

Organization of the Cross-filaments in Intestinal Microvilli

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ABSTRACT We studied the arrangement of the cross-filaments in intestinal microvilli to understand how microfilaments interact with the membrane. Observations on thin-sectioned or negatively stained microvilli with the electron microscope demonstrate that the cross-filaments on the core bundle lie opposite to one another and are spaced 32.5 nm apart. In sections grazing through the membranes, the cross-filaments appear as transverse stripes in a barber-polelike arrangement. The cross-filaments point away from the microvillus tip. This orientation appears similar to that seen when microvilli are decorated with muscle myosin subfragments S1 or HMM. The cross filaments are associated not only with the microfilaments but also with electron-dense patches on the inside surface of the membrane. These results suggest the cross-filaments are arranged as a double helix around the core bundle. Furthermore, the cross-filaments can serve as *in situ* markers for microvillar polarity. Lastly, the cross-filaments interact not only with specific portions on the actin filaments but also with dense patches on the membrane. These observations are summarized in a model of the microvillus cytoskeleton.

The free surfaces of many cells are covered by fingerlike processes called microvilli. Microvilli can vary in length from the 2 μm long microvilli of the intestine brush border (1) to the 31 μm long stereocilia of hair cells (14). One feature of all microvilli is the presence of a central core of bundled microfilaments. Core bundles have been found in microvilli of the intestine brush border (6, 21, 23), the kidney brush border (3), sea urchin eggs (8, 26), setae (25), and hair cells (12, 14, 28). While optical diffraction methods have described the organization of the filament bundle (12, 26), little is known of how these bundles are attached to the membrane.

The core bundle is associated with the membrane by two types of attachments. In the first type, the core bundle appears to insert end-on into an electron-dense cap at the tip of the microvillus (6, 21, 23). These distal attachments resemble the membrane attachment sites for stress fibers in cultured cells and the Z-line attachment sites for thin filaments in muscle (21). However, the mechanism by which microfilaments are attached to these membrane sites has not been discerned.

The second type of attachment is composed of cross-filaments which measure 15–30 nm long and 2–8 nm wide (19, 21, 23). The cross-filaments appear to laterally attach the core bundle to the membrane (20) and are arranged around the core bundle like spokes in a wheel (21, 23). Several studies have described some details of the attachments of these cross-filaments to the core bundle. In Mg^{++} -treated microvilli, the cross-

filaments appear spaced with a 33 nm repeat along the length of the core bundle (21) and appear to link the core bundle directly to integral membrane particles (23). Because of the periodicity of the helical cross-over points in the actin filaments, Mooseker and Tilney (21) suggested that the cross-filaments were bound to specific portions on the actin filaments.

As shown in a recent study (18), the periodic array of the cross-filaments in the microvilli was preserved without the need for Mg^{++} incubation if tannic acid was included in the fixation protocol. In tangential sections through the microvilli, the cross-filaments formed transverse stripes spaced every 33 nm along the length of the core bundle. The orientation of these stripes suggested that the cross-filaments spiraled around the core bundle in a helical manner.

The observations that cross-filaments are attached directly to membrane particles (23) together with the repeating arrangement of cross-filaments suggest that the membrane particles should be arranged in the membrane in an orderly manner. However, the particle distributions observed in the membrane do not overlap with the arrangement of the underlying cross-filaments. Neutra (24) observed that in a minority of the microvilli in primate large intestines there is a linear particle array along the length of the microvillus. This distribution is not seen in the large or small intestines of nonprimates (24). Tilney and Mooseker (27) described a helical pattern of membrane particles in Mg^{++} -treated brush borders. The spacing

between successive bands of the helix was 90 nm, which is three times larger than the cross-filament periodicity. These two special cases of an orderly particle distribution are exceptions to the majority of freeze-fracture studies which instead show a random particle distribution (19, 23, 24, 27). The conclusion reached from the freeze-fracture studies is that the observed particle distribution is not correlated with the arrangement of the underlying cross-filaments.

Here we describe additional features of the cross-filaments. The cross-filaments spiral around the core bundle as a double helix with a lateral spacing of 32.5 nm. A model of the helically arranged cross-filaments predicts that the actin filaments in the core bundle are packed in lateral register. The cross-filaments are tilted with respect to the core bundle and can serve as *in situ* markers for microvillar polarity. The cross-filaments also appear to be attached to electron-dense patches on the inside surface of the membrane.

MATERIALS AND METHODS

All experiments were performed on ice except where noted. To prevent proteolysis, freshly prepared phenylmethylsulfonyl fluoride (PMSF) from a 0.1 M stock in isopropanol was added, at a final concentration of 0.1 mM, immediately to each resuspended pellet.

