

Organization of the Actin Filament Cytoskeleton in the Intestinal Brush Border: A Quantitative and Qualitative Immunoelectron Microscope Study

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Abstract. In the present study we have used immunogold labeling of ultrathin sections of the intact chicken and human intestinal epithelium to obtain further insight into the molecular structure of the brush-border cytoskeleton. Actin, villin, and fimbrin were found within the entire microvillus filament bundle, from the tip to the basal end of the rootlets, but were virtually absent from the space between the rootlets. This suggests that the bulk of actin in the brush border is kept in a polymerized and cross-linked state and that horizontally deployed actin filaments are virtually absent. About 70% of the label specific for the 110-kD protein that links the microvillus core bundle to the lipid bilayer was found overlying the microvilli. The remaining label was associated with rootlets and the interrootlet space, where some label was regularly observed in association with vesicles. Since the terminal web did not contain any significant amounts of tubulin and microtubules, the present findings would

support a recently proposed hypothesis that the 110-kD protein (which displays properties of an actin-activated, myosin-like ATPase) might also be involved in the transport of vesicles through the terminal web. Label specific for myosin and α -actinin was confined to the interrootlet space and was absent from the rootlets. About 10–15% of the myosin label and 70–80% of the α -actinin label was observed within the circumferential band of actin filaments at the zonula adherens, where myosin and α -actinin displayed a clustered, interrupted pattern that resembles the spacing of these proteins observed in other contractile systems. This circular filament ring did not contain villin, fimbrin, or the 110-kD protein. Finally, actin-specific label was observed in close association with the cytoplasmic aspect of the zonula occludens, suggesting that tight junctions are structurally connected to the microfilament system.

THE resorptive surface of the intestinal epithelium, called brush border, is increased 15–30-fold by numerous microvilli that are 1–2 μm long and 100 nm in diameter (64). Each microvillus is supported by an axial bundle of actin filaments that are in parallel alignment and display identical polarity (51, 53, 59; for review see references 8, 52). The microvillus core bundle is attached to the surrounding membrane by periodically arranged lateral bridges that consist of a complex of a rod-shaped 110-kD protein and calmodulin (13, 31, 37, 46, 47, 69). The actin filament core bundle is extensively cross-linked by two proteins, fimbrin (68 kD) and villin (95 kD; 2, 4, 5, 6, 25, 55). In the presence of Ca^{++} ($>10^{-6}$ M), villin causes fragmentation of the core bundle whereas the bundling activity of fimbrin appears to be independent of Ca^{++} (9, 17, 48, 49, 55). Villin is also present in the rootlets (23). In contrast to the core bundle, the rootlets contain tropomyosin (21). There is a report indicating that the muscular Z-line protein, α -actinin, may also be present in the rootlets and absent from the microvilli (27).

The space between the rootlets contains numerous fine

filaments that are mainly oriented perpendicular to the axis of the rootlets (33). This filamentous web appears to be largely comprised of myosin (21, 35, 36) and isoforms of spectrin (29, 30, 34, 38). A third set of filaments extends along the cytoplasmic aspect of the zonula adherens to form a contractile circumferential filament band (7, 39) that is composed of actin filaments (9, 33, 35, 36) and contains α -actinin (4, 16, 26, 27), tropomyosin (4, 26), and, as judged by immunofluorescence, myosin (36).

Despite this rather detailed knowledge about the structural organization of the intestinal brush border, there are still several fundamental questions about the molecular structure of this specialized cytoskeleton–membrane complex. A main question concerns the state of polymerization of actin. In homogenates of whole enterocytes $\sim 30\%$ of the actin appears to be monomeric (65). But there are presently no data available that allow an estimate of the amount of unpolymerized actin (G-actin) in the brush border in vivo. Moreover, it is still not clear whether actin filaments are absent from the interrootlet space in vivo. It is possible that the absence

of actin filaments from the interrootlet space in isolated and extracted brush borders (33–36) may be due to artifactual removal or destruction of the filaments.

Another question is whether the rootlets contain fimbrin and the 110-kD protein. Since villin has been shown to be a component of the rootlets (23), it is possible that fimbrin and the 110-kD protein are also present at this site. Recent studies indicate that bundling of actin by villin is inhibited by tropomyosin (10), which is also present in the rootlets but absent from microvilli. Thus, the rootlets should contain further cross-linking proteins that maintain the actin filaments bundled. With respect to the 110-kD protein, there is increasing evidence that it shares several properties of a myosin-related mechanoprotein (12, 14). This has led to the suggestion that the 110-kD protein may not only link the core bundle to the plasma membrane (13, 31, 37, 46, 47) but may also connect vesicles to the rootlet bundles and thus may play a role in transport of vesicles through the terminal web (14).

Finally, little is known about the ultrastructural distribution of α -actinin and myosin in the circumferential filament band along the zonula adherens (26, 27, 35, 36). Immunofluorescence microscopy of contracted and uncontracted brush borders suggests that myosin is present within this filament ring (36); α -Actinin has also been found at this site (26, 27). However, these studies have not addressed whether or not myosin and α -actinin display some kind of supramolecular order analogous to other contractile systems, such as muscle or stress fibers which show periodic spacing of these proteins.

To address these questions we have conducted ultrastructural immunogold studies using formaldehyde- and glutaraldehyde-fixed intact chicken and human intestinal epithelium. The results presented in this paper are based on immunogold labeling of both ultrathin frozen sections and ultrathin sections of resin-embedded tissue. These techniques allowed us to obtain detailed qualitative as well as quantitative data about the subcellular distribution of actin, myosin, α -actinin, villin, fimbrin, the 110-kD protein, and tubulin in the different microdomains of the intact intestinal brush border region.

Materials and Methods

Antibodies and Immunoblotting

All antibodies used in this study were raised in rabbits. Specificity of the antibodies to actin from chicken gizzard (21) and intestinal epithelial brush border (24), to α -actinin from chicken gizzard (20), and to villin (23) and fimbrin (24) from chicken intestinal brush border has been described in the references indicated. A further antibody to native chicken brush border fimbrin was the kind gift of Dr. P. Matsudaira (Whitehead Institute, Cambridge, MA). Antibodies to chicken intestinal brush border myosin were prepared against the purified native protein (the kind gift of Dr. Kendrick-Jones, MRC Laboratory for Molecular Biology, Cambridge, United Kingdom) and against the protein isolated according to reference 40, separated by SDS-PAGE, and excised from the gel for immunization. Antibodies to chicken intestinal brush border 110-kD protein (62) were a kind gift from Dr. M. Mooseker (Yale University, New Haven, CT). A further antibody was prepared against SDS-denatured, purified 110-kD protein (prepared according to reference 28) excised from SDS-PAGE. Antibodies to tubulin were raised against native porcine brain tubulin dimer that was prepared according to reference 57.

All antisera were affinity purified using the respective antigens immobilized by transfer to nitrocellulose paper (66). The bound IgG was eluted either in PBS, pH 7.4, warmed to 56°C (20) or with 0.2 M glycine-HCl (pH 2.8) at 4°C (66).

Isolated chicken intestinal brush borders (3) or isolated intact human

intestinal epithelium (20) were dissolved in sample buffer and subjected to SDS-PAGE in the presence of 2% β -mercaptoethanol. The separated proteins were electrophoretically transferred to nitrocellulose paper (11) (Schleicher and Schuell GmbH, Darmstadt, Federal Republic of Germany [FRG]) which was then processed for antibody staining using the peroxidase-antiperoxidase method as described (23).

