ORGANIZATION OF AN ACTIN FILAMENT-MEMBRANE COMPLEX

Filament Polarity and Membrane Attachment in the Microvilli of Intestinal Epithelial Cells

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ABSTRACT

The association of actin filaments with membranes is now recognized as an important parameter in the motility of nonmuscle cells. We have investigated the organization of one of the most extensive and highly ordered actin filament-membrane complexes in nature, the brush border of intestinal epithelial cells. Through the analysis of isolated, demembranated brush borders decorated with the myosin subfragment, S_1, we have determined that all the microvillar actin filaments have the same polarity. The S_1 arrowhead complexes point away from the site of attachment of actin filaments at the apical tip of the microvillar membrane. In addition to the end-on attachment of actin filaments at the tip of the microvillus, these filaments are also connected to the plasma membrane all along their lengths by periodic (33 nm) cross bridges. These bridges were best observed in isolated brush borders incubated in high concentrations of Mg^{++}. Their visibility is attributed to the induction of actin paracrystals in the filament bundles of the microvilli. Finally, we present evidence for the presence of myosinlike filaments in the terminal web region of the brush border. A model for the functional organization of actin and myosin in the brush border is presented.

Actin has now been identified as a major component of eucaryotic cells. Myosin seems to be present in many of these cells as well (see ref. 44 for a recent review of actomyosin-mediated motility in nonmuscle cells). Unlike the situation in skeletal muscle, however, there is very little known about the organization and function of these proteins in nonmuscle cells.

Most investigators assume that the mechanochemical basis for motility associated with actin and myosin is similar to, or at least includes, that established for skeletal muscle. If so, actin and/or myosin must be anchored for the generation of force. It is no surprise, then, that most recent models of motility mediated by actin and myosin are essentially extensions of the sliding filament model for muscle contraction in which membrane replaces the Z band as the anchorage site for actin filaments (25, 42, 44, 52). [Bray's recent model for motility in the nerve growth cone is less specific and predicts a membrane attachment of either actin or myosin (7).]

These models are based on the observation that in many systems actin filaments are associated with membranes at sites of active motility. In these systems, observed filaments have been identified as

actin by at least the criterion of heavy meromyosin (HMM) or myosin subfragment 1 (S_1) binding. Examples of motility associated with actin filaments and membrane include cytokinesis in amphibian eggs (40) and in HeLa cells (50), amoeboid movement (13, 42, 43), chloroplast streaming in *Nitella* (28, 38), motility of cultured fibroblasts (9, 11, 26, 32, 39, 61) and cultured nerve cells (31), the motility and assembly of microvilli in the brush border (26, 33, 58, 60), the generation of the acrosomal process in echinoderm sperm (59) and in *Limulus* sperm (57), morphogenetic movements of embryonic epithelia (51), platelet contraction (63, 64), and endocytosis in macrophages (2–4, 45). Among the above examples there are only two in which the direct attachment of actin filaments to membranes has been observed—in *Acanthamoeba* and in the brush border of intestinal epithelial cells (6, 8, 34, 37, 58).

There is also biochemical evidence for the association of actin with membranes. For example, actin has been shown to be a constituent protein of synaptosome fractions isolated from mammalian brain (5) and of plasma membrane fractions isolated from *Dictyostelium* amoebae (52) and tissue culture cells (19, 20). From this evidence, however, the association of actin with membranes cannot be defined. For example, the actin may be attached to the membrane in a filamentous state, in a nonfilamentous state (55, 56), or may in fact be an integral part of the membrane itself.

Some critical questions about the interaction of actin filaments with membranes include the following: how is the attachment of actin filaments to membrane effected? Where along the length of an actin filament can attachment to membranes occur? What is the polarity of the actin filaments relative to the membrane? The answer to this last question is particularly significant as it should provide clues into the mechanochemical basis for motility, since in skeletal muscle, the interaction of actin and myosin to produce force is a polarized process (24). For example, the sliding filament models mentioned above for actomyosin-mediated motility in nonmuscle cells (25, 42, 44, 52) carry the assumption that actin filaments are attached to membrane with the same polarity as are the actin filaments attached to the Z line, i.e., arrowhead complexes should point away from the membrane. In the two nonmuscle systems in which direct attachment of actin filaments to membrane has been observed (the brush border and *Acanthamoeba*), suggestive evidence from *in situ* HMM-binding studies (26, 44, 60) supports this assumption.

We have sought answers to these questions through the investigation of the brush border of intestinal epithelial cells. The brush border is ideal for such an investigation because, unlike most nonmuscle systems where the contractile apparatus is loosely organized and/or labile, the brush border is a highly ordered, easily isolated and stable structure, allowing detailed analysis of membrane-actin filament interaction. The results presented here provide an unequivocal determination of the polarity of actin filaments with respect to membrane. In addition, we will present evidence which extends earlier observations (34) that microvillar actin filaments are associated with the plasma membrane not only at their ends, but all along their lengths. These observations, coupled with the recent characterization of the brush border as a discrete, Ca^{++} regulated contractile apparatus (33) allow us to present a molecular model for the involvement of actin and myosin in microvillar movement.

MATERIALS AND METHODS

(All procedures described below were carried out at 0–4°C.)

Brush Border Isolation

Epithelial cells from the chicken intestine were isolated by a method modified from Evans et al. (14). The small intestines from one-two chickens were each cut into four segments and then flushed with cold 0.15 M NaCl containing 0.02% sodium azide. The segments were filled sausage-fashion with 0.2 M sucrose, 0.076 M Na_2HPO_4, 0.019 M KH_2PO_4, 0.012 M ethylene diaminetetraacetate (EDTA), and 0.1 mg/ml soybean trypsin inhibitor (SBTI, Sigma Chemical Co., St. Louis, Mo.). String was used to tie off the ends. The segments were then placed in cold saline and incubated for 10–15 min. Each segment was gently rubbed along its length between a thumb and two fingers to help free the epithelial cells. The contents of the segments were collected and each segment was washed with an additional 50 ml of the above cold sucrose solution. These washes were added to the suspension of cells already obtained. The cells were pelleted at 400 *g* for 5 min. Brush borders were isolated from the epithelial cells by a method modified from Forstner et al. (16). The cells were suspended in 100 ml of 4 mM EDTA, 1 mM ethylene glycol-bis-*N*,*N*′-tetraacetate (EGTA), 10 mM imidazole buffer at pH 7.3. 10 mM Tosyl arginine methyl ester (TAME, Sigma), and 0.1 mg/ml SBTI were added to the above solution to inhibit proteolysis. The suspension was homogenized for 10–15 s in an Omni-Mixer (Dupont Instruments, Sorvall Opera-

tions, Newtown, Conn.) at setting 7. Isolated brush borders were collected from this homogenate by centrifugation at 800 *g* for 5 min. The isolated brush borders were resuspended in 50 ml of solution A: 60 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM TAME, 10 mM imidazole buffer at pH 7.3. Centrifugation and resuspension in solution A were repeated several times.

Preparation of Demembranated Brush Borders

Membranes were removed by repeated washing (two–four times) in solution A which contained 1% Triton X-100 (a nonionic detergent, Sigma) and collected by centrifugation at 1,000 *g* for 10 min. We often added extra Mg^{++} (5–10 mM) to this solution to enhance the stability of the demembranated brush borders.

Preparation of Myosin Subfragment 1 (S_1)

Subfragment 1 was prepared from chicken breast muscle by the method of Lowey et al. using insoluble papain (29). S_1 was stored at −20°C in 50% glycerol buffered with 10 mM phosphate buffer at pH 7.0. Before use the S_1 solutions were dialysed against solution A for 2 h to remove the glycerol; the protein was then concentrated by ammonium sulfate precipitation (60% saturation; Schwartz-Mann, Div. Becton, Dickenson & Co., Orangeburg, N. Y.). The S_1 precipitate was collected by centrifugation (10,000 *g* for 10 min) and dissolved in a volume of solution A to achieve a final S_1 concentration of about 10 mg/ml. The protein concentration was determined by the method of Lowry et al. (30). The solution was then dialysed against solution A to remove residual ammonium sulfate.

S_1 Binding In Situ

Pellets of isolated, demembranated brush borders were suspended in 5 vol of the concentrated S_1 solution. This suspension was dialysed against solution A for 4–6 h. Brush borders were then collected by centrifugation (1,000 *g* for 10 min) and immediately processed for electron microscopy.

