Characterization of Intestinal Microvillar Membrane Disks: Detergent-resistant Membrane Sheets Enriched in Associated Brush Border Myosin I (110K-Calmodulin)

Mark S. Mooseker,* Karen A. Conzelman,* Thomas R. Coleman,* John E. Heuser,‡ and Michael P. Sheetz‡

*Department of Biology, Yale University, New Haven, Connecticut 06511; and ‡Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. The actin bundle within each microvillus of the intestinal brush border (BB) is tethered laterally to the membrane by bridges composed of BB myosin I. Avian BB myosin I, formerly termed 110K-calmodulin, consists of a heavy chain with an apparent M_r of 110 kD and three to four molecules of calmodulin "light chains." Recent studies have shown that this complex shares many properties with myosin including mechanochemical activity. In this report, the isolation and characterization of a membrane fraction enriched in bound BB myosin I is described. This membrane fraction, termed microvillar membrane disks, was purified from ATP extracts of nonionic detergent-treated microvilli prepared from avian intestinal BBs. Ultrastructural analysis revealed that these membranes are flat, disk-shaped sheets with protrusions which are identical in morphology to purified BB myosin I. The disks exhibit actin-activated Mg-ATPase activity and bind and cross-link actin filaments in an ATP-dependent fashion. The mechanochemical activity of the membrane disks was assessed using the *Nitella* bead movement assay (Sheetz, M. P., and J. A. Spudich. 1983. *Nature [Lond.].* 303:31–35). These preparations were shown to be free of significant contamination by conventional BB myosin. Latex beads coated with microvillar membrane disks move in a myosin-like fashion along *Nitella* actin cables at rates of 12–60 nm/s (average rate of 33 nm/s); unlike purified BB myosin I, the movement of membrane disk–coated beads was most reproducibly observed in buffers containing low Ca^{2+}.

THE actin bundle within each microvillus of the intestinal brush border (BB)[1] is tethered laterally to the plasma membrane by spirally arranged bridges (Mooseker, 1985). These bridges are composed of 110K-calmodulin (CM), a complex consisting of an ~110-kD protein and multiple molecules of CM (see Coluccio and Bretscher, 1989 for review; this recent study demonstrates the reconstitution of bridges by addition of 110K-CM to actin bundles in vitro). Studies from several laboratories have shown that 110K-CM purified from chicken BBs shares many properties with myosin (Collins and Borysenko, 1984; Howe and Mooseker, 1983; Conzelman and Mooseker, 1987; Coluccio and Bretscher, 1987, 1988; Krizek et al., 1987; Swanljung-Collins et al., 1987; Carboni et al., 1988) including mechanochemical activity (Mooseker and Coleman, 1989). Because 110K-CM is structurally analogous to the tailless, single-headed myosin I's first characterized in amoeboid cells (for review see Korn and Hammer, 1988), this complex has been named BB myosin I. Recent studies on the domain structure of the 110-kD subunit indicate that it consists of two discrete domains: an ~80–90-kD myosin head domain; and a 20–30-kD CM-binding domain at the presumed COOH-terminal end of the molecule (Coluccio and Bretscher, 1988; Carboni et al., 1988). Additional support for the conclusion that the 110K-CM isolated from avian BBs is a member of a family of myosin I mechanoenzymes is provided by the identification of a gene expressed in bovine intestine which is homologous in sequence (within the myosin head domain) to *Acanthamoeba* myosin Ib heavy chain (Hoshimaru and Nakanishi, 1987). This gene most probably encodes the heavy chain of bovine BB myosin I since it is 74% homologous to the sequence deduced from a partial-length, cDNA-encoding 1,000 amino acids of the avian BB myosin I heavy chain (Garcia, A., E. Coudrier, J. Carboni, J. Anderson, J. Vanderckhove, M. Mooseker, D. Louvard, and M. Arpin, manuscript submitted for publication).

While the studies outlined above provide convincing data that the actin bundle of the intestinal microvillus is linked to the membrane by a mechanoenzyme of the myosin I class, there is little information concerning how the interaction of BB myosin I with the membrane is effected. It has been suggested (based on the hydrophobic properties of the 110-kD

1. *Abbreviations used in this paper*: BB, intestinal brush border; CM, calmodulin.

subunit) that the heavy chain of BB myosin I is an integral membrane protein (Glenney and Glenney, 1984). However, most data available support the conclusion that this complex is peripherally associated with the membrane (for review and discussion see Mooseker, 1985). The latter conclusion is also consistent with the deduced amino acid sequence of the protein encoded by the bovine myosin I heavy chain gene (Hoshimaru and Nakanishi, 1987) which lacks a hydrophobic stretch of amino acids that could serve as a membrane-spanning domain; the (partial) deduced primary structure of the avian BB myosin I heavy chain also lacks such a domain. Studies by Coudrier et al. (1983) have provided indirect evidence for the involvement of a glycoprotein in the interaction of BB myosin I with the membrane. These workers identified a 140-kD membrane protein (derived by proteolysis of a 200-kD protein) that remains associated with the microvillar cytoskeleton of porcine BBs after detergent treatment; this same protein binds to the porcine 110-kD subunit immobilized on nitrocellulose. However, further investigation will be required to ascertain the specificity of this interaction given the apparent hydrophobic nature of the 110-kD protein (Glenney and Glenney, 1984; Conzelman and Mooseker, 1986).

In the present report, we describe a membrane fraction isolated from detergent-treated microvilli which should facilitate the molecular dissection of BB myosin I–membrane interaction. This preparation, termed microvillar membrane disks, consists of small, circular sheets of membrane which are highly enriched in bound BB myosin I. In this initial study we have assessed the ultrastructural organization of BB myosin I present of the disks, and have characterized the actin-binding properties, and ATPase and mechanochemical activities associated with the microvillar membrane disk preparation.

