The Spectrin-related Molecule, TW-260/240, Cross-links the Actin Bundles of the Microvillus Rootlets in the Brush Borders of Intestinal Epithelial Cells

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ABSTRACT   Previous studies have shown that molecules related to erythrocyte spectrin are present in the cortical cytoplasm of nonerythroid cells. We report here the localization by immunoelectron microscopy of one such molecule, TW-260/240, in the brush border of intestinal epithelial cells. Using highly specific antibodies against TW-260 and TW-240 as well as antibodies against fodrin, another spectrinlike molecule, we have found that the TW-260/240 molecules are displayed between rootlets at all levels of the terminal web. Occasionally, extended structures appear labeled suggestive of the fine filaments known to cross-link actin bundles. These results are in line with previous in vitro studies showing that TW-260/240 binds to, and cross-links, actin filaments. The results are discussed in terms of a model in which rootlets are immobilized in the terminal web in a matrix of TW-260/240.

Recent studies have established the existence of spectrin-related molecules in nonerythroid cells (1-7). Made up of two nonidentical subunits, they are termed TW-260/240 (260 kdaltons and 240 kdaltons, isolated from brush borders of chicken intestinal epithelial cells (2), and fodrin (240 kdaltons and 235 kdaltons) isolated from brain. The similarity between these molecules and erythrocyte spectrin has been documented by immunological cross-reactivity (1, 3, 6, 7, 7a), limited proteolytic digestion patterns (1, 3), and electron microscopy (2, 3, 4, 7, 7a). Not only are the 240-kdalton subunits of spectrin, fodrin, and TW-260/240 structurally similar, but they have been shown to bind calmodulin calcium dependently (2, 3, 5) and therefore account for one of the major calmodulin-binding activities found in a variety of tissues (5). All three proteins have been characterized as elongated, flexible, double-stranded molecules that bind to, and cross-link, actin filaments (2, 4, 7). Immunofluorescence microscopy reveals that spectrin-related molecules are present in the cortical cytoplasm of many cell types (2, 6, 8). This finding correlates well with immunoelectron microscopic observations showing that erythroid spectrin appears confined to the membrane (9). Given the current interest in membrane anchorage of microfilaments and the possible role of spectrin-related molecules in these interactions, we have, by using immunoelectron microscopy, analyzed the distribution of one such molecule, TW-260/240, in isolated brush borders of intestinal epithelial cells.

The brush borders of intestinal epithelial cells are a particularly useful system for the study of microfilament architecture. They can be isolated free of other organelles and retain most of the filamentous organization observed in situ. The catalogue of most major cytoskeletal proteins present in the isolated brush border is now well understood (10), and highly improved electron microscopic images are available by the quick-freeze, deep-etch, and rotary replication method (11, 12). Here we show that TW-260/240 molecules are situated between the microvillus core rootlets cross-linking neighboring actin bundles. They are found at all levels of the terminal web. This finding is in line with the known actin-binding and cross-linking activity of TW-260/240 found in vitro (2). We propose that TW-260/240 molecules account, at least in part, for the fine filaments previously observed connecting neighboring rootlet structures.

MATERIALS AND METHODS

Antibodies to TW-260/TW-240 and Fodrin: Chicken TW-260/240 and chicken fodrin were purified as described (2). Further purification was accomplished by preparative SDS-gel electrophoresis using 4% gels. After electrophoresis was completed, protein bands were visualized by exposure to a cold 0.3 M KCl solution (13). The bands were excised and the proteins electrophoretically eluted into dialysis tubing and used for antibody preparation in rabbits. This provided separated TW-260 and TW-240 polypeptides, whereas no attempt was made to fractionate the 240-kdalton and 235-kdalton polypeptides of fodrin. Since some anti-TW antibody preparations showed reactivity with both TW-260 and TW-240 (3), we cross-absorbed the antibodies on Sepharose-conjugated TW-260 or TW-240 or fodrin. Briefly, anti-TW-260 antiserum was first applied to a column of immobilized fodrin. The unretarded flow-through fractions that were depleted in TW-240 reactivity were then applied to a column of TW-260 bound to Sepharose. Specific antibodies were released with neutral 4 M MgCl₂ and dialyzed into PBS. Similarly, anti-240 antisera were applied first to a TW-260-Sepharose column, and the flow-through was subsequently fractionated

