CHARACTERIZATION AND LOCALIZATION OF MYOSIN IN THE BRUSH BORDER OF INTESTINAL EPITHELIAL CELLS

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ABSTRACT

The brush border of intestinal epithelial cells consists of a tightly packed array of microvilli, each of which contains a core of actin filaments. It has been postulated that microvillar movements are mediated by myosin interactions in the terminal web with the basal ends of these actin cores (Mooseker, M. S. 1976. *J. Cell Biol.* **71**:417–433). We report here that two predictions of this model are correct: (*a*) The brush border contains myosin, and (*b*) myosin is located in the terminal web. Myosin is isolated in 70% purity by solubilization of Triton-treated brush borders in 0.6 M KI, and separation of the components by gel filtration. Most of the remaining contaminants can be removed by precipitation of the myosin at low ionic strength. The yield is ~1 mg of myosin/30 mg of solubilized brush border protein. The molecule consists of three subunits with molecular weights of 200,000, 19,000, and 17,000 daltons in a 1:1:1 M ratio. At low ionic strength, the myosin forms small, bipolar filaments with dimensions of 300 × 11 nm, that are similar to filaments seen previously in the terminal web of isolated brush borders. Like that of other vertebrate, nonmuscle myosins, the ATPase activity of isolated brush border myosin in 0.6 M KCl is highest with EDTA (1 μmol P_i/mg-min; 37°C), intermediate with Ca^{++} (0.4 μmol P_i/mg-min), and low with Mg^{++} (0.01 μmol P_i/mg-min). Actin does not stimulate the Mg-ATPase activity of the isolated enzyme. Antibodies against the rod fragment of human platelet myosin cross-react by immunodiffusion with brush border myosin. Staining of isolated mouse or chicken brush borders with rhodamine-antimyosin demonstrates that myosin is localized exclusively in the terminal web.

KEY WORDS antibody · brush border · intestine · myosin · terminal web

The arrangement of actin and its companion proteins in the cortex of cells must be highly ordered, given the anisotropic nature of most movements involving the cell surface. Cytokinesis is a classic example (26, 35). From "guilt by association" evidence, we surmise that a key feature of this arrangement is the interaction of actin with the plasma membrane (4, 6, 20, 22–25, 32, 33, 37, 40). It seems reasonable to assume that the ani-

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sotropy of cell surface movements is provided for, at least in part, by the precise positioning of actin filament-membrane attachments with respect to both location and filament polarity. This assumption holds for all the systems that have been examined so far (5, 8, 13, 21, 23–25, 32, 40). In these examples, actin filaments attached to the plasma membrane always have the same polarity: the heavy meromyosin or myosin fragment S_1 "arrowheads" always point away from the membrane attachment site. This means that the membrane is the structural equivalent of the Z line of skeletal muscle in the anchorage of actin filaments.

If actin filaments in the cell cortex are precisely arranged in this way, then the distribution of myosin must also be defined. Although a myosin molecule can bind anywhere along the length of an actin filament, random interactions of this kind yield only isodiametric contractions such as that seen in the superprecipitation of actomyosin or the contraction of gelled cytoplasmic extracts (28, 39). To produce anisotropic movements such as the contraction of a sarcomere, actin and myosin are positioned nonrandomly with respect to each other. If the paradigm of skeletal muscle holds for the production of force and the resultant movements observed in nonmuscle cells, we would predict that myosin is positioned so that interaction with actin filaments would be efficient. This is particularly important because there simply is not enough myosin to bind to all the available actin in nonmuscle cells (27). It follows that if we know how the actin filaments are arranged in the cell cortex, and also know the patterns of movements in which those filament arrays are involved, we should be able to predict the position of myosin. Once we know the polarity of an actin filament and the way it is moved, the probable location of myosin can be surmised because the polarity of an actin filament defines the direction in which that filament can be moved by an interaction with myosin (19). In this report we have tested this hypothesis, using the only system available in which we know both how the actin filaments are organized and how those filaments redistribute during a movement. This system is the apical cortex of intestinal epithelial cells, the brush border (22–24, 33). It was predicted, on the basis of the polarity of actin filaments in the microvillus and the nature of microvillar contraction observed in vitro in isolated brush borders (22, 23), that microvillar movements were mediated by myosin interactions with the basal ends of microvillar actin filaments in the terminal web. The results presented here demonstrate that two aspects of this prediction are correct: (*a*) the brush border contains myosin; and (*b*) this myosin is located exclusively in the terminal web.