Materials and Methods

Isolation of BBs and Microvilli

BBs were isolated from chicken intestines by methods described in Keller and Mooseker (1982). Microvilli were isolated from BBs by a modification of the method of Bretscher and Weber (1978) essentially as described in Mooseker and Howe (1982). In brief, pellets of purified BBs were suspended in 10 vol of solution A consisting of 75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole-Cl, pH 7.2, 0.4 mM DTT, and 0.2 mM PMSF. Microvilli were released from the BBs by homogenization (40–80 strokes) in a tight fitting, stainless steel Dounce homogenizer (Kontes Glass Co., Vineland, NJ). Microvilli were separated from intact BBs and BB fragments by differential sedimentation. The homogenate was spun at 5,000 *g* for 10 min to pellet BBs; the free microvilli were collected from the supernate by sedimentation at 40,000 *g* for 15 min. The microvillar pellet was resuspended in 10 vol of solution A and the above sedimentation protocol was repeated two to three times. Preparation purity was monitored by phase-contrast light microscopy. Although the repeated cycles of differential sedimentation resulted in a substantial reduction in yield of microvilli (particularly long microvilli), this protocol greatly reduced contamination with small BB fragments which contain conventional BB myosin.

Preparation of BB Myosin I–enriched Microvillar Membrane Disks

Pellets of purified microvilli were suspended in 20–30 vol of solution B (same as solution A with 2 mM $MgCl_2$) containing 1.5% NP-40 (wt/vol; Particle Data Laboratories, Elmhurst, IL). After incubation on ice for 5 min, the demembranated microvillar cores were collected by sedimentation at 40,000 *g* for 15 min. Residual detergent was removed by washing the microvillar cores two times with 20–30 vol of solution B; microvillar cores were collected by sedimentation at 40,000 *g* for 15 min. The microvillar core pellet was then resuspended in 4 vol of solution B containing 8 mM ATP and the suspension immediately spun at 40,000 *g* for 15 min. The supernate, containing both free and membrane-associated BB myosin I, was applied to a 1.5 × 40-cm (S-200; Pharmacia Fine Chemicals, Piscataway, NJ) gel-filtration column equilibrated with solution B. Void volume fractions containing the BB myosin I–enriched membrane disks were identified by SDS-PAGE, pooled, and spun at 15,000 *g* for 10 min. This low speed spin was included to remove trace amounts of F-actin-containing aggregates. The membrane disks were collected from the supernate by sedimentation at 100,000 *g* for 30 min. The resulting membrane disk pellet was resuspended in solution B and stored on ice until use. Contamination of the disk preparations by conventional BB myosin was quantitatively assessed immunochemically as described in Mooseker and Coleman (1989).

Characterization of Microvillar–Membrane Disk: Interaction with F-Actin

The interaction of membrane disks with F-actin was assessed by a low speed sedimentation assay. F-actin (0.2 mg/ml) in solution B, was mixed with membrane disks (0.4 mg/ml) in the presence and absence of 5 mM ATP. The suspensions were then sedimented at 14,000 *g* for 15 min and the supernate and pellet fractions examined by SDS-PAGE. Before sedimentation, aliquots were removed for examination by dark-field light microscopy to assess the presence of aggregated filaments.

ATPase Activity of Microvillar Membrane Disks

The K-EDTA and Mg-ATPase activity of BB myosin I–enriched membrane disk preparations in the presence and absence of F-actin was assayed by the method of Taussky and Shorr (1953). Assay conditions were as described in Conzelman and Mooseker (1987) using membrane disks at a final concentration of ~100 μg/ml. The Mg-ATPase assays were carried out in the absence or presence of F-actin (0.9 mg/ml).

Electron Microscopy

Negatively stained images of membrane disks were obtained by application of disk suspensions to parloidian–carbon-coated grids and staining with 1% uranyl acetate. Pellets of membrane disks were processed for thin section electron microscopy exactly as described in Mooseker and Howe (1982) for fixation and embedding of isolated BBs. Rotary-shadowed specimens of quick-freeze, deep-etch preparations of disks adsorbed to mica chips were prepared by the method of Heuser (1983).

In Vitro Motility Assay

The movement of latex beads (Covaspheres, 1-μm diameter; Covalent Technologies, Ann Arbor, MI) coated with BB myosin I–enriched membrane disks was examined using the *Nitella* bead movement assay exactly as described in Sheetz et al. (1986). Disk-coated beads were prepared by addition of 1 vol of Covasphere suspension to 9 vol of membrane disks (0.1–1 mg/ml). Bead movements were documented by video light microscopy (bright-field optics) using a 1/2 in. time-lapse video recorder equipped with a time/date generator. Bead velocities were determined by measurement of transit distances traced on the screen of the video monitor. The same methods were used to test the motility of beads coated with BB myosin prepared by incubation of Covaspheres with suspensions of BB myosin filaments at a concentration of 0.2–0.5 mg/ml. To examine the motility of phosphorylated BB myosin, a preparation of BB myosin (0.5 mg/ml) was incubated for 30 min at room temperature in the presence of 50 μg/ml CM and 25 μg/ml myosin light chain kinase isolated from chicken gizzard as described in Keller et al. (1985) in a buffer containing 0.2 mM $CaCl_2$ and 1 mM ATP before addition to beads.

Other Methods

SDS-PAGE was performed on 5–15% gradient gels by the method of Matsudaira and Burgess (1978). Actin was purified from acetone powders of chicken breast muscle by the method of Spudich and Watt (1971). BB myosin was purified by gel filtration of ATP extracts of BBs as described for purification of BB myosin I (Coluccio and Bretscher, 1987). Fractions containing BB myosin were dialyzed against solution A to induce thick filament formation. The myosin filaments were collected by sedimentation (50,000 *g* for 20 min) and stored on ice as a suspension in solution A. Protein concen-

trations were determined either by the method of Lowry et al. (1951) or by the BCA method following directions of the manufacturer (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as standard. The concentration of BB myosin I relative to total protein concentration in the membrane disk preparation was estimated by densitometric analysis of gels containing the disks and known amounts of BB myosin I purified by the method of Coluccio and Bretscher (1987).