Isolation of Intestinal Epithelial Cells

1-yr-old laying hens, obtained from local farms, were killed by decapitation. An incision was made in the body wall on the right side of the abdomen just posterior to the sternum. The portion of the intestine from the pylorus to the caecum was excised, slit down the side, rinsed in ice-cold saline, and cut into 4- to 5-cm-long pieces. The intestinal pieces were stirred in phosphate-buffered saline (made 0.1 M in sucrose and 20 mM in EDTA) for 30 min at room temperature. The segments of intestine were separated from the cell suspension by filtration through coarse fiberglass screening. The segments of intestine were then discarded and the remaining cell suspension (primarily consisting of sheets of epithelial cells) was centrifuged for 10 min at 750 g in a Sorvall RC-5B centrifuge (DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Newtown, CT).

Isolation of Brush Borders

The pellet of cells was resuspended in 4 mM EDTA, 1 mM EGTA, 10 mM imidazole, pH 7.3, and homogenized by four 15-s bursts at full speed in a Sorvall Omnimixer. The homogenate was centrifuged for 10 min at 750 g to pellet the brush borders. The pellet was washed again in the EDTA-EGTA-imidazole solution and once in solution I (75 mM KCl, 1 mM EGTA, 0.1 mM MgCl₂, 10 mM imidazole, pH 7.3). The pellet (consisting of brush borders, nuclei, and cellular debris) was layered on a 40%/50% sucrose step gradient made up in solution I and centrifuged in a Sorvall AH-627 swinging bucket rotor at 10,000 g for 2 h. The brush borders banded at the 40%/50% interface and were collected by centrifugation at 12,000 g for 10 min and washed free of sucrose by centrifuging at 750 g for 10 min. From approximately 1.0 g of cells, 40 mg of pure brush borders was isolated in 5 h.

Isolation of Microvilli

Microvilli were isolated from brush borders using the protocol outlined by Bretscher and Weber (4). This involves shearing the brush borders forcefully through a 20-gauge needle. The sheared brush borders are separated from the microvilli by centrifugation at 500 g for 5 min. The microvilli in the supernatant were pelleted at 10,000 g for 10 min. Modifications of their method included omission of the polyethylene glycol from the solutions and the inclusion of PMSF at all steps during the microvillar isolation.

Demembration of Brush Borders and Microvilli

Isolated brush borders or microvilli were demembrated by resuspending them in solution I containing 1% Triton X-100 for 15 min at room temperature. The demembrated brush borders or microvilli were collected by centrifugation for 10 min at 12,000 g in a Sorvall SS-34 rotor, resuspended in solution I, divided

into aliquots, and recentrifuged. The brush borders and microvilli were stored on ice and used within 2 h after demembration.

Electron Microscopy

For negative staining, demembrated microvilli were pipetted onto Formvar- and carbon-coated grids, rinsed with five drops of solution I, and five drops of unbuffered uranyl acetate (pH 4.1).

The brush borders or microvilli were fixed as pellets using the fixative described by Begg et al. (2) and processed for embedding in Epon-Araldite. Thin sections (silver in color) were cut with diamond knives on a Sorvall MT-1 or MT-5000 ultramicrotome and stained with uranyl acetate and lead citrate.

Grids were examined at 60 or 80 kV in a JEOL 100CX electron microscope using a rotating-tilt stage. The microvilli were rotated until their long axis was perpendicular to the tilt axis of the stage. Micrographs were taken at 0, ±10, and ±20 degrees tilt. The distance between the cross-filaments was measured directly from the micrographs in the tilt series which represented a longitudinal section through the microvillus.

RESULTS

In longitudinal section, the cross-filaments appear spaced with a periodicity along the length of the core bundle (Fig. 1). The measured spacing between the cross-filaments is 32.5 nm (SD 1.5 nm, $n = 56$) which is in close agreement with the original observations of Mooseker and Tilney (21). This number is uncorrected for specimen shrinkage during the dehydration or embedding for electron microscopy. In tangential sections the periodicity between the cross-filaments is smaller, as expected from simple geometry (Fig. 2, double arrows). In both thin-sectioned and negatively stained microvilli, the cross-filaments lie directly opposite to one another (Figs. 1, 2, and 3, arrowheads). The cross-filaments also appear to attach to periodically spaced electron-dense patches on the inside of the membrane (Figs. 1 and 4).