Immunoelectron Microscopy

Pieces of the intestinal mucosa of 6-wk chicken and biopsies of the human jejunal mucosa were fixed for 3 h at 21°C in either McClean and Nakane's fixative (50) that contained 2% (wt/vol) paraformaldehyde, 0.75 M lysine, 0.01 M NaIO₄ in 0.0375 M NaPi (pH 6.2) or the tissues were fixed at 4°C for 3 h in a mixture of 2% paraformaldehyde and 0.1–0.2% glutaraldehyde in PBS (pH 7.4). After fixation, the tissue blocks were washed in PBS (three times for 15 min), then treated with 0.5 mg/ml sodium borohydride, and freshly dissolved in PBS (three times for 15 min) to reduce free aldehyde groups. Small pieces of the fixed tissue were either immersed in 2.3 M sucrose in PBS and then processed for ultracryotomy on an ultracryotome (model FC-4D, Reichert Jung, Vienna, Austria) (18) or the tissue samples were embedded in LR-White (London Resin Co., Woking, United Kingdom) at 4°C as described in detail previously (22). Polymerization was performed in closed gelatine capsules at 4°C with UV light (360 nm). Ultrathin frozen sections were transferred to Formvar carbon-coated grids. Ultrathin sections of the resin-embedded tissue were collected on water and retrieved to gold grids.

Antibody labeling of the grids and processing for EM was done at room temperature exactly as described in previous papers (18, 22, 68). All affinity-purified primary antibodies were used at an IgG concentration of 10–50 μ g/ml. Colloidal gold- (10-nm or 5-nm) labeled goat anti-rabbit IgG was prepared as described (60) or purchased from Janssen Pharmaceutica (Beerse, Belgium). The gold-tagged IgG was used at dilutions of 1:50–1:100 in PBS containing 0.1% gelatine. After processing for immunogold labeling the ultrathin frozen sections were stained by the adsorption technique (67). The staining solution consisted of a mixture of 2% Carbowax and 0.2% methylcellulose with 0.4% uranyl acetate as a staining additive. Resin-embedded sections were stained for 5 min in 2% aqueous uranyl acetate.

Results

Immunoblotting

In the brush border of the chicken and human intestinal epithelium, antibodies to the 110-kD protein, α -actinin, villin, fimbrin, and actin bound to polypeptide bands of 110, 95, 68, and 42 kD, respectively (Fig. 1). Antibodies to chicken brush border myosin did not show any detectable affinity for human brush border but reacted strongly with chicken brush border myosin heavy chain (200 kD). No other cross-reactive polypeptide bands were detected by immunoblotting with the antibodies tested (Fig. 1).

Technical Considerations

In the present study we have used goat anti-rabbit IgG coupled to colloidal gold (IgG-gold). We have also tried protein A-colloidal gold complexes. However we found that the background with protein A was somewhat higher than with IgG-gold. Two sizes of IgG-gold particles were applied: (a) particles 10 nm in diameter were used to allow estimation and documentation of the overall distribution and specificity of the immunogold label at low magnification; and (b) IgG-gold particles 5 nm in diameter were chosen to analyze the binding pattern of antibodies at high magnification. Using the same antibody, the density of label counted at a particular antigenic site (e.g., the rootlets) was three to five times higher with 5-nm-diam particles than with 10-nm-diam particles. Only 5-nm-diam IgG-gold particles were used for quantitative determination of the binding sites.

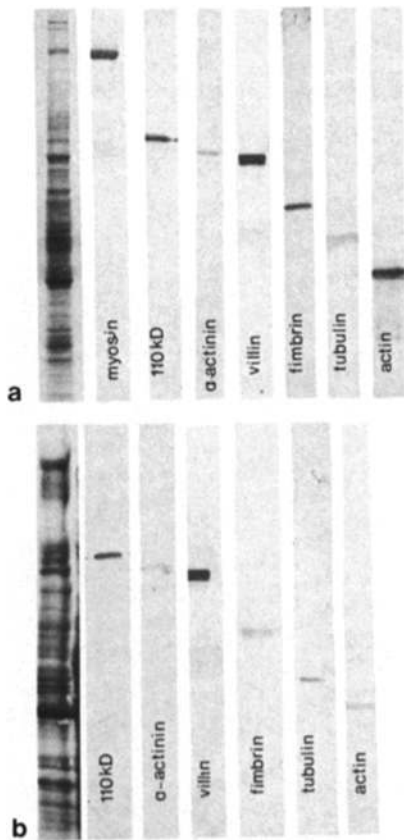


Figure 1. Specificity of antibodies determined by Western blot analysis of crude chicken intestinal brush border (a) and homogenized whole human intestinal (duodenal) epithelium (b). Lanes transferred to nitrocellulose paper were stained with either Amido Black (left lanes in a and b, respectively) or with antibodies to the various proteins indicated.

Interpretation of the pattern of IgG-gold label in the brush border is complicated by the fact that the diameter of the rootlets and the microvillus core bundle is approximately half the thickness of the section that is normally ~ 100 nm. Since in sections of resin-embedded tissue and, to some extent, also in ultrathin frozen sections the IgG molecules cannot infiltrate into the section (60, 63), only those core bun-

dles and rootlets which are exposed to the surface of the section will be labeled. Fig. 2 illustrates the typical patterns of immunogold label seen in projection on the screen. While some microvilli will be labeled along their entire length, adjacent microvilli may be completely unlabeled. Filament bundles that were only labeled along the rootlets or parts of the rootlets but were completely unlabeled along the microvillus core bundle or vice versa were frequently observed (see Figs. 3 b, 4 a, and 5 e). In cross sections of microvilli and rootlets, virtually all profiles of the filament bundles were labeled. However, the situation was different in obliquely sectioned microvilli or rootlets. In these cases, the gold label was confined to one or both ends of the bundle depending on whether only one (5-nm-diam gold particles) or both surfaces (10-nm-diam gold particles) of the section were exposed to the antibody. Similar conditions apply to vesicles. An antigen at the surface of a vesicle will only be labeled if the vesicle is at least tangentially sectioned. In tangentially cut vesicles, the label will be projected on the screen as if the gold particles are located in the center of the vesicle.

Actin

With both antibodies to actin used in this study, we obtained an identical pattern of the immunogold label (Fig. 3, a-d). No differences were observed between the pattern of label in chicken (Fig. 3, a, c, and d) and human brush border (Fig. 3 b); there were also no significant differences between the label on ultracryotome sections (Fig. 3 d) and sections of LR-White-embedded tissue (Fig. 3, a-c). The antibodies labeled the microvillus actin filament bundle from its tip to the end of the rootlets (Table I). In cross sections of the chicken brush border the density of IgG-gold label was virtually the same in microvilli and rootlets. The label observed in the terminal web space between the rootlets was negligible: 96% of the IgG-gold particles were associated with the rootlets, whereas only 4% were encountered in the interrootlet space (Table II).

A second site of significant immunolabel was the entire lateral plasma membrane of the zonula occludens and adherens. The macula adherens was unlabeled. As seen in Fig. 3, a and c, the dense filamentous material that is associated with the cytoplasmic surface of the tight junction area shows a similar density of label as the circumferential actin filament band of the zonula adherens.