Results

Isolation and Characterization of Microvillar Membrane Disks

The microvillar membrane disk preparation described in this report was purified by gel filtration of ATP extracts of microvillar cores which have had the bulk of their membranes removed by treatment with nonionic detergent. As first shown by Matsudaira and Burgess (1979), ATP extraction of demembranated microvillar cores results in a loss of the lateral bridges and a concomitant solubilization of BB myosin I. Such extracts contained variable amounts of "aggregated" BB myosin I as assessed by either high speed sedimentation (100,000 *g* for 30 min) or gel filtration (10–30% based on estimation from SDS-PAGE of supernate and pellet fractions; results not shown). Ultrastructural analysis of void volume fractions from an S-200 column containing the high molecular weight form of BB myosin I revealed the presence of a morphologically uniform population of membrane fragments. By negative staining, these membranes appeared to be in the form of vesicles (Fig. 1 *a*). The surface of these membranes was studded with particles. Particles protruding from the edge of these membranes had a length of ~5–8 nm. Close examination of these negatively stained void volume fractions indicated that very little material was present on the grid surface between the membranes. Examination of pelleted membranes by thin-section techniques (Fig. 1 *b*) revealed that the membranes were actually flat sheets which must be roughly circular or disk-shaped, given their morphology by negative staining. Cross-sectional profiles of these disks often revealed the presence of projections predominantly on a single surface of the membrane (Fig. 1 *b*).

The protein composition of these membrane disks was relatively simple, at least with respect to constituents detectable by Coomassie blue staining of SDS gels (Fig. 2). Major proteins included the 110-kD and CM subunits of BB myosin I and a prominent, diffusely migrating band at 120–140 kD. Less prominent bands of 85 and 220 kD were also present, but the amount of these bands relative to BB myosin I and the 120–140-kD band was variable. Trace amounts of actin were often present. The 85-kD band increased with time of preparation storage and was probably a proteolytic fragment of a protein or protein(s) in the 120–140-kD band since these two bands contain the only glycoproteins present, as detected by staining of gels with periodic acid–Schiff reagent (results not shown). The 120–140-kD glycoprotein band comigrates with the major periodic acid–Schiff reagent–positive band of the isolated avian BB (Mooseker and Stephens, 1980) which probably contains numerous microvillar membrane proteins assuming a membrane composition similar to that of mammalian BBs (Semenza, 1986). Densitometric analysis of SDS gels containing disk preparations and known amounts of purified BB myosin I indicated that the BB myosin I in the disks contributed 60–70% of the total disk protein.

Evidence that the BB myosin I present in these preparations is associated with the membrane disks is provided by ultrastructural examination of disks using the quick-freeze, deep-etch technique (Fig. 1 *c*). By this technique, the membrane disks appear to have a distinct polarity, with respect to projections on their surfaces. The surface facing "up" on the mica surface is generally smooth except at the edges of the disk where tadpole-shaped protrusions can be seen. These protrusions are identical in size and morphology to purified BB myosin I visualized using this same technique (Conzelman and Mooseker, 1987). Two plausible interpretations of these images are that the BB myosin I is associated either with the edges of the disks, or with the whole surface of the disk adsorbed to the mica substrate.

Interaction of Microvillar Membrane Disks with Actin

If the membrane-bound BB myosin I is functional, it would be expected to effect the association of the membrane disks with actin in a ATP-dependent fashion. Addition of membrane disks to solutions of F-actin, in the absence but not presence of ATP, caused an immediate formation of aggregates discernable by eye or by light microscopy (Fig. 3 *b*). Low speed sedimentation of such F-actin–membrane disk mixtures showed that a sedimentable complex of F-actin and the membrane disks was formed in the absence but not presence of ATP (Fig. 3 *a*). The fact that all the protein constituents of the membrane disk fraction sedimented with F-actin, provides additional evidence that the BB myosin I in these preparations is associated with the membrane disks.

Electron microscopy of disk–F-actin mixtures using the quick-freeze, deep-etch technique provided further evidence that the observed aggregation is due to cross-linking of F-actin by the membrane disks (Fig. 4). The disks appear to be associated with F-actin; occasionally, bridges emanating from the edge of a disk can be seen linked to actin filaments. The surface of the disks facing up on the mica substrate appeared free of protrusions providing evidence that the BB myosin I molecules are restricted either to a single side of the disk, or to the disk edges. These observations are consistent with the functional integrity of BB myosin I.

If the observed filament cross-linking is mediated by disk-bound BB myosin I, it should be noted that the presumed mechanism of cross-linking is probably distinct from that observed with purified BB myosin I (Conzelman and Mooseker, 1987). The disks are multivalent complexes of BB myosin I and filament cross-linking could be promoted solely by filament binding to the ATP sensitive site in the head domain of the bound BB myosin I molecules. Filament cross-linking by free BB myosin I, if analogous to *Acanthamoeba* myosin I (see Korn and Hammer, 1988), may be effected by a second, ATP-insensitive site located elsewhere on the BB myosin I heavy chain.

Another way to assess the functional properties of BB myosin I in the disk preparations is to compare the ATPase activity of the disks to that of the purified protein, although interpretation of such results could be confounded by the presence of other ATPases present in the disk preparation. For these studies, both actin activation of Mg-ATPase and K-EDTA ATPase in the absence of actin were examined. (The Mg-ATPase assays were done in the presence of EGTA; average values for assays from three different disk preparations are reported here.) Unlike purified BB myosin I, the membrane disk preparations exhibited relatively high activ-

