibody did slow the reaction slightly, its effect on this ATPase activity was not because it prevented phosphorylation of the myosin regulatory light chain (data not shown). However, it is possible that the antibody simply precipitated the myosin from solution, thereby indirectly preventing its interaction with actin.

To help circumvent this problem, the effect of the antibody on the K^+-EDTA-ATPase activity of the myosin was determined. This activity was assumed to be less sensitive to myosin precipitation and more indicative of a direct effect on the actual active site of the myosin-ATPase. As indicated (Table I), the antibody also inhibited the K^+-EDTA-ATPase activity, but much higher molar ratios of antibody to myosin were required to obtain effects equivalent to those on the Mg^{++}-ATPase activity. For example, at an antibody to myosin ratio as high as 9:1, the antibody had little or no effect on K^+-EDTA-ATPase activity. However, at ratios of >10:1, the antibody inhibited the K^+-EDTA-ATPase activity and this inhibition increased linearly with increasing antibody concentration, so that at a ratio of 35:1 the inhibition was 50% (Table I). Extrapolation of these results indicates that ratios of >60:1 would be required for complete inhibition of this activity.

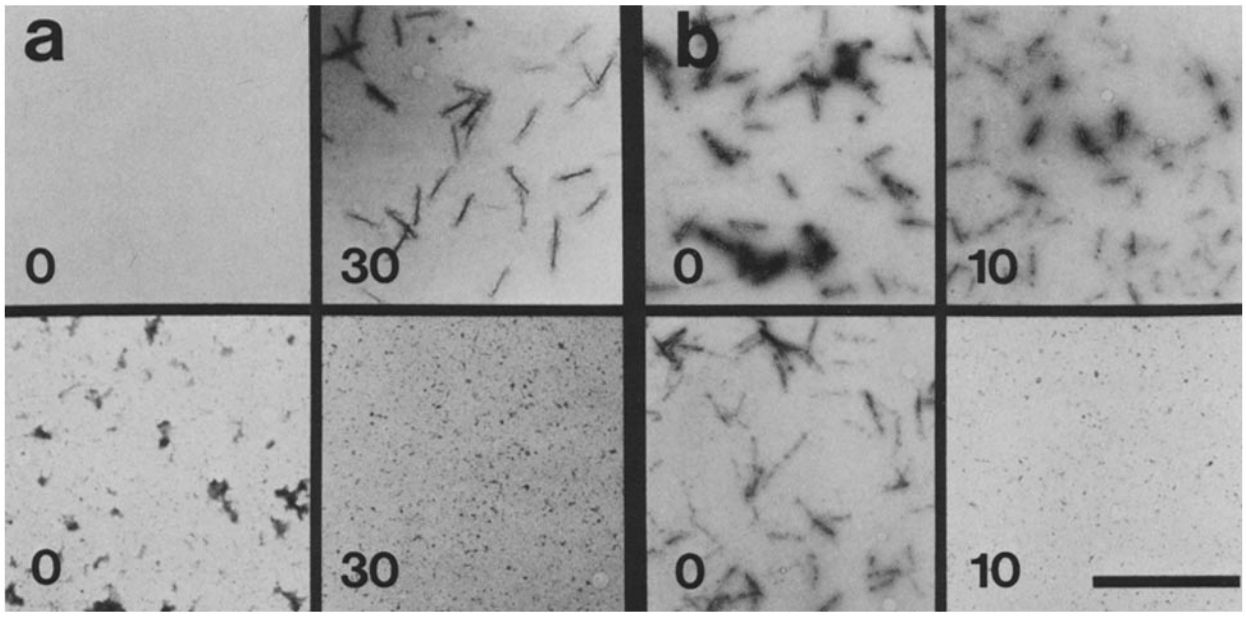

FIGURE 5 Effects of anti-brush border myosin on the assembly and on the stability of myosin bipolar filaments. (*a*) Purified brush border myosin (0.32 mg/ml) in MSB containing 0.6 M KCl was diluted with (*bottom*) and without (*top*) an affinity-purified antibody to brush border myosin, yielding final concentrations of myosin of 0.16 mg/ml, KCl of 0.3 M, and antibody of 2 mg/ml (a ratio of antibody to myosin of ~20:1) and incubated 10 min. Each mixture was then diluted twofold again so that the final concentration of KCl was 0.15 M. Immediately after the final dilution (*0*) and after 30 min of incubation at room temperature (*30*), samples of each condition were negatively stained for analysis with electron microscopy. (*b*) Purified brush border myosin was stimulated to assemble by diluting it to a final concentration of 0.11 mg/ml in MSB containing 0.15 M KCl. Then the antibody was added to one sample (*bottom*) and an appropriate buffer control solution was added to the other sample (*top*) so that the final concentrations of myosin, antibody, and KCl were equivalent to those in *a*. After 10 min, samples of each were negative stained. In the absence of antibody, myosin assembled into numerous stable bipolar filaments. In the presence of antibody, myosin assembly was completely inhibited and previously formed bipolar filaments were completely disassembled.