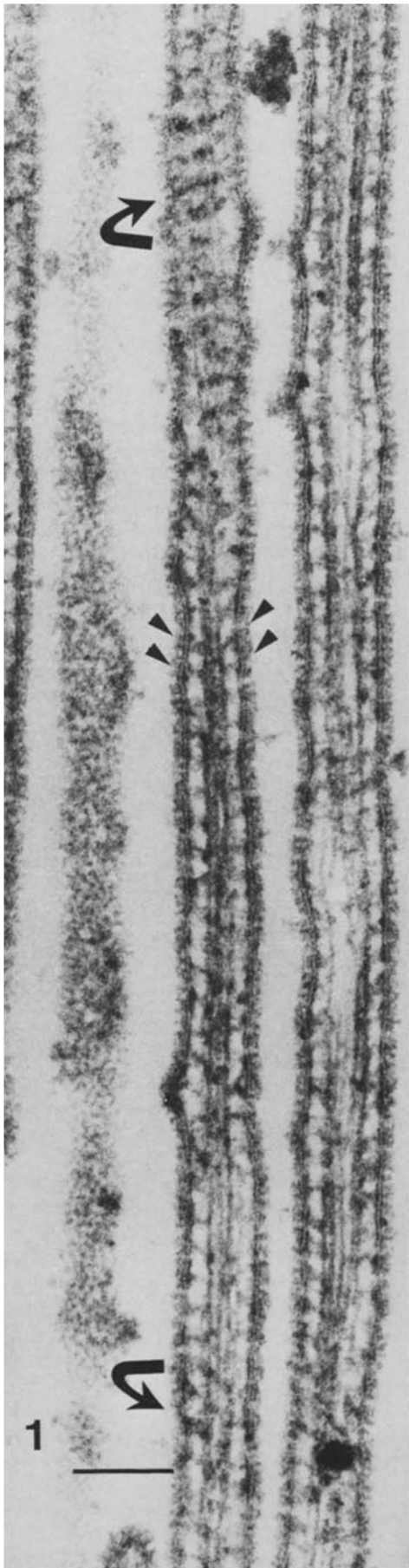
The cross-filaments are tilted with respect to the core bundle (Figs. 1 and 4, arrowheads). This feature is still seen in both thin sections or negative stains of demembrated microvilli (Figs. 2 and 3, arrowheads). The canted cross-filaments appear to decorate the core bundle in an arrowheadlike manner and point away from the tips of the microvillus (Figs. 1 and 2) like S1- or HMM-decorated microvilli (2, 21).

The arrangement of cross-filaments around the core bundle is most clearly seen in tangential thin sections (Figs. 1 and 2) and negatively stained preparations of demembrated microvilli (Fig. 3). As the plane of section travels toward the membrane, the tips of the cross-filaments appear in cross-section as a barber-polelike arrangement of transverse strips (Fig. 1, arrows). This banding pattern is retained after the membrane is removed and is seen in both thin-sectioned (Fig. 2, arrows) and negatively stained (Fig. 3, arrows) cytoskeletons. As previously noted (18), the handedness of the banding changes in some cases along the length of the core bundle as the section travels through the microvillus (Fig. 1, curved arrows). In negatively stained microvilli, a cross-hatched striping pattern can also be detected (Fig. 3, double arrowheads).

DISCUSSION

Helical Arrangement of the Cross-filaments

There are three possible ways in which cross-filaments can be arranged to form a transverse striping pattern with a 33-nm repeat. In the first arrangement (Fig. 5a), the cross-filaments can be arranged around the core bundle like spokes in a wheel. This possible arrangement was first suggested by earlier freeze-fracture studies on microvillar structure (23, 27). In this model the stripes formed by the cross-filaments would be perpendic-



ular to the long axis of the bundle. This model can be eliminated because the transverse stripes are not perpendicular to the core bundle but are slanted with respect to the long axis of the bundles.

A second possible arrangement for the cross-filaments is seen in Fig. 5 *b*. Here, the cross-filaments form stacks of tilted rings. When viewed from one side of the bundle, these rings would appear as barber-polelike stripes. However, if the core bundle is rotated 90° around its axis, then the barber-pole arrangement would be transformed into a series of stacked chevrons. This model is eliminated by the observation that in sections through the microvillus the angle of the stripes changes direction. If the cross-filaments are arranged as tilted rings as depicted in Fig. 5 *b*, then the angle of the stripes should remain the same on both sides of the core bundle as the plane of section cuts through the microvillus. Furthermore, different patterns of the cross-filaments such as stripes or chevrons should be seen in random grazing sections through the microvilli. Neither pattern is observed.

In the third arrangement (Fig. 5 *c*), the cross-filaments spiral around the core bundle as a helix. The striping pattern formed by the cross-filaments would remain the same when the bundle is rotated. The change in the angle of the stripes can only be explained by a helical arrangement of the cross-filaments (Fig. 5 *c*) and is the strongest evidence for a helical distribution of the cross-filaments around the core bundle.