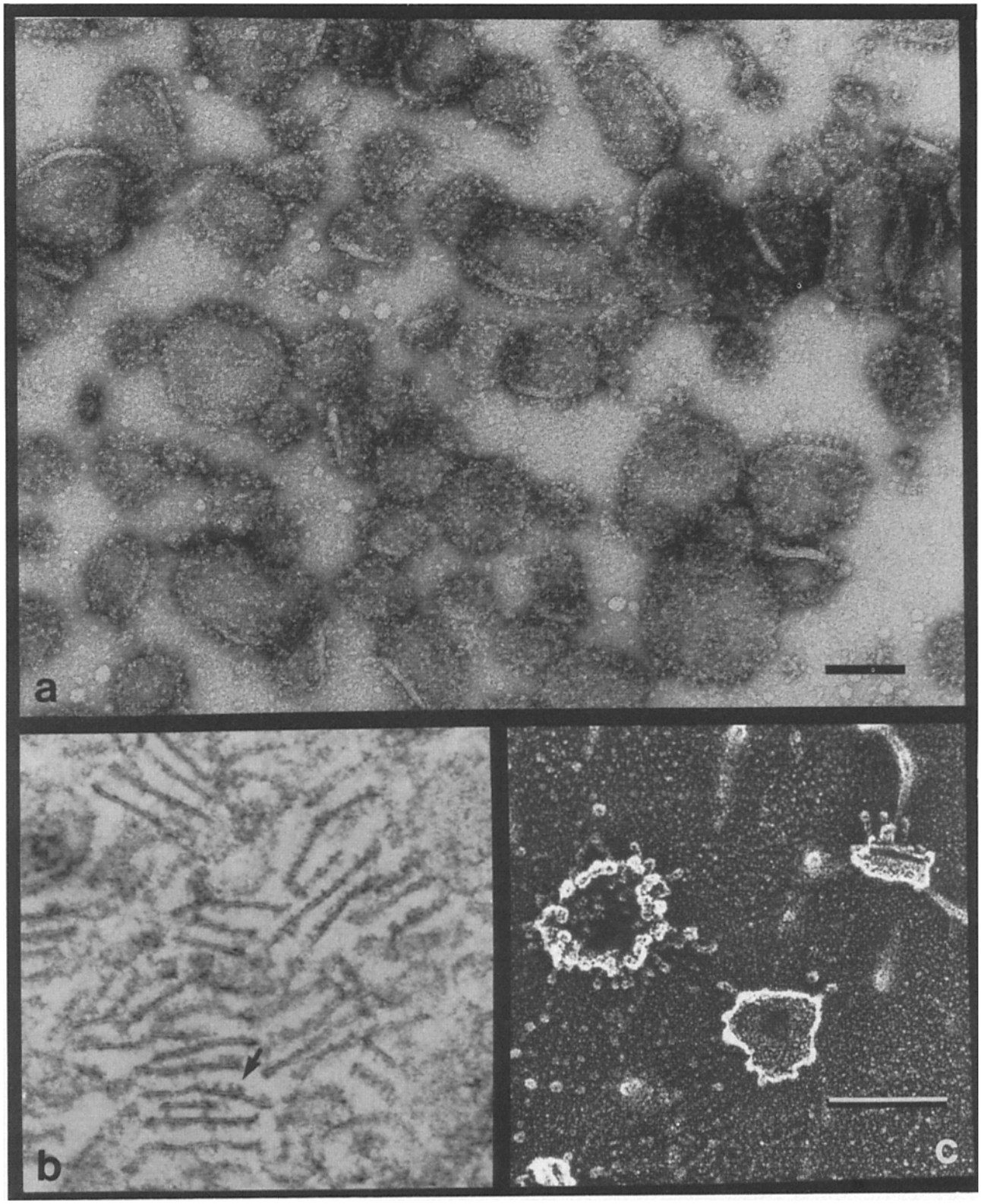

Figure 1. Ultrastructural characterization of BB myosin I-enriched membrane disks. (*a*) Negatively stained preparation of microvillar membrane disks purified by gel filtration of ATP extracts of isolated, detergent-treated microvillar cores. (*b*) Thin section of a membrane disk pellet comparable to that shown in *a*. Note the protrusions on some of the membranes (*arrow*). (*c*) Quick-freeze, deep-etch replica of microvillar membrane disks. The protrusions emanating from the edge of the disks are morphologically identical to purified BB myosin I visualized by this same technique (see Conzelman and Mooseker, 1987). Bars, 100 nm.

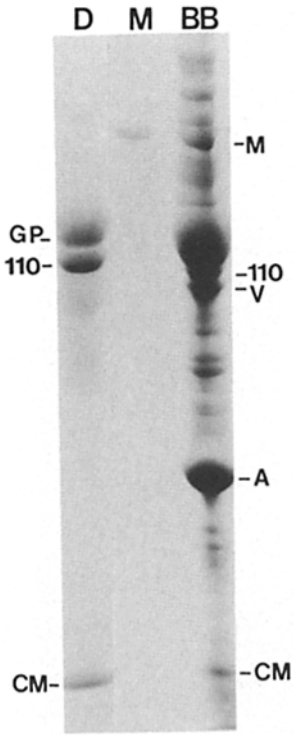

Figure 2. SDS-PAGE analysis of purified microvillar membrane disks. The Coomassie-stained gel shows the electrophoretic profile of loadings of the purified membrane disk preparation (*D*), purified conventional BB myosin (*M*, ~100 ng), and isolated BBs (*BB*). The migration positions (shown to the right of the figure) of myosin heavy chain (*M*), villin (*V*), actin (*A*), and the 110-kD (*110*) and calmodulin (*CM*) subunits of BB myosin I are indicated. The disk preparation contains, in addition to BB myosin I, a prominent 120–140-kD band (*GP*) which is periodic acid–Schiff reagent positive (result not shown).

ity in the presence of Mg^{2+} (0.46 μmol P_i/mg per min). Nevertheless, similar levels of actin activation, compared to the purified enzyme (~1.4-fold) were observed (0.65 μmol P_i/mg per min). The K-EDTA ATPase of these preparations was substantially lower (0.08 μmol P_i/mg per min) than one would expect for the purified enzyme (Conzelman and Mooseker, 1987; Swanljung-Collins et al., 1987; Krizek et al., 1987). The observed actin activation of the Mg-ATPase is consistant with the presence of a functionally active myosin in the disk preparation. If this activity is due to BB myosin I, the relatively low K-EDTA ATPase and high Mg-ATPase of the disks in the absence of actin suggest that the association of BB myosin I with the membrane alters its activity. Alternatively, there may be additional enzymes present which could contribute either solely or additively to the observed disk ATPase activities.

Movement of Membrane Disk-coated Beads along Nitella Actin Cables

Ultrastructural analysis of the membrane disks suggested that the BB myosin I in these preparations was associated with the disk membranes in a configuration reminiscent of its presumed orientation in vivo; i.e., with the "head" domain of the complex facing out from the membrane. If so, we reasoned that this preparation might be a useful means to assess the motile properties of membrane-associated BB myosin I. To this end, we used the *Nitella* bead movement assay of Sheetz and Spudich (1983; Sheetz et al., 1986). Using conditions exactly as described in Sheetz et al. (1986), we observed slow movements of membrane disk–coated beads along *Nitella* actin cables (Fig. 5). This movement was in the same direction as that for myosin, as determined by injection of chicken skeletal muscle myosin–coated beads onto the same region where membrane disk movement was observed (results not shown). The rates of movement observed were quite variable; for this report, the rates of movement of 50 beads or bead aggregates from three different preparations of membrane disks were measured. The range of rates measured was 13–60 nm/s, with an average rate of 33 nm/s (SD = 13.4 nm/s). Most of the movements documented by time-lapse video microscopy were of bead clusters, since addition of the membrane disks to the latex spheres caused aggregation of the bulk of the beads present. However, when single bead movements were observed, these generally moved at a faster rate (average rate, 44 nm/s; SD = 15 nm/s; n = 10) than bead clusters (average rate, 26 nm/s; SD = 9 nm/s). The rates of both single beads and bead clusters were, on average, substantially faster than that measured for beads coated with