MATERIALS AND METHODS

Brush Border Isolation

We isolated brush borders from the small intestines of chickens by a modification of the procedures of Mooseker and Tilney (24). In brief, the brush borders were prepared from isolated epithelial cells by a simple homogenization procedure. The isolated brush borders were separated from most contaminating cell debris by repeated washing and centrifugation in the following solution referred to as Solution A: 75 mM KCl, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetate (EGTA), 4 mM Na_3N, 0.1 mM dithiothreitol (DTT), and 10 mM imidazole, pH 7.3. Phenylmethyl sulfonyl fluoride (PMSF) was added to all solutions used in these experiments by adding 2 ml/l of a 0.1 M stock in ethanol. A fresh stock solution was prepared immediately before use. Brush borders prepared by the method of Mooseker and Tilney (24) are fairly pure except for contaminating nuclei. The nuclei were removed by centrifugation of the brush borders on a sucrose step gradient. Pellets of isolated brush borders were suspended in 7–10 vol of Solution A containing 55% sucrose (692 g/l). Solution A was added to dilute the sucrose concentration to 35%. With a syringe, the brush border suspension was layered onto a step gradient consisting of 50% sucrose (615 g/l), 45% sucrose (541 g/l), and 40% sucrose (471 g/l). All sucrose solutions were made in Solution A.[1]

The gradients were centrifuged for 2 h at 25,000 rpm in a Beckman SW 27 (113,000 *g*-max) (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The brush borders which collect at the 40–45% and 45–50% interfaces were removed with a syringe, diluted with 10 vol of Solution A, and pelleted by centrifugation at 10,000 *g* for 10 min. It was difficult to remove the brush borders from the gradient without some contamination from the vesicular material that concentrates at the 35–40% interface. This material was removed by washing the brush border preparation several times in Solution A, using low-speed centrifugation (1,000 *g* for 5 min). The yield from the intestines of five chickens is ~30–60 mg of purified brush border protein (2–3 g wet weight). The yield varies considerably, and the major variables seem to be the age, diet, and sex of the birds used. For example, a young, well-fed hen will give a much better

[1] The sucrose solutions were made by adding the dry sugar to double-strength Solution A and adjusting to the final desired volume with water.

preparation than an old, slender rooster. After storage on ice overnight as pellets, the brush borders show no change in morphology by light microscopy nor any degradation of their proteins as detected by gel electrophoresis in sodium dodecyl sulfate.

For antibody staining experiments, brush borders were isolated from the small intestines of rabbit, chicken and mouse, and from the spiral intestines of dogfish by the procedures of Moosker and Tilney (24) and were used without further purification. Brush border preparations to be used for antibody experiments were stored at −20°C as a suspension in 50% glycerol in Solution A.

Purification of Brush Border Myosin

Pellets of purified brush borders were suspended in Solution A containing 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) and collected by centrifugation (1500 *g* for 5 min). This washing procedure was repeated three times to insure complete removal of the brush border membrane (24). The detergent washes were followed by three washes in Solution A. The demembranated brush borders were homogenized for 15–30 s with a Teflon pestle homogenizer driven with a low-speed electric motor in 3–5 vol of KI buffer (0.6 M KI, 5 mM ATP, 1 mM MgCl, 1 mM DTT, 0.1 mM PMSF, and 30 mM imidazole buffer, pH 7.3). The homogenate was centrifuged at 35,000 rpm in a Beckman 42.1 rotor at 4°C for 30 min. The supernate applied immediately to a 2.5 × 50 cm column of 4% agarose (A 15 m [200–400 mesh], Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.6 M KCl, 1 mM ATP, 1 mM DTT, and 10 mM imidazole, pH 7.2. A 10-ml front of KI buffer was added to the column just ahead of the brush border sample (31). Fractions containing myosin were identified by ATPase assay. Final purification and concentration of the myosin was achieved by dialysis of the column fractions containing myosin against a low ionic strength buffer (10 mM imidazole, and 0.1 mM DTT, pH 7.0) and collection of the precipitated myosin by centrifugation at 5,000 *g* for 10 min. The purified myosin was stored as pellets, on ice, until use.

Biochemical Methods

Protein concentrations were estimated by the method of Hartree (17), using bovine serum albumin as a standard. ATPase activity was measured at 37°C (30). Phosphate production was proportional to time and enzyme concentration. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out with Tris glycine buffer (38). Gels were stained with Coomassie Brilliant Blue R by the methods of Fairbanks et al. (14). Stained gels were scanned with a Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio), at a wavelength of 550 nm. Scans were made of gels in which the most intense bands had an optical densitivity of <1.2. The areas under protein peaks were measured by cutting and weighing.