on a column of TW-240-Sepharose eluting again with MgCl₂. Antifodrin antibodies were isolated by affinity chromatography on fodrin-conjugated Sepharose as described above. All affinity columns contained 300–500 μg protein bound per ml of settled Sepharose 4B supplied in the activated form (Pharmacia Fine Chemicals, Piscataway, NJ). Cross-absorbed antibodies at 10 μg/ml were tested for specificity by the immune blotting procedure using electrophoretic transfer (14) of total brush border cytoskeletal proteins prepared as described (15). Thus, three purified antibody preparations were used: anti-TW-260, anti-TW-240, and antifodrin (240/235).

Immunoelectron microscopy: Chicken intestinal brush borders (15) were fixed with a Triton X-100/glutaraldehyde mixture and further reduced with NaBH₄ (Sigma Chemical Co., St. Louis, MO) as described previously (16) with minor modifications. Briefly, brush borders were treated at room temperature with a solution of 10 mM imidazole pH 7.3, 75 mM KCl, 0.1 mM MgCl₂, 1 mM EGTA (solution I) containing 0.1% Triton X-100 (Sigma Chemical Co.) and 0.1% glutaraldehyde (Polysciences, Inc. Warrington, PA). After 15 min, brush borders were collected by centrifugation (12,000 g, 5 min) and washed two times with solution I containing 10 mM NaBH₄. Brush borders were washed two times with PBS containing 1 mM EGTA and incubated with the affinity-purified antibody at 75 μg/ml in PBS containing 1 mM EGTA and 1 mg/ml BSA, or with PBS/BSA/EGTA solution without antibody. After 1 h of incubation at 37°C, brush borders were washed two times with PBS/EGTA by low-speed centrifugation and further incubated for 1 h at 37°C with 500 μg/ml ferritin-conjugated sheep anti-rabbit IgG (a gift from Dr. D. Henderson, Max-Planck-Institute) in PBS/EGTA/BSA. Brush borders were washed five times with PBS/EGTA as before and finally fixed as pellet in 2% glutaraldehyde in 50 mM cacodylate, 75 mM KCl, 0.1 mM MgCl₂, 1 mM EGTA, pH 7.3, at 4°C overnight. Pellets were postfixed in 0.5% OsO₄ for 20 min on ice and processed by standard EM procedures.

RESULTS

Characterization of Antibodies

In previous studies we showed that antibodies against chicken TW-260 or TW-240 often displayed cross-reactivity between both polypeptides (2, 3). To insure that the antibody preparations were as specific as possible for the polypeptide of interest, we cross-absorbed the antisera on the corresponding immobilized polypeptides before the isolation of antigen affinity-purified antibodies (see Materials and Methods). The preparations obtained showed a marked specificity for only one polypeptide (Fig. 1). When such purified antibodies were tested on brush border cytoskeletal proteins electrophoretically transferred to nitrocellulose, anti-TW-260 antibodies reacted strongly and almost exclusively with TW-260, and anti-TW-240 antibodies showed a similar preference for TW-240 (Fig. 1). As reported previously, antifodrin antibodies cross-reacted significantly with the TW-240 polypeptide (Fig. 1), presumably due to the highly related calmodulin-binding 240-kdalton subunit present in TW-260/240 and fodrin (3). Both antifodrin and anti-TW-240 antibodies also reacted with a minor component of 140 kdaltons, which is present in varying amounts even in purified TW-260/240 preparations and is thought to arise by proteolytic degradation of the TW protein (2).