TABLE I

Effects of Antibody on ATPase Activity

Condition	Ratio of antibody to myosin	Inhibition
		%
Mg^{++} ATPase + actin + Ab-BBM	5:1	73
Mg^{++} ATPase + actin + IgG	5:1	3
K^+-EDTA + Ab-BBM	9:1	0
K^+-EDTA + Ab-BBM	35:1	50

The effect of an affinity-purified antibody to brush border myosin (Ab-BBM) was determined on the actin-activated Mg^{++} ATPase activity of brush border myosin whose regulatory light chain had been phosphorylated (as described in Materials and Methods). The Mg^{++} ATPase activity of the phosphorylated myosin in the presence of 0.15 M KCl, 5 mM MgCl, and 0.2 mM CaCl, pH 7.0, at 37°C was activated 10-fold by actin to a value of 0.28 μmol P_i/min per mg in the absence of antibody. Nonimmune IgG (from rat) was used to control for any nonspecific effect on actin-myosin that IgG might have because of its affinity for actin (see reference 12). The effects of two different concentrations of Ab-BBM on the K^+-EDTA-ATPase activity of brush border myosin was determined. The K^+-EDTA-ATPase activity of the myosin alone in the presence of 0.5 M KCl and 10 mM EDTA, pH 7.0, at 37°C was 0.32 μmol P_i/min per mg.

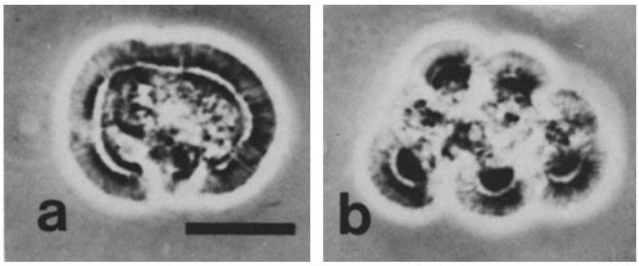

FIGURE 6 Antibody inhibition of terminal web contraction in isolated brush borders. Isolated brush borders that had been previously extracted with CS-150 containing 4 mM ATP at 0°C were incubated in CS-150/ATP for 60 min on ice in the presence of either (*a*) affinity-purified antibody (6.4 mg/ml) to brush border myosin or (*b*) antibody that had been preincubated with excess brush border myosin. The brush borders were then warmed to 37°C for 10 min and processed for light microscopy. The affinity-purified antibody to brush border myosin completely inhibited contraction whereas preabsorption of the antibody with brush border myosin relieved this inhibition. Phase-contrast optics. × 41,000; bar, 0.5 μm.

Inhibition of Brush Border Contraction with Antimyosin

Although the brush border extraction results outlined above strongly indicate that some but not all of the myosin in brush borders is necessary for contraction, we directly demonstrated the active role of myosin in brush border contraction by using the affinity-purified antibody to brush border myosin. Contraction was completely inhibited when brush borders were incubated in the presence of the antibody under conditions that normally stimulate contraction (Fig. 6*a*). Complete inhibition of contraction (for the duration of a 30-min stimulation) required high concentrations of antibody. Contraction was inhibited by antibody concentrations of 6.4 and 0.53 mg/

ml, which correspond to ratios of antibody to the myosin estimated to be present in the assay of 2,400:1 and 200:1, respectively, whereas contraction was not inhibited by 0.053 mg/ml, a ratio of 20:1.

In addition, the inhibition was completely relieved by adding excess brush border myosin to the antibody before testing its effect on contraction (Fig. 6*b*). Purified nonimmune rat IgG at the same concentrations, used as a control for nonspecific effects of high concentrations of IgG, had little or no effect on brush border contraction (not shown), indicating that the inhibition by the myosin antibody was a specific effect.