Electron Microscopy

The formation of myosin filaments was induced by brief dialysis (1 h) of column fractions containing myosin against 10 mM imidazole, pH 7.0, with and without 1.0 mM $MgCl_2$. Judging from its conductivity, the concentration of KCl in the dialysis bag after 1 h was ~10 mM. Drops of the dialyzed fraction (0.05–0.1 mg/ml) were negatively stained with 0.5% uranyl acetate on Formvar-carbon-coated grids. Platinum-shadowed replicas of individual molecules were made in a Balzers freeze-fracture machine (Balzers High Vacuum Corp., Santa Ana, Calif.).[2] Electron micrographs were made with a Siemens Elmiskop 101.

Staining of Brush Borders with Fluorescent Antimyosin

Myosin localization studies were conducted with tetramethylrhodamine-labeled immune IgG from rabbits immunized with the rod fragment of human platelet myosin. The preparation and characterization of this labeled antibody is described in detail elsewhere (15). Cross reactivity of this antibody with chicken brush border myosin was determined by double diffusion in 0.5% agarose buffered with 20 mm sodium pyrophosphate, using as test antigens the KI extract of brush borders described above and purified brush border myosin.

Antibody staining of brush borders from mouse, chicken, rabbit, and dogfish was performed as follows: Pellets of glycerinated brush borders (see above) were washed once with Solution A containing 0.5% Triton X-100, fixed for 10–15 min with 0.4% formalin in Solution A, and washed four–six times with Solution A to remove the fixative. Before the final wash, the brush border preparations were divided among 15 ml conical centrifuge tubes so that the final pellets had volumes of ~10 μl. For antibody staining, these small pellets were suspended in 25 μl of rhodamine-antimyosin (0.3 mg/ml) diluted with either 75 μl of pre-immune serum (20 mg/ml) or 75 μl of Solution A. As controls, pellets were suspended in either 25 μl of rhodamine-antimyosin diluted with 75 μl of unlabeled antimyosin or 25 μl of rhodamine-labeled preimmune IgG (0.3 mg/ml) diluted with 75 μl of Solution A. The suspended pellets were incubated for 45–60 min on ice, washed five times with Solution A, and observed with a Leitz Orthoplan microscope equipped for visualization of rhodamine fluorescence (15). Fluorescence and phase contrast light micrographs were made on Tri-X film developed in Microdol X developer (Eastman Kodak Co., Rochester, N. Y.).

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[2] Pollard, T. D., W. F. Stafford, and M. E. Porter. 1978. Characterization of a second form of myosin from *Acanthamoeba castellanii*. *J. Biol. Chem.* **253**:4798–4808.

RESULTS

Purity of Brush Border Preparations

The preparations of intestinal brush borders used for myosin purification are free of contaminants visible in the light microscope (Fig. 1). It seems unlikely that the myosin described here comes from other cells such as smooth muscle from the lamina propria.

Purification of Brush Border Myosin

Myosin is purified from demembranated brush borders by three steps: solubilization in KI, gel permeation chromatography on a 4% agarose column, and low ionic strength precipitation (31). ~90% of the brush border contractile proteins are solubilized in 0.6 M KI. Since both the myosin and actin are depolymerized in the extraction buffer, they can be separated by gel permeation chromatography (Fig. 2). A discontinuous buffer system is used for chromatography to separate rapidly the myosin from the KI which has deleterious effects on the enzymatic properties of myosin. The myosin heavy and light chains elute from the column together with the K^+-EDTA ATPase activity, which is characteristic of myosins (Figs. 2 and 3). At this stage the myosin is free of actin and is 50–70% pure, judging by gel electrophoresis. The major contaminants are several polypeptides with molecular weights greater than that of the heavy chain of myosin. These contaminants elute along with some Ca^{++}-Mg^{++} ATPase activity which is ahead of, but usually overlapping, the myosin peak (Fig. 2). Precipitation of the myosin by low ionic strength dialysis improves the purity to 80–90% by removing most of the high molecular weight contaminants and also doubles the K^+-EDTA ATPase specific activity while removing the contaminating Mg^{++} ATPase activity (Fig. 3, Table I). After precipitation, the main contaminants of the myosin preparations are proteins with subunit molecular weights below that of the myosin heavy chain (150,000, 130,000, and 70,000). Most of these "contaminants" may be proteolytic fragments of the myosin heavy chain because their proportion increases as the preparation ages. The yield of purified myosin is 0.5–1.0 mg per 20–30 mg of starting brush border protein. If proteolysis is not controlled by including PMSF in all solutions, the yield and enzymatic activity are lower and, of course, the amount of intact myosin is reduced. Even with the inclusion of PMSF in all solutions, proteolysis remains a problem. After a few days of storage as high-salt solutions, myosin preparations are almost completely degraded into head and tail fragments as determined both by electron microscope examination of platinum-shadowed replicas of the myosin molecules in these solutions and by gel electrophoresis in sodium dodecyl sulfate.