Incubation of the Isolated Brush Borders in High Concentrations of Mg^{++}

Isolated brush borders, either having intact membranes or demembranated with Triton X-100, were suspended in 10 vol of solution A plus additional Mg^{++} (10–40 mM) and dialysed against the same solution for 30 min to 1 h. The Mg^{++}-incubated brush borders were then collected by centrifugation and prepared for electron microscopy.

Electron Microscopy

Brush border preparations in the early stages of this investigation were fixed in 2% glutaraldehyde (Electron Microscopy Services, Fort Washington, Pa.) in 0.1 M cacodylate buffer at pH 7.3 for 1 h and postfixed with 1% OsO_4 in 0.1 M cacodylate buffer at pH 7.3 for 1 h. Later preparations were fixed in 1% glutaraldehyde, 0.1 M phosphate buffer at pH 7.0 for 30 min and postfixed in 1% OsO_4, in 0.1 M phosphate buffer at pH 6.0 for 45 min (17). This latter procedure is preferable for the preservation of actin filaments. The preparations were then washed two–three times with distilled water, stained *en bloc* with 0.5% uranyl acetate (aqueous) for 2–4 h and rapidly dehydrated with acetone and embedded in Araldite. Thin sections were cut with a diamond knife on a Sorvall Porter-Blum I or II ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with a Philips 200 electron microscope. The microscope was calibrated using a replica grating (Ernest Fullham, Inc., Schenectady, N. Y.).

Freeze-Etch Techniques

The isolated brush borders were fixed for 10–30 min in 1% glutaraldehyde, 0.1 M phosphate buffer at pH 7.0. They were then transferred through a graded series of glycerol concentrations of 10, 20, and 30% for 20 min each. They were concentrated by centrifugation. All operations were carried out at 4°C. The resultant pellets of brush borders in 30% glycerol were transferred to specimen holders (Denton Vacuum Company, Cherry Hill, N. J.). They were rapidly frozen in Freon 22 cooled with liquid N_2. The specimens were fractured in a Denton freeze-fracture apparatus at −115°C and etched for 1 min at −100°C.

RESULTS

Morphology of the Isolated Brush Border

There are numerous reports which describe the morphology of the brush border of absorptive epithelial cells, both in intact tissue and as an isolated organelle (6, 8, 10, 27, 34, 36, 37, 46, 47, 60). We would like to review here the ultrastructural features of the brush border which are relevant to an analysis of this organelle as a membrane-associated contractile apparatus.

It is clear from Figs. 1 and 2 that the structural integrity of the brush border is maintained in the isolated state. The microvilli of chicken intestinal epithelial cells are usually 1–2 μm in length and 0.1 μm in diameter. Each microvillus contains a bundle of 20–30 actin filaments (Fig. 2); this bundle is usually 50–60 nm in diameter. It is attached to the inner surface of the plasma membrane at the tip of the microvillus in a matrix of

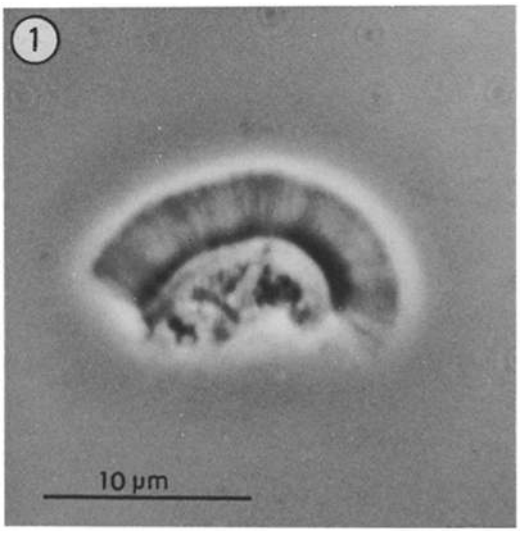

FIGURE 1 Brush border isolated from chicken intestinal epithelium. Phase-contrast light micrograph. × 2,700.

densely staining material. This material is composed at least in part of the Z-band protein, α-actinin, as identified by *in situ* staining with antibody to this protein (49). The filament bundle extends the entire length of the microvillus and continues below it into the terminal web region (Fig. 2). The main structural component of the terminal web is the basal ends of the microvillar filament bundles. There are also numerous filaments between the actin filament bundles; however, the number and appearance of these filaments vary in different preparations. One variable in this regard is the presence or absence of Mg^{++} in the brush border preparation before fixation. The brush border in Fig. 2 was prepared in the absence of Mg^{++}. Many of the interstitial filaments are actin filaments which have splayed off from the microvillar filament bundles (Fig. 2). The terminal web also contains 10-nm filaments (tonofilaments), although it is difficult to distinguish between thin (actin) and intermediate (10 nm) filaments in this micrograph. The terminal web region of brush borders prepared in the presence of Mg^{++} (5–10 mM) contains a third class of filaments (Fig. 3); these filaments are shorter and thicker than actin filaments (11 nm × 150–200 nm). The opposite ends of these filaments often connect adjacent microvillar filament bundles. The dimensions of these filaments are comparable to those reported for filaments formed in vitro from myosins isolated from a variety of nonmuscle sources (44), e.g. fibroblast myosin, 10 nm × 250 nm (1) and platelet myosin, 11 nm × 320 nm (35). The morphology of these filaments and their association with the actin filament bundles suggests to us that they may be comprised of brush border myosin.

Morphology of the Demembranated Brush Border

In order to determine the polarity of actin filaments in the brush border through *in situ* S_1 decoration, it is first necessary to disrupt the brush border membrane. Earlier work using Ishikawa's technique for *in situ* HMM decoration (26) relied on glycerol for this purpose (26, 60). Unfortunately, glycerol treatment has a deleterious effect on the structural integrity of the filamentous superstructure underlying the brush border membrane. In these preparations, the apical ends of most microvillar filament cores were lost; only their basal ends in the terminal web remained intact. As a result, reliable determination of polarity for all the actin filaments in the brush border was impossible. Furthermore, the direction of the arrowheads was extremely difficult to discern. By using the detergent, Triton X-100, to remove the brush border membrane, rather than glycerol, the filamentous superstructure remains essentially intact (see Fig. 4). The fact that the brush border does not fall apart as the result of membrane removal indicates that this structure is held together by components within the terminal web and within microvillar filament bundles, rather than by its attachment to the brush border membrane. High concentrations of Mg^{++} (5–10 mM) increase the stability of isolated brush borders after membrane removal.

Decoration of Actin Filaments in the Demembranated Brush Border

Figs. 5–7 are electron micrographs of demembranated brush borders incubated with S_1. The direction of arrowheads is easily determined on all the filaments which are in good longitudinal section and which are not too close to neighboring actin filaments. All such filaments in the micrographs shown here, and in the hundreds we have examined, have the same polarity; the arrowhead complexes point in a basal direction toward the terminal web. These results confirm the suggestion made from earlier work that the actin filaments