DISCUSSION

The results presented here suggest that the brush border contains two subsets of myosin: one (~80% of the total myosin) that is dissociated from brush borders by ATP in the presence of physiologic salt concentrations and another (the other 20%) that remains stably associated under these conditions. They also demonstrate that the ATP-dependent dissociation from the brush border of myosin does not require phosphorylation of its regulatory light chain. In addition, our results show that loss of the ATP-dissociable subset of myosin has no effect on the ability of brush borders to contract, but that contraction is completely inhibited both by an antibody to brush border myosin and by removal of all of the myosin from the brush border structure.

These results raise a number of interesting questions about the role of myosin in the brush border. For example, what is the basis for the difference between the two myosin subsets in binding to the brush border cytoskeleton? Does the difference in dissociability of these two subsets reflect a difference in localization and even a difference in function? If so, then what are the functions of the two subsets of myosin in the brush border?

It is likely that, in the absence of ATP, the ATP-dissociable myosin is associated directly with the brush border cytoskeleton only through an ATP-sensitive, rigorlike complex with actin. Disruption of this type of interaction by ATP should not necessarily require phosphorylation of the myosin's regulatory light chain (see reference 43), and we have found that extraction of this subset of myosin from the brush border does not. Moreover, our results indicate that phosphorylation of its regulatory light chain during contraction in ATP appears to enhance the association of the myosin with the brush border. This is in accordance with the observation that platelet myosin increases its association with the Triton-insoluble platelet cytoskeleton after phosphorylation of its regulatory light chain (13). Consistent with its being mostly unphosphorylated, ATP-dissociable brush border myosin is found to be predominantly kink-tailed dimers and monomers (31).

When extracted from brush borders, the regulatory light chain of the brush border myosin becomes phosphorylated when the ATP supernatant is warmed to 37°C. In fact, urea-glycerol PAGE indicates that at least some of the regulatory light chain undergoes a double shift in migration, which appears to be due to the incorporation of two phosphates per light chain. Multiple phosphorylation of myosin regulatory light chains has been reported previously (14, 27, 34). However, the validity of using only the migration of the light chain on nondenaturing gels as an indication of its state of phosphorylation has recently been called into question (18), because of the potential for similar shifts in migration on these gel systems in the absence of phosphate incorporation. Nevertheless, it is clear from our results that the initial shift in migration of most, if not all, of the light chain corresponds to incorporation of phosphate (Fig. 4), and that the second shift in migration of the light chain corresponds to a doubling of the specific activity of ^{32}P-labeling. Although proteolysis, which could account for both a shift in migration and a change in specific activity, is always a concern in preparations of brush borders, we have never observed a change in the migration of the light chain on SDS PAGE that would reflect the large loss of protein from the light chain that would be necessary to account for the observed changes in specific activity. Therefore, although neither proteolysis nor anomalous migration behavior can be rigorously ruled out, it is unlikely that either is contributing significantly to these results. Because the ATP supernatant is a complex mixture of proteins, it is possible that more than one type of brush border kinase can phosphorylate the myosin light chain at different positions. In this regard, it is interesting to note that phosphorylation of smooth muscle myosin light chain by protein kinase C actually inhibits the myosin's actin-activated ATPase that is stimulated by myosin light chain kinase phosphorylation of the light chain (34). We have also observed the apparent double light chain phosphorylation when purified brush border myosin was incubated with purified chicken gizzard myosin light chain kinase (unpublished observations), indicating that the second site of phosphorylation in the brush border light chain can be phosphorylated by a myosin light chain kinase. Further studies are now in progress to determine whether the addition of more than one phosphate on the regulatory light chain influences any of the known properties of brush border myosin.

Based on the difference in its dissociability, the myosin resistant to ATP extraction appears to have a very different association with the brush border cytoskeleton. Nevertheless, it is possible that this myosin has an ATP-sensitive interaction with actin in the brush border, in addition to an interaction with another brush border component that is sensitive only to high concentrations of salt. A double-binding interaction of this kind would require both ATP and high concentrations of salt in order to be disrupted, which is exactly what is observed for this subset of myosin. We are currently investigating the molecular basis for the difference in ATP dissociability to determine whether interactions between myosin and proteins other than actin are important in the function of this subset of myosin. The fact that only this subset of myosin is necessary for the one apparent function of brush border myosin—terminal web contraction—suggests that myosin has more than this one function in the brush border.