Localization of TW-260 and TW-240 Antigens in Isolated Brush Border Preparations

Isolated chicken brush border preparations retain a significant amount of structural organization (Fig. 2). Particularly striking is a system of fine filaments displayed between the well-preserved microvillus core bundles in the terminal web. In order to preserve the structure for antibody localization experiments, we lightly fixed brush borders with a mixture of Triton and glutaraldehyde followed by a treatment with NaBH₄ to reduce free aldehyde groups and Schiff's bases. Control experiments showed that this treatment performed in a buffer known to stabilize brush borders during their isolation had no effect on the pattern of TW antigens in intestinal epithelial cells or various cultured cells when assessed by immunofluo-
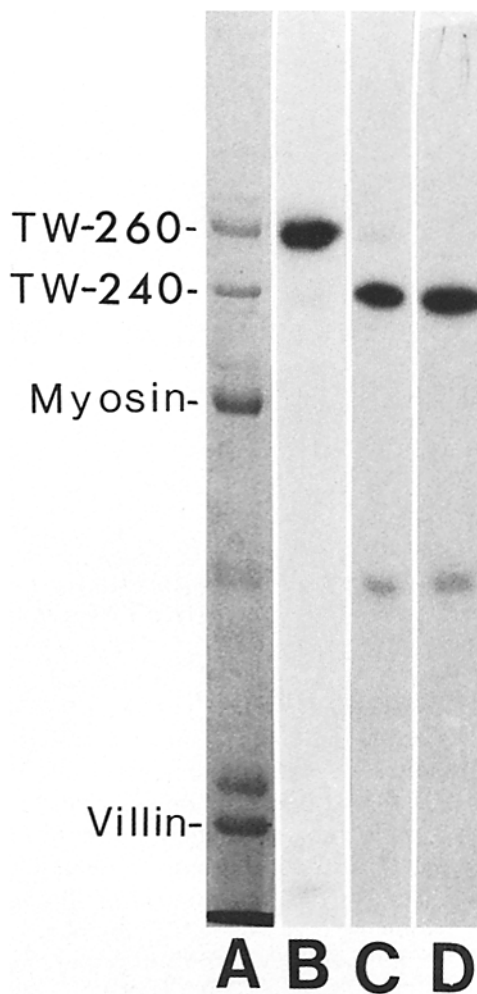

FIGURE 1 Immune blot using affinity-purified rabbit antibodies directed against TW-260, TW-240, or fodrin used in this study. Chicken brush border cytoskeletal proteins were resolved by SDS PAGE (5% gel) and either stained with Coomassie Blue (lane A) or transferred to nitrocellulose and reacted with anti-TW-260 (lane B), anti-TW-240 (lane C), or antifodrin (lane D), followed by ¹²⁵I-protein A and detection by autoradiography. Note the decoration of TW-240 by the antifodrin and the lack of cross reactivity of anti-TW-260 and TW-240.

rescence microscopy (data not shown). Fixed brush borders were treated with rabbit anti-TW-260, anti-TW-240 or antifodrin antibodies followed by a ferritin-conjugated sheep anti-rabbit antibody before being processed for thin-section analysis. Electron micrographs showed that the ferritin localization patterns for the three antibody preparations were indistinguishable (Fig. 3). Ferritin was observed between the rootlets in the terminal web region, generally extending from the level of the apical plasma membrane to the base of the rootlets. In addition, in optimal specimens several ferritin particles were observed as a row displayed between neighboring rootlets as if a fine filament were decorated (Fig. 3). Since the ferritin-antibody complex was prepared by molecular sieve chromatography, this is not the result of preformed aggregates of second antibody, but rather indicates to us the decoration of an extended structure. Occasionally, regions of sparse ferritin labeling were observed in the center of the terminal web, surrounded by regions of much heavier antibody decoration. The lower labeling index in the central region of the terminal web was not a consistent property of all brush borders and even neighboring brush borders in a section differed in this respect. This is

FIGURE 2 Thin-section electron microscopic image of an isolated brush border showing a meshwork of thin filaments between rootlets. Micrograph courtesy of Dr. P. Matsudaira, MRC Laboratory, Cambridge, England. × 75,000.

probably not due to antibody accessibility since a central zone of sparse antibody labeling is not observed when antimyosin is used (D. Henderson and K. Weber, unpublished observation). No ferritin was found associated with microvillus cores extending from the apical surface.

