

A THIN-SECTION AND FREEZE-FRACTURE STUDY OF MICROFILAMENT-MEMBRANE ATTACHMENTS IN CHOROID PLEXUS AND INTESTINAL MICROVILLI

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ABSTRACT

Choroid plexus and intestinal microvilli in thin sections have microfilaments in the cytoplasm adjacent to the membranes, and in replicas have broken strands of filaments in both cytoplasm and on E faces of plasma membranes. The microfilaments contain actin as indicated by their binding of heavy meromyosin (HMM). In sections of choroid plexus, the microfilaments are 7–8 nm in diameter and form a loose meshwork which lies parallel to the membrane and which is connected to the membranes both by short, connecting filaments (8×30 nm) and dense globules (~15–20 nm). The filamentous strands seen in replicas are ~8 nm in diameter. Because they are similar in diameter and are connected to the membrane, these filamentous strands seen in replicas apparently represent the connecting structures, portions of the microfilaments, or both. The filamentous strands attached to the membrane are usually associated with the E face and appear to be pulled through the P half-membrane.

In replicas of intestinal brush border microvilli, the connecting strands attaching core microfilaments to the membrane are readily visualized. In contrast, regions of attachment of core microfilaments to dense material at the tips of microvilli are associated with few particles on P faces and with few filamentous strands on the E faces of the membranes.

Freeze-fracture replicas suggest a morphologically similar type of connecting strand attachment for microfilament-membrane binding in both choroid plexus and intestinal microvilli, despite the lack of a prominent core bundle of microfilaments in choroid plexus microvilli.

KEY WORDS choroid plexus · intestine · brush border · microvilli · freeze-fracture · microfilaments · membrane

Filaments, which have diameters between 4 and 8 nm and indeterminate lengths, are prominent components of the peripheral cytoplasm of many

cell types and are commonly named microfilaments (27). Microfilaments contain F-actin, as indicated by their ability to bind heavy meromyosin (HMM) and subfragment I in a manner similar to purified F-actin; specifically, they produce “arrowhead complexes” whose formation is inhibited by ATP or pyrophosphate (13, 23).

Filaments with a larger diameter, 10–12 nm, lack this ability to bind heavy meromyosin (13). Microfilaments attach directly or indirectly to the cytoplasmic surface of plasma membranes, examples of which occur in certain types of cell-to-cell junctions, i.e., 70F-macula adherens in cultured cells (11, 18–20), zonula adherens in intestinal epithelial cells (29), and fascia adherens in the intercalated disk of cardiac muscle (10, 20).

The nature of the interaction between microfilaments and membranes has been the subject of several studies (8, 23, 25, 26, 34, 36, 37). At the fascia adherens, the actin filaments, i.e., “thin filaments,” of the terminal sarcomeres insert into a dense material, named the filamentous mat (15, 16), which coats the cytoplasmic surface of the plasma membrane. Similarly, in intestinal epithelium, terminal web microfilaments insert into dense filamentous material on the cytoplasmic surface of the membrane at the zonula adherens (29). The precise nature of the microfilament-membrane attachments has yet to be defined. The possibility that there may be several different types of microfilament-membrane attachments has not been emphasized often, but it should be considered because a dense, filamentous mat is notably absent in some regions of microfilament-membrane attachment (26).

To learn whether freeze-fracture techniques can be used to characterize the attachment of microfilaments to membranes, a systematic examination was made of fine filaments that are seen to bridge between cytoplasm and membrane faces in replicas. Several authors have obtained freeze-fracture images which suggest a visualization of microfilaments attached to membranes either directly or by intermediary filaments (15, 16, 23, 24). However, these observations have been puzzling because a detailed interpretation of how such images are produced has not been possible. In this manuscript, the microfilament-membrane attachments in guinea pig choroid plexus microvilli and intestinal microvilli are compared in thin sections and freeze-fracture replicas.

MATERIALS AND METHODS

Tissue

Specimens of choroid plexus were excised from the ventricles of outbred male albino guinea pigs (500–700 g) anesthetized with sodium pentobarbital. After craniotomy and rapid excision, portions of choroid plexus were fixed for 4 h in 2% glutaraldehyde-2% formalde-

hyde in 0.1 M sodium cacodylate buffer, pH 7.4 at 20°C. Other portions were rapidly processed unfixed by the freeze-fracture technique.

Segments of intestine were removed from male Balb/c mice (20–40 g) or Wistar rats (400 g) which were given water and Purina Chow (Ralston Purina Co., St. Louis, Mo.) ad libitum before sacrifice by cervical dislocation or decapitation. The intestine was removed quickly and placed in Hanks' balanced salt solution (BSS) in an ice bath. Segments were rinsed in cold BSS and were cut into rings, each 5–10 mm in thickness. Contraction of the longitudinal smooth muscle layer caused the edges of the rings to roll back, exposing the villi at the edge to the free bathing solution. The tissues were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 30 min at 0°C and processed for thin-section and freeze-fracture study. Some specimens were freeze-fractured without prior fixation.

Thin-Section Preparation Procedure

After glutaraldehyde fixation, the tissues were rinsed in 0.1 M sodium cacodylate buffer, pH 7.4, and fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 3 h at 4°C. The tissues were rinsed in 0.1 M veronal acetate buffer, pH 5.0, and stained en bloc with 1% uranyl acetate in veronal acetate buffer, pH 4.0, for 1 h at 20°C. The specimens were dehydrated in a graded series of ethanol-water mixtures and embedded in Epon 812 or Spurr's epoxy resin (35). Sections were stained with uranyl acetate and lead citrate and were photographed on a Philips EM 201 electron microscope.

Stereo pairs were obtained by photographing the same specimen area at +6° and –6° and preparing matching prints. The stereo images were examined in a CF-8 stereoscope (Abrams Instrument Corp., Lansing, Mich.).