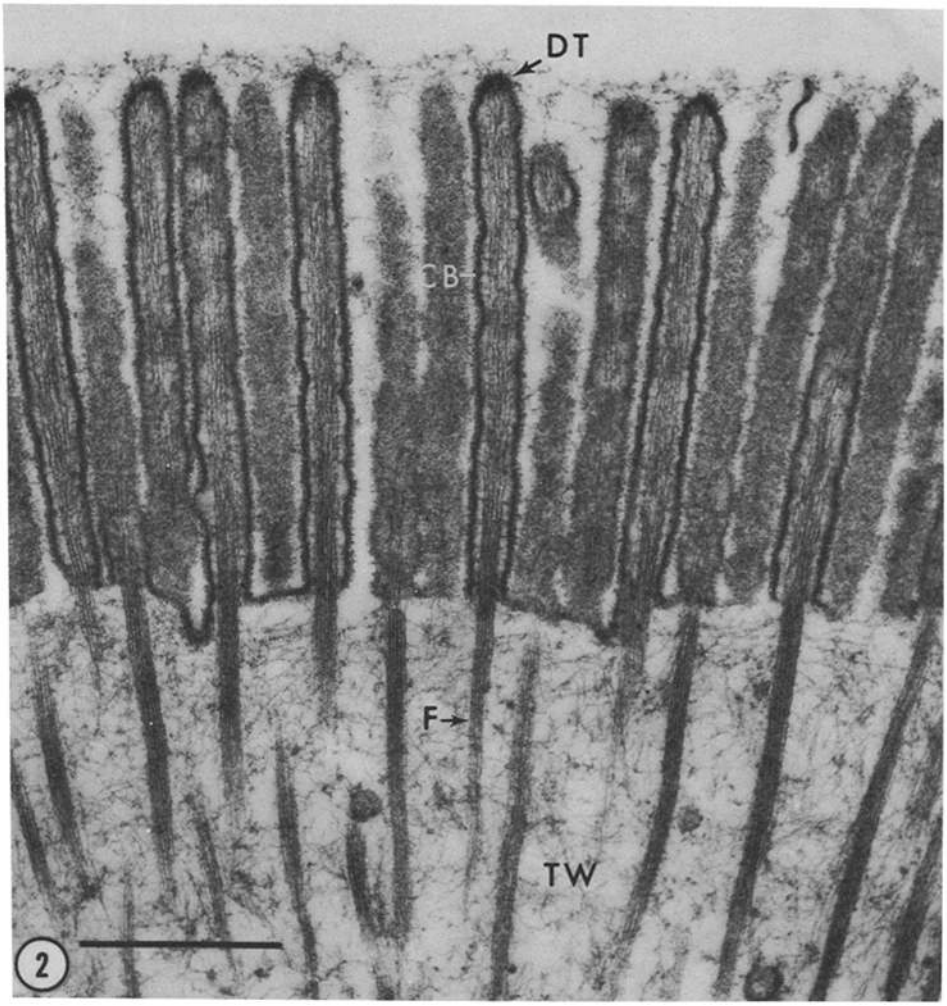

FIGURE 2 Isolated brush border. Each microvillus contains a core of actin filaments (F) which extends below the plasma membrane into the terminal web (TW) region. The apical tip of each filament core is embedded in a dense matrix (DT) which effects the attachment of the core to the plasma membrane. Cross bridges (CB) connecting the filament core laterally to the membrane are also detectable. Bar, 0.5 μm. × 52,000.

within a microvillus have the same polarity. As one would expect, the arrowhead complexes occur periodically along the length of the actin filaments. The periodicity measured from longitudinally sectioned filaments averages only 30 nm (the periodicity in negatively stained preparations is about 37 nm) indicating that rather severe shrinkage may occur during specimen preparation.

A critical factor in the visualization of the arrowheads on actin filaments is the use of high concentrations of S_1. At concentrations of S_1 above 5–6 mg/ml dramatic splaying of the filament bundles occurs. This is presumably due to the removal of the dense material at the tips of microvilli as well as of the cross bridges which link filaments together within the bundle (34) (Fig. 11). This splaying aids tremendously in the visualization of arrowheads on the actin filaments, as can be observed in Figs. 5 and 6, where arrowhead direction is easily distinguishable only on those filaments free from the intact bundles. This is due to a reduction in superposition. Although splaying is advantageous for the determination of filament polarity, one is left with the remote possibility that the shredding of filament bundles allows the release of a set of actin filaments with opposing

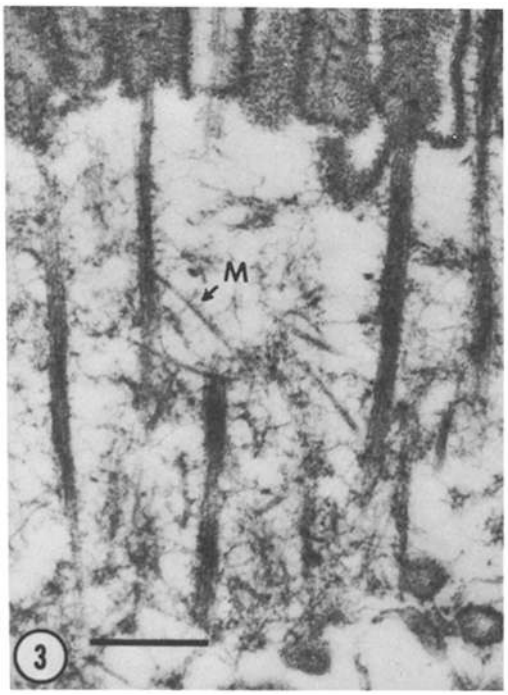

FIGURE 3 Terminal web region of an isolated brush border prepared in the presence of Mg^{++} (incubated for 20 min in solution A before fixation). Note the thick, myosinlike filaments (M) associated with the microvillar filament bundles. Bar, 0.2 μm. × 75,000.

polarity. The fact that we have never seen an actin filament in a decorated brush border with arrows pointing up the microvillus toward the distal tip indicates that such a situation is highly unlikely. Furthermore, we present evidence in Fig. 7 which clearly negates this possibility. This micrograph is unusual in that the downward direction of S_1 arrowheads is readily visible on many of the microvillar actin filaments even though the filament bundles are relatively intact and unsplayed. In fact, several of the microvilli still have the dense matrix at their tips, indicating that a set of antiparallel filaments could not have slipped off the ends of the filament bundles.

An examination of cross sections through the terminal web of S_1-decorated brush borders (Fig. 8) indicates that there are fewer nonmicrovillar terminal web filaments than in undecorated brush borders (Figs. 2, 3, and 4. Brush borders from S_1 control experiments are similar to the demembranated brush border in Fig. 4). There are very few nonmicrovillar actin filaments or 10 nm tonofilaments. There are, however, numerous thick filaments similar to the myosinlike filaments in Fig. 3. Since these filaments are the only remaining structural elements in the terminal web which connect adjacent microvillar filament cores, it is reasonable to assume that they are critical in maintaining the structural integrity of the isolated brush border.

The Attachment of Actin Filaments along their Lengths to the Plasma Membrane

Some time ago, Mukherjee and Staehelin (34) made the exciting observation that the filament cores in the microvilli of mouse intestinal epithelial cells are attached along their lengths to the plasma membrane by cross bridges. These bridges were detected in freeze-etched preparations of both longitudinally and cross-fractured microvilli. Using the same technique, we have observed similar cross bridges in the microvilli of brush borders isolated from chicken intestine. Cross bridges which form spokelike connections between the filament core and the plasma membrane are present in all the microvilli in Fig. 9. We have calculated an average of three–four bridges per cross section, but it is obvious from this micrograph that there is considerable variation in this number (1–9 in this micrograph). The dimenions of the cross bridges measured from micrographs of freeze-etched preparations are 3–5 nm × 10–25 nm. The variation in length may be due to intrinsic length differences. Alternatively, since we cannot resolve whether or not a given bridge originates somewhere within the core or is attached to its surface, the variation in length may reflect differences in the distance between the filament core and the membrane. Although we assume that the bridges attach to individual filaments in the core, it is impossible to resolve the bridge-filament attachment site because individual actin filaments cannot be easily distinguished in micrographs of freeze-etched preparations.

Unfortunately, in sectioned material, where actin filaments in the microvillus core are easily discernible, the cross bridges are not. For example, cross bridges are barely detectable in the isolated brush border shown in Fig. 2 even though the preservation of the actin filaments and other structures is unusually good. We have discovered that the visualization of cross bridges in sectioned material is dramatically improved by the incubation of isolated brush borders in high concentra-