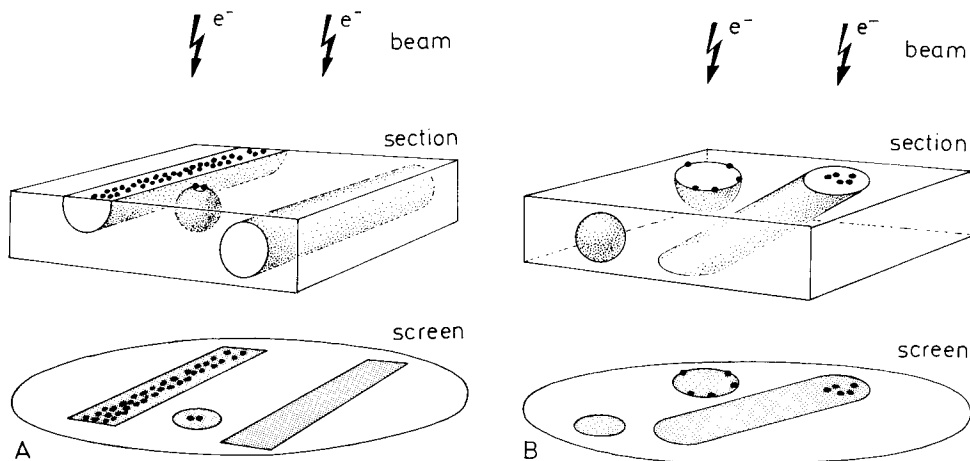


Figure 2. Diagrams illustrating patterns of immunogold label on ultrathin tissue sections seen in projection on the screen. Vesicles and rod-shaped structures (symbolizing microvilli) are drawn in frequently observed planes of section and orientation. In these examples only one surface of the section has been exposed to the antibody (refer to text).

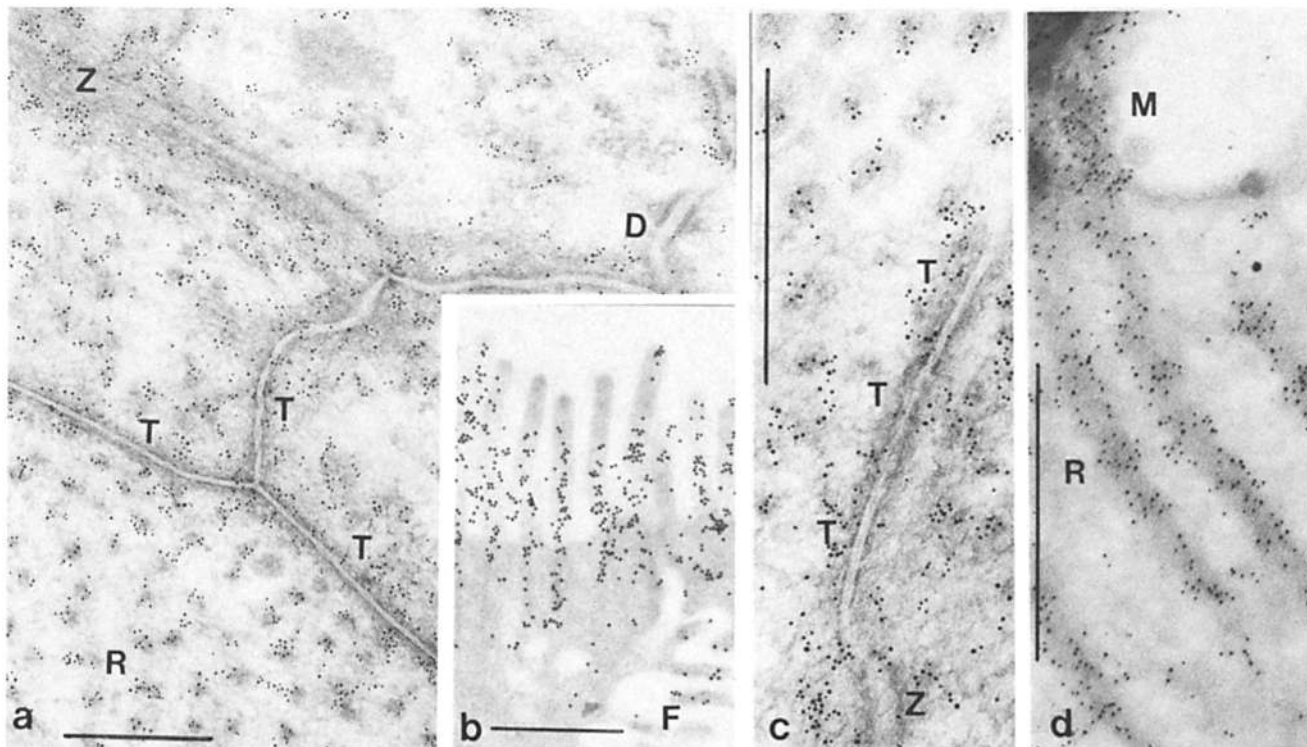


Figure 3. Section of the chicken (*a*, *c*, and *d*) and human (*b*) intestinal epithelium incubated with antibodies to actin of chicken brush border (*a*, *c*, and *d*) and gizzard (*b*). *d* is an ultracryotome section; *a*–*c* show sections of resin-embedded tissue. *b* was incubated with second antibodies coupled to 10-nm gold particles and sections *a*, *c*, and *d* with 5-nm IgG-gold particles. Note restriction of the label to microvilli (*M*), rootlets (*R*), and the filaments associated with filamentous material at the zonula occludens (*T*) and zonula adherens (*Z*). Spot desmosomes (*D*) are unlabeled. In *b* label is also seen in the lateral folds (*F*). Bars, 0.5 μm .

Villin and Fimbrin

The antibodies to chicken villin and fimbrin reacted with ultrathin sections of frozen and resin-embedded tissue of both the human (Fig. 4, *a* and *c*) and chicken brush border (Fig. 4, *b* and *d*). The immunolabel obtained with both antibodies was restricted to the microvillus core bundle and the rootlets. Both antibodies differed from the antibodies to actin in that the label associated with the rootlets was $\sim 20\%$ less than the label counted in cross sections of microvilli of the same tissue section (Table I).

Labeling of the interrootlet space was insignificant (2.7% for villin and 4.6% for fimbrin; Table II). No label was seen in association with the lateral plasma membrane and the zonula adherens.

Table I. Relative Density of IgG-Gold Label Overlying Cross Sections of Chicken Brush Border Microvilli and Rootlets

Antibodies to	Microvilli	Microvilli counted	Rootlets	Rootlets counted
	%	<i>n</i>	%	<i>n</i>
Actin	100	121	102	184
Fimbrin	100	143	81.2	142
Villin	100	154	79.4	154
110-kD protein	100	163	28.1	152

The average density of gold particles counted on cross-sectioned microvilli was set at 100%.

110-kD Protein

The label obtained with the antibody to the native protein was stronger than that obtained with the antibody to the denatured protein. Fig. 5, *a*–*f*, and the data given in Tables I and II were obtained with the antibody to the native protein. In ultrathin sections of frozen and resin-embedded human and chicken intestinal epithelium, the antibodies to native and denatured 110-kD protein bound to the microvilli and, to a lesser extent, to the terminal web. No label was associated with the junctional complex (Fig. 5, *a* and *c*). In ultrathin frozen sections of microvilli the label was typically arranged in clusters (Fig. 5 *b*) which may represent lateral bridges exposed to the surface of the section. Within the apical cyto-

Table II. IgG-Gold Label Overlying Rootlets and the Interrootlet Space of the Terminal Web of the Chicken Brush Border

Antibodies to	Rootlets	Interrootlet space	IgG-gold particles counted
	%	%	<i>n</i>
Actin	96	4.0	944
Fimbrin	95.4	4.6	295
Villin	97.3	2.7	715
110-kD protein	89.4	10.6	655
α -Actinin	10.2	89.8	137
Myosin	6.4	93.6	319

The total number of IgG-gold particles (*n*) was set at 100% for each antibody.