Measurements were made on photographic prints at × 120,000 or 200,000 with an ocular micrometer. Electron microscope negatives were calibrated with a waffle grid with 54,864 lines per inch (Ladd Research Industries, Inc., Burlington, Vt.).

Freeze-Fracture Procedure

Except as indicated in the figure legends, the tissues were glycerinated to either 20 to 40% glycerol in 0.9% sodium chloride for 15 min at room temperature for fixed specimens, and at 0°C for unfixed specimens. Small portions were mounted on metal specimen carriers and frozen by immersion in monochlorodifluoromethane at –150°C. Some of the specimens were fractured in a Balzers BAF-301 freeze-etch machine (Balzers High Vacuum Corp., Santa Ana, Calif.) at –100°C. Replicas were prepared immediately after fracture by evaporation of platinum and carbon and retrieved by standard technique (17).

A few choroid plexus specimens were prepared with a modification of the brass block technique of Bullivant (4).

Several unfixed, unglycerinated specimens of intestine were frozen rapidly by pressing fresh intestine against the surface of a highly polished copper block at liquid nitrogen temperature according to the method of Dempsey and Bullivant (9).

HMM Labeling Studies

HMM was used as a test reagent (13, 28) for determining whether cytoplasmic microfilaments resembled fibrous actin (F-actin). Choroid plexus was rapidly removed and placed unfixed in a solution of 1% Triton X-100 in 0.1 M KCl, 0.005 M MgCl₂, and 0.006 M Na₂HPO₄ at pH 7.1 (hereafter called SPB). This detergent treatment was carried out at ice bath temperature for 15–30 min. The tissues were rinsed in SPB and either: (a) fixed in 2% glutaraldehyde-2% formaldehyde in 0.1 M sodium cacodylate, pH 7.4; (b) incubated overnight in 2 mg/ml HMM solution in SPB; or (c) incubated in HMM solution as above with the addition of 0.006 M sodium pyrophosphate.

Incubated specimens were rinsed in several changes of SPB and fixed for 30 min in the above aldehyde fixative at 0°C. This was followed by 30-min fixation in 1% osmium tetroxide in sodium veronal buffer, pH 7.2, initially at 0°C and then allowed to warm to room temperature. The tissues were stained with 1% uranyl acetate in veronal buffer, pH 4, for 30 min before dehydration and embedment in epoxy plastic as described above in the section on thin-section procedures.

RESULTS

Choroid Plexus

The apical surface of choroid plexus epithelial cells forms numerous pleomorphic microvilli which are called bulbous or clavate microvilli (1) (Fig. 1). These microvilli lack a well-developed core of parallel microfilaments and, instead, have a loose meshwork of microfilaments which are attached to the plasma membrane at numerous sites (Fig. 2). The microfilaments are ~7 nm in diameter in regions of minimal superimposition with other microfilaments. Microfilaments are often parallel to the membrane but are attached at dense globules (12–20 nm in diameter) which merge with the inner leaflet of the unit membrane (Fig. 3). Also, fine filaments, ~8 × 30 nm, connect the microfilaments to the membrane (Fig. 2).

The microfilaments present in the choroid plexus microvilli bind HMM to form decorated complexes (Fig. 4). This binding is inhibited by the addition of sodium pyrophosphate (Fig. 5). Choroid plexus microvilli are extremely fragile structures, which are easily disrupted by the brief

detergent treatment used to allow penetration of HMM. After detergent treatment, relatively few microfilaments survive osmium tetroxide fixation, unless protected by HMM binding, as previously noted by others (27).

In freeze-fracture replicas of choroid plexus, the pleomorphic and branched nature of the microvilli can be appreciated. On the P face, the plasma membranes contain numerous intramembrane particles ~10 nm in diameter, but few such particles are seen on the E face (Fig. 6), according to current terminology (3). In preparations processed without glutaraldehyde fixation, the E faces of the membranes have occasional short strands of material, 2.5–10 nm in diameter and 30–80 nm in length. These strands are most abundant where the fracture plane reveals cytoplasm at the edges of the E face (Figs. 6 and 7). At these cytoplasm-to-membrane transitions, the strands of material frequently have one end in the fractured cytoplasm and the other end on the membrane E face. The strands are not all oriented in the same direction, a feature which might be expected if they resulted from distortion due to the force of fracturing with the cold knife applied from a single direction. Instead, when different areas of the same replica are compared (Figs. 6 and 7), the strands may lie at a variety of angles relative to each other. At a given individual cytoplasm-to-E-face transition, the strands are oriented perpendicular to the edge of the E face. Some of the strands are uniform in diameter (~10 nm), and others vary in diameter by tapering to a fine point. When tapered, the fine point is usually on the E face of the membrane, but occasional strands are tapered toward the cytoplasm. Because membranes are split during freeze-fracture (2, 6), the complementary image for strands would be grooves. Consequently, replicas have been examined carefully for evidence of grooves at transitions from cytoplasm to P face, and such evidence has been lacking. Occasional strands are at transitions between P face and extracellular space, but this appears less frequently than the strands at E face and cytoplasm transitions (Fig. 8). Large grooves are not present in membrane P faces, in which the resolution is adequate to demonstrate small, punctate depressions (Fig. 8). In choroid plexus cells fixed in glutaraldehyde before freeze-fracture, these strands are not as prominent.