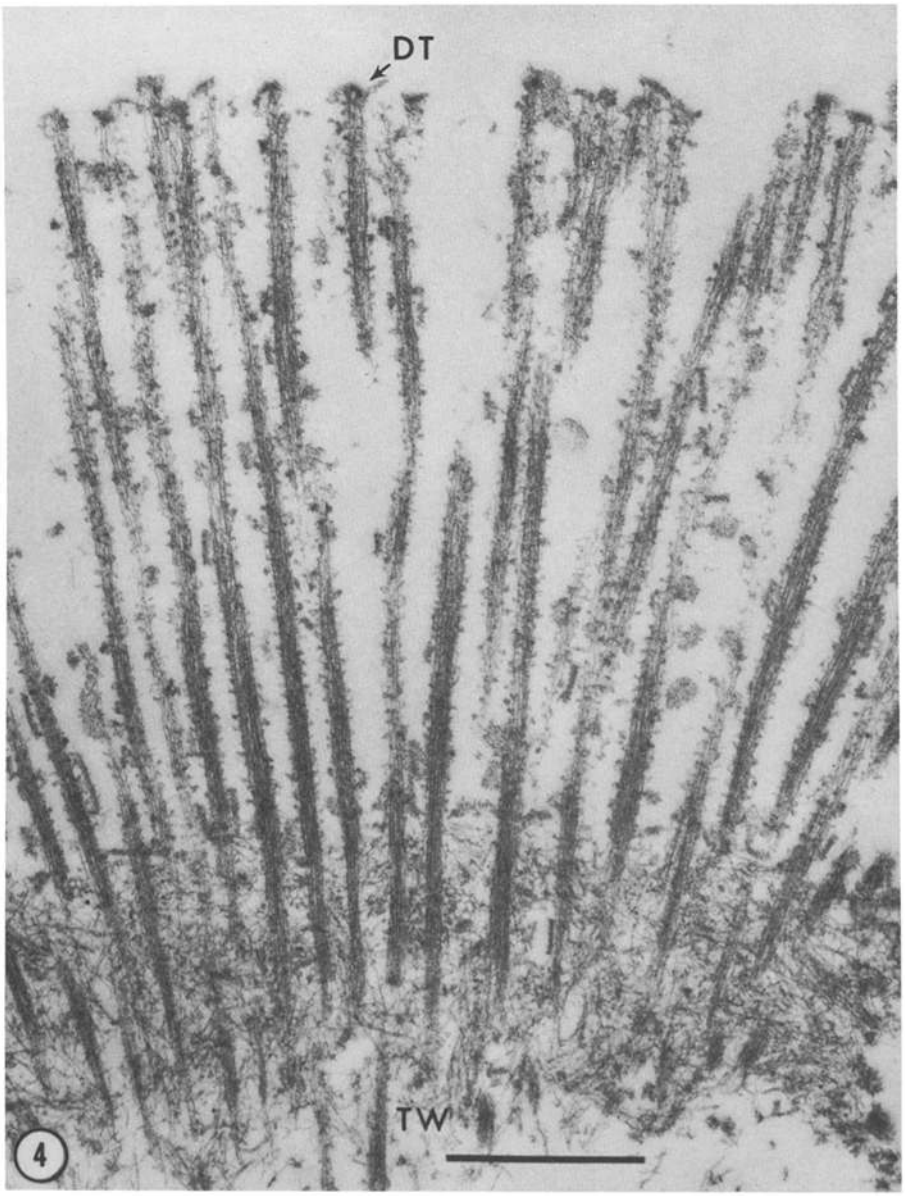

FIGURE 4 Isolated brush border demembranated with Triton X-100. The terminal web (TW), filament cores, and dense tip material (DT) remain intact after membrane removal. Note the numerous cross bridges along the length of the filament cores. Bar, 0.5 μm. × 51,000.

tions of Mg^{++}. This discovery was made as a result of a series of experiments in which we attempted to induce the actin filaments in the microvilli to form paracrystals *in situ*. Figs. 10 and 11 illustrate the extensive and highly organized distribution of cross bridges which link actin filaments in the microvillus core laterally to the plasma membrane. The cross bridges are attached directly, and in many regions periodically, to individual actin filaments. The center-to-center spacing between adjacent bridges in those regions where attachment is periodic is about 33 nm. Occasionally, lateral striations can be seen on the surface of the filament bundle. These striations occur with the same axial periodicity (33 nm) as the bridges and are presumably due to the radial disposition of the cross

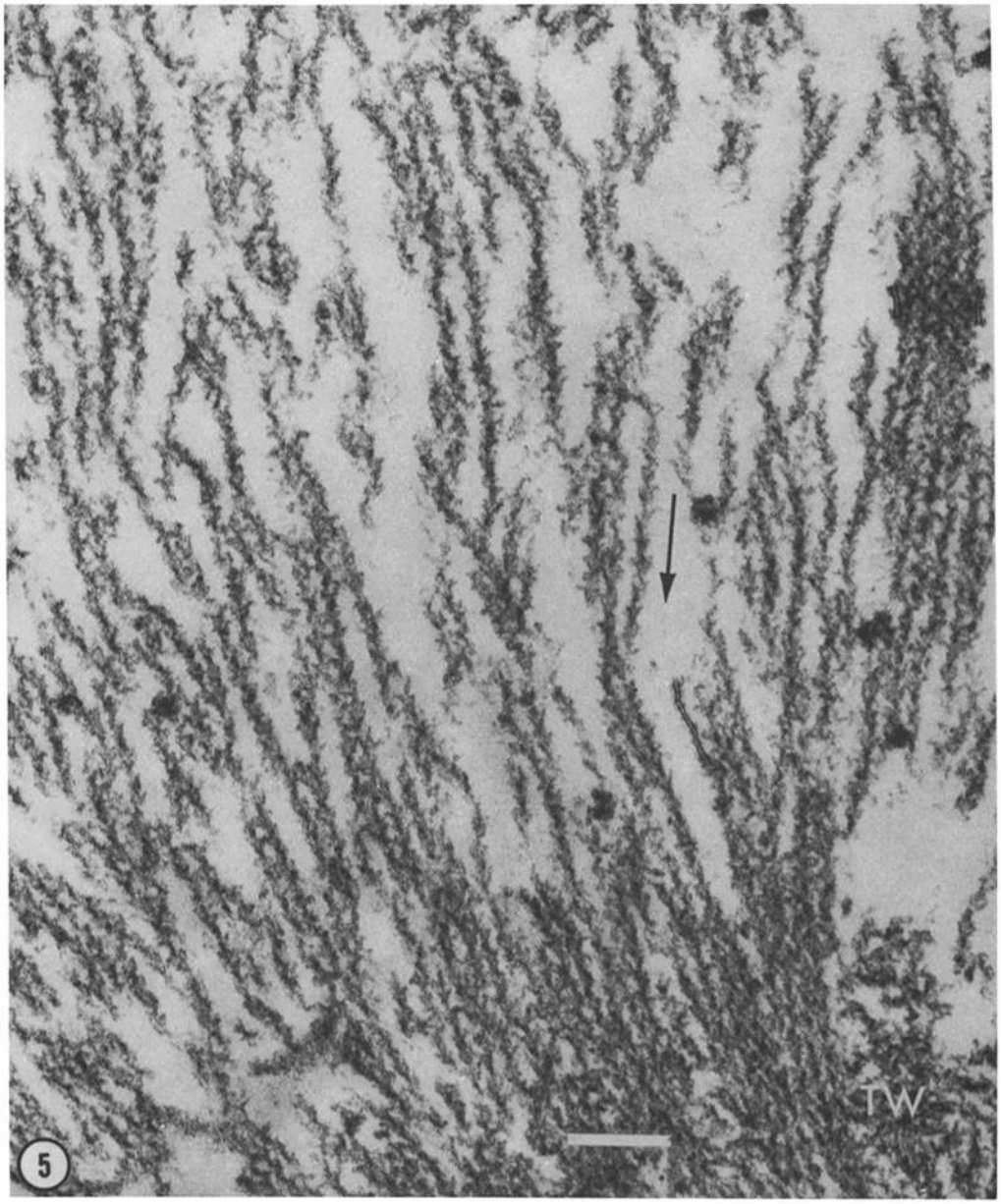

FIGURE 5 **Demembranated brush border decorated with S_1. The microvillar filament bundles are splayed** as a result of S_1 binding. Arrowheads pointing downward toward the terminal web (TW) are visible on many of the actin filaments. The arrow indicates the polarity of one such filament. Bar, 0.1 μm. × 131,000.

bridges on the filament bundle. The periodicity of the bridges or striations is equivalent to the axial periodicity (34 nm measured in sectioned material) of actin paracrystals formed in vitro (21, 22).