In all experiments the labeling pattern did not extend to the lateral membrane at the level of the terminal web (Fig. 4) where a dense meshwork of microfilaments exists, which is thought to reflect an organization related to a circumferential belt or "contractile ring" (17). Since indirect immunofluorescence microscopy has revealed either TW (2) or fodrin antigens (8) in the lateral membranes of unextracted intestinal cells, it is possible that this anti-TW reactive material is either below the level of the terminal web, extracted upon brush border isolation, or inaccessible to the ferritin-labeled antibodies used here.

DISCUSSION

The brush border of chicken intestinal epithelial cells provides a unique model system for the combined biochemical and morphological analysis of microfilament organization. Eight major polypeptides have been identified as structural elements of isolated chicken brush border cytoskeletons. Five of these (villin, fimbrin, 110,000-mol wt protein, calmodulin, and actin) have been assigned to the microvillus core filament bundle (see reference 18 for a recent review). Biochemical fractionation experiments also suggest that the additional three high molecular weight polypeptides TW-260, TW-240, and myosin are present in the terminal web and excluded from the microvillus. This conclusion is fully supported by immunofluorescence microscopy, which has documented the presence of the com-

plex TW-260/240 (2) and myosin (19, 20) in the terminal web in addition to α-actinin (20), vinculin (21), tropomyosin (20), and filamin (20), which are either lost upon brush border isolation or present as only minor components in the structure.

It has been known for some time that the terminal web, an organelle-excluding zone into which the microfilament bundles extend as rootlets, is laced with a fine filamentous network that seems to connect actin bundles (17, 22, 23). It has been proposed that these filaments are myosin, based on (a) the large amount of myosin present in the terminal web of isolated brush borders, (b) decoration of the terminal web region with antimyosin antibodies (although labeled fine filaments as such have not been observed (12, 24, 25), and (c) extraction of many of these filaments with a high salt solution containing ATP that was correlated with the biochemical extraction of myosin heavy chain (12).