Intestinal Epithelium

In tissue processed for freeze-fracture within

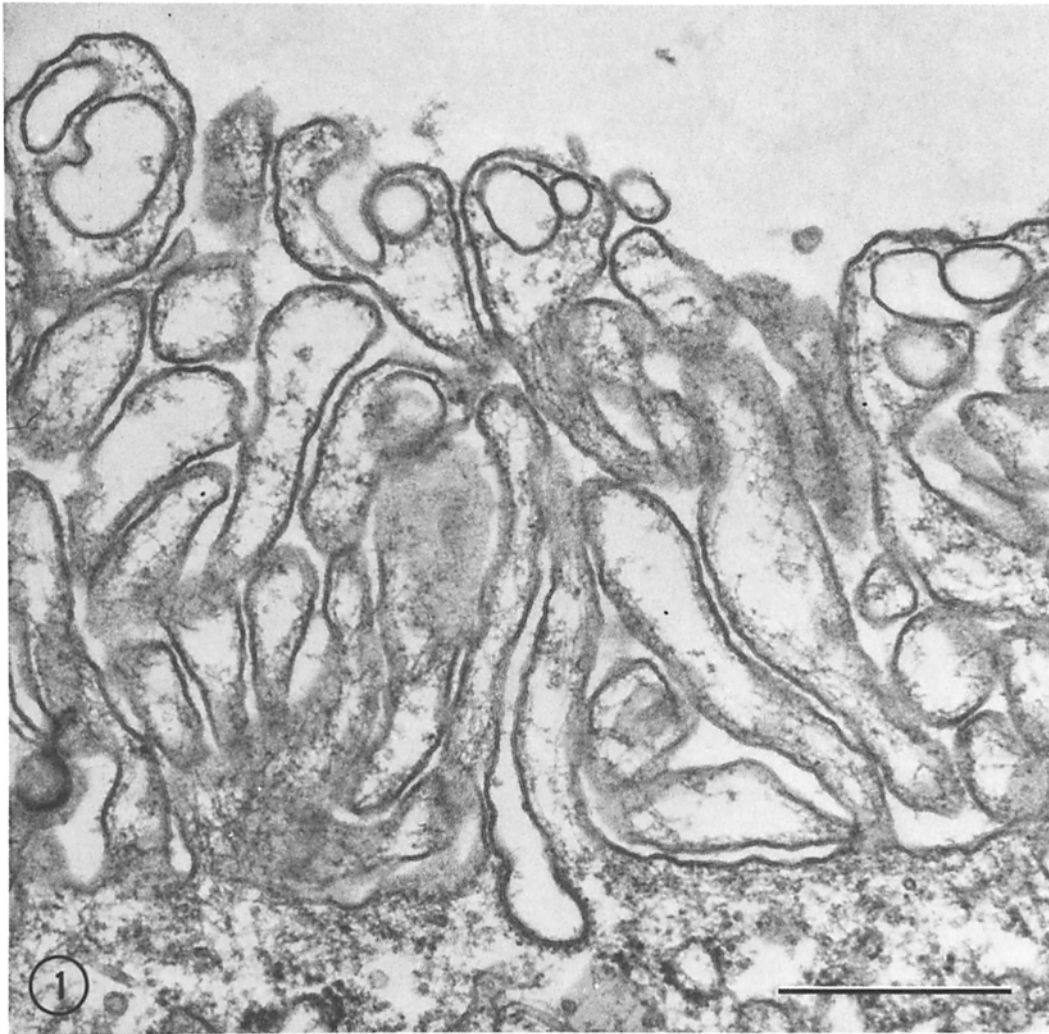


FIGURE 1 Pleomorphic microvilli of guinea pig choroid plexus epithelial cells. The microvilli branch and have a fusiform swollen appearance. The background cytoplasmic matrix is very electron-lucent, a feature which facilitates the study of the microfilamentous network within the microvilli. This tissue is from the same preparation as used for Figs. 6-8. Bar, $0.5 \mu\text{m}$. $\times 61,200$.

the first 1-2 h after removal from the animal, replicas reliably demonstrate the presence of broken strands of a filamentous component in the terminal web region and in microvilli of the intestinal epithelial cells (Figs. 9-11). As judged from the maximal diameter of the strands, the filaments are $\sim 8-10$ nm in diameter uncorrected for replica thickness, or 6-8 nm in diameter if corrected for a platinum cap 2-nm thickness (which was not measured directly but is estimated based on the resolution of the replicas). The filaments appear similar to those visualized in choroid plexus epithelium

in size as well as in distribution, i.e., at regions where the fracture plane passes from cytoplasm to plasma membrane so that it exposes the E face and on the E face of plasma membranes of the microvilli at a distance from cytoplasm. (Figs. 9 and 11). Also in the unfixed unglycerinated specimens prepared by rapid freezing on a copper block, the filamentous strands are prominent on both E faces of the membranes and at cytoplasm-to-membrane transitions (Fig. 11), indicating that the strands are not just a glycerination artifact. The core bundle of microfilaments usually is not

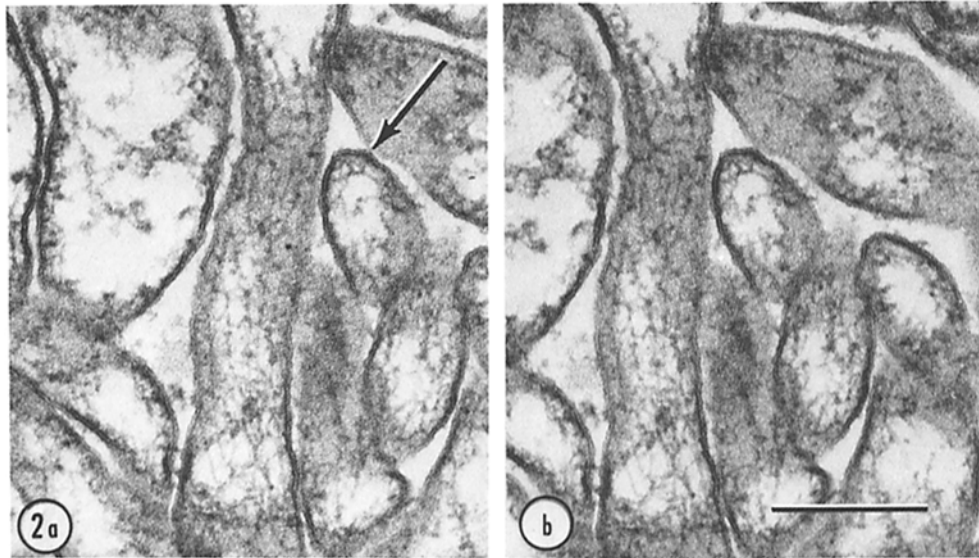


FIGURE 2 Stereo pair of electron micrographs illustrating the filamentous network beneath the plasma membrane of choroid plexus microvilli. The microfilamentous network has many branches and interconnections. In some regions, short filaments can be seen to attach the network to the plasma membrane (at arrow). Bar, $0.25 \mu\text{m}$. $\times 82,500$.