The precise 32.5 nm periodicity of the cross-filaments along the length of the bundle together with the helical arrangement of the cross-filaments suggests that the cross-filaments are bound to the core bundle in a nonrandom manner. This arrangement is interesting from a structural point of view because it comments on the organization of the underlying actin filaments. Assuming that the actin filaments in the core bundle are arranged in the same manner as the filaments in actin needles from extracts of sea urchin eggs (11), sea urchin microvilli (26), and in hair cell stereocilia (12), it is possible then to predict the helical arrangement of the cross-filaments around the core bundle.

To understand how this helical arrangement can be generated, we must first understand how actin filaments are bundled. Diffraction studies on actin bundles (11, 12, 26) have shown that the actin filaments within a bundle are in lateral register; that is, the cross-over points in the two-stranded actin filament lie in the same plane (depicted in Fig. 6 *a*). Secondly, these studies have shown that the actin monomers in the filaments have the same azimuthal orientation, that is, the actin monomers in different filaments at any level of bundle are all pointing in the same direction (Fig. 6 *a*). DeRosier et al. (11, 12) have formulated a quasi-equivalent actin cross-linker bonding rule in which an actin cross-linking protein can only bind two adjacent actin filaments when the monomers in the actin filaments are oriented with respect to one another in a strictly specified way (see Figs. 2 and 3 of DeRosier et al., reference

FIGURE 1 Thin section through the isolated brush border. The core bundle of microfilaments is connected laterally to the membrane by cross-filaments. The cross-filaments are canted (arrowheads) with respect to the membrane and with respect to the core bundle point toward the terminal web (toward the bottom of the page). As the plane of section travels toward the membrane, the cross-filaments are seen as punctated transverse stripes (arrows). The curved arrows mark the areas where the handedness of the helix changes along the length of microvillus. Bar, 100 nm. $\times 150,000$.