To determine whether there is a differential localization of the two subsets of myosin, we have compared brush borders before and after ATP extraction using both electron microscopy and immunofluorescent localization. Although electron microscopy of ATP-extracted brush borders (21, 31) indicates that there is a great loss of filamentous material which we assume to be myosin from the interrootlet region, it is difficult to judge whether there is an equivalent loss of material from the circumferential ring, which appears to remain intact. Disappointingly, attempts so far to localize myosin immunofluorescently before and after ATP extraction have not conclusively reflected an 80% loss of antigen. This indicates either that more antigenic sites become available for reaction after ATP extraction or, more likely, that the technique is not

sufficiently quantitative to reflect a significant loss of myosin. There may also be a problem with access of the antibody to the brush border myosin especially in the ring. This may be the reason that a ratio of antibody to myosin (20:1) that disrupts myosin bipolar filaments in vitro does not inhibit brush border contraction. Alternatively, this lack of inhibition may be due to a stabilization of bipolar myosin filaments by other brush border components in the circumferential ring. Nevertheless, it is reasonable to speculate that the function of the myosin in the circumferential ring may be different from the function of the myosin in the interrootlet region.

Previously, we have suggested that contraction of the circumferential ring could effect an increased tensile force on tight junctions, thereby regulating their permeability (32). Since then, studies have appeared demonstrating that factors that change the tensile force on tight junctions, e.g., osmotic load (28) and mechanical tension (39), change the orientation of the intramembranous strands in the junctions. Intramembranous strand orientation appears to be directly correlated to tight junction permeability, at least in some systems (1, 10). Therefore, it is possible that tension development within the circumferential ring which in vitro can rip tight junctions apart (5, 21, 26), may have much more subtle effects in vivo when subjected to the physical constraints imposed by the whole cell. In order to ensure the maintenance and stability of the terminal web contractile system, it is possible that a certain subset of myosin is tethered into the brush border circumferential ring in an ATP-insensitive fashion.

On the other hand, the myosin that is not required for terminal web contraction might depend on a certain degree of freedom in ability to translocate in order to perform its functions. For example, myosin in the interrootlet region of the terminal web may be involved in moving vesicles (for more discussion of this possibility, see reference 32). Recently, it has been shown that myosin covalently coupled to beads will move them along tracks of actin (44). In the brush border terminal web, vesicular traffic may depend on myosin that is free to translocate and only transiently associated with the cytoskeleton under physiological conditions of salt and ATP. Elucidation of the exciting potential that myosin plays a role in vesicle movement through the brush border terminal web awaits further investigation.

While this manuscript was being prepared, another report analyzing the association of myosin with isolated brush borders was published. In that study, Broschat et al. (4), reported that the myosin in brush borders as isolated is unphosphorylated, but that in the presence of ATP or ATP-γ-S at room temperature some of that myosin becomes phosphorylated, is released from the brush border cytoskeleton, and assembles into large bipolar filaments. From these results, they conclude that phosphorylation of the regulatory light chain is required for dissociation of myosin from brush borders and that formation of large bipolar filaments is an intermediate step in the terminal web contraction process.

There are numerous obvious differences between their conclusions and ours about the structure and function of myosin in the brush border. These differences stem mainly from differences in the experiments performed. Although our results are consistent with their observation that in the presence of ATP at room temperature, the regulatory light chain on the solubilized myosin becomes phosphorylated, we have demonstrated that phosphorylation is not required for ATP extraction of up to 80% of the total myosin. More importantly, we have demonstrated that the myosin that is extracted by ATP under these conditions is not even required for terminal web contraction. Because of this, we suggest that it is premature to make any conclusions from the available data about changes that might occur in the structural state of myosin during brush border contraction and about the role of ATP-dissociable myosin in brush border function. Nevertheless, the results of both of these studies indicate that the brush border remains an fruitful model system with which to investigate the structure and function of an actin-based cytoskeleton.

We thank C. L. Howe for important observations especially during the initial stages of this work. We also thank G. Conzelman and J. Morrow for technical advice on immunological procedures and E. Bonder, D. Fishkind, H. Alicea, and B. Stevenson for their support and numerous helpful discussions. Thanks also to S. Lee and A. Goglia for excellent technical support.

This research was carried out partly during the tenure of a Muscular Dystrophy Association Postdoctoral Fellowship to Dr. Keller and a National Science Foundation Predoctoral Fellowship to M. Conzelman. This work was supported by grant AM-25387 from the National Institutes of Health and a Basil O'Conner Starter Grant from the March of Dimes Foundation.

Received for publication 4 October 1984, and in revised form 25 January 1985.