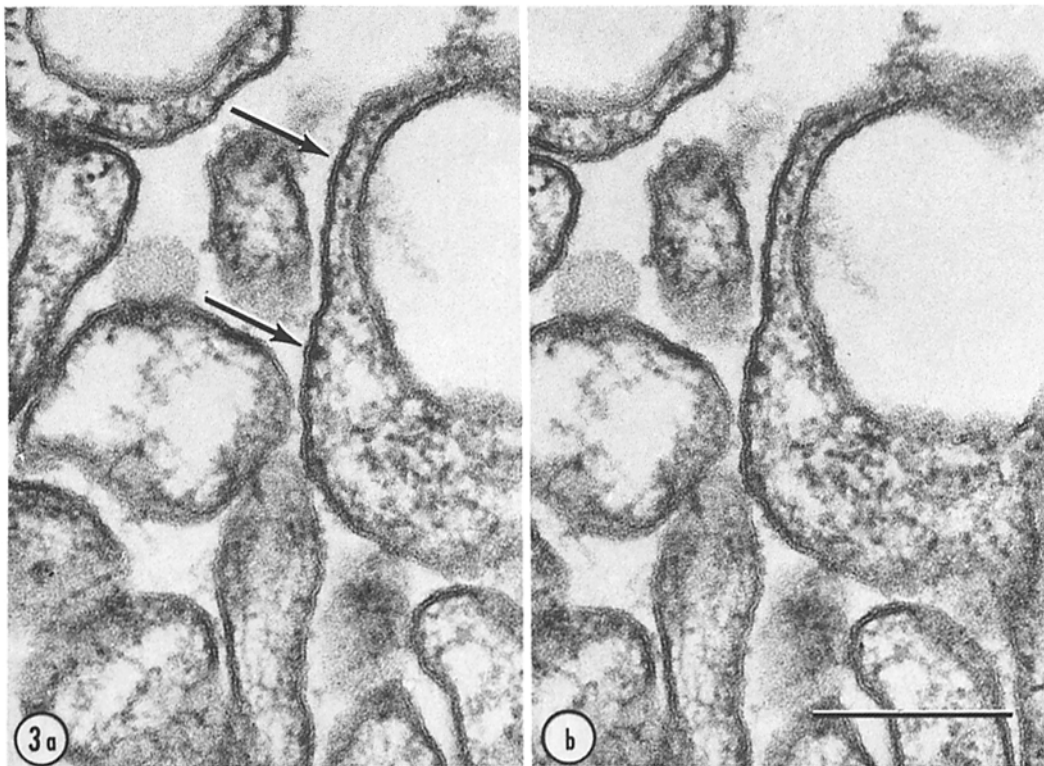


FIGURE 3 Stereo pair of electron micrographs showing dense globules (15–20 nm in diameter) attached to the cytoplasmic leaflet of the plasma membrane in choroid plexus microvilli. The filamentous network attaches to these dense globules (at arrows). Bar, $0.25 \mu\text{m}$. $\times 107,000$.

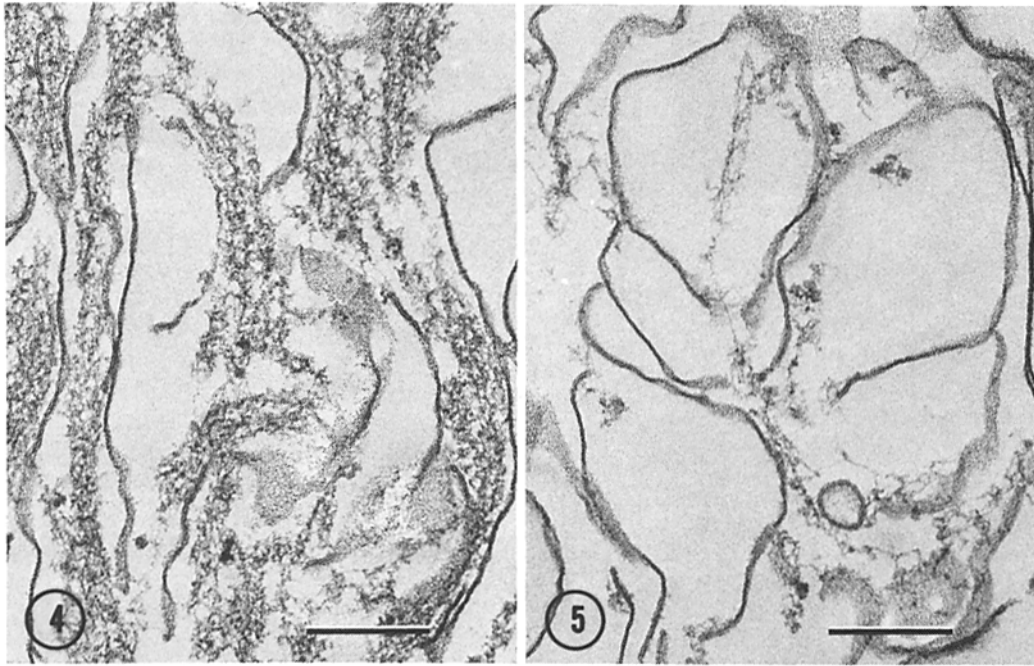


FIGURE 4 Detergent-treated choroid plexus microvilli contain numerous decorated filaments after exposure to HMM. Compare to Fig. 5. Bar, $0.25 \mu\text{m}$. $\times 64,300$.