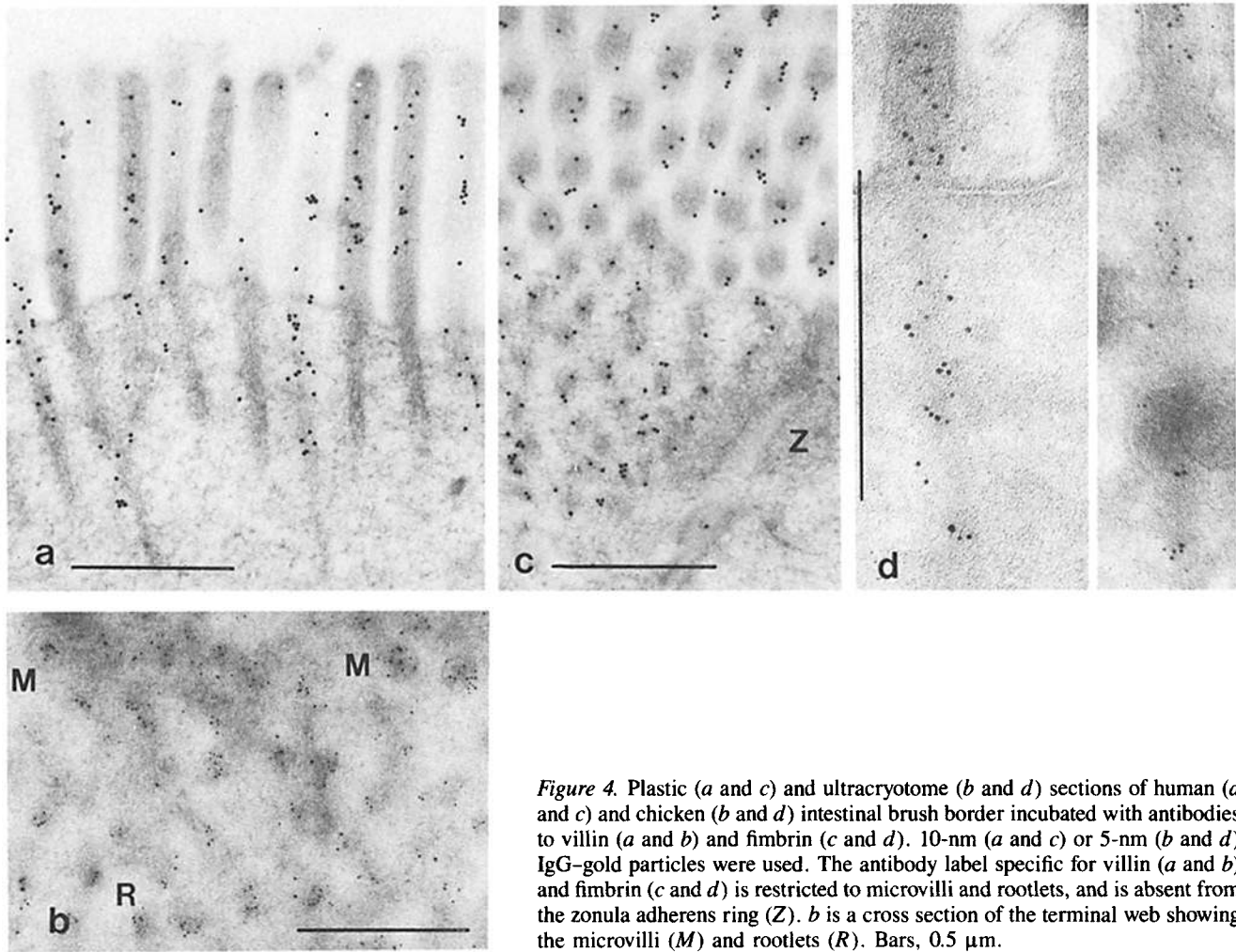


Figure 4. Plastic (*a* and *c*) and ultracryotome (*b* and *d*) sections of human (*a* and *c*) and chicken (*b* and *d*) intestinal brush border incubated with antibodies to villin (*a* and *b*) and fimbrin (*c* and *d*). 10-nm (*a* and *c*) or 5-nm (*b* and *d*) IgG-gold particles were used. The antibody label specific for villin (*a* and *b*) and fimbrin (*c* and *d*) is restricted to microvilli and rootlets, and is absent from the zonula adherens ring (*Z*). *b* is a cross section of the terminal web showing the microvilli (*M*) and rootlets (*R*). Bars, 0.5 μm .

plasm, ~90% of the gold particles were associated with the rootlets or their surface and only 10% of the label was clearly present in the interrootlet space (Table II). The density of label that was associated with the rootlets was only 28% of the label counted on sections of the microvilli (Table I). Gold particles close to vesicles in the terminal web were regularly found (Fig. 5, *a* and *c-f*). Whether the gold particles seen in the center of some vesicles are surface-bound antibodies of tangentially sectioned vesicles (as illustrated in Fig. 2 *A*) could not be decided.

Myosin

Antibodies to chicken brush border myosin did not cross-react with human tissue. In ultrathin frozen sections of the chicken, the label was much weaker than in sections of resin-embedded tissue. Therefore, the present immunocytochemical data refer to myosin in resin-embedded chicken brush border. As shown in Figs. 6 and 7 *a*, the immunolabel is confined to the terminal web and the zonula adherens, but is absent from the microvilli. The basal half of the terminal web (level of the zonula adherens) was more strongly labeled than the apical half (level of zonula occludens). The apical half contained only 19% of the IgG-gold label of the terminal

web ($n = 480$ gold particles). Within the terminal web some 94% of the IgG-gold particles were located in the interrootlet space whereas only 6% appeared to be associated with the periphery or center of rootlets. Typically, the IgG-gold complexes formed clusters. Myosin-like clusters were also observed in tangential sections of the zonula adherens where, locally, an interrupted pattern of the clusters was visible (Fig. 6). The dense filamentous material associated with tight junctions did not show any significant label. Rough calculation of the amount of myosin label associated with the terminal web and the zonula adherens ring indicate that only 10–15% of the brush border myosin is confined to the circumferential filament band, whereas 85–90% of the total myosin is located between the rootlets. This calculation is based on thin sections of the terminal web perpendicular to the cell surface. The average width of random profiles of 52 chicken brush borders was 7.6 μm (corresponding to a diameter of a cylindrical cell of ~9 μm); the average density of 5-nm gold particles per μm of terminal web profile was 65 and the number of gold particles overlying one profile of the zonula adherens ring was 34. Thus the average label of two profiles of the zonula adherens ring is 12.3% of the total label overlying an average profile of the intestinal brush border.