REFERENCES

1. Bentzel, C. J., B. Hainau, S. Ho, S. W. Hui, A. Edelman, T. Anagnostopoulos, and E. L. Benedetti. 1980. Cytoplasmic regulation of tight junction permeability: effect of plant cytokinins. *Am. J. Physiol.* 239:C75–C89.
2. Bretscher, A. 1983. Microfilament organization in the cytoskeleton of the intestinal brush border. *Cell Muscle Motil.* 4:239–268.
3. Bretscher, A., and K. Weber. 1978. Localization of actin and microfilament associated proteins in the microvilli and terminal web of the intestinal brush border by immunofluorescent microscopy. *J. Cell Biol.* 79:839–845.
4. Broschat, K. O., R. P. Stidwell, and D. R. Burgess. 1983. Phosphorylation controls brush border motility by regulating myosin structure and association with the cytoskeleton. *Cell.* 35:561–571.
5. Burgess, D. R. 1982. Reactivation of intestinal epithelial brush border motility: ATP-dependent contraction via a terminal web contractile ring. *J. Cell Biol.* 95:853–863.
6. Burgess, W. H., D. K. Jemiolo, and R. H. Kretsinger. 1980. Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochem. Biophys. Acta.* 623:257–270.
7. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.
8. Centrifuge desalting. *In* Sephadex Gel Filtration in Theory and Practice. Pharmacia Fine Chemicals, Inc., New Jersey.
9. Drenckhahn, D., and U. Groschel-Stewart. 1980. Localization of myosin, actin, and tropomyosin in rat intestinal epithelium: immunohistochemical studies at the light and electron microscope levels. *J. Cell Biol.* 86:475–482.
10. Duffy, M. E., B. Hainau, S. Ho, and C. J. Bentzel. 1981. Regulation of epithelial tight junction permeability by cyclic AMP. *Nature (Lond.).* 203:451–453.
11. Fairbanks, G. T., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
12. Fechheimer, M., J. L. Dais, and J. J. Cebra. 1979. Interaction of immunoglobulin with actin. *Mol. Immunol.* 16:881–888.
13. Fox, J. E. B., and D. R. Phillips. 1982. Role of phosphorylation in mediating the association of myosin with the cytoskeletal structures of human platelets. *J. Biol. Chem.* 257:4120–4126.
14. Gagelmann, M., J. C. Ruegg, and J. DiSalvo. 1984. Phosphorylation of the myosin light chains and satelite proteins in detergent-skinned arterial smooth muscle. *Biochem. Biophys. Res. Commun.* 120:933–938.
15. Glenney, J. R., Jr., P. Glenney, M. Osborne, and K. Weber. 1982. An F-actin and calmodulin binding protein from isolated intestinal brush borders has a morphology related to spectrin. *Cell.* 28:843–854.
16. Glenney, J. R., Jr., P. Glenney, and K. Weber. 1983. The spectrin-related TW 260/240 cross-links bundles of the microvillus rootlets in the brush borders of intestinal epithelial cells. *J. Cell Biol.* 96:1491–1496.
17. Guerriero, V., D. R. Rowley, and A. R. Means. 1981. Production and characterization of an antibody to myosin light chain kinase and intracellular localization of the enzyme. *Cell.* 27:229–458.
18. Haeberle, J. R., J. W. Hott, and D. W. Hathaway. 1984. Pseudophosphorylation of the smooth muscle 20,000 dalton myosin light chain. *Biochim. Biophys. Acta.* 790:78–86.
19. Herman, I., and T. D. Pollard. 1981. Electron microscopic localization of cytoplasmic myosin with ferritin-labeled antibodies. *J. Cell Biol.* 88:346–351.
20. Hirokawa, N., R. E. Cheney, and M. Willard. 1983. Location of a protein of the fodrin-spectrin-TW 260/240 family in the mouse intestinal brush border. *Cell.* 32:953–965.
21. Hirokawa, N., T. C. S. Keller, R. Chasan, and M. S. Mooseker. 1983. Mechanism of