FIGURE 5 Detergent-treated choroid plexus microvilli exposed to HMM plus pyrophosphate show inhibition of HMM binding by the microfilaments in the presence of pyrophosphate. Compare to Fig. 4. Bar, $0.25 \mu\text{m}$. $\times 64,000$.

visible as such in longitudinal fracture unless "etching" or ice sublimation has been allowed to occur before replication. In oblique fractures of microvilli, strands of filamentous material connect the central core regions of the microvilli to the E faces of the plasma membrane (Figs. 11 and 12), as previously described in etched preparations (23, 24). These connecting filaments closely resemble the filamentous strands described above in the terminal web cytoplasm and at cytoplasm-membrane transitions, as well as the strands observed in choroid plexus microvilli. Replicas of terminal web cytoplasm and microvillous membrane P faces do not contain grooves that could be complementary to the many filaments.

The ability to visualize the filamentous strands diminishes with increasing time of glutaraldehyde fixation (beyond 30 min for 2% glutaraldehyde) and with increasing time of storage in glycerol (beyond 4–5 h). Prolongation of these times results in a loss of filaments on E faces, in terminal web cytoplasm, and at cytoplasm–E-face transitions, so that most of the E faces appear to have

only a few large globular "intramembrane" particles (~ 10 – 12 nm in diameter), and the cytoplasm appeared finely granular. It is unclear whether the pleomorphic E-face particles are a separate structure or whether they represent only the broken ends of filamentous strands attached to the membrane.

In confirmation of the results of Mooseker and Tilney (23), we also observed that there were fewer intramembrane particles on the P faces of membranes at the tips of the microvilli than along the sides of the microvilli. Also, in our preparations, the E faces of the membranes had very few particles and strands at the tips of the microvilli (Figs. 11 and 12).

DISCUSSION

Despite differences in the overall organization of choroid plexus and intestinal microvilli as seen in thin sections, the freeze-fracture replicas show remarkable similarities in the appearance of filamentous strands in the cytoplasm, at cytoplasm-

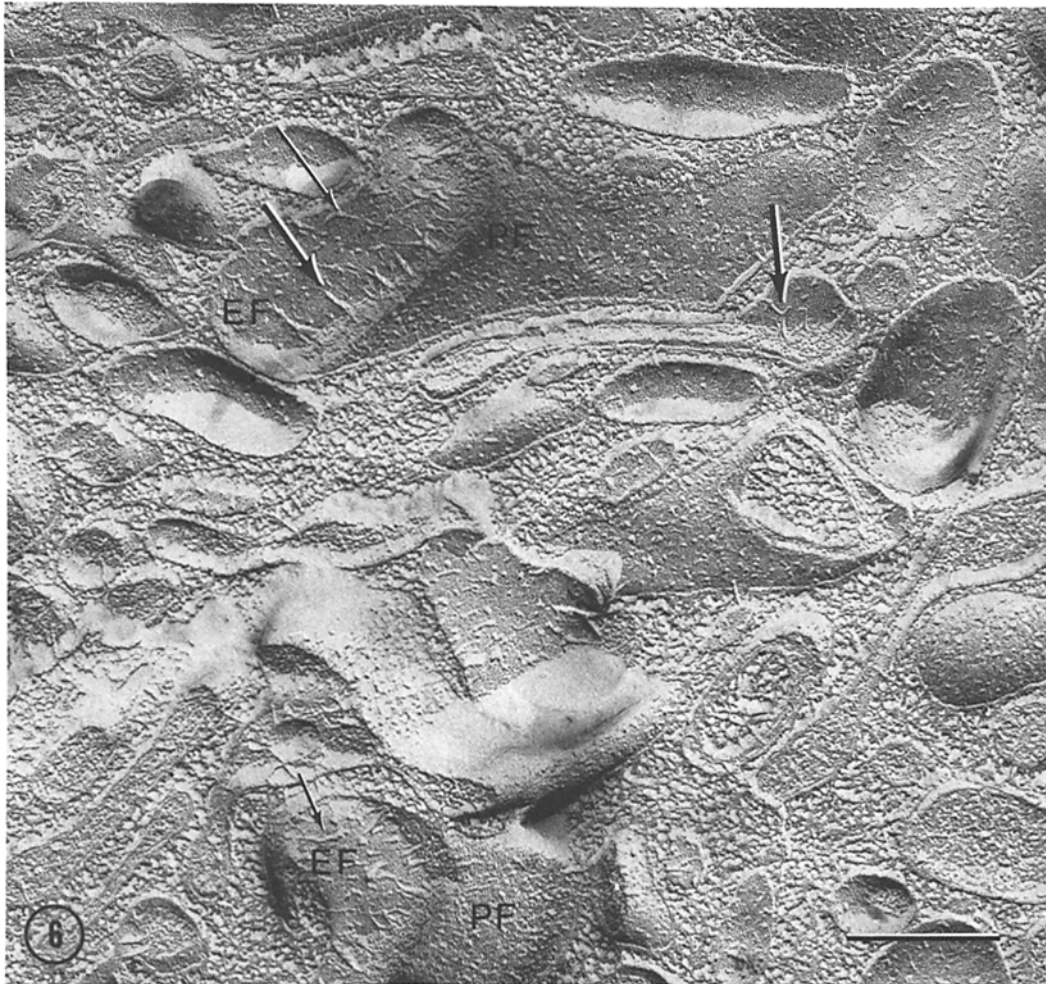


FIGURE 6 Freeze-fracture replica of choroid plexus microvilli (unfixed, 20% glycerol, replicated at -105°C). Numerous filamentous strands interconnect cytoplasm and the E face (EF) of the plasma membrane (at arrows). P face (PF) images lack grooves although small pits are resolved. Occasional E face strands branch (at asterisk) in a manner resembling the thin sections of the filamentous network (see Fig. 2). Also some strands appear on E faces at a distance from cytoplasm (small arrows). Note that the filamentous strands are not all oriented in the same direction. Bar, $0.25\ \mu\text{m}$. $\times 80,000$.

to-membrane transition zones, and on membrane E faces in both types of microvilli.