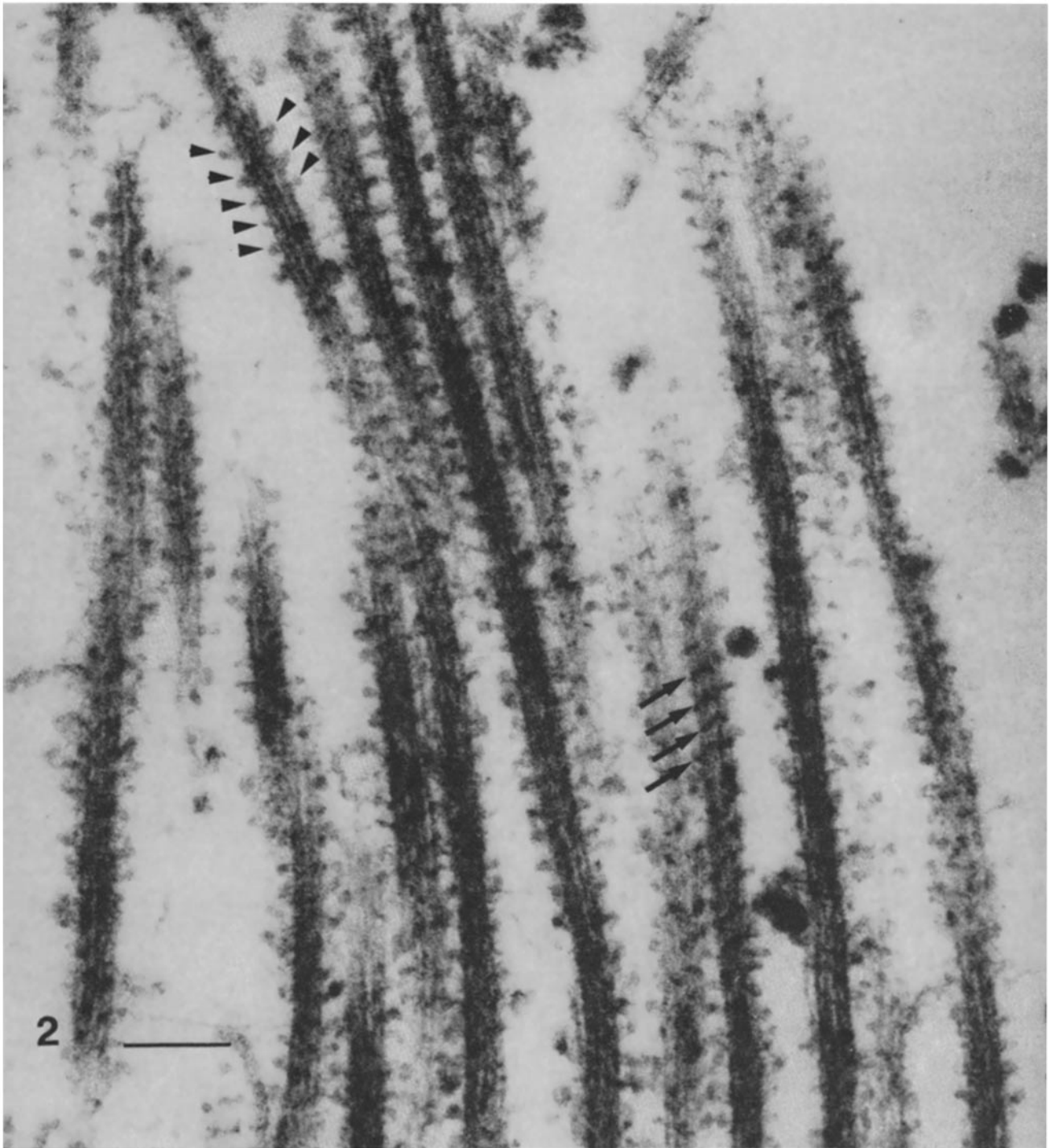


FIGURE 2 Thin section through the demembrated brush border. The cross-filaments retain their transverse striping patterns (arrows) as well as their arrowheadlike decoration (arrowheads) of the core bundle after the membranes are removed. The periodicity of the striping pattern is similar (24 nm) in oblique sections through the core bundle (arrows). Bar 100 nm. $\times 175,000$.

12). In addition, the distance between the actin subunits must be close enough for the cross-linker to form a bond. With this arrangement, the cross-over points of the filaments within a bundle would appear as transverse stripes spaced 37.5 nm apart like those seen in actin paracrystals (15). However, the level in the bundle at which the cross-over points are in register is solely dependent on the perspective from which the bundle is viewed. Thus, if we moved around the perimeter of the bundle, the cross-over points would appear to translate along the length

of the bundle. The cross-over points have appeared to move only because the perspective from which we have viewed the bundle has changed.

To generate a helical array of cross-filaments around the bundle, two assumptions must be made. First, the actin filaments must be organized with their cross-over points in lateral register as just described. Second, bonds between the filaments in the bundle and the overlying membrane must be specified by similar bonding rules as described for bundle structure. This