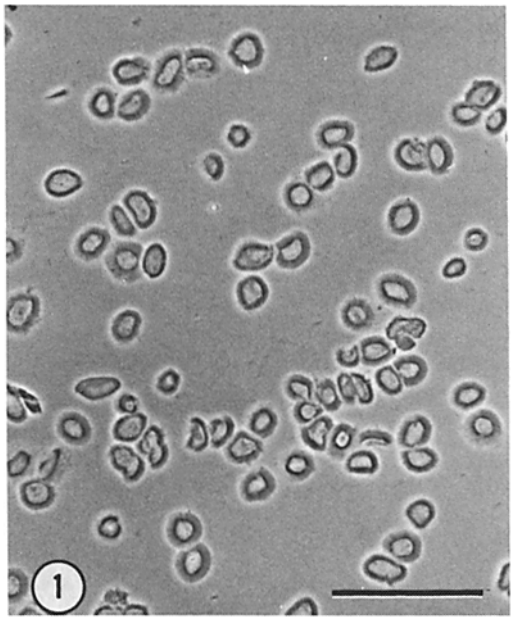

FIGURE 1 Phase-contrast light micrograph of purified chicken brush borders. Bar, 40 μm. × 500.

Properties of Brush Border Myosin

Brush border myosin is similar to other vertebrate, nonmuscle myosins with respect to its subunit composition, its size and shape, and the filaments it forms (3, 9, 32). Brush border myosin consists of three polypeptide chains with molecular weights of 200,000, 19,000, and 16,000 daltons in a molar ratio of approx. 1:1:1 (Fig. 3). The molecule has a Stokes' radius of 17.5 nm like skeletal muscle myosin and all other vertebrate, nonmuscle myosins studied.[3] We have obtained electron micrographs of a few brush border myosin molecules. They appear to have two globular heads attached to a slender tail ~130 nm

[3] A figure for the myosin Stokes's radius of 19.2 nm has frequently been quoted in the literature (1), but is not correct. The value of 17.5 nm was calculated from the accepted intrinsic sedimentation coefficient and molecular weight and agrees with new experimental data (see footnote 2).