In the choroid plexus microvilli, the morphology of the association of microfilaments with membrane can be studied somewhat more easily than in the intestine because the microfilaments are not organized into a closely packed core bundle and because the clavate microvilli have a background cytoplasm of low staining density. The microfilament-membrane associations are studied best in stereo pairs of electron micrographs to reduce the effects of superimposition of

images within the section thickness (26). In favorable sections, the microfilaments are seen to attach to discrete, dense globules and short, connecting filaments which merge with the inner leaflet of the unit membrane. Similar attachment of microfilaments to dense globules on the inner surface of the membrane has been observed by Kidd in studies of the isolated plasma membrane of eggs of the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* (14). In Kidd's electron micrographs, two sizes of globules were visualized, 11–14 nm and 40–44 nm, where mi-

crofilaments attached to membranes. The smaller variety of globules was more frequently observed and is approximately the same size as the globules found in the choroid plexus. Kidd was unable to observe these structures in intact sea urchin eggs, possibly due to the obscuring effect of cytoplasmic background. Our success with choroid plexus clavate microvilli is probably due to the very low background density of the cytoplasm, which may result from bulk flow of cerebrospinal fluid secretion across the microvillar border (21, 30). Similar submembranous filaments have been shown in platelets with decreased background cytoplasmic staining after hypotonic shock (39).

By examining choroid plexus specimens with

both thin-section and fracture techniques, we hoped to learn whether the microfilament-membrane attachments are represented in freeze-fracture replicas. In these microvilli, striking freeze-fracture images are obtained which show filamentous strands of varying lengths attached to E faces and in a few instances to P faces as well. These filaments are 5–10 nm in diameter, uncorrected for replica thickness. The filamentous strands are predominantly on E faces or are attached at one end to E faces. Because the E face is the exposed aspect of the E half-membrane or outer half-membrane (3), it is puzzling why the filaments are not broken off by P half-membranes which would be interposed between the E half-membrane and



FIGURE 7 Freeze-fracture replica of apical cytoplasm and microvilli of choroid plexus epithelium (unfixed, 20% glycerol, -105°C). Numerous filamentous strands connect cytoplasm to the E face (EF) of the microvillar membrane (at arrows). Note that the filamentous strands are always perpendicular to the edge of the E face but lie at a variety of angles relative to each other when different areas of the replica are compared. A few similar filaments are seen lying on the E face and apparently isolated from cytoplasm (at asterisk). Bar, $0.25\ \mu\text{m}$. $\times 82,000$.



FIGURE 8 High magnification of an unusual area of choroid plexus microvilli in which the replica shows filamentous strands which have nontapered ends on the P face (PF) of one microvillus. Some of these strands have tapered ends lying on the E face (EF) of the membrane of an adjacent microvillus (at arrow). Such images suggest that the filamentous strands become exposed for replication by a process called plastic deformation (see text). Bar, $0.25 \mu\text{m}$. $\times 150,000$.

the cytoplasm. Because this type of freeze-fracture image has not been discussed extensively in previous publications, several possible interpretations must be presented here.

One possible hypothesis regarding E-face strands and P half-membranes would be that there is no real relationship between them. This situation might arise if one of the initial phases of nucleation and condensation of water vapor or other contaminants in the vacuum system produced interconnections between particles which would then become fused into strands. Although this is a theoretical possibility that is almost impossible to exclude, it seems highly unlikely to be the explanation because (a) the replicas lack other evidence of water vapor contamination, (b) the strands are present on E faces of membranes replicated in either the Balzers apparatus or in the Bullivant cold-block device, and (c) it is difficult to understand how any such contamination would vary with glutaraldehyde fixation.

Another hypothesis might be that the strands are pulled artifactually out of the cytoplasm and

simply flop down onto the E face of any membrane nearby or onto the fractured surface of the cytoplasm. If the filaments spanning the E face and cytoplasm are pulled out by the force of the knife, the filaments might be expected to be oriented parallel to the direction of fracturing. Because these filaments are found oriented at various angles relative to each other in different areas of the replica, it is difficult to comprehend



FIGURE 9 Low magnification electron micrograph of a freeze-fracture replica showing the microvilli and apical cytoplasm (brush border) in control unincubated mouse intestine (unfixed, 40% glycerol, replicated at -100°C). Numerous segments of filamentous strands are visible (at arrows) in the apical cytoplasm, which corresponds to the terminal web region in thin sections. Also portions of similar filamentous strands are present on the E face (EF) of the microvillar membranes and are often oriented circumferentially on the microvilli (at asterisk). Bar, $0.5 \mu\text{m}$. $\times 55,000$.