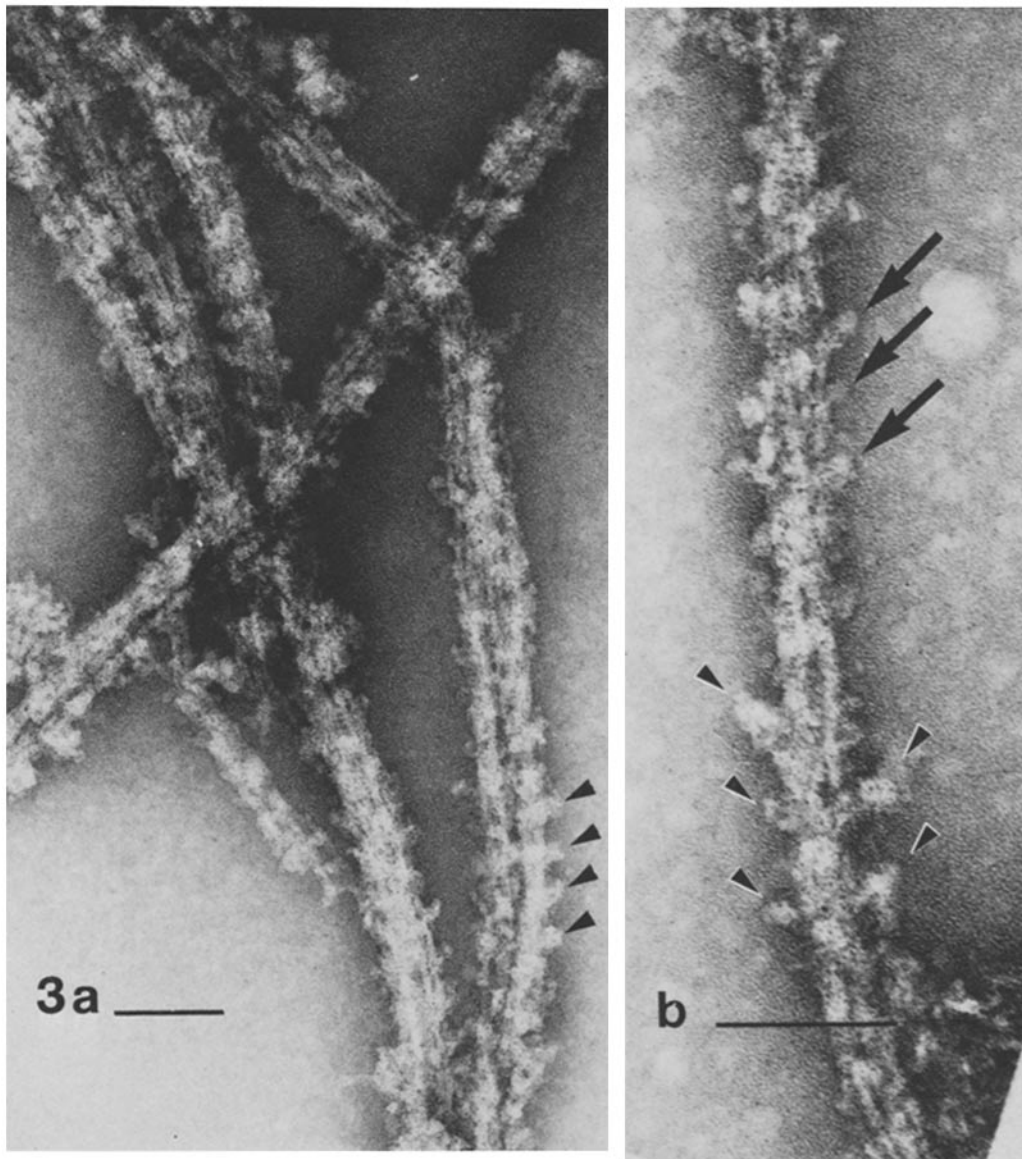


FIGURE 3 Negatively stained microvilli. When demembranated microvilli are negatively stained, the helical (arrows) and polar (arrowheads) features of the cross-filaments are clearly seen. Bar 100 nm. (a) $\times 145,000$. (b) $\times 240,000$.

means that the cross-filaments must bind simultaneously to specific portions of both the actin filament and the membrane. These assumptions seem reasonable since they are based on previous studies of actin bundle structure. In fact, the 32.5-nm periodicity of the cross-filaments along the length of the bundle strongly suggests that cross-filament binding to the filament is restricted to a repeating portion of the actin filament. For the sake of illustration, let us pick the helical cross-over points in the actin filaments on the periphery of the bundle as the sites where the cross-filaments bind. From the standpoint of the membrane, the cross-over points in the actin filament bundle will depend on, once again, the perspective from which the bundle is viewed (in this case, by the membrane). Fig. 6a depicts the view of the actin bundle with filament 1 in the front. As can be seen, the cross-over points in the bundle all lie in the same plane. If we move around the bundle so that filament 2 is now in the foreground, then we would see that the cross-over points would have shifted up the filament by approximately four subunits. As we continue around the bundle, we would see the cross-over points continually shifting in

their position on the bundle so that when we return to filament 1 again, we would find ourselves 75 nm from our original starting point. Moving around the bundle or, instead, rotating the bundle around its axis is functionally equivalent to unrolling the bundle into a flat sheet as seen in Fig. 6b. Once this operation is performed, the peripheral filaments in the bundle would be arranged as in Fig. 7a. The stippled subunits would be the subunits to which the cross-filaments would bind. With this arrangement, the cross-filaments would form a single helix around the core bundle with a 75-nm periodicity. However, the periodicity of the cross-filaments along the length of the bundle is 32.5 nm (approximately half that distance) and not 75 nm as predicted by this single helical model. In addition, this model would predict that the cross-filaments on one side of the bundle would lie staggered with respect to the cross-filaments on the other side of the bundle. This prediction is not supported by the observation that the cross-filaments lie directly opposite to one another across the core bundle. Also, the single helical model cannot explain the cross-hatched appearance of *negatively stained* microvilli. This model does not adequately explain