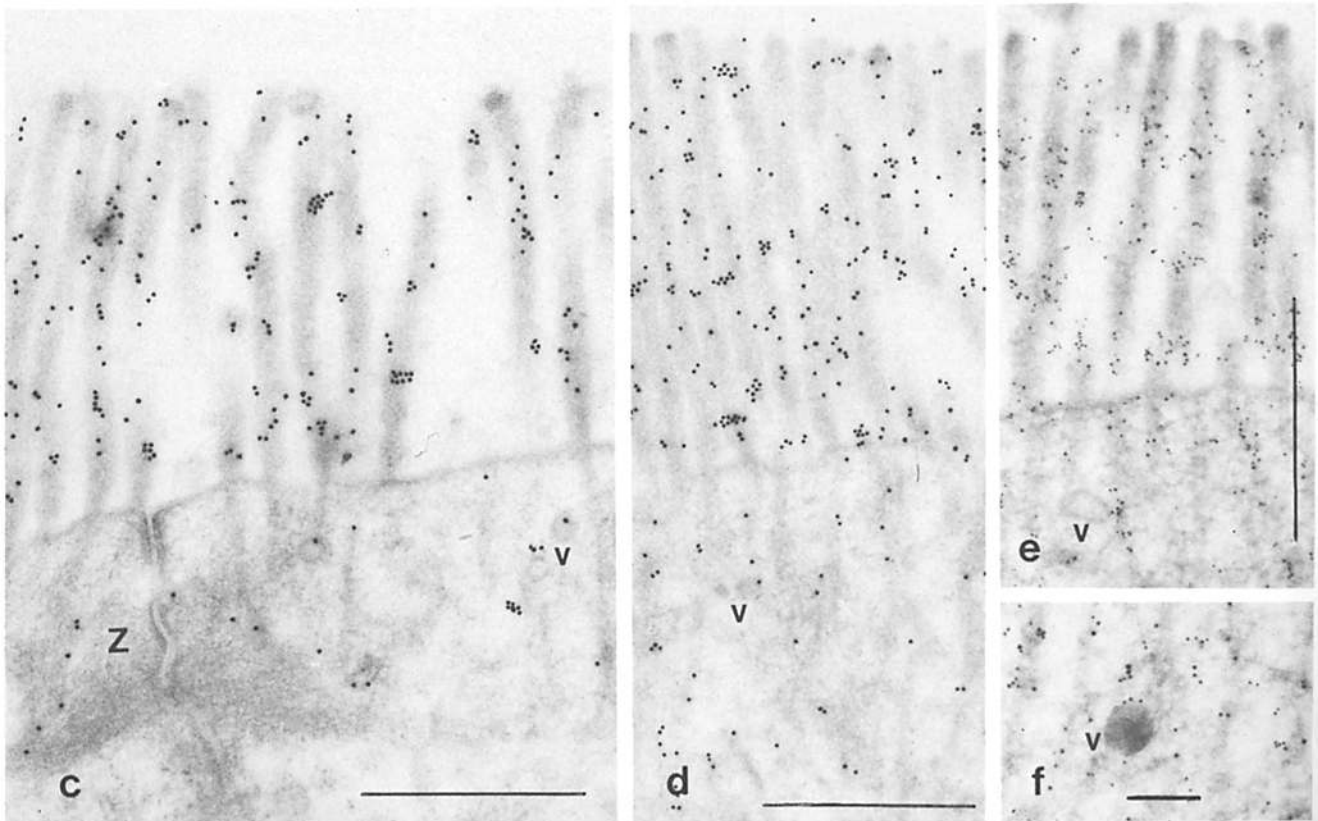
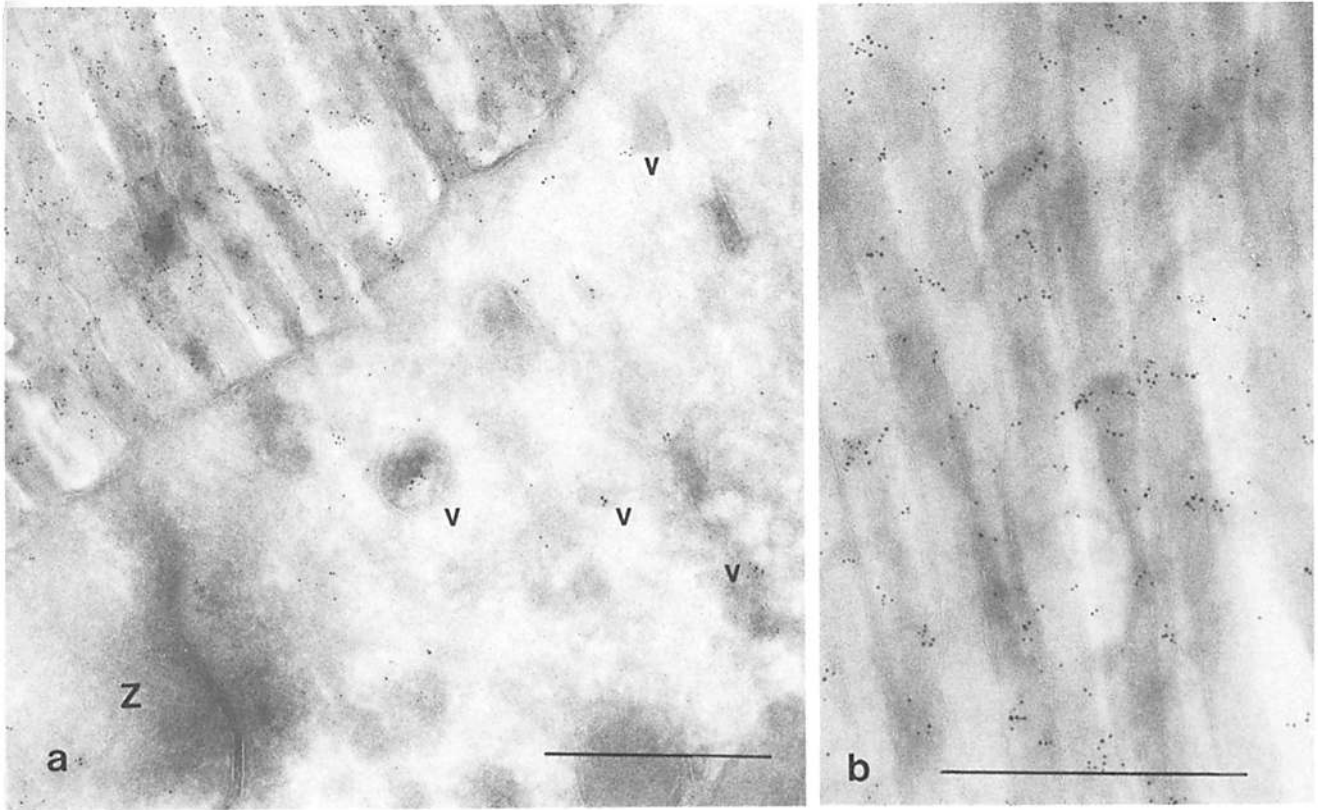


Figure 5. Ultracryotome (*a* and *b*) and plastic sections (*c*-*f*) of the chicken (*a*, *b*, and *d*-*f*) and human (*c*) intestinal brush border incubated with antibodies to the 110-kD protein. Note association of the immunogold label (*a*, *b*, and *d*-*f* 5-nm particles; *c*, 10-nm particles) with both microvilli and rootlets. There is also some label at vesicles (*v*) in the terminal web. The zonula adherens (*Z*) is unlabeled. Bars: (*a*-*e*) 0.5 μ m; (*f*) 0.1 μ m.