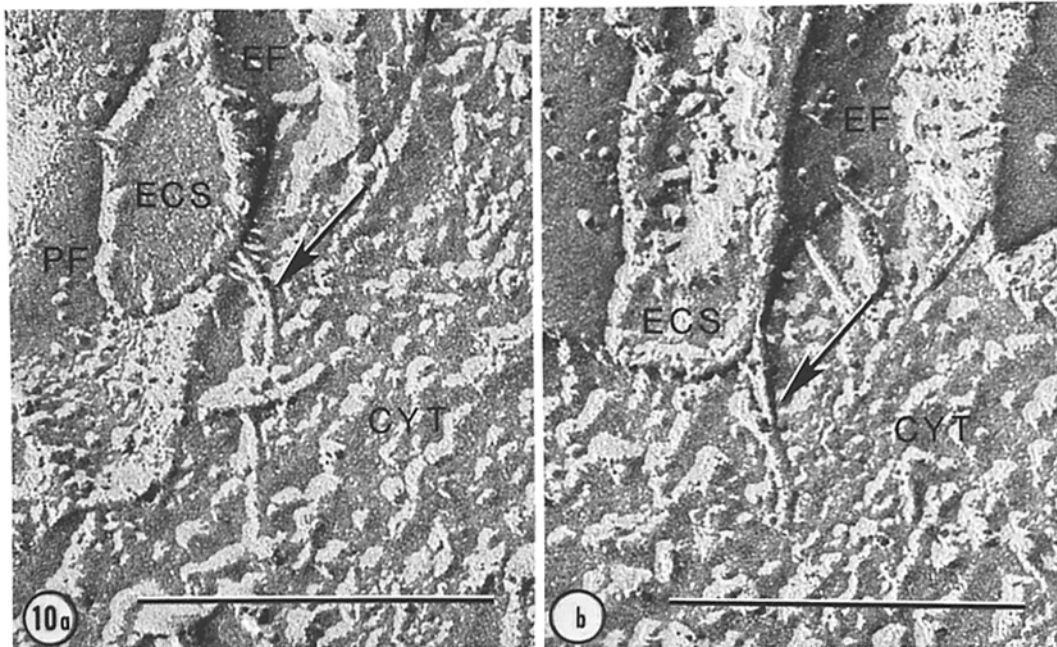


FIGURE 10 High magnification of terminal web cytoplasm showing filamentous strands, which are particularly evident at the edges of plasma membrane E face (*EF*) adjacent to cytoplasm. The cytoplasm also has similar filaments at a distance from the E face. Fig. 10*a* and *b* are filaments revealed by etching so that extensive lengths are visible (at arrows). In both Fig. 10*a* and *b*, the insertion of filaments at the membrane edge can be seen. P face (*PF*), E face (*EF*), extracellular space (*ECS*), and cytoplasm (*CYT*) are shown. Bars, 0.25 μm . (*a*) $\times 190,000$; (*b*) $\times 190,000$.

how a shearing force might produce such images. This hypothetical explanation might be true for some of these filaments which have one end on cytoplasm and one end on E face. Indeed, such a mechanism appears to be the explanation for the observation of collagen fibrils which have one end lying on the P face of fibroblast membranes in connective tissues (7). Also, fibrils which connect the cell wall to the plasmalemma P face of *Saccharomyces cerevisiae* probably are pulled out of the inner portion of the cell wall and fall onto the P face of the membrane (31). However, such a hypothetical mechanism would fail to account for the filaments seen entirely on E faces in this study. The similarities in size and occurrence in preparations suggest a common derivation for filaments on E faces, at E face-to-cytoplasm transitions and on fractured cytoplasm.

Deformation is very likely to be involved in the production of the filaments seen on E faces. This is evident in the lack of exact complementarity of P and E faces of the membranes. The complement of E-face filaments would be P-face grooves, which are not observed. Because the shape of the filaments may be molded by the fracturing proc-

ess, the name "plastic deformation," is commonly applied to such a mechanical distortion in shape (31). Plastic deformation has been shown to affect the freeze-fracturing of polystyrene spheres, so that the spheres are pulled into elongated conical shapes (7, 31). Plastic deformation is also used to refer to the pulling of collagen fibers onto P faces of membranes (7) and the pulling of myosin molecules out of sarcoplasm (5). However, in some cases, the name "plastic" may be inappropriate, because the diameter of the collagen fiber remains approximately constant along the exposed length, which indicates that it has undergone slippage relative to the surrounding frozen matrix rather than intra-assembly slippage or intramolecular unravelling of the tertiary structure of the proteins. The fact that some of the E-face filaments in this study have tapered ends implies that there has been intramolecular slippage or unravelling during the plastic deformation.

The fracturing properties of some proteins are dependent on the exact angle at which the fracture takes place relative to the protein bonding forces. Willison (38) has demonstrated a good example of this phenomenon in the fracture of crystals of

the "fraction 1" protein from chloroplasts. When these crystals were fractured through a hexagonally packed axis, there was no evidence of plastic deformation. In contrast, when the crystals were fractured along an axis of square packing, long, wormlike rods of protein were pulled up during the fracture and exposed for replication. These observations imply that the study of conditions under which proteins exhibit plastic deformation may provide data on the types and strengths of bonding forces holding the protein in place. This suggestion may relate to the observations made in the present study that the filamentous strands were frequently oriented perpendicular to regions

of transition where the fracture plane passed from cytoplasm to an intramembrane plane to reveal its E face. In these regions the fracture plane would have an orientation close to parallel to the filament axis.

Sleytr and Umrath (32, 33) have demonstrated that plastic deformation is responsible for the appearance of small fibrils connecting the cell wall and P face of *Saccharomyces cerevisiae*. These look remarkably similar to the fine strands connecting cytoplasm and E face in the present study. Complementary replicas failed to show any complementary grooves at the edges of the E faces in *S. cerevisiae* but did not settle the question of how



FIGURE 11 Microvilli of intestinal epithelium rapidly frozen without pretreatment. Long, filamentous strands attach central core regions to the E faces of the microvillar membrane (at arrows). Tips (*T*) of microvilli appear free of such strands. *Inset* shows strands which attach cores of microvilli to the membrane at higher magnification. *CYT*, cytoplasm. Bar, 0.25 μm . $\times 94,700$; *inset*, $\times 112,300$.