The enhanced visibility of the cross bridges after incubation in high Mg^{++} is probably due to a reenforcement effect as a result of that paracrystal formation. Another effect of paracrystal formation which would result in the enhanced visibility of the bridges is that lateral aggregation of the actin filaments. This should reduce the superposition of actin filaments and cross bridges in the zone between the filament core and the membrane and as a result, improve the image contrast of the

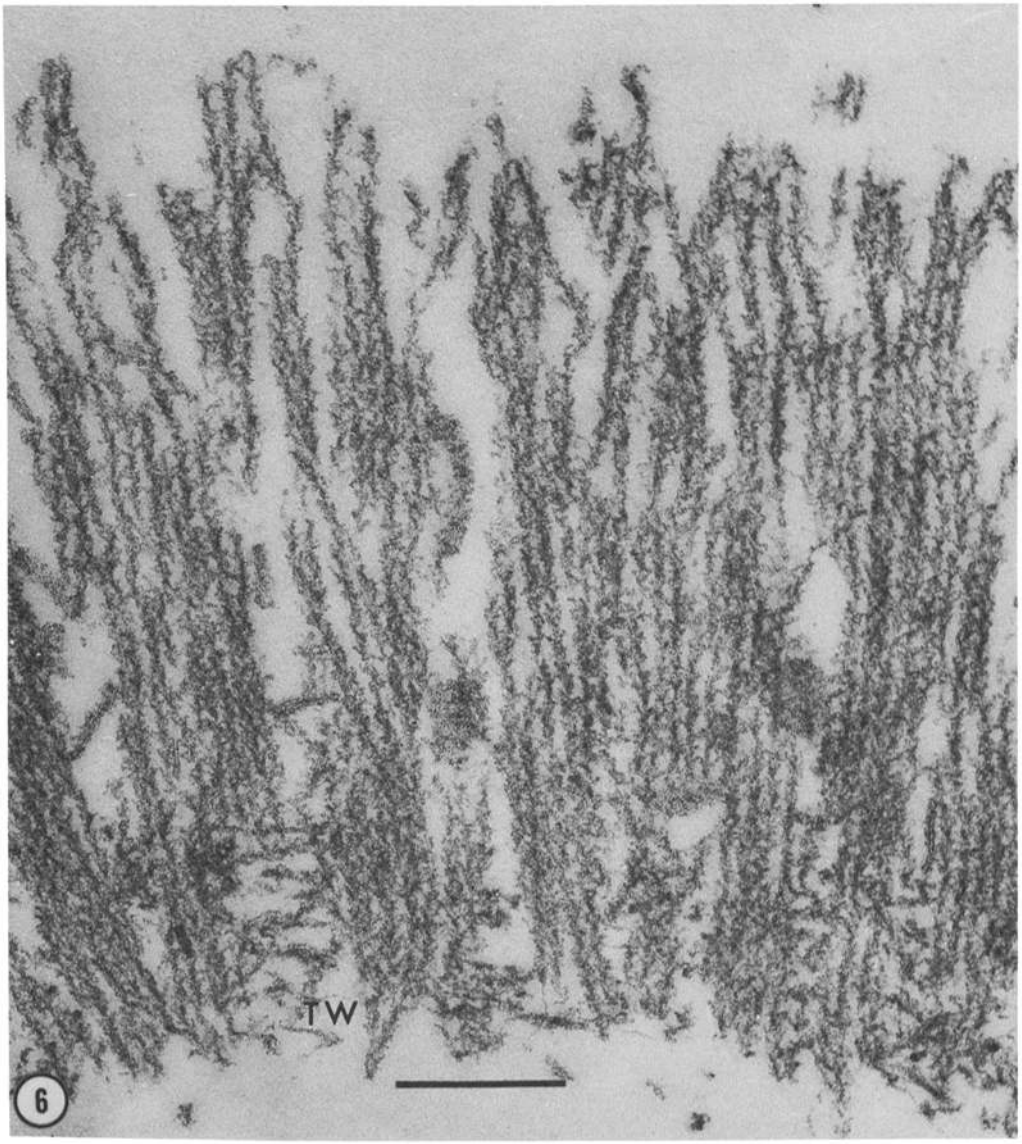

FIGURE 6 Demembranated brush border decorated with S_1. As in Fig. 4, all filaments on which arrowheads can be discerned have the same polarity, S_1 arrowhead complexes point toward the terminal web (TW). The microvillar bundles remain relatively unsplayed in the terminal web. Bar, 0.2 μm. × 109,000.

bridges viewed in sectioned material. This tightening of the filament bundles is obvious in a comparison of the filament packing in the microvilli in Fig. 2 (low Mg^{++}) with that of Fig. 10 (high Mg^{++}).

Demembranation with Triton does not remove the cross bridges from the microvillar filament bundles (Figs. 4 and 12). In demembranated brush borders incubated in high concentrations of Mg^{++} (15–40 mM) the bridges are attached along the length of the actin filaments with the same periodicity as above—33 nm. However, there is less interruption in this periodicity than in membrane-intact brush borders incubated in high Mg^{++} (see Fig. 12).

The cross bridges have varied dimensions. For those bridges that are attached to the membrane and to an actin filament, the length of a given bridge obviously depends on the distance it must

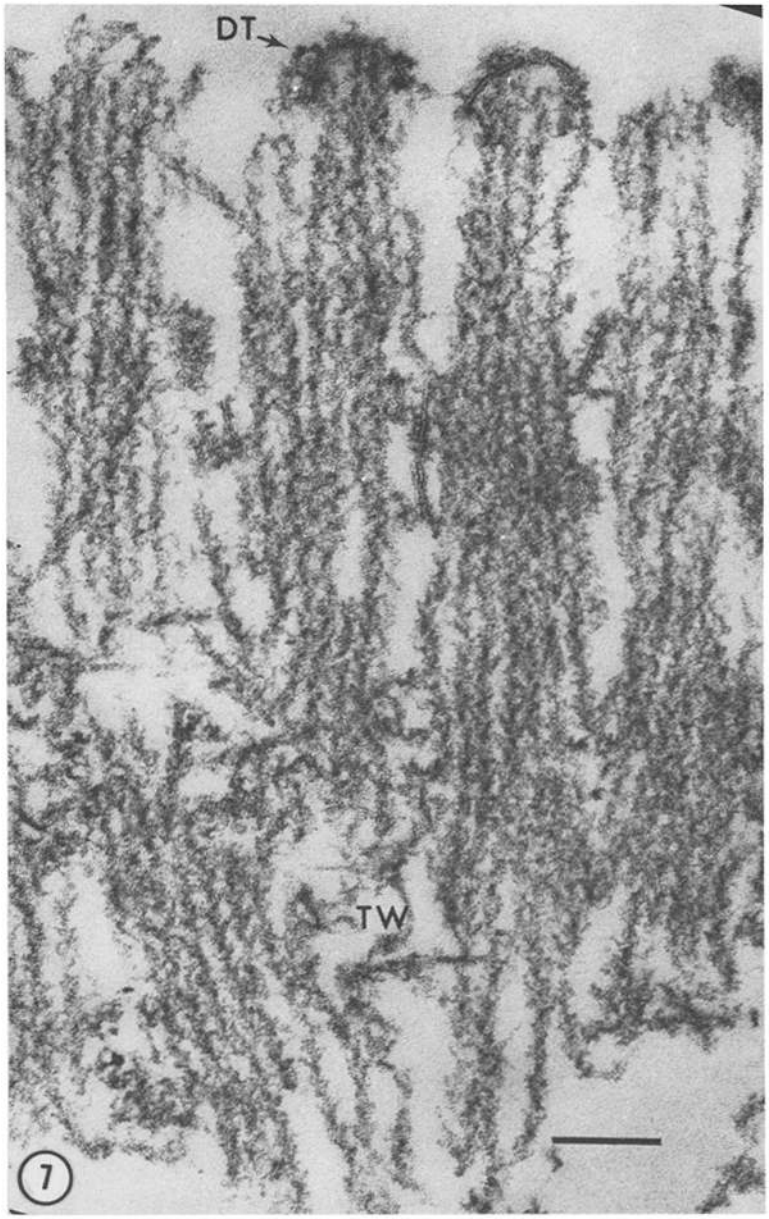

FIGURE 7 **Demembranated brush border decorated with S_1. The downward polarity of the microvillar** actin filaments is readily visualized even though the filament bundles are relatively intact. The dense tip material (DT) remains attached to several of the filament bundles. Bar, 0.1 μm. × 138,000.

span to maintain the connection. Generally this distance is about 15–30 nm (average, 20 nm). However, if the plasma membrane separates from the filament core by a distance greater than about 35 nm, the filament-membrane connection is broken. The diameter of these bridges also varies (2–7 nm), and for most of the bridges measured the diameter seems to depend inversely on bridge length. For example, the longest bridge in Fig. 10 is also one of the thinnest (30 nm × 2 nm). The cross bridges on the demembranated brush border have more uniform dimensions (average, 15 nm ×

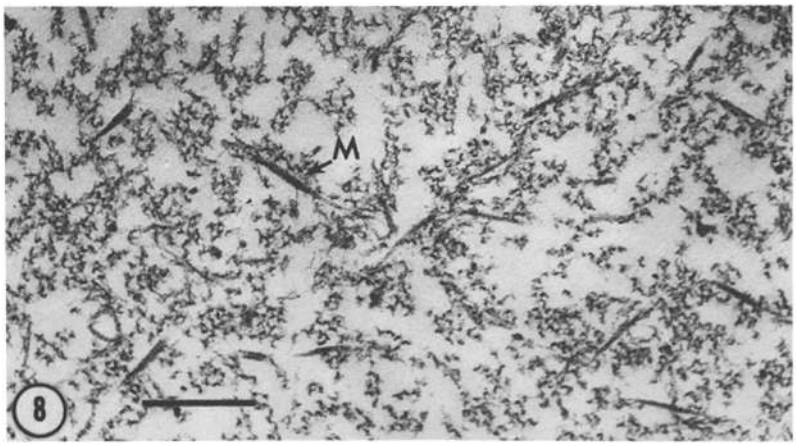

FIGURE 8 Transverse section through the terminal web of a brush border decorated with S_1. Microvillar actin filaments are seen in oblique section. Note the tapered myosinlike filaments (M), similar to those in Fig. 3 which are associated with the microvillar filament bundles. Bar, 0.2 μm. × 70,000.