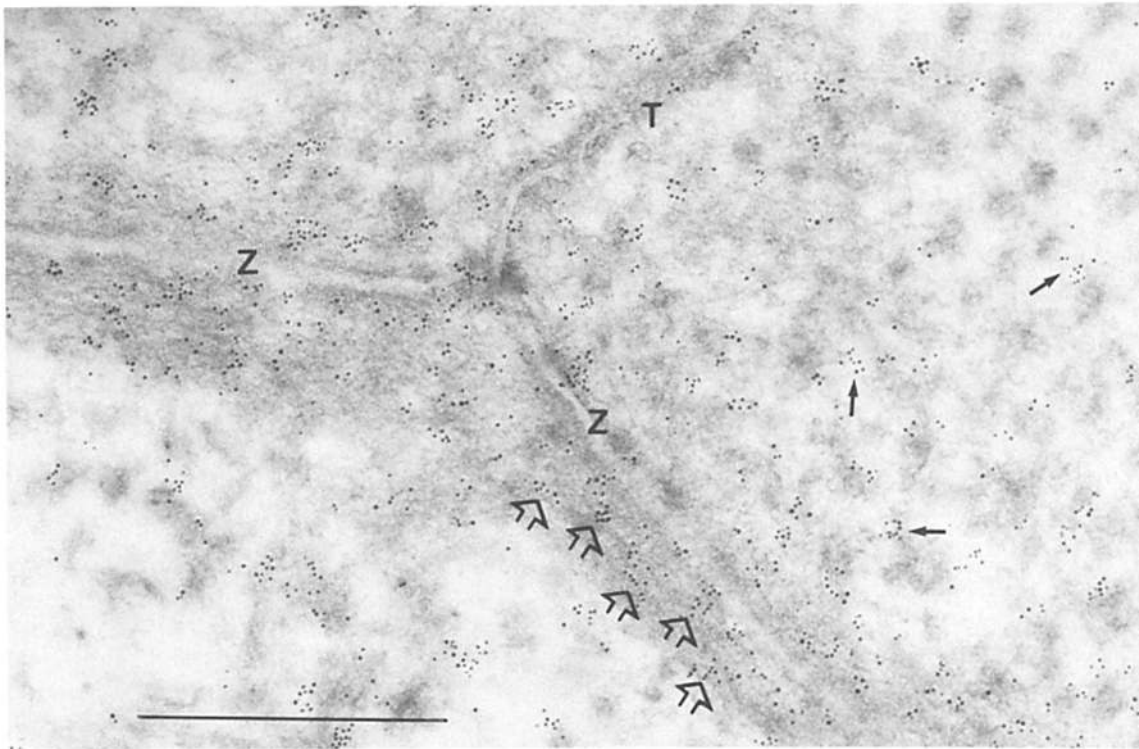


Figure 6. Section of resin-embedded chicken intestinal epithelium labeled with antibodies to chicken brush border myosin and 5-nm immunogold particles. The label is concentrated in the terminal web and the filament band associated with the zonula adherens (Z). No significant label is found in the area of the tight junctions (T). Small arrows point to clusters of IgG-label in the interrootlet space. Large open arrows indicate immunogold clusters within the zonula adherens ring. Bar, 0.5 μm .

α -Actinin

Like the antibodies to myosin, the α -actinin antibody showed a higher affinity for sections of resin-embedded tissue than for ultrathin frozen sections. Both human and chicken intestinal epithelium were labeled by the antibody to a similar extent. The highest density of label was observed in the filament band along the zonula adherens, whereas the terminal web was only weakly labeled (Fig. 7, *b* and *c*). The density of label of the terminal web decreased with the distance from the zonula adherens (Fig. 7 *c*). Only 10% of the gold particles in the terminal web were associated with rootlets while 90% were located in the interrootlet space (Table II). No significant label was seen close to the plasma membrane of the tight junction region. Within the zonula adherens, the label was more or less diffuse in human but often arranged in clusters in the chicken brush border. In favorable planes of section, a periodic arrangement of clusters was observed (Fig. 7 *b*).

Tubulin

The present findings obtained with antibodies to the 110-kD protein prompted us to look for tubulin. If the 110-kD protein plays any role in vesicle transport through the terminal web, it would be interesting to know whether microtubules, the main candidate for intracellular transport of organelles, are also present in the terminal web. As shown in Fig. 8, the terminal web is virtually unlabeled with the tubulin antibody. The bulk of microtubules and tubulin is found immediately below the terminal web where the tubules appear to be pref-

erentially oriented parallel to the plane of terminal web. The centrioles were always located in the apical cytoplasm below the web of tonofilaments and were absent from the perinuclear area (Fig. 8).

Discussion

In the present study we have applied recently developed techniques (60, 68) that allow antibody labeling of ultrathin sections of resin-embedded intact intestinal epithelium. This technique minimizes the possibility of artifactual displacement of the antigens and allows direct access of the antibodies to the antigens that are exposed at the surface of the section. For the first time, these conditions allow quantitative calculations about the relative concentration of a given cytoskeletal protein in different subcellular compartments of the brush border. A model for the structural organization of the brush border is given in Fig. 9. It should be stressed at this point that brush border cytoskeletal proteins are turning over very rapidly (8, 52). These findings may have significant relevance when considering the implications of G- and F-actin distribution; the localization of fimbriin, 110-kD protein, and villin to the rootlets; and the possible role of the 110-kD protein in vesicle movement.

Rootlets and the Terminal Web

The rootlets appear to contain all the proteins that are associated with the microvillus core bundle; i.e., villin, fimbriin, and also the 110-kD protein. In addition, the rootlets contain tropomyosin (21) and there is a report (27) indicating

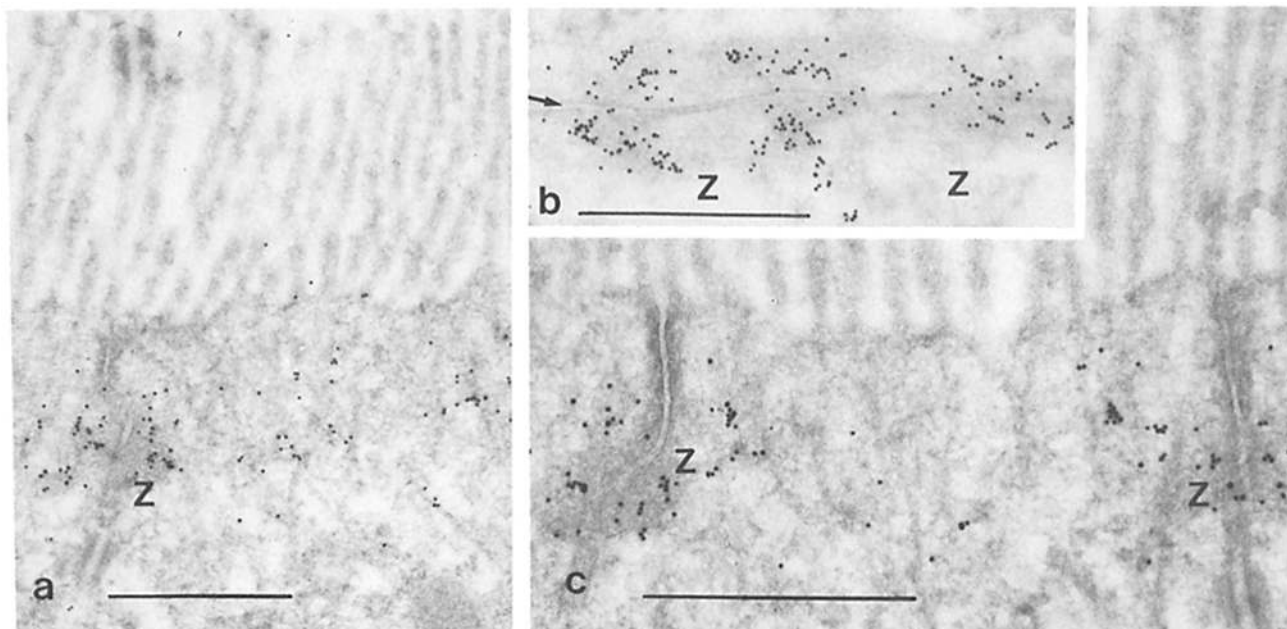


Figure 7. Plastic sections of chicken intestinal brush border incubated with anti-brush border myosin (*a*), anti-gizzard α -actinin (*b* and *c*), and 10-nm immunogold particles. The terminal web is more densely labeled with antimyosin (*a*) than with anti- α -actinin (*b* and *c*). The zonula adherens (Z) displays a similar density of antibody label with both antibodies. In *b*, a longitudinal section of the zonula adherens is shown in which the antibody label specific for α -actinin shows periodically arranged clusters (arrow points to the intercellular cleft). Bars, 0.5 μ m.