There are a number of observations not readily consistent with models in which myosin is cross-linking the rootlets. The rootlet-connecting filaments are thinner than actin (12), whereas the classical myosin filament is clearly thicker (26). Although "minifilaments" of myosin have been proposed to account for this structure (12), such "minifilaments" of an appropriate length (~0.2 μm) and width (<6 nm) have not been directly demonstrated in situ. In addition, as a first approximation one would expect the geometry of the actin-myosin interaction to be the same as in the well-known skeletal muscle model (i.e., the myosin tail to be parallel with the actin filament and cross-linking antiparallel actin filaments (26). It seems unlikely, then, that brush border myosin filaments could violate this geometry of interaction and have the tail extend at about right angles from the rootlet. Since actin filaments do not splay out from the rootlet (11, 23) and since free actin
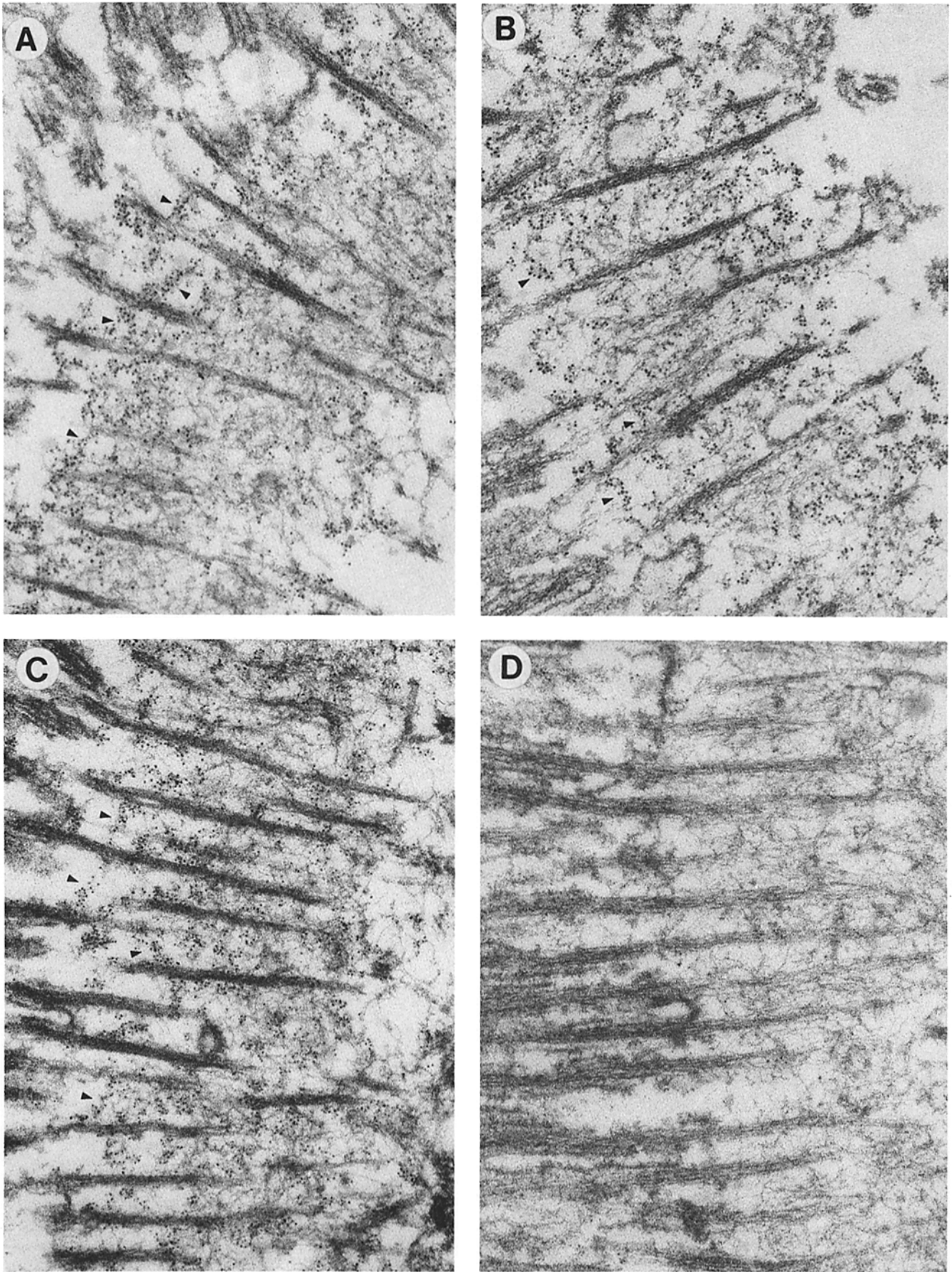
FIGURE 3 Localization of TW-260 and TW-240 antigens in isolated brush borders. Isolated chicken brush borders were treated with rabbit anti-TW-260 (A), anti-TW-240 (B), antifodrin (C), or buffer alone (D) followed by ferritin-conjugated sheep anti-rabbit IgG. After washing out unbound ferritin, brush border pellets were fixed in 2% glutaraldehyde and processed for thin-section analysis. Note the specific decoration of extended structures (arrowheads in A–C) which may represent rootlet connection filaments. × 83,000.
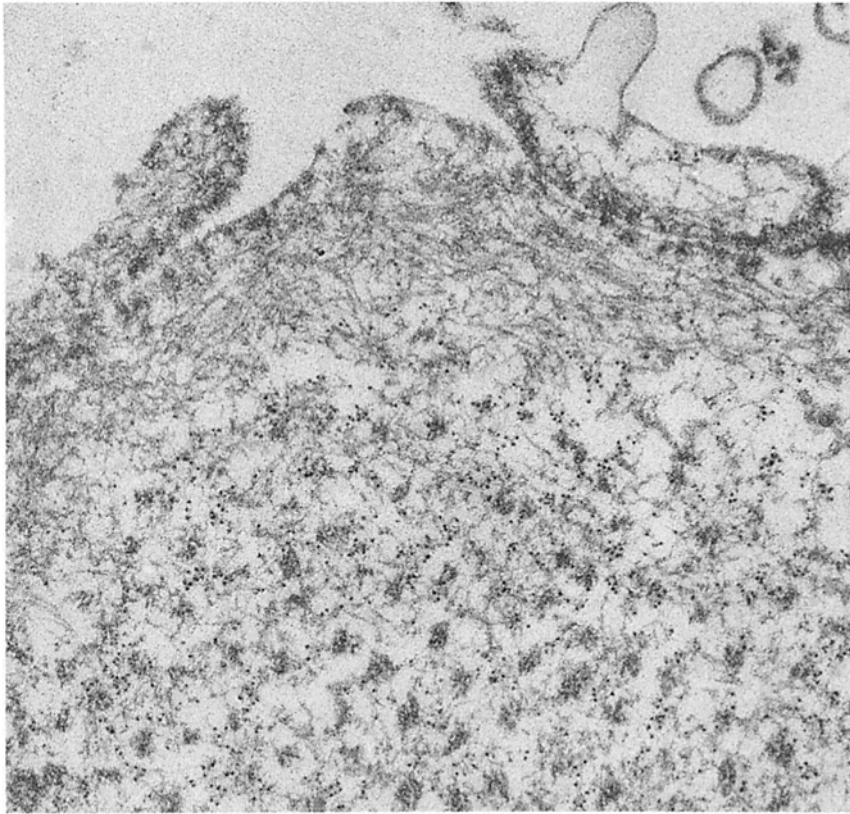
FIGURE 4 Localization of TW-240 antigens in a cross section through the terminal web at the level of the circumferential band of microfilaments. Note the labeling in the region of the rootlets, but the absence of ferritin within the circumferential band or near the lateral membrane.