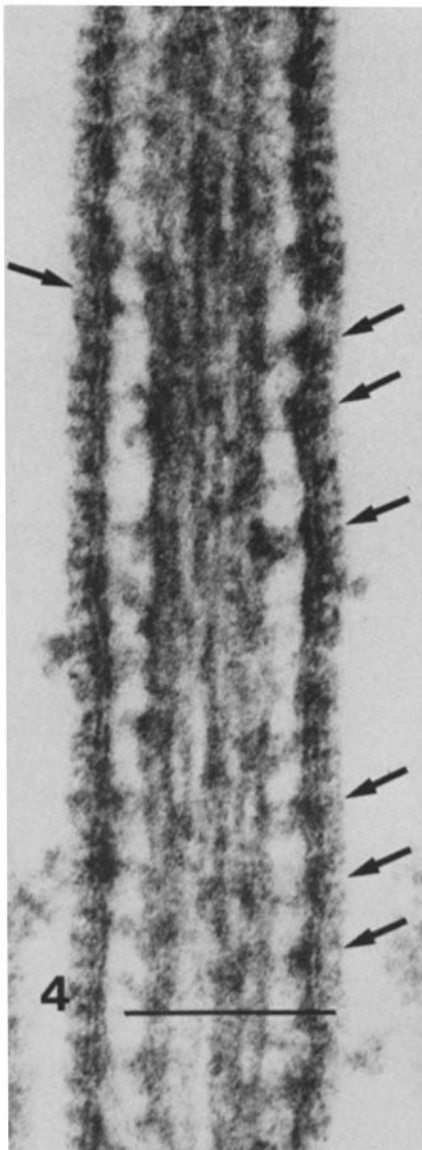


FIGURE 4 Cross-filaments associated with membrane patches. In thin sections, the cross-filaments connect the core bundle to the membrane at electron-dense patches (arrows) on the inside surfaces of the membrane. These patches are also periodic in their spacing. Bar 100 nm. $\times 280,000$.

all the observations and has to be amended.

The structure of the actin bundle as depicted in Fig. 7a provides several additional clues to the arrangement of the cross-filaments in the microvillar cytoskeletons. Between the rows of cross-over points is another row of cross-over points (marked by x) which are part of the other strand of the actin filament. Since both strands of the actin filament are polarized in the same direction, that is, the strands are parallel to one another in their orientation, then each monomer on one strand is related to the monomers on the other strand by a twofold screw axis of symmetry. This means that the cross-over points on the actin filaments are structurally equivalent. Thus we could build a model (Fig. 8) in which all the cross-over points, as seen from the membrane, are occupied by cross-filaments. The important feature of this model is that the cross-filaments would form a double helix around the core bundle with a 37.5-

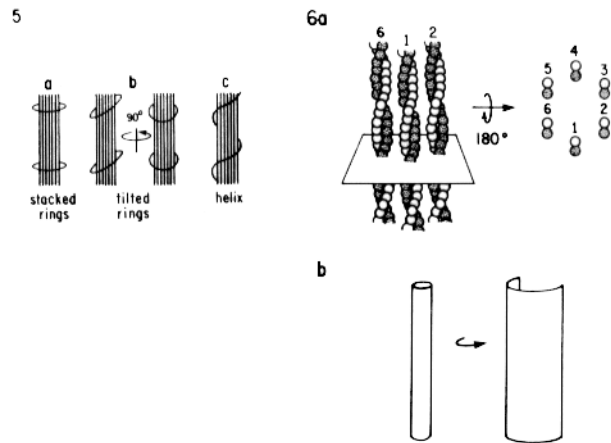


FIGURE 5 The possible arrangements of transverse stripes. Transverse stripes can be arranged as rings (*a* and *b*) or as helices (*c*). The rings could lie perpendicular to the axis of the bundle (*a*) or tilted with respect to the bundle (*b*). In the latter case, rotating the bundle would result in a transformation of the slanted striping pattern into the chevronlike arrangement. Rotation of the stacked ring or the helical arrangements of the cross-filaments would not result in any change in the observed striping patterns.

FIGURE 6 The arrangement of the actin filaments in the bundle. The actin filaments are depicted as having their cross-over point in lateral register, i.e., the cross-over points of the actin filaments (stippled subunits) all lie in the same plane (*a*). In this arrangement, the actin monomers are oriented in the same direction at any level in the bundle. For the purposes of illustration, we will restrict the model to six filaments in hexagonal packing. The filaments are numbered from 1 to 6 in a counterclockwise direction. To visualize the relationship of the cross-over points of the filaments in the bundle, the bundle is opened into a flat sheet (*b*). This is spatially equivalent to viewing a rotating bundle.

nm periodicity and that, arrayed in this manner, the cross-filaments would lie directly opposite one another on the core bundle. Our observations are consistent with the predictions of this model. The measured periodicity of the cross-filaments is 32.5 nm, which is close to the predicted value of 37.5 nm. The discrepancy between these two numbers could easily be a result of specimen shrinkage during embedding. Because of superposition effects, one would expect to see a cross-hatched pattern of the cross-filaments in negatively stained microvilli but not in thin-sectioned microvilli. This model predicts that the pitch of the cross-filament helices would be dependent on the bundle width. As the bundle width increases, the angle of the helices would decrease. On the other hand, the lateral spacing between the cross-filaments would be independent of the bundle width because spacing is specified by the cross-over points in the actin filament.