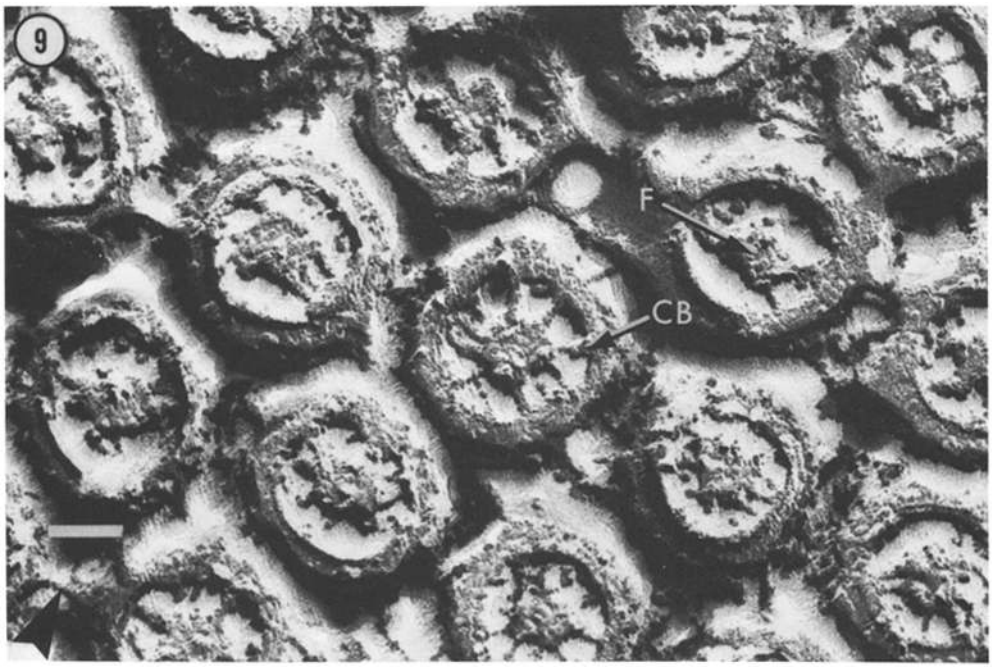

FIGURE 9 Electron micrograph of cross-fractured microvilli. This preparation was etched for 1 min. A variable number of cross bridges (CB) which form spoke-like connections between the filament core (F) and the microvillar membrane are present in all the microvilli. Arrow at lower left indicates direction of platinum shadowing. Bar, 0.05 μm. × 195,000.

7 nm), and are shorter and thicker than those bridges which are attached to the plasma membrane and an actin filament (see Fig. 12).

Another important structural feature of the microvillus visible in the micrographs of brush borders incubated in high concentrations of Mg^{++}, are the bridges which connect actin filaments to each other within the core, rather than to the plasma membrane (see Fig. 11). These bridges, which are morphologically similar to those attach-

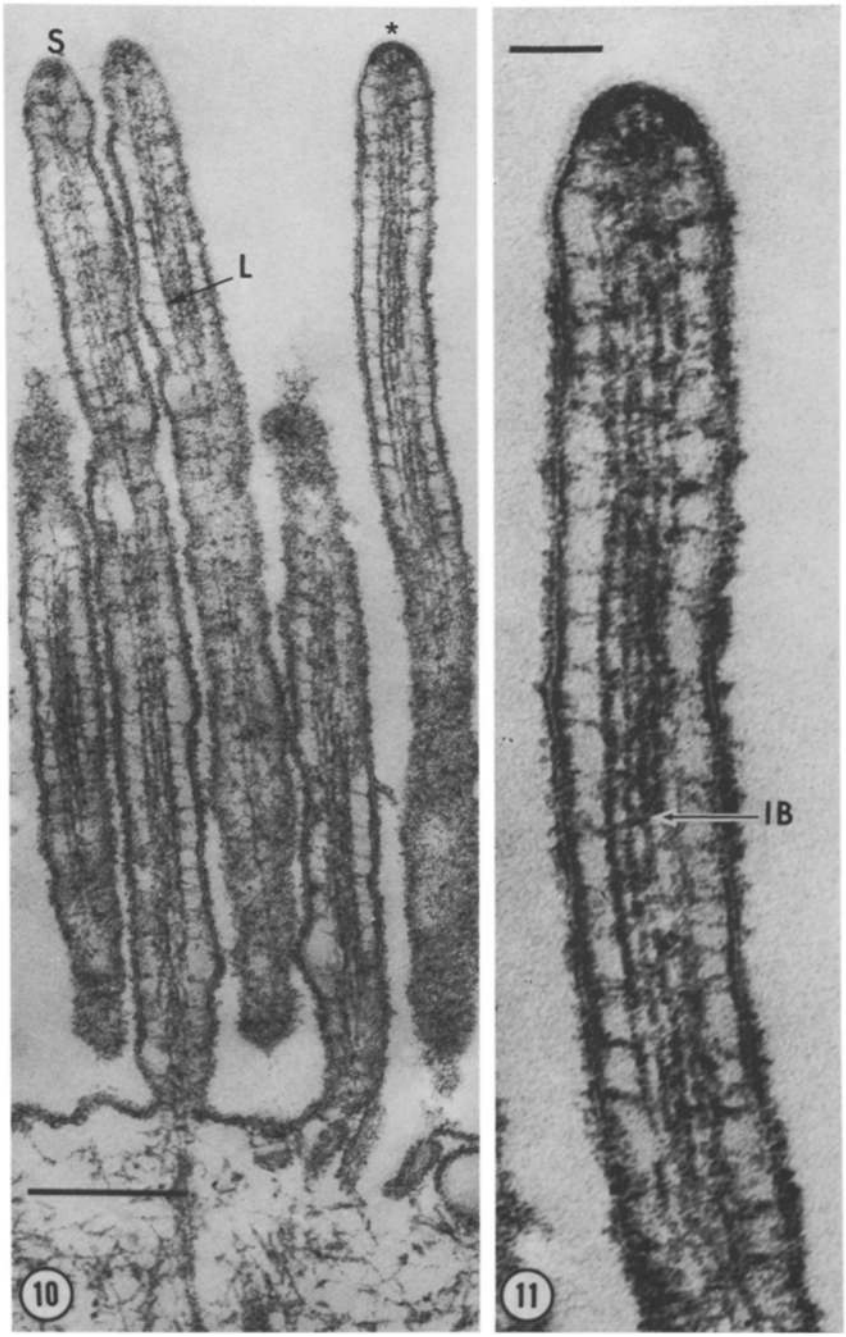

FIGURE 10 Thin section of an isolated brush border incubated in 15 mM Mg^{++}. Cross bridges connecting the filament cores along their lengths to the microvillus membrane are visible. In some regions the bridges are periodic (33 nm). Lateral striations with the same periodicity are visible on the filament bundle in the microvillus indicated (S). Bridges of various lengths and diameters can be seen. The diameter usually is inversely proportional to length. An arrow indicates the longest and thinnest bridge. Bar, 0.2 μm. × 102,000.

FIGURE 11 A higher magnification of the microvillus starred in Fig. 9. In addition to membrane-filament cross bridges, bridges (IB) connecting actin filaments to each other within the bundle can be seen. Bar, 0.05 μm. × 240,000.