filaments do not seem to traverse the terminal web horizontally (11, 27) in isolated brush borders (except for the circumferential band), then a direct interaction of myosin with rootlet actin would be required. We do not dispute previous findings of myosin throughout the terminal web region (12, 24, 25), but we only question the ability of such myosin molecules to cross-link rootlets and to account for all connecting filaments seen.

Recently, a large rodlike molecule that fulfills the requirements necessary to cross-link rootlets in the terminal web, has been isolated from brush borders (TW-260/240). It has been shown both biochemically and by immunofluorescence microscopy that TW-260/240 is a major component of this organization (2). TW-260/240 has a defined length (263 nm) that could easily traverse the intra-rootlet distance. Although a length of 180 nm for the cross-linking fibrils (12) seems somewhat lower than that of isolated TW-260/240, such in situ measurements may represent a minimum due to the difficulty inherent in finding the ends of the molecules among the bulky rootlets. Optimal images of slightly extracted, quick-frozen, deep-etched, rotary-replicated intestinal epithelial cells give the impression that at least some rootlet-connecting fibrils have a double-stranded flexible morphology (see, for instance, Fig. 9 of reference 11), which is strikingly similar to the morpholgy of the isolated TW-260/240 complex (2). TW-260/240 has been shown biochemically to bind to, and cross-link, actin filaments (2). In accordance with this model, we now report the immunoelectron microscopic localization of TW-260/240 between rootlets at all levels of the terminal web. In addition, some micrographs give the impression of the decoration of an extended structure that we interpret to be the fine fibrils of the terminal web (Fig. 3). Previous studies on TW-260/240 revealed that the isolated molecule is a complex of two polypeptides with molecular weights of 260,000 and 240,000, with the 240,000 component structurally and antigenically related to the 240,000 subunit of fodrin (2, 3). In agreement with this result

we show here that antisera specific for TW-260, TW-240, or fodrin result in a similar decoration pattern. We cannot conclude that all such rootlet anchoring structures represent TW-260/240 molecules, but at least some of these can be assigned to the TW-protein.