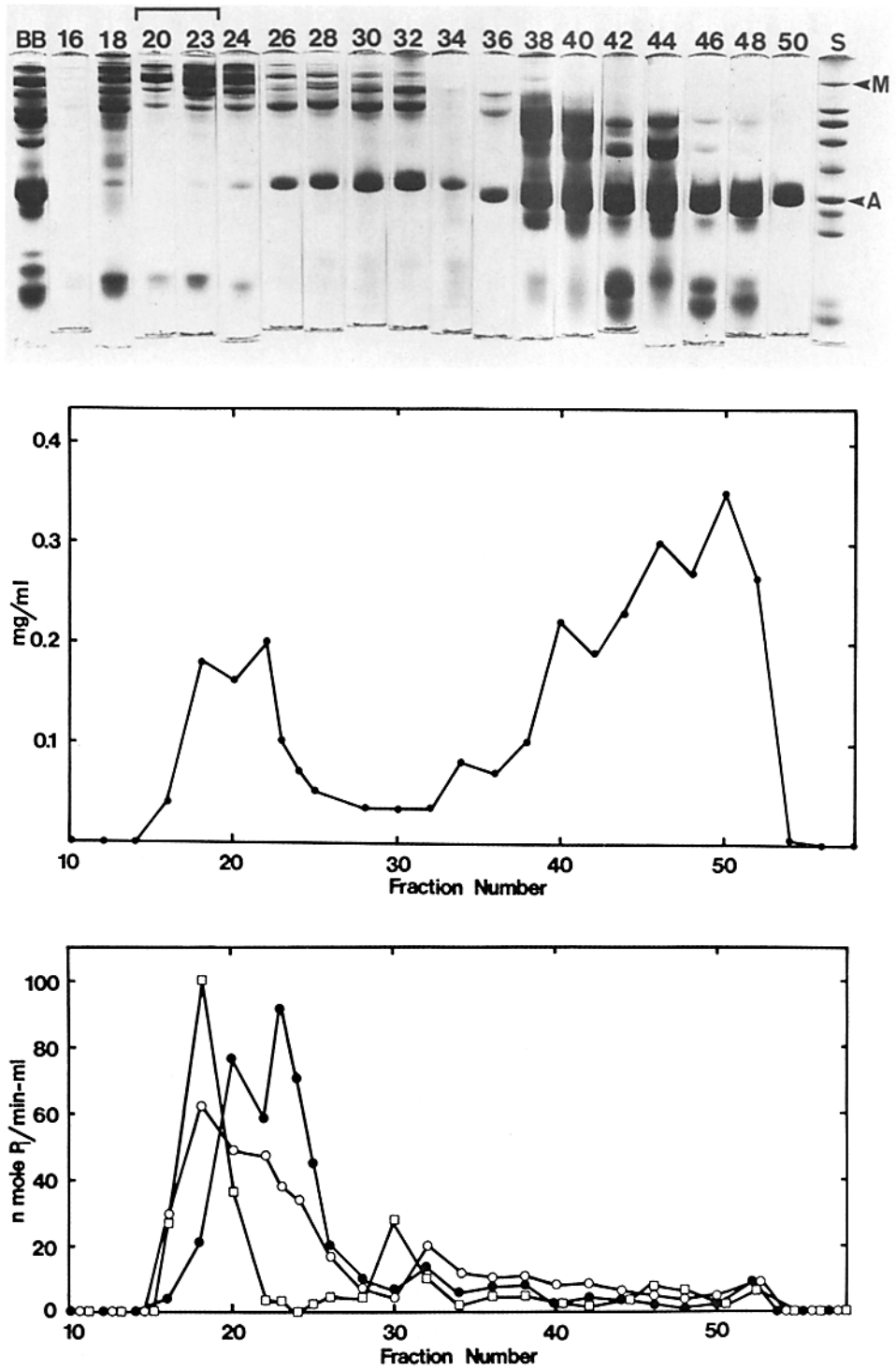

FIGURE 2 Fractionation of brush border proteins solubilized in 0.6 M KI by gel permeation chromatography on 4% agarose. (*Top*) 7.5% Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of selected fractions. The column sample (*BB*) is on the left. The mobilities of the myosin heavy chain (*M*) and actin (*A*) are indicated on the standard gel on the right, which also includes phosphorylase A (95,000), serum albumin (68,000), catalase (60,000), immunoglobulin G (50,000), aldolase (40,000), carbonic anhydrase (29,000), ribonuclease (13,500), and cytochrome *c* (11,200). (*Middle*) Protein concentration of each fraction. (*Bottom*) K^+-EDTA ATPase (●—●). Ca^{++} ATPase (○—○). Mg^{++} ATPase (□—□). Brush border myosin K^+-EDTA ATPase elutes in fractions 20–24, just behind a peak of Mg^{++} ATPase in the void volume.

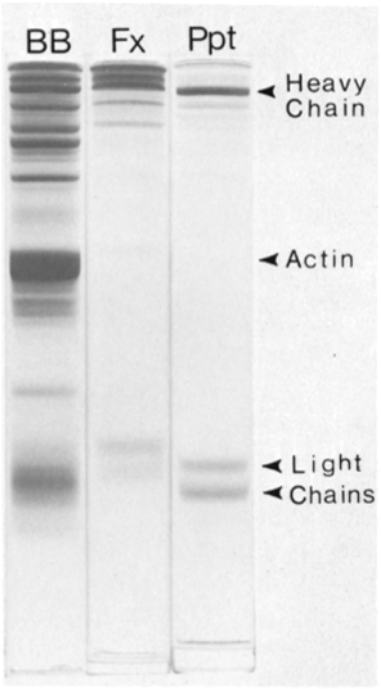

FIGURE 3 Final purification of brush border myosin. Analysis by 10% polyacrylamide gel electrophoresis in sodium dodecyl sulfate. *BB*: KI solubilized brush border proteins. *Fx*: myosin peak from the agarose column. *Ppt*: purified brush border myosin obtained by low ionic strength precipitation of the agarose fraction.