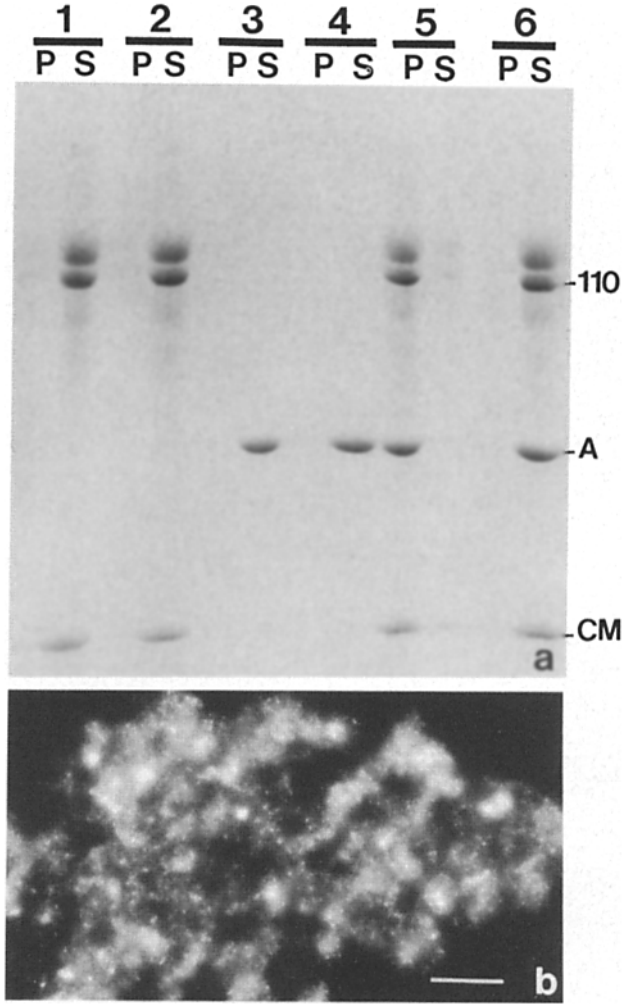

Figure 3. ATP-dependent cross-linking of actin filaments by BB myosin I–enriched membrane disks. (*a*) SDS-PAGE analysis of supernate (*S*) and pellet (*P*) fractions from a low speed cosedimentation assay to examine the ATP-dependent binding and cross-linking of membrane disks to actin. Neither the disks (lanes *1* and *2*) nor actin alone (lanes *3* and *4*) are pelleted at low speed in either the absence (lanes *1* and *3*) or presence (lanes *2* and *4*) of ATP. Addition of disks to actin causes the pelleting of both actin and disk proteins in the absence (lanes *5*) but not the presence (lanes *6*) of ATP. (*b*) Dark-field light micrograph of a mixture of membrane disks and actin in the absence of ATP. Addition of the disks to F-actin induces the formation of large, macromolecular aggregates. Bar, 20 μm.

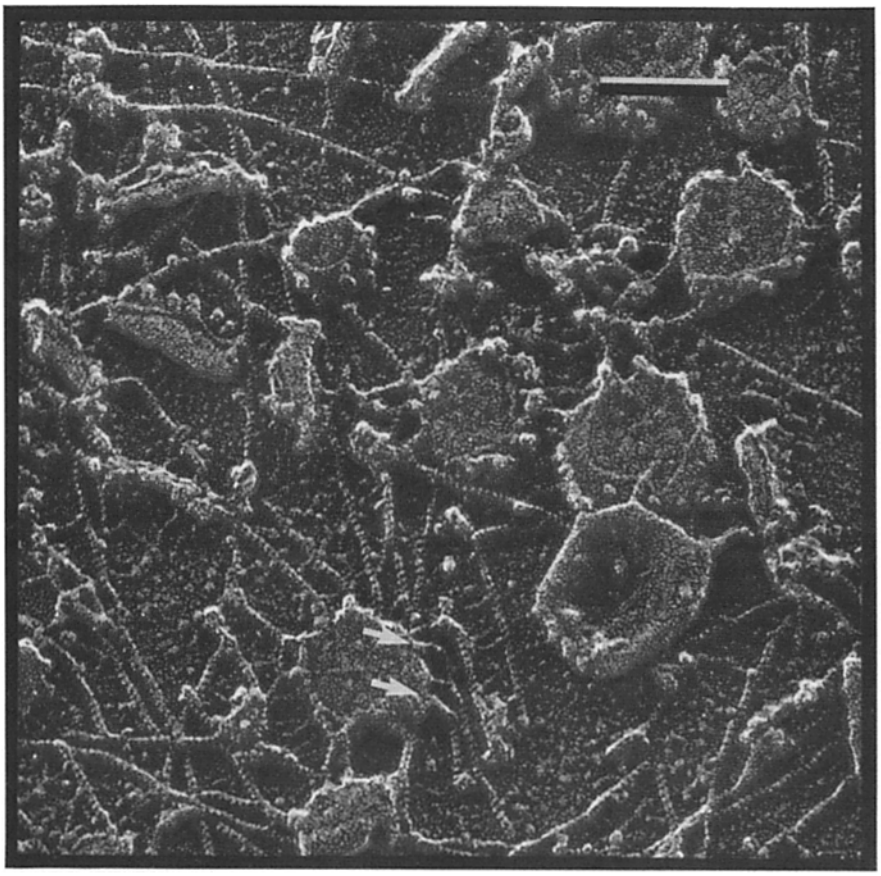

Figure 4. Ultrastructural analysis of disk–actin interaction in the absence of ATP; rotary shadowed metal replica of a quick-freeze, deep-etched specimen absorbed to mica. Arrows indicate links between the disks and actin filaments. Bar, 100 nm.

purified BB myosin I (average rate, ~8 nm/s; Mooseker and Coleman, 1989). All the measurements reported above were from experiments using motility buffers containing 1 mM EGTA. In two of the five motile disk preparations assayed, occasional bead movements were observed using buffers containing Ca^{2+} (1 mM EGTA, 1 mM $CaCl_2$, ~10 μM Ca^{2+}; Portzehl et al., 1964); however, in both of these preparations, optimal activity was observed in the absence of Ca^{2+}.