This model is based on assumptions gathered from structural studies on the actin bundles in sea urchin actin needles (11), sea urchin microvilli (26), and hair cell stereocilia (12). One feature seen in these actin bundles is a striking transverse striping pattern formed by the cross-links within the bundles (7, 8). In the actin bundles of the intestinal microvilli, such transverse striping patterns are not seen in microvilli from which the cross-filaments were removed by ATP (18). As a result, it is not known whether the actin filaments within the intestinal microvilli are in lateral register as assumed for the model and as shown in other systems. Optical diffraction studies currently in progress should provide some information

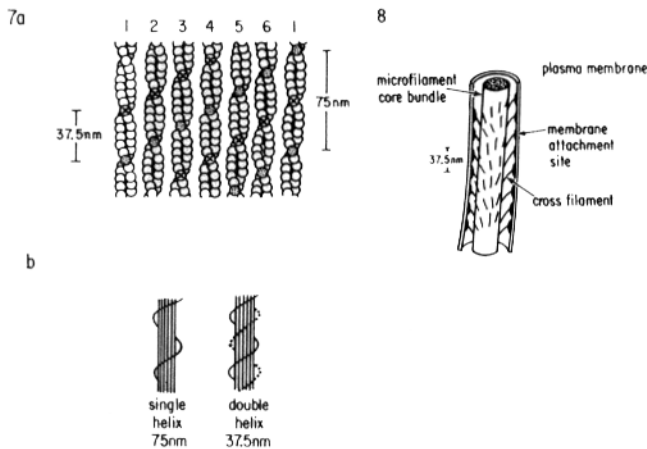


FIGURE 7 The possible arrangement of the cross-filaments around the bundle. The cross-over points on one strand of the filaments (stippled) translate up the bundle as we would view each successive filament around the periphery of the bundle. By following the cross-over points, we would arrive at filament 1 but 75 nm from our original starting point. This is depicted in (b) as the single helical model. However, if the cross-filaments are also attached to the cross-over points on the remaining strand of the actin filament in the bundle (x's), then the double helical model could be constructed (b). In this model, the cross filaments would be spaced every 37.5 nm apart along the length of the bundle.

FIGURE 8 A model of the microvillar cytoskeleton. The microvillus is composed of a core of bundled microfilaments which are connected to electron-dense patches on the inside surface of the membrane by a double-helical array of membrane linkages called cross filaments. The cross-filaments are canted with respect to the axis of the core bundle and point away from the tip of the microvillus and toward the underlying terminal web.

on the organization of the actin filaments in intestinal microvillar bundles.

Polarity of the Cross-filaments

An intriguing observation in this study is the polar orientation of the cross-filaments with respect to the core bundle. Because the cross-filaments point away from the tips of the microvilli just as the arrowheads of S1 decorated microvilli, the cross-filaments serve as *in situ* markers for microvillar polarity. This orientation could be exploited by those studying the assembly of actin onto actin bundles where it is necessary to know the polarity of the actin in the bundle. With the cross-filaments present, there would be no need to add exogenous polarity markers such as the myosin head fragments HMM or S1.

The cross-filaments are similar to S1 or HMM in two respects. First, both are released from actin by ATP (18). Second, the cross-filaments are oriented with respect to the bundle with the same polarity as S1. In light of these similarities, one could ask whether these arrowheadlike structures are proteolytic fragments of myosin from the terminal web. This possibility is remote since cross-filaments which are attached to the membrane still retain their canted orientation. In addition, immunocytochemical studies have shown that myosin antibodies stain only the terminal web and not the microvilli (5, 13, 16, 22). Finally, freeze-fracture studies (19, 21, 23) have amply documented the presence of the cross-filaments linking the core bundle to the membrane.

Cross-filament-Membrane Attachments

Although freeze-fracture studies have failed to demonstrate any correlation between the distribution of membrane particles and the underlying arrangement of the cross-filaments, the possibility still exists that such interaction might be undetected for several reasons. If the membrane particles are small, then the build-up of metal during shadowing might obscure these particles. Also, the particle distribution might be affected by external factors such as the Mg^{++} concentration as shown by Tilney and Mooseker (27) or by fixation and glycerol treatment before freezing (9).

Our observations suggest that membrane/cross-filament interaction can exist since the cross-filaments are associated with electron-dense patches on the inside surface of the membrane. Through interaction with the cross-filaments, the underlying actin filaments could play a role in controlling the organization of membrane proteins. Such actin/membrane interactions have been reported for the H-2 antigen (17) and the capping of membrane receptors (10).

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