TABLE I

Purification of BB Myosin ATPase Activity

	EDTA	Ca^{++}	Mg^{++}
BB extract	0.04	0.06	0.06
fraction no. 20	0.50	0.31	0.23
low salt precipitate	1.2	0.43	0.01

Units: μmol P_i/mg-min at 37°C; all assays in 0.6 M KCl.

long. Dialysis of brush border myosin into solutions of low ionic strength induces the formation of small, bipolar filaments (Fig. 4*a*, *b*, and *c*) with dimensions similar to those of the myosinlike filaments observed in the terminal web of isolated brush borders (24). Most of the synthetic myosin filaments formed are ~300 nm long and have a central bare zone ~170 nm long and 11 nm wide. We have observed some variability in the dimensions of filaments formed in several preparations (Fig. 4*b*). Because proteolysis is so difficult to control, this variability is probably because of the presence of myosin tail fragments. Like other synthetic nonmuscle myosin filaments, the brush border myosin filaments have globular material, presumably the myosin heads, projecting from the backbone at both ends. Although we have not conducted an extensive study of the effects of ionic conditions on filament formation, we do observe that the addition of 1 mM $MgCl_2$ to the low ionic strength dialysis buffer causes considerable head-to-head aggregation of the myosin filaments (Fig. 4*d*). Similar aggregation occurs if the dialysis is allowed to proceed to equilibrium, in which the final KCl concentration in the dialysis bag is less than 1 mM.

The influence of ionic conditions on the ATPase activity of brush border myosin is summarized in Table II. Like that of most other myosins, the ATPase activity in 0.6 M KCl is highest in the presence of EDTA, intermediate in Ca^{++}, and very low in Mg^{++}. The "EDTA" ATPase activity is actually K^+ ATPase activity, as shown by its dependence upon the K^+ concentration and the inability of Na^+ to substitute for K^+. We are unable to demonstrate any activation of the low ionic strength Mg^{++} ATPase by muscle actin.

Given the similarities between brush border myosin and the other nonmuscle myosins enumerated above, it is not surprising to find that it cross-reacts with antibodies against human platelet myosin (Fig. 5). Among the many proteins in the brush border extract, only the myosin forms a strong precipitin line with this antiserum, indicating that these antibodies can be used as a reliable probe for the localization work described in the next section.

Localization of Brush Border Myosin with Fluorescent Antibody

Fluorescent antimyosin stains the terminal web region of brush borders isolated from either chicken or mouse intestine (Fig. 6). No staining of the microvilli is detected. This staining is presumably indicative of the location of myosin, because (*a*) the staining can be blocked by incubation of the brush borders in excess unlabeled antibody, (*b*) rhodamine labeled pre-immune IgG does not stain the brush border, and (*c*) brush borders isolated from rabbit intestine do not stain with rhodamine-antimyosin (Fig. 7). The rabbit is the species which produced the antimyosin. We have attempted without success to stain brush borders from the spiral intestine of the dogfish (Fig. 7). Although these brush borders contain a myosin-