Since the intestinal BB contains high concentrations of

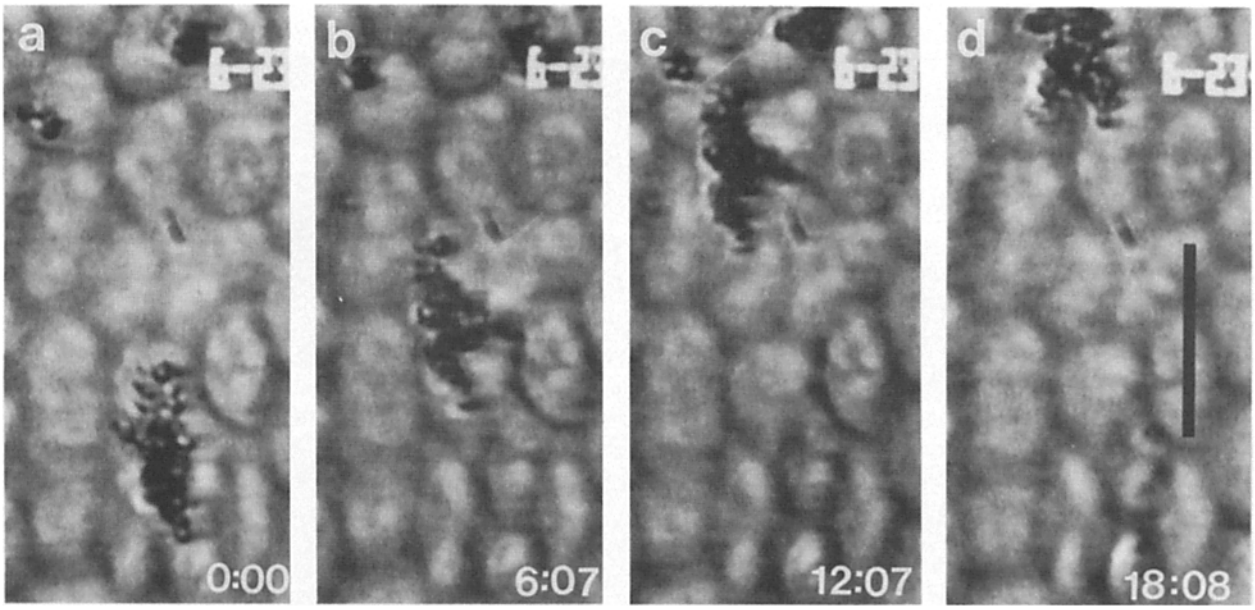

Figure 5. Movement of membrane disk–coated latex beads along *Nitella* actin cables. This bead cluster moved at a rate of ~23 nm/s. Time intervals for each successive time-lapse video frame are indicated at the lower right of each frame in minutes:seconds. This motile preparation of disk membranes contained <0.3 μg/ml contamination by conventional BB myosin. Bar, 10 μm.

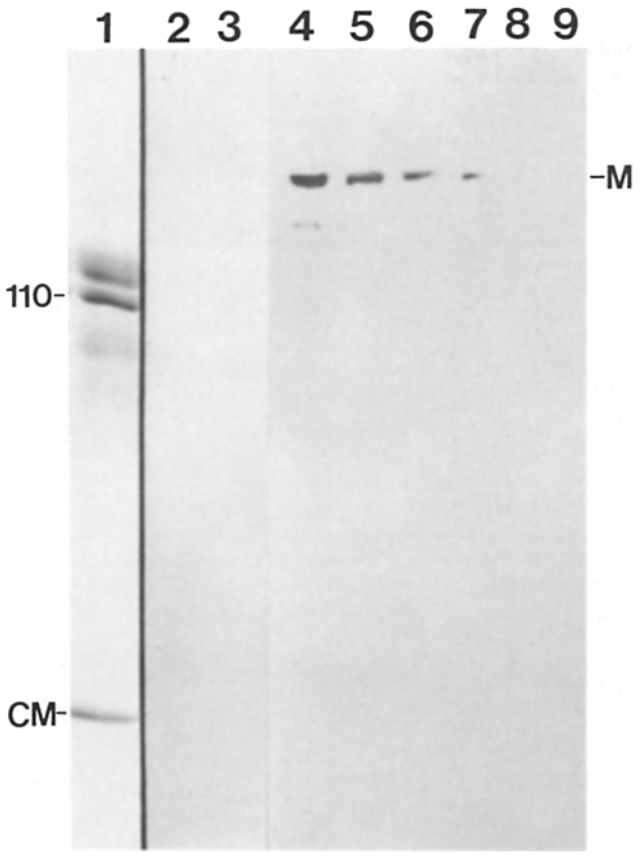

Figure 6. Assessment of conventional BB myosin contamination in motile preparations of membrane disks. Lane *1*, Coomassie blue-stained SDS-PAGE of a membrane disk preparation. Lanes *2–9*, Immunoblot of an identical gel to that in lane *1* stained with antimyosin heavy chain. Lanes *2* and *3* contain 5-μl loads of membrane disks at the same (lane *2*) and three times the concentration (lane *3*) used for motility assay. Lanes *4–9* contain 5-μl loadings of a serial dilution of purified BB myosin from 9, 4.5, 2.3, 1.7, 1, to 0.8 μg/ml. Immunostaining of myosin heavy chain (*M*) was visually detected in all the myosin lanes (lanes *4–9*) but not photographically documentable in lanes *8* or *9*. No myosin heavy chain staining was detected in either of the membrane disk lanes.