The fodrin molecule does not seem to be present in isolated chicken brush borders. Fodrin consists of a 235-kdalton subunit bound to the 240-kdalton polypeptide to form a tetramer (4) with an overall organization similar to erythroid spectrin (7a). Although antifodrin antibodies used in the present study reacted with a 240-kdalton component of brush border cytoskeletons and decorated a filamentous structure in the terminal web, this is very likely due to a reaction with TW-240 polypeptide, which is known to be structurally and antigenically related to the 240-kdalton subunit of fodrin and spectrin. In SDS gels of brush border cytoskeletons the TW-260 and TW-240 subunits are present at an approximate 1:1 molar ratio and the 235-kdalton polypeptide characteristic of fodrin is not observed (reference 2; see also Fig. 1). We cannot, however, rule out the possibility that fodrin is also present in the intestinal epithelial cell and lost upon isolation of the brush border.

The concept of the brush border as a "motile organelle" has been repeatedly discussed in recent years based primarily on experiments in which microvillus cores were observed contracting into, and through the level of the terminal web (28). Not only has the original experimental basis recently proven to be due to the Ca^{++}-dependent microvillus disintegration (19), but the finding that microvillus cores are highly cross-linked by TW-260/240 molecules again leads us to doubt that microvilli could move. Taken in the context of previous studies by Ito (29), who showed that an extensive glycocalyx envelops the luminal side of intestinal microvilli, it would appear that these microvilli are immobilized from both inside and outside the cells making movement highly unlikely. This does not exclude a potential contraction in the circumferential band of microfil-
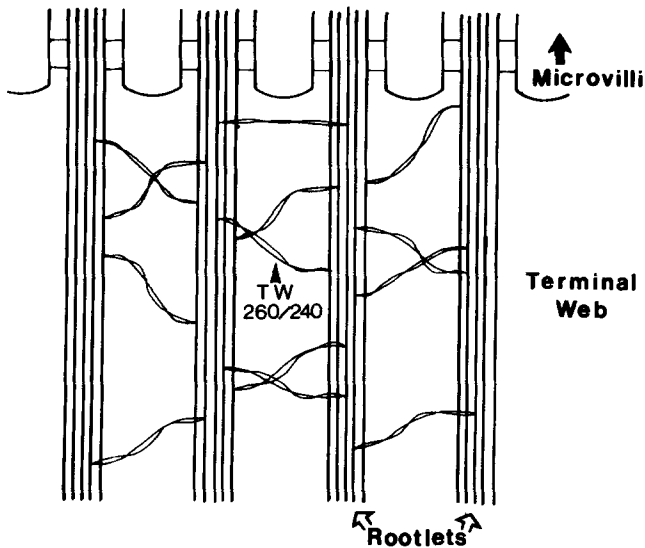
Figure 5 One possible orientation of TW-260/240 within the terminal web. The TW-260/240 molecule that is known to cross-link actin filaments in vitro (see reference 2) is depicted here anchoring neighboring rootlets. The flexible double-stranded morphology of TW-260/240 would allow a small amount of rootlet positional flexibility without the need to interrupt actin-TW-260/240 interactions.

aments (30, 31), however in a junctionally connected epithelial sheet, such contractility would not result in net movement.

Finally, we draw attention to a possible related function between erythroid spectrin and TW-260/240. Whereas the submembranous spectrin seems to cross-link short actin oligomers in the erythrocyte, TW-260/240 cross-links actin bundles in the brush border. Given the striking difference in the amounts of actin in the two systems and the presence of specific actin-associated proteins that bundle F-actin in the case of the brush borders, the two cross-linking molecules in each case seem to provide flexible connections between filamentous actin over an extended distance in the cortical cytoplasm of the respective cell types. Whereas spectrin links actin to the plasma membrane in erythrocytes, however, the present study would suggest that the spectrin-related molecule, TW-260/240, is too far away from the membrane to serve such a function in brush borders.

Note Added in Proof: Recently a report by Hirokawa et. al. (1983, *Cell,* 32:953–965) has shown that anti-fodrin antibodies decorate the fine filament system in mouse intestinal brush borders.

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