that α -actinin is also a constituent of the rootlets. However, in the present study we did not find any indication of significant amounts of α -actinin in the rootlets. The presence of tropomyosin in the rootlets is probably one reason why the rootlets do not depolymerize in the presence of increased levels of Ca^{++} (1, 9, 10, 52, 54, 71). There is experimental evidence from studies *in vitro* that tropomyosin blocks one binding site of villin at the actin filament. Thus, villin can no longer bundle the actin filaments (10) and can no longer cause depolymerization of the filaments at elevated levels of Ca^{++} (1, 10, 54, 71). Given that the bundling activity of fimbrin is not inhibited by tropomyosin, and considering that the 110-kD protein causes lateral association between actin filaments *in vitro* (13, 14), the present demonstration of fimbrin and 110-kD protein in the rootlets may help to explain why rootlet filaments are still in a bundled state.

Surprisingly, IgG-gold label specific for villin and fimbrin was $\sim 20\%$ less concentrated in the rootlets than in the microvillus core bundle although the density of anti-actin label was the same in both the rootlets and microvilli. Again this finding could be explained by the presence of tropomyosin in the rootlets. Recently published *in vitro* studies on the effect of tropomyosin on the affinity of villin and fimbrin for F-actin have shown that tropomyosin can cause dissociation of $\leq 30\%$ of villin and fimbrin from actin filaments (10).

A further finding was the absence of any significant actin-specific IgG-gold label from the terminal web space between the rootlets. Since the present study was performed on the intact epithelium, any loss of soluble proteins that occurs in isolated brush borders cannot account for the absence of actin from the interrootlet space. This observation is of particular interest for the following reasons.

Given that the affinity of the actin antibodies for mono-(oligo)meric actin (G-actin) is the same as for polymeric actin (F-actin), then it would be reasonable to conclude that the concentration of G-actin in the intact brush border is extremely low and that the bulk of the actin pool of the brush border is kept in a polymerized state. On the other hand, estimation of the G-actin contents in homogenates of whole intestinal epithelial cells indicated that $\sim 33\%$ of the cellular actin is monomeric (65). However, this pool of G-actin may be associated with the basolateral cortical cytoplasm which contains rather low amounts of visible actin filaments but which was always rather strongly labeled with actin antibodies (21; Drenckhahn, D., and R. Dermietzel, unpublished observations). Another explanation for this apparent discrepancy would be that G-actin or the G-actin-profilin complex are concentrated by a still unknown mechanism within the core bundle. Experimental addition of G-actin to isolated brush borders and isolated microvillus cores has been shown to cause concentration-dependent elongation of the core bundle and rootlets (56, 58). It is possible, therefore, that the length of the microvillus filament bundle is mainly regulated by controlling the actin monomer concentration in the cell. Shortening of the microvilli in intestinal tissue treated with cycloheximide would support this idea (43). To show that the actin antibodies used in this study also bind to G-actin in resin-embedded tissue, we have loaded leaky human erythrocyte ghosts with 10 μ M of muscle G-actin or G-actin-DNase I complex and processed the vesicles for immunocytochemistry. Under these conditions a moderate degree of actin-specific IgG-gold label was observed within the loaded vesicles (not shown). Although we have not yet determined the critical concentration of G-actin that still can be detected

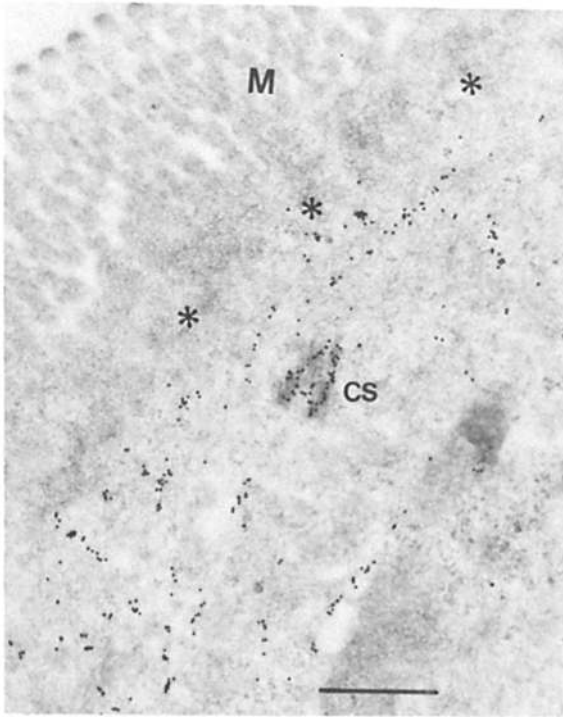


Figure 8. Distribution of tubulin on a plastic section of the apical cytoplasm of the human intestinal epithelium (10-nm immunogold particles). The antibody label is confined to the cytoplasm below the terminal web. The terminal web (*) and microvilli (*M*) are unlabeled. A centrosome (*cs*) is also seen. Bar, 0.5 μ m.

by immunogold labeling, these experiments show that the antibodies still bind to G-actin after fixation and embedding in resin.

The absence of any significant actin-like immunolabel from the interrootlet space supports recently published quick-freeze deep-etch studies on isolated brush borders in which horizontally deployed actin-like filaments were difficult to document (35, 36). Selective extraction and antibody labeling of the terminal web suggested that in the isolated brush border the bulk of the 5–8-nm filaments consist of non-erythroid spectrin (fodrin, TW 260/240) and myosin (30, 34–36) rather than actin. This conclusion is supported by the present study on the intact epithelium where the myosin label was clearly confined to the interrootlet space, whereas the actin label was absent from this space. Thus, these findings speak in favor of the prevalent view that terminal web contraction does not depend on interaction of myosin with horizontally oriented actin filaments (for review see reference 52).