conventional myosin, the possibility exists that the observed movements were due to the presence of conventional BB myosin contaminating the membrane disk preparations. SDS-PAGE analysis of the disk preparations (e.g., Fig. 1) indicated that contamination by BB myosin was below the limits detectable by Coomassie blue staining, although silver staining sometimes revealed the presence of a faint band comigrating with the heavy chain of BB myosin (results not shown). To quantitate the levels of BB myosin contamination, each disk preparation used for motility studies was assayed by immunoblotting with an antibody reactive with BB myosin heavy chain. A serial dilution of known amounts of BB myosin was run on the same gel to assess the detection limits of this assay. By this technique, motile preparations of membrane disks contained variable amounts of BB myosin contamination from ~3 μg/ml to less than detectable amounts (<0.3 μg/ml; e.g., Fig. 6). It should be noted that we failed to observe movement of beads coated with conventional BB myosin (three preparations of BB myosin were tested for motility) purified from ATP extracts of BBs using concentrations of myosin up to 0.5 mg/ml. One of these BB myosin preparations was assayed for motility after preincubation with gizzard myosin light chain kinase and CM. This preparation did exhibit bead movements (~20 nm/s), a result consistent with the observations of Umemoto et al. (1989), who have demonstrated that nonmuscle myosins promote bead movement in the *Nitella* assay only in the phosphorylated state. Because we have eliminated the possibility that the observed movement is due to contamination by conventional BB myosin, we conclude that it is mediated either by BB myosin I, which constitutes >60% of the total disk protein, or by an as yet uncharacterized mechanoenzyme present in the disk preparation.

Discussion

In this report we have described a novel preparation of detergent-resistant microvillar membrane fragments which is highly enriched in associated BB myosin I. Several types of data are presented which indicate that the microvillar membrane disk preparation will be a useful model system for investigating the interaction of BB myosin I with the membrane. First, ultrastructural evidence is provided (Fig. 1) which indicates that the association of BB myosin I with the disk membrane is topographically similar to its presumed orientation within the mirovillus. Second, we demonstrate that microvillar disks devoid of contamination by conventional BB myosin are functionally active with respect to ATP-dependent actin-binding and enzymatic activities. Finally, we demonstrate that the disk preparation is mechanochemically active. Based on these observations, we consider it most likely that the BB myosin I present in the disks is responsible for the observed movements. However, given the differences in enzymatic and motile properties of disks vs. purified BB myosin I (see Table I) we cannot exclude the possibility that the observed motility is mediated not by BB myosin I, but by a different mechanoenzyme present in the preparation.

Assuming that the observed motility of disk-coated beads is mediated by BB myosin I, then the interaction of this motor with the membrane may be critical in regulating its activity. This is because of the dramatic differences in both Ca^{2+} requirements and rate, compared to that observed for movement of beads coated with the purified enzyme; i.e., the disk-coated beads move faster, and with opposite requirements for Ca^{2+}. Precedent for such a regulatory role is provided by the observations of Adams and Pollard (1986) who reported that *Acanthamoeba* myosin I–mediated movement of organelles occurs at rates over 10-fold faster than that observed using purified myosin I (Albanesi et al., 1985). We must consider, however, two alternative explanations for the observed motility. First, the observed movements could be mediated by conventional BB myosin contaminating the preparation; this is particularly worrisome since the observed rates are comparable to that reported by Umemoto et al. (1989) for vertebrate nonmuscle myosins using the same assay. However, three lines of evidence are presented here which strongly argue against this possibility. First, we demonstrate that disk preparations containing undetectable levels of BB myosin contamination (<0.3 μg/ml) exhibit motility. Second, any contaminating BB myosin would most probably be unphosphorylated (Keller et al., 1985), and thus unable to promote

Table I. Microvillus Disk ATPase and Motility: Comparison with BB Myosin I and II

	ATPase activity*			Motility rate‡	
	K-EDTA	Mg^{2+}	Mg^{2+} + actin	$-Ca^{2+}$	$+Ca^{2+}$
BB myosin (II)§	320	30	280	20–40 nm/s	ND
BB myosin I‖	240	14	94	nonmotile	8 nm/s
Microvillar membrane disks	80	460	650	33 mm/s	nonmotile

* Activities are given as *n* moles P_i/mg per min. Mg-ATPase activities reported were measured under comparable conditions to those used for microvillus disks except for the Mg-ATPase of BB myosin which was assayed in the presence of 150 mM KCl rather than 75 mM KCl.
‡ Motility rates indicate average velocities for motor-coated beads along Nitella actin cables. See Material and Methods for assay conditions.
§ Values for ATPase of chicken BB myosin (II) are from Keller et al. (1985), using BB myosin phosphorylated by purified gizzard myosin light chain kinase. The range of rates indicated for bead movement include the observations reported here (~20 nm/s) and that of a detailed study by Umemoto et al. (1989) on myosin from human platelets and turkey BBs (40 nm/s).
‖ Values for Mg-ATPase and motility rates from Mooseker and Coleman (1989). K-EDTA ATPase values are taken from Conzelman and Mooseker (1987).

bead movement (Umemoto et al., 1989); it should be noted that preparations of isolated microvilli from which the disks are prepared do not contain detectable levels of BB myosin light chain kinase (Keller, T. C. S., and M. S. Mooseker, unpublished observations). Third, freshly isolated preparations of conventional BB myosin, made under solution conditions comparable to that used for disk preparation, failed to promote bead movement unless those preparations were preincubated with kinase. The second alternative is that the observed motility may be mediated by a second, as yet uncharacterized, mechanoenzyme present in the preparation (e.g., the 120–140-kD glycoprotein[s]). This possibility cannot be formally excluded, particularly given the disparity in rates of movement and Ca^{2+} dependence between pure BB myosin I and the disk preparation (see Table I).

Our goals in future studies using the disk preparation will be to directly establish the presumed role of BB myosin I in disk motility, elucidate the molecular basis of BB myosin I interaction with the membrane, and determine how this association affects its various activities including mechanochemistry. One question of immediate concern will be to determine if the glycoprotein band present in the disk preparation is a random mixture of detergent-insoluble BB membrane glycoproteins or a distinct protein involved in the interaction of BB myosin I with the membrane. Specifically, does this band contain the avian form of the 140-kD glycoprotein in pig microvillar that has been implicated in the association of porcine BB myosin I with the membrane (Coudrier et al., 1983). Although the disk preparation should help resolve this question, it is important to note that the disks may be representative of only one of multiple modes of BB myosin I–membrane interaction. Detergent treatment and subsequent ATP extraction of microvillar cores releases two different populations of BB myosin I: disk-associated myosin; and soluble BB myosin I. The hydrodynamic and functional properties of the soluble BB myosin I are comparable to BB myosin I purified by standard techniques from membrane-intact BBs (results not shown). Future studies will address whether these operationally defined fractions of BB myosin I are reflective of two distinct populations of BB myosin I within the microvillus differing in their interaction with the membrane, or if these fractions are simply an artifact of detergent treatment. Clearly, once we understand how and to what the BB myosin I is bound on the microvillar membrane, we will be in a better position to understand the function of this myosin in vivo.