brush border contractility studied by the quick-freeze-deep-etch method. *J. Cell Biol.* 96:1325–1336.
22. Hirokawa, N., L. G. Tilney, K. Fujiwara, and J. E. Heuser. 1982. The organization of actin, myosin, and intermediate filaments, in the brush border of intestinal epithelial cells. *J. Cell Biol.* 94:425–443.
23. Howe, C. L., and M. S. Mooseker. 1983. Characterization of the 110-kDalton actin-calmodulin-and-membrane-binding protein from microvilli of intestinal epithelial cells. *J. Cell Biol.* 97:974–985.
24. Hull, B. E., and L. A. Staehlin. 1979. The terminal web. A re-evaluation of its structure and function. *J. Cell Biol.* 81:67–82.
25. Keller, T. C. S., K. Conzelman, R. Chasan, and M. S. Mooseker. 1983. Terminal web contraction in isolated brush borders requires little of the brush border myosin. *J. Cell Biol.* 97:295a. (Abstr.)
26. Keller, T. C. S., 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.
27. Ledvora, R. F., K. Barany, D. L. VanderMeulen, J. T. Barron, and M. Barany. 1983. Stretch-induced phosphorylation of the 20,000-dalton light chain of myosin in arterial smooth muscle. *J. Cell Biol.* 258:14080–14083.
28. Madara, J. L. 1983. Increases in Guinea pig small intestinal transepithelial resistance induced by osmotic loads are accompanied by rapid alterations in absorptive-cell tight-junction structure. *J. Cell Biol.* 97:125–136.
29. Mooseker, M. S. 1976. Brush border motility. Microvillar contraction in Triton-treated brush borders isolated from intestinal epithelium. *J. Cell Biol.* 71:417–432.
30. Mooseker, M. S. 1983. Actin binding proteins of the brush border. *Cell.* 35:11–13.
31. Mooseker, M. S., E. Bonder, K. A. Conzelman, D. J. Fishkind, C. L. Howe, and T. C. S. Keller. 1984. Brush border cytoskeleton and integration of cellular functions. *J. Cell Biol.* 99:104s–112s.
32. Mooseker, M. S., T. C. S. Keller, and N. Hirokawa. 1983. Regulation of contractility and cytoskeletal structure in the brush border. *In* Brush Border Membranes. *CIBA Found. Symp.* 95:195–215.
33. 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.
34. Nishikawa, M., H. Hidaka, and R. S. Adelstein. 1983. Phosphorylation of smooth muscle heavy meromyosin by calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* 258:14069–14072.
35. Owaribe, K., R. Kodama, and G. Eguchi. 1981. Demonstration of contractility of circumferential actin bundles and its morphogenic significance in pigmented epithelium in vitro and in vivo. *J. Cell Biol.* 90:507–514.
36. Owaribe, K., and H. Masuda. 1982. Isolation and characterization of circumferential microfilament bundles from retinal pigmented epithelial cells. *J. Cell Biol.* 95:310–315.
37. Pearl, M., D. J. Fishkind, M. S. Mooseker, D. Keene, and T. C. S. Keller. 1984. Studies on the spectrin-like protein from the intestinal brush border, TW 260/240, and characterization of its binding with the cytoskeleton and actin. *J. Cell Biol.* 98:66–78.
38. Perrie, M. T., and S. V. Perry. 1970. An electrophoretic study of the low molecular weight components of myosin. *Biochem. J.* 119:31–38.
39. Pitelka, D. R., and B. N. Taggart. 1983. Mechanical tension induces lateral movement of intramembrane components of the tight junction: studies on mouse mammary cells in culture. *J. Cell Biol.* 96:606–612.
40. Pollard, T. D., and E. D. Korn. 1973. Acantamoeba myosin. I. Isolation from Acanthamoeba castellanii of an enzyme similar to muscle myosin. *J. Biol. Chem.* 248:4682–4690.
41. Rodewald, R., S. B. Newman, and M. J. Karnovsky. 1976. Contraction of isolated brush borders from the intestinal epithelium. *J. Cell Biol.* 70:541–554.
42. Scholey, J. M., R. C. Smith, D. Drenkhahn, U. Groschel-Stewart, and J. Kendrick-Jones. 1982. Thymus myosin. Isolation and characterization of myosin from calf thymus and thymic lymphocytes, and studies on the effect of phsophorylation of its Mr 20,000 light chain. *J. Cell Biol.* 257:7737–7745.
43. Sellers, J. R., E. Eisenberg, and R. S. Adelstein. 1982. The binding of smooth muscle heavy meromyosin to actin in the presence of ATP. *J. Biol. Chem.* 257:13880–13883.
44. Sheetz, M. P., and J. A. Spudich. 1983. Movement of myosin-coated fluorescent beads on actin cables in vitro. *Nature (Lond.).* 303:31–35.