FIGURE 12 Replica of obliquely fractured mouse intestinal microvilli which demonstrates filamentous strands on E faces of microvillar membranes and connecting E faces to cytoplasm within the microvilli (at arrows). At the tips (T) of the microvilli, there are few particles or strands on the E face. (Unfixed, 40% glycerol, -100°C .) Bar, $0.25\ \mu\text{m}$. $\times 74,000$.

these fibrils arise (32). If there are cytoplasmic filaments attached to membrane, as in choroid plexus, which can be visualized in thin sections, then similar filaments in freeze-fracture replicas are likely to represent the same structure, even if somewhat deformed during the fracture process. Because deformation of globular intramembrane particles 8 nm in diameter cannot be expected to create filaments up to 100 nm in length and 7 nm in diameter, the additional material needed for the filament must come from some tissue compartment. The cytoplasm contains such filaments and is the probable source of the filamentous strands in these freeze-fracture replicas.

To this point, it may be concluded that the production of E-face filaments appears to involve membrane splitting and plastic deformation. E-face filaments may represent segments of cytoplasmic filaments pulled through the P face because of their attachment to structures in the E half-membrane. This interpretation is supported by the

occasional observation of filaments with a broad end on the P face and a tapered end at a distance, as shown in Fig. 8. Although this may be the proper explanation, it remains to be elucidated what type of binding would be responsible for such strong linkage of cytoplasmic filaments to the E half-membrane.

Because the structures and chemical compositions of the filamentous components of intestinal microvilli have been investigated thoroughly by others (22, 23), this study has attempted to relate the freeze-fracture appearance of these microvilli to what is known about their composition. Freeze-fracture of intestinal epithelium is capable of demonstrating an abundance of filamentous strands in the terminal web cytoplasm, on E faces of microvillar membranes, and at transition zones where the fracture plane passes from cytoplasm to reveal the membrane E face, as also was previously found in choroid plexus microvilli. The filamentous strands were observed in unpretreated tissue

frozen directly and could be preserved in tissue after certain conditions of glutaraldehyde fixation and glycerination before freezing.

At least two types of microfilament membrane attachments have been described in thin-section studies. In previous studies of microfilament attachments to membranes, Pollard and Korn (26) noted that the microfilaments attached to the inner leaflet of the plasma membranes of *Acanthamoeba castellanii* but they did not observe any dense specialization at the sites of attachment. In contrast, perpendicular attachments of actin filaments to membranes in mammalian cells are often associated with a cytoplasmic dense material in the zone of attachment, e.g., in myocardial cells (15). In intestinal microvilli, two types of microfilament-membrane associations have been defined: perpendicular attachments with dense material at the tips of microvilli and parallel attachments without dense material along the sides of the microvilli. In the parallel type, microfilaments lie parallel to the plane of the membranes and are separated by an interspace which is spanned by fine filaments which have been called "connecting filaments" (24), "cross bridges," or "lateral filaments" (23). On the basis of high magnification studies of standard thin sections, Mukherjee and Staehelin (24) concluded that the connecting filaments were probably not actin, because they were unable to demonstrate in thin sections any periodicity similar to the 5-nm periodicity seen in actin filaments that have been negatively stained. Moesker and Tilney (23) state that the cross bridges are probably alpha-actinin; this conclusion was based on unpublished immunocytochemical evidence that alpha-actinin is present along the lateral borders of the microvilli as well as at their tips (23) and on negative stain evidence that purified alpha-actinin is a rod-shaped molecule, 30×2 nm in size. Other studies raise the possibility that actin binds directly to membrane phospholipids, as shown by Ostlund et al. (25). They found that actin bound to the membrane of pituitary secretion granules in vitro by a mechanism which was not trypsin-sensitive and may not involve an intermediary protein.

In previous studies, freeze-etch images have been shown that apparently represent the connecting filaments (24) or cross bridges (23). However, whether these structures become exposed by fracturing or etching was not studied. Also, Isenberg et al. (12) isolated cytoplasmic veins from *Physarum polycephalum* that contain acto-

myosin fibrils and demonstrated by freeze-etching that a filamentous component could be visualized in the replicas. They speculated that the replicated strands were F actin; however, they did not identify the filamentous component or relate it to the plasma membrane.

What appears significant in this study is that the freeze-fractured filamentous strands are in terminal web cytoplasm that is rich in microfilaments and on E faces of membranes in regions of microvilli where microfilaments are attached to the membranes by means of connecting filaments (24) or cross bridges (23). Because the strands were not visualized on E faces at the tips of microvilli, they appear to be unrelated to the type of attachment of microfilaments to a dense plaque of material on the cytoplasmic surface of the membrane, as is seen at the tips of the microvilli. The fact that freeze-fracture shows these two regions of attachment differently is further evidence that there are several types of microfilament-membrane attachments and that freeze-fracture may be useful for the study of one of them, namely the cross-bridge type.

It is tempting to speculate that the differences in organization between choroid plexus microvilli and intestinal microvilli are related to the differences in the degree of shape changes which these structures undergo. Choroid plexus microvilli are capable of dramatic shape changes in response to increased secretory demand (30), whereas intestinal brush borders are rather rigid structures which survive homogenization and isolation procedures (22, 23). Perhaps the connecting filament type of attachment which predominates in choroid plexus microvilli, as well as the lack of a tightly bundled core of microfilaments, allows the membrane to undergo the requisite shape changes.

The question of whether the microfilaments are attached to a separate intramembrane particle is not definitively resolved by this study. In some replicas the connecting filaments appear to attach to a pleomorphic intramembrane particle on the E face (e.g., inset of Fig. 11). However, such particles are not clearly recognizable as different structures from the connecting filaments themselves. Biochemical studies directed toward the identification of these structures are needed to resolve these components.

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