110-kD Protein and Tubulin

The 110-kD protein is a main component of the lateral bridges that connect the core bundle to the surrounding plasma membrane (13, 31, 37, 46, 47, 69). Two recent studies indicate that the 110-kD protein shares several properties of a myosin-like mechanoenzyme (12, 14). Its ability to bind with one end to actin filaments and with the other to components of the lipid bilayer has led to the suggestion that this

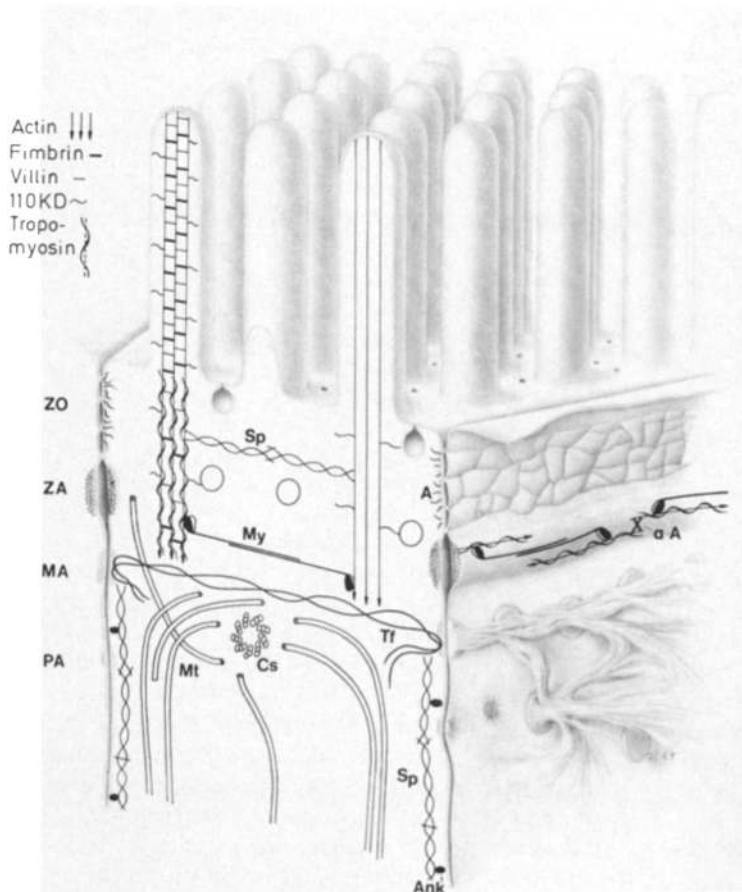


Figure 9. Hypothetical model for the molecular organization of the cytoskeleton in the apical cytoplasm of the intestinal epithelium. The basolateral ankyrin-spectrin lattice that is located below the most apical spot desmosome (19, 41) is also shown. *A*, actin; *a-A*, α -actinin; *Ank*, ankyrin; *Cs*, centrosome; *MA*, macula adherens; *Mt*, microtubules; *My*, myosin; *PA*, punctum adherens or actin-associated spot desmosome (20); *Sp*, spectrin isoforms; *Tf*, tonofilaments; *ZA*, zonula adherens; *ZO*, zonula occludens.

protein may cause movements of the microvillus core bundle relative to the surrounding plasma membrane or that the 110-kD protein may mediate directional transport of vesicles through the terminal web (12). In the present study, we observed significant IgG-gold label specific for the 110-kD protein in the terminal web where ~90% of the label was located along the rootlets and 10% in the interrootlet space. It should be stressed at this point that tropomyosin does not affect actin binding of the 110-kD protein (10) so that there is no obvious reason why the rootlets should not serve as a binding site for the 110-kD protein. Since tropomyosin interferes with binding of fimbrin and villin to actin filaments, it is possible that the 100-kD protein serves as an additional cross-linker of the rootlets.

A fraction of the IgG-gold particles in the terminal web was associated with vesicles. At present we cannot decide whether association of the label with vesicles is only incidental or whether this location might reflect functions of the 110-kD protein in terms of vesicle transport through the terminal web. Deep-etch replicas of the mouse brush border have demonstrated fine connecting filaments between vesicles and rootlets (34). It is presently unknown whether these cross-bridges consist of nonerythroid spectrin (as do the remaining 5-nm filaments in the terminal web) or whether at least a fraction of these bridges consist of the 110-kD protein. Recent immunocytochemical studies on isolated and Triton-extracted chicken brush borders have not revealed any significant immunolabel for the 110-kD protein in the terminal web (31). However, it is possible that under these rather harsh conditions of tissue processing the 110-kD protein was removed from the terminal web.

In a previous study on the pig intestinal epithelium, Coudrier et al. (15) observed that antibodies to pig brush border 110-kD protein label both microvilli and terminal web. But the antibody also reacted with the intermyofibrillar space in striated muscle in which we did not detect any immunostain.

One problem with antibodies to the 110-kD protein is the possibility of cross-reactivity with myosin (14). Although unlikely, the lack of cross-reactivity with brush border myosin on Western blots (Fig. 1) might be explained by epitopes that are not exposed on nitrocellulose blots but might be accessible in tissue sections of resin-embedded and frozen epithelium. Such shared epitopes between 110-kD and myosin are assumed to occur in the head region of myosin (14). Since the head regions of myosin probably attach to the surface of the rootlets, staining of brush border myosin by the 110-kD antibody would be located close to the surface of the rootlets, as is the label for the 110-kD protein in the chicken and human brush border. However, for several reasons we do not believe that the 110-kD label within the terminal web is due to cross-reactivity with myosin: (a) both 110-kD antibodies used in this study did not label the zonula adherens ring (Fig. 5, a and c) which, however, is a main site for myosin in the brush border (Figs. 6 and 7 a); (b) in contrast to myosin, the label specific for the 110-kD protein did not show any significant differences in density between the apical and basal half of the terminal web; and (c) extensive absorption of the 110-kD antibodies with purified myosin from chicken gizzard and brush border did not change the affinity of the antibodies for the terminal web.

With respect to the question of how endo- or exocytotic vesicles are transported through the terminal web, we have

also looked for tubulin in the chicken and human brush border. Recent thin section studies indicated that microtubules may only rarely project into the interrootlet space (32, 61). About one microtubule profile was found in thin sections of the terminal web per number of cell profile (32). However, it could be possible that the number of microtubules in the terminal web is underestimated because they may be difficult to discover within the dense meshwork of filaments in this area of the cell apex or because they may be extremely unstable. As shown in this study, tubulin is virtually absent from the terminal web. Microtubules do not extend in any significant number beyond the basal web of tonofilaments.

Junctional Complex

The cytoplasm adjacent to the junctional complex including the zonula occludens and zonula adherens was densely labeled with antibodies to actin. At the zonula occludens the label was associated with dense filamentous material that was easily visible under the present conditions of embedding and contrasting. In a recent study on detergent-extracted intestinal epithelial cells of the guinea pig, Madara (44) observed S₁-decorated microfilaments frequently located in the vicinity of tight junctions. However, it was difficult to decide whether these filaments were derived from adjacent rootlets or from elsewhere in the terminal web of the extracted brush borders. In view of the observation that cytochalasin B and D increase ion flow through the paracellular pathway of various epithelial cells in vitro and in situ (44, 45, and further studies cited in these references) it is tempting to speculate that the actin filament system plays a role in regulation of the selective permeability of tight junctions.

The continuous band of actin filaments that surrounds the cell apex at the level of the zonula adherens has been shown by deep-etched freeze-fracture replicas to consist of actin filaments with mixed polarity (35). In addition this filament band contains shorter and thicker filaments that resemble the myosin-like filaments of the terminal web (35). It is possible that these nonactin filaments provide the structural basis for the clusters of the antimyosin label that we observed in longitudinal sections of the zonula adherens ring. Recent studies have provided evidence that extraction of ~80% of the myosin in the brush border does not interfere with the contraction of the circumferential filament band (40). It is likely that the remaining 20% of myosin corresponds to the 10-15% of the myosin label that we found to be associated with the zonula adherens. IgG-gold label specific for α -actinin was also arranged in clusters. Occasionally these clusters displayed a periodic pattern that resembled the distribution of α -actinin in stress fibers of cultured cells (42) or the periodic pattern of the densities in the zonula adherens ring in Madin-Darby bovine kidney cells in vitro (70). These clusters of the α -actinin label might represent specialized sites within the circumferential band where the actin filaments are cross-linked by α -actinin to form arrays of actin filaments of opposite polarities (Fig. 9). These sites may be related to the Z-line in striated muscle or to the densities in smooth muscle or stress fibers.

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