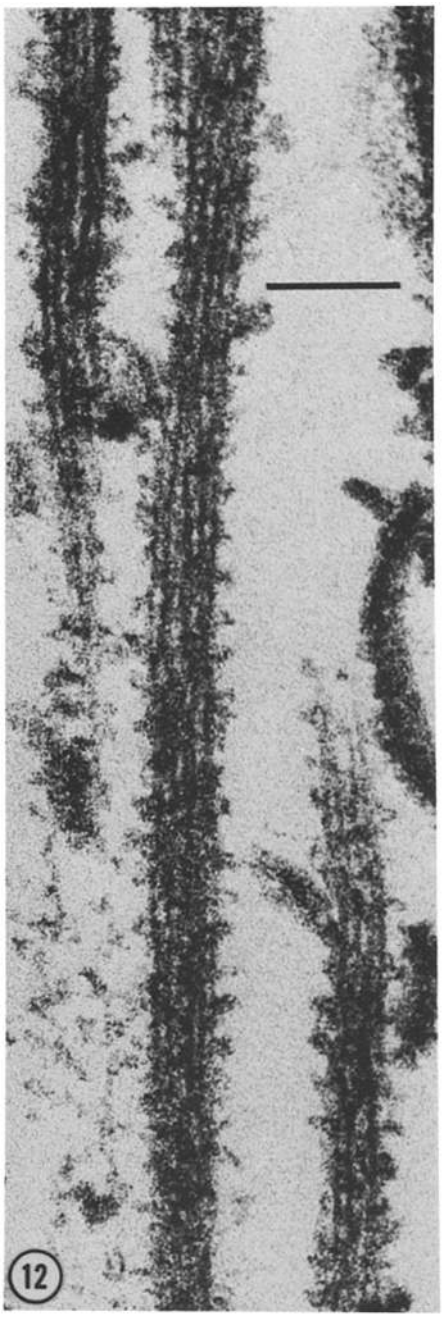

FIGURE 12 High magnification electron micrograph of microvillar filament bundles in a demembranated brush border incubated in 40 mM Mg^{++}. Note the lateral protrusions along the length of the filament bundles. These protrusions have the same periodicity (33 nm) as the membrane-filament bridges in Figs. 10 and 11. Bar, 0.1 μm. × 168,000.

ing actin filaments to the membrane, have also observed by Mukherjee and Staehelin (34) and ourselves in freeze-etched preparations of longitudinally fractured microvilli. Because of superposition problems, we cannot tell from these micrographs if the "internal" bridges are as numerous or as periodic as those attached to the membrane.

DISCUSSION

Unidirectional Filament Polarity: Its Significance in the Motility and Assembly of Microvilli

In this report we demonstrated that all the actin filaments within each microvillus have the same polarity. This was determined by examining the arrowhead complexes produced by the addition of S_1 to the actin filaments. The insight this information provides into understanding how the brush border contractile apparatus functions to generate microvillar movement is best discussed within a framework of what is known about that movement.

There are two reports, unfortunately not well documented, of in vivo microvillar motility in the brush border of epithelial cells (48, 54). The exact nature of this movement cannot be determined from these reports, but it is presumably some kind of cycled, perhaps cilia-like motility. The existence of cycled motility in vivo is supported by our observations in vitro that microvillar movements can be induced in isolated (membrane-intact) brush borders (unpublished observations). In addition, Rodewald has observed, in isolated rat brush borders, a divalent cation and ATP-dependent contraction of the terminal web region. This contraction does not involve microvillar movement or shortening (R. Rodewald, Dept. of Biology, University of Virginia, Charlottesville, Va., personal communication). Finally, we have shown that the isolated, demembranated brush border, in a fashion analogous to the isolated muscle myofibril, retains contractile potential. The microvillar filament bundles plunge rapidly into and through the terminal web upon the addition of Ca^{++} and ATP (33). Although the one-shot contraction seen in demembranated brush borders is clearly a simplified version of in vivo motility which is cycled and does not involve such dramatic microvillar length changes, the in vitro phenomenon does indicate that one force-generating element in the

production of in vivo movement is the basal displacement of microvillar actin filaments toward the terminal web. Because in vivo motility is cycled, there must be a second element as well, a relaxation, to return displaced microvillar actin filaments to their original positions. The basis for this relaxation could be mechanochemically active (e.g. actomyosin interaction) or passive (e.g. elastic).

The presence of actin, myosin, and other contractile proteins in the brush border indicates that an actomyosin interaction is undoubtedly responsible for one or both force-generating elements (i.e. contraction and relaxation) in the production of in vivo microvillar motility. If the interaction of brush border actin and mysoin is similar to that described in skeletal muscle, certain predictions can be made concerning the polarity of the actin filaments and the localization of brush border myosin. In order to generate the microvillar contraction elicited in demembranated brush borders by ATP and Ca^{++} in vitro, there must be a set of actin filaments in each microvillus polarized so that if decorated with S_1, the arrowheads would point toward the terminal web. There must also be myosin localized in the terminal web regions since the entire filament core moves into this region. If relaxation were mediated by an actomyosin interaction, there would have to be a set of actin filaments in each microvillus with polarity opposite to that needed for contraction.

Since we have shown in this report that all the actin filaments in a microvillus have the same polarity, with the S_1 arrowheads pointing toward the terminal web, these filaments are thus organized with the correct polarity to generate a myosin-mediated contraction of the filament bundles toward the terminal web (see Fig. 13 which schematically compares the polarity of actin filaments in the microvillus and the myofibril). Also, the presence of myosinlike filaments in the terminal web (Figs. 4 and 8) is supportive evidence for the predicted localization of brush border myosin in that region. Since there are no actin filaments in the microvillus with the opposite polarity (arrowheads pointing toward the tip of the microvillus), the relaxation component cannot be due to an actomyosin interaction.

In order to generate movement of an actin filament, the myosin molecule or molecules must be anchored. Fixation of the myosin molecules in skeletal muscle is achieved by the polymerization of individual myosin molecules into a polymeric, bipolar myosin filament which is itself anchored by interaction with actin filaments of opposite polarity. The presence of myosinlike filaments in the terminal web suggests that a similar mechanism for the anchorage of myosin is operative in the brush border, although one cannot eliminate the possibility that myosin is anchored through its attachment to some structural element other than actin filaments, e.g. to the plasma membrane. If the anchorage of brush border myosin is effected by its interaction with actin filaments of opposite polarity, then this anchorage cannot be achieved through the binding of a myosin unit to actin filaments in the same microvillus, because all the actin filaments have the same polarity. The anchorage of the myosin unit could be achieved, however, by its interaction with actin filaments of adjacent microvilli, or between the actin filaments in a microvillus and a set of nonmicrovillar actin filaments located in the terminal web. The first alternative is supported by our electron micrographs which show that the myosinlike filaments in the terminal web often connect adjacent microvillar filament bundles. The second alternative is also possible since Rodewald has observed actinlike filaments in the terminal web of neonatal rat brush borders which originate from the *zonula adherans* (R. Rodewald, personal communication). Our proposal for the functional organization of actin and myosin in the contractile apparatus of the brush

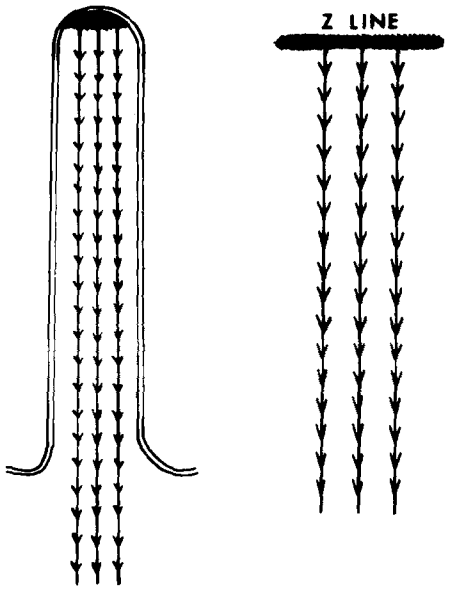

FIGURE 13 A drawing comparing the polarity of the actin filaments in the microvillus and in skeletal muscle.

border is depicted in Fig. 14. This illustration is consistent with the observed morphology of brush borders which have been induced to contract with Ca^{++} and ATP (33). If we ignore, for the moment, the cross bridges along the length of the actin filaments, this drawing (Fig. 14) points out that each microvillus is functionally analogous to half a sarcomere. This is consistent with the speculation of Tilney and Cardell (58). Thus two adjacent microvilli linked by a myosin filament(s) in the terminal web are equivalent to a whole sarcomere. This homology is strengthened by the fact that the Z-band protein, α-actinin, has been identified by antibody staining techniques (49) as a constituent of the dense material at the tips of the microvilli. In the model, this material is the functional and positional equivalent of the Z band in muscle in that it effects the end-on anchorage of the microvillar actin filaments. There are, however, two major differences between the organization of the microvillus and that of the sarcomere. First, since actin filaments in the microvillus are connected to each other (Fig. 11), the movement of the entire filament bundle could be effected by the interaction of one of the actin filaments in the bundle with myosin. Secondly, the filaments in the bundle are attached laterally as well as at the tip of the microvillus.