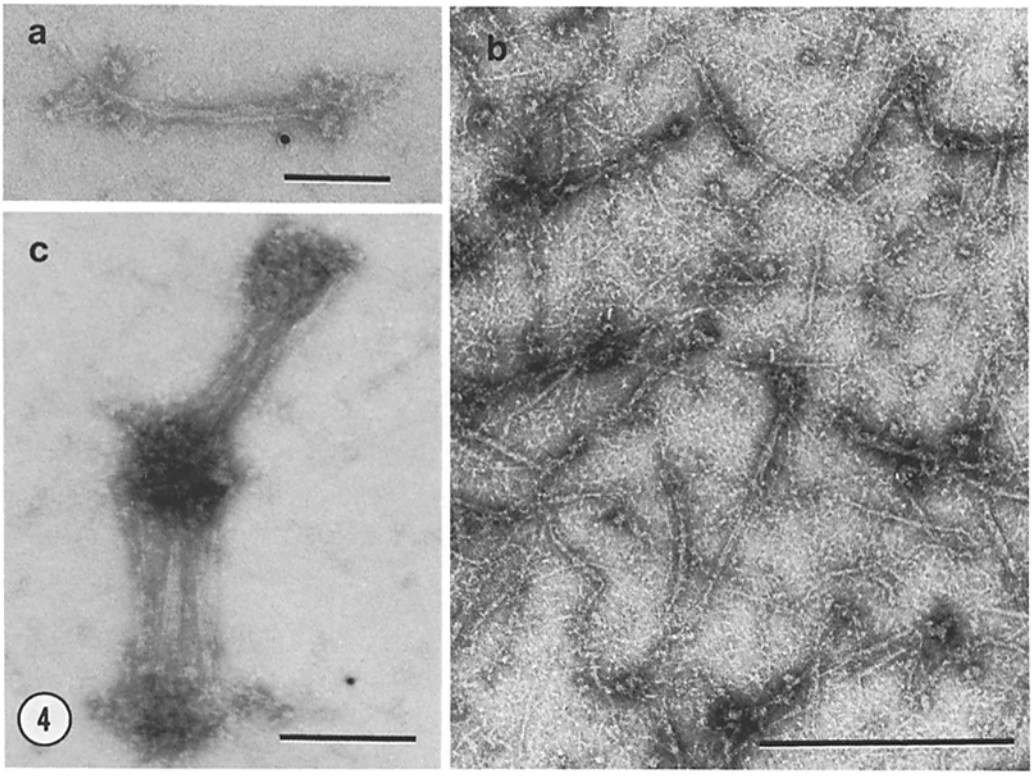

FIGURE 4 Electron micrographs of negatively stained brush border myosin filaments. (*a* and *b*) Myosin filaments formed by dialysis to 10 mM KCl at pH 7.1 in 10 mM imidazole buffer. (*c*) Side-to-side and head-to-head aggregation of myosin filaments in 1 mM $MgCl_2$, 10 mM imidazole, pH 7. (*a*) Bar, 1 μm; × 140,000. (*b*) Bar, 0.5 μm; × 70,000. (*c*) Bar, 0.2 μm; × 90,000.

TABLE II

ATPase Activity of Brush Border Myosin

	0.6 M KCl	0.06 M KCl	0.6 M NaCl
EDTA	1.0	0.5	0.01
Ca^{++}	0.4	0.08	0.5
Mg^{++}	0.01	0.01	0.02

Units: μmol P_i/mg-min at 37°C. Actin does not activate the Mg ATPase activity of BB myosin.

like ATPase,[4] it may have failed to cross-react with our antibody because of the evolutionary distance between humans and dogfish. The absence of staining of the microvilli is not a result of difficulty in antibody penetration because the microvillar membrane has been removed. Furthermore, both fluorescent antiactin and fluorescent heavy meromyosin stain the microvilli of isolated, demembranated brush borders.[5]

[4] Mooseker. Unpublished observation.

[5] Herman and Pollard. Unpublished observation.

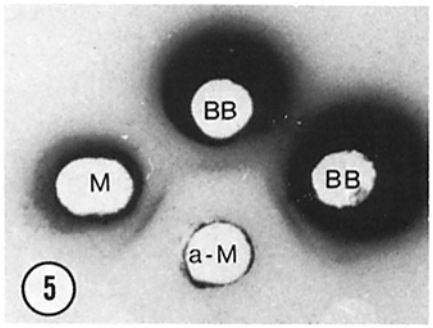

FIGURE 5 Double immunodiffusion reaction of anti-human platelet myosin (*a - M*; rabbit no. 8 from reference 15) against purified chicken brush border myosin (*M*; 0.5 mg/ml) and KI solubilized extract of brush borders (*BB*) (13 mg/ml).

DISCUSSION

Our results establish that myosin is a constituent of the intestinal brush border and confirm previous electrophoretic, enzymatic, and electron microscopic evidence for brush border myosin (22-

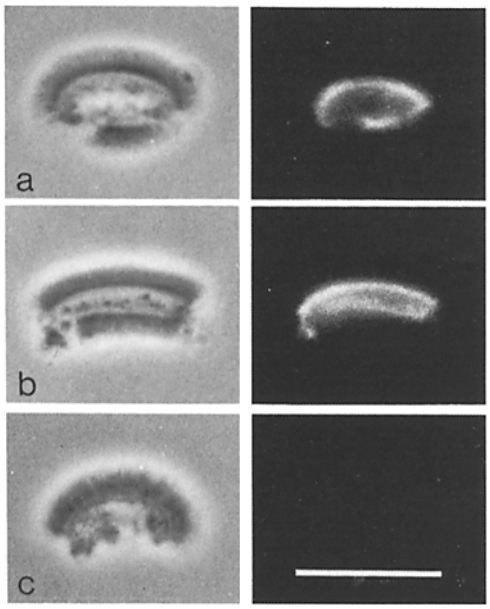

FIGURE 6 Isolated demembranated murine brush borders stained with rhodamine-antiplatelet myosin (rabbit no. 8 from reference 15; 0.3 mg/ml; 4–5 rhodamines per IgG). (*a* and *b*) Pairs of phase-contrast (*left*) and fluorescence (*right*) micrographs of the same stained brush borders. (*c*) A pair of micrographs of a brush border stained with rhodamine-antimyosin in the presence of excess unlabeled antiserum. Bar, 10 μm. × 2,000.