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References

Adams, R. A., and T. D. Pollard. 1986. Propulsion of organelles isolated from *Acanthamoeba* along actin filaments by myosin I. *Nature (Lond.).* 322: 754–756.

Albanesi, J. P., H. Fujisaki, J. A. Hammer III, E. D. Korn, R. Jones, and M. P. Sheetz. 1985. Monomeric *Acanthamoeba* myosin Is support movement in vitro. *J. Biol. Chem.* 260:8649–8652.

Bretscher, A., and K. Weber. 1978. Purification of microvilli and an analysis of the protein components of the microfilament core bundle. *Exp. Cell Res.* 116:397–407.

Carboni, J. M., K. A. Conzelman, R. A. Adams, D. A. Kaiser, T. D. Pollard, and M. S. Mooseker. 1988. Structural and immunological characterization of the myosin-like 110-kD subunit of the intestinal microvillar 110K-calmodulin complex: evidence for discrete myosin head and calmodulin-binding domains. *J. Cell Biol.* 107:1749–1757.

Collins, J. H., and C. W. Borysenko. 1984. The 110,000-dalton actin- and calmodulin-binding protein from intestinal brush border is a myosin-like ATPase. *J. Biol. Chem.* 259:14128–14135.

Coluccio, L. M., and A. Bretscher. 1987. Calcium-regulated cooperative binding of the microvillar 110K-calmodulin complex of F-actin: formation of decorated filaments. *J. Cell Biol.* 105:325–333.

Coluccio, L. M., and A. Bretscher. 1988. Mapping of the microvillar 110K-calmodulin complex: calmodulin-associated or-free fragments of the 110-kD polypeptide bind F-actin and retain ATPase activity. *J. Cell Biol.* 106: 367–373.

Coluccio, L. M., and A. Bretscher. 1989. Reassociation of microvillar core proteins: making a microvillar core in vitro. *J. Cell Biol.* 108:495–502.

Conzelman, K. A., and M. S. Mooseker. 1986. Reevaluation of the hydrophobic nature of the 110kD-calmodulin-, actin-, and membrane binding protein of the intestinal microvillus. *J. Cell. Biochem.* 30:271–279.

Conzelman, K. A., and M. S. Mooseker. 1987. The 110-kD protein–calmodulin complex of the intestinal microvillus is an actin-activated MgATPase. *J. Cell Biol.* 105:313–324.

Coudrier, E., H. Reggio, and D. Louvard. 1983. Characterization of an integral membrane glycoprotein associated with microfilaments of pig intestinal microvilli. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:469–474.

Glenney, J. R., and P. Glenney. 1984. The microvillus 110kD cytoskeletal protein is an integral membrane protein. *Cell.* 37:743–751.

Heuser, J. E. 1983. Procedure for freeze-drying molecules adsorbed to mica flakes. *J. Mol. Biol.* 169:155–195.

Hoshimaru, M., and S. Nakanishi. 1987. Identification of a new type of mammalian myosin heavy chain by molecular cloning: overlap of its mRNA with preprotachykinin B mRNA. *J. Biol. Chem.* 262:14625–14632.

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.

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.

Keller, T. C. S., III, K. A. Conzelman, R. Chasan, and M. S. Mooseker. 1985. Role of myosin in terminal web contraction in isolated intestinal epithelial brush borders. *J. Cell Biol.* 100:1647–1655.

Korn, E. D., and J. A. Hammer III. 1988. Myosins of nonmuscle cells. *Annu. Rev. Biophys. Biophys. Chem.* 17:23–45.

Krizek, J., L. M. Coluccio, and A. Bretscher. 1987. ATPase activity of the microvillar 110 kDa polypeptide-calmodulin complex is activated in Mg^{2+} and inhibited in K^{+}-EDTA by F-actin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 225:269–272.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin biuret reagent. *J. Biol. Chem.* 193:265–271.

Matsudaira, P., and D. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386–396.

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.

Mooseker, M. S. 1985. Organization, chemistry and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu. Rev. Cell Biol.* 1:261–293.

Mooseker, M. S., and T. R. Coleman. 1989. The 110-kD protein-calmodulin complex of the intestinal microvillus (brush border myosin I) is a mechanoenzyme. *J. Cell Biol.* 108:2395–2400.

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. *Methods Cell Biol.* 25:144–175.

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.

Portzehl, H., P. C. Caldwell, and J. C. Ruegg. 1964. The dependence of contraction and relaxation of muscle fibres from the crab *Mara squinado*. *Biochim. Biophys. Acta.* 79:581–591.

Semenza, G. 1986. Anchoring and biosynthesis of stalked brush border membrane proteins: glycosidases and peptidases of enterocytes and renal tubuli. *Annu. Rev. Cell Biol.* 2:255–314.

Sheetz, M. P., and J. A. Spudich. 1983. Movement of myosin-coated fluorescent beads on actin cables in vitro. *Nature (Lond.).* 303:31–35.

Sheetz, M. P., S. M. Block, and J. A. Spudich. 1986. Myosin movement in vitro: a quantitative assay using oriented actin cables for *Nitella*. *Methods Enzymol.* 134:531–543.

Spudich, J. A., and S. Watt. 1971. The regulation of 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.

Swanljung-Collins, H., J. Montibeller, and J. H. Collins. 1987. Purification and characterization of the 110-kDa actin- and calmodulin-binding protein from intestinal brush border: a myosin-like ATPase. *Methods Enzymol.* 139:137–148.

Taussky, H. H., and S. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorous. *J. Biol. Chem.* 202:675–686.

Umemoto, S., A. R. Bengur, and J. R. Sellers. 1989. Effect of multiple phosphorylations of smooth muscle and cytoplasmic myosins on movement in an in vitro motility assay. *J. Biol. Chem.* 264:1431–1436.