The determination of filament polarity may also help us to understand factors controlling the assembly of microvilli. Recent results from Woodrum et al. (62) and Hayashi and Ip (23) indicate that the polymerization of actin appears to involve polarized, end-on addition of monomer. They demonstrated that the addition of monomeric actin to F-actin filaments which had been decorated with HMM occurs preferentially on the barbed end of these filaments, or on the end of the actin filaments which would be embedded in the Z line of muscle, or in the dense plaque material at the tips of the microvilli. The addition of monomer to the opposite ends of decorated filaments occurred only at high concentrations of actin monomer. The significance of these experiments is clouded by the fact that there is no way of knowing what effect the presence of bound myosin has on the directionality of polymerization or whether myosin blocks interstitial growth. These results suggest, with these reservations, that actin polymerization appears to be a polarized process, with the preferred direction of growth opposite to the direction one would predict for the growth of actin filaments in the brush border. This prediction is based on the experiments of Tilney and Cardell (58). Using hydrostatic pressure which results in the retraction of microvilli, these investigators demonstrated that the reextension of microvilli after the release of pressure is the result of polymerization of actin from the dense plaque material at the tips of the microvilli. It is reasonable to assume that the elongation of the filaments and thus the microvillus as well, occurs by the addition of monomeric actin to the free ends (i.e., basal ends) of the actin filaments, not to the ends embedded in the dense tip material. If so, then polymerization is proceeding in the nonpreferred direction, given the observed polarity of these filaments. This immediately suggests two possible control factors in the assembly of an actin filament-membrane complex with the correct filament polarity to generate a myosin-mediated contraction. One is the availability of membrane-bound nucleating sites for polymerization, and the second is an actin monomer concentration high enough to promote polymerization in the nonpreferred direction.

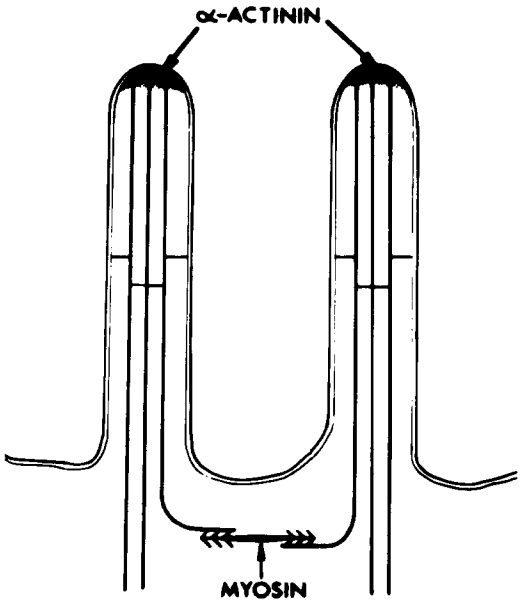

FIGURE 14 A model for the functional organization of actin and myosin in the brush border. (Refer to the text for an explanation of the model.)

The Significance of Lateral Attachments between Actin Filaments and the Membrane

We have shown that the filaments in the brush border are attached to the membrane by bridges all

along their lengths. This observation demonstrates the existence of a mechanism in nonmuscle cells for extensive lateral anchorage of actin filaments which lie parallel, not perpendicular, to membranes. Such parallel association of actin filaments with membrane is characteristic of many, if not most, types of motility in which membrane-associated actin filaments have been implicated. The contractile "ring" of actin filaments responsible for cytokinesis is a notable example. The lateral attachment of actin filaments to membranes should facilitate the coupling of such a contractile apparatus to the plasma membrane, and also greatly increase the available degrees of freedom for actomyosin mediated force generation.

This parameter of actin filament-membrane interaction has not been considered in the recent models of nonmuscle motility extrapolated from the functional organization of actin and myosin in the sarcomere, which incorporate only the end-on attachment of actin filaments to membranes (25, 42, 44, 52). [Bray's model for motility in the nerve growth cone does include "actomyosin units" which lie parallel to the membrane, but does not specify how these "units" are coupled to it (7)]. We do not wish to imply that these models are inoperative, but rather that they are incomplete. In fact, the results we present here on the polarity of actin filaments in the microvillus constitute strong experimental evidence in support of these hypotheses; the polarity of the micovillar actin filaments with respect to the membrane is the same as that required by these models, where membrane replaces the Z line as the end-on attachment site for actin filaments. We do feel, however, that these models are somewhat naive given the wide spread occurrence of motility associated with parallel, not perpendicular, arrays of actin filaments. The demonstration of actin filaments laterally attached to the brush border membrane indicates that the added degrees of freedom this makes available for force generation have to be considered in further model building.

The potential advantage the lateral attachment of actin filaments to membrane may impart to nonmuscle motile systems is exemplified by the possible roles the lateral bridges may play in the generation of microvillar movement in the brush border. The obvious role is that of transmitting the force from the contractile apparatus to the membrane allowing the membrane to move in concert with the contractile apparatus. The bridges could also participate in the generation of complex bending patterns if actomyosin contraction induced differential shear among individual filaments within the filament bundle. Alternatively, they could participate in the production of an elastic component responsible for the relaxation phase of microvillar movement as a result of intrinsic bridge elasticity and/or as transducers of membrane elasticity. All three functions may, in fact, be operative in the brush border.

The assignment of structural functions to these bridges is supported by evidence discussed below which indicates that the bridges are composed of the structural protein, α-actinin. However, until this is rigorously determined, we cannot rule out the possibility that the bridges may have enzymatic properties as well (structural and enzymatic functions need not be mutually exclusive). For example, given the periodic placement of the bridges along the length of actin filaments, they could perform a regulatory function analogous to troponin, which through its interaction with tropomyosin is attached with a similar period to thin filaments in muscle. Alternatively, the bridges could be membrane-bound myosin, although as such, the myosin clearly could not participate in the generation of the microvillar contraction observed in demembranated brush borders.

The Identification of the Bridge Protein as α-Actinin

The evidence that α-actinin is a constituent protein of the lateral bridges is as follows: (*a*) Fluorescent- or peroxidase-conjugated antibodies prepared against α-actinin not only stain the tips of the microvilli, but also along the length of the filament bundle (49). Unfortunately, the cross bridges cannot be resolved in these preparations. (*b*) The 95,000 dalton protein (this protein co-electrophoreses with α-actinin) is quite prominent on SDS gels of demembranated brush borders. There seems to be too much of this protein to be accounted for by the dense tip material alone (the molar ratio of actin/95,000 dalton protein is roughly 8:1, on the basis of gel scans (33; unpublished observations)). (*c*) Recently Podlubnaya et al. (41), using negatively stained preparations of purified α-actinin, demonstrated that this protein is a rodlike molecule with dimensions of 30 nm $\times$ 2 nm. The bridges in the brush border have comparable dimensions. These authors have confirmed the results from Goll's

laboratory (18, 53) that α-actinin can bind laterally to actin filaments in vitro and effect their cross linking in the absence of tropomyosin. Thus, although α-actinin in the sarcomere is restricted to the end-on attachment of actin filaments, it can, in the absence of tropomyosin bind anywhere along the length of these filaments. The fact that α-actinin can cross-link actin filaments laterally in vitro suggests that the internal cross bridges which connect actin filaments to one another rather than to membrane are also comprised of α-actinin (Fig. 11).

Obviously, a problem is raised by the presence of tropomyosin in the brush border. However, like tropomyosins isolated from other nonmuscle sources (12, 15), brush border tropomyosin is smaller (30,000 dalton subunit vs. 35,000 for muscle) and presumably shorter than muscle tropomyosin (although we have not yet made length measurements, platelet and brain tropomyosin have lengths of about 34 nm; muscle tropomyosin is 40 nm in length). It is possible that lateral association of α-actinin with actin filaments is not prevented by brush border tropomyosin, either because of sequence differences or because of its shorter length. It may be significant that the lateral bridge periodicity is equivalent to the presumed length of the tropomyosin molecule.

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