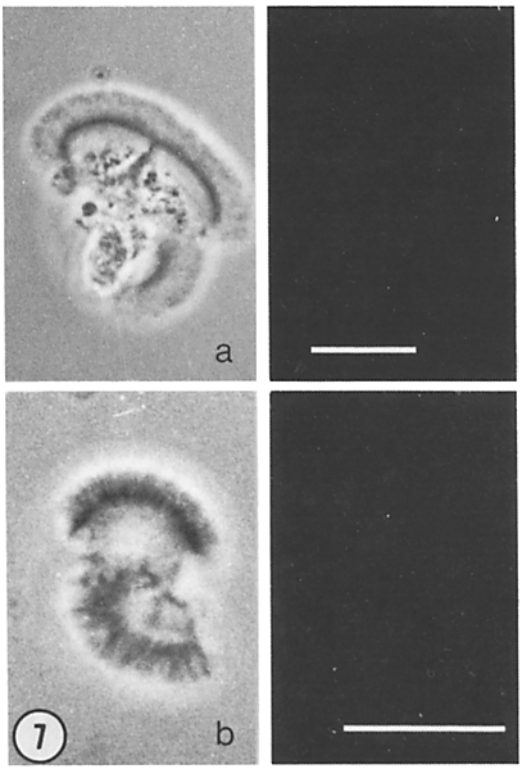

FIGURE 7 Isolated, demembranated brush borders from rabbit (*a*) and dogfish (*b*) stained with rhodamine-antiplatelet myosin. Pairs of phase-contrast (*left*) and fluorescence (*right*) micrographs. (*a*) Bar, 10 μm; × 1,400. (*b*) Bar, 10 μm; × 2,000.

24, 42). The characterization of brush border myosin uncovered no surprises. The physical and enzymatic properties of the purified myosin are similar to those of other vertebrate, nonmuscle myosins, including the lack of activation of the MgATPase by actin. If the intestinal epithelium is similar to other cells which require phosphorylation of a myosin light chain for actin-myosin ATPase activity (2, 10, 12, 16, 35, 36), the light chain kinase must either be lost during isolation of the brush borders or overwhelmed by phosphatases. Furthermore, we have been unable to detect the presence of any kinase activity in isolated brush border preparations.[6]

The main significance of our work on brush border myosin does not lie in the fact that we have characterized yet another nonmuscle myosin, but, rather, in the insight we gain from its presence and location in the brush border with respect to the molecular architecture of the cell cortex. The brush border is, of course, a poor source of myosin for detailed biochemical analysis, but it is the ideal system to test the hypothesis that actin and myosin in the cortex of nonmuscle cells are arranged in configurations functionally homologous to their organization in muscle. Thus, having ascertained the presence of myosin by independent biochemical criteria, we used antibodies to ask the more significant question: Where is myosin located in the brush border? As predicted (22–24, 33), myosin is confined to the terminal web, confirming that the brush border contractile proteins are arranged in the shape of a folded sarcomere with the tip of the microvillus analogous to the Z-line, the actin bundle within the microvillus analogous to the I-band, and the terminal web analogous to the A-band. The contractile interaction of the actin filaments with the myosin in the terminal web can explain how microvilli might shorten or wave from side to side. We have not examined whole epithelial cells, but we expect that myosin will be found in other parts of these cells in addition to the terminal web.

[6] Mooseker. Unpublished observations.

There is now enough evidence from other cells to indicate that it is possible to extrapolate a general model of the cell cortex from the arrangement of actin and myosin in the brush border. On one hand, there are several examples of polar attachments of actin filaments to the plasma membrane (5, 8, 13, 21, 23–25, 32, 40). On the other hand, antibody localization has shown that myosin is found at the base rather than within actin-containing cell surface extensions such as tissue culture cell filopodia or lamellipodia (11, 15, 18) and platelet microspikes (29). In these cases, where the molecular architecture seems to be similar to that of the brush border, a contractile mechanism can easily explain how the surface extensions are withdrawn or wiggled from side to side. Likewise, a contractile mechanism explains the constriction of the cell surface during cytokinesis where the actin (26, 34) and myosin (15) are found together in the cortex.

Although a folded sarcomere in the cortex can explain the contractions discussed above, it cannot explain all cell surface movements. For example, the active protrusion of an actin-containing cellular process from the cell body, such as the extension (7, 22–24) or formation (41) of brush border microvilli, must involve some other mechanism.

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