Localization of Myosin, Actin, and Tropomyosin in Rat Intestinal Epithelium: Immunohistochemical Studies at the Light and Electron Microscope Levels

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ABSTRACT Myosin, tropomyosin, and actin were localized in the epithelial cells of rat intestine by means of specific antibodies to chicken gizzard smooth muscle myosin, tropomyosin, and actin by immunohistochemical studies at both the light and electron microscope levels (unlabeled antibody enzyme technique). The pattern of antibody staining was the following: (a) Anti-actin was associated with the microfilament bundles of the microvilli in their entire length, as well as with the microfilament network in the terminal web. (b) Anti-myosin was concentrated along the rootlets of the microvillar microfilament bundles and within the filamentous feltwork forming the terminal web. (c) Anti-tropomyosin showed a distribution similar to that of anti-myosin. In addition, the three antibodies also labeled the subplasmalemmal web underneath the cell membrane bordering on the basal lamina. Utilizing the above ultrastructural findings, we wish to propose a functional model of microvillar contraction.

In recent years much interest has been focused on the force-generating apparatus responsible for the motility of intestinal epithelial brush border. This highly ordered structure proved to be an ideal model for studying the structural basis of nonmuscle cell motility.

By phase-contrast microscopy, epithelia of rat intestine and of kidney tubules were shown to perform fast microvillar movements (31, 35). Studies on isolated apical segments of intestinal epithelium demonstrated rapid microvillar retraction or contraction of the whole apical segments in response to ATP, Ca$^{2+}$, and Mg$^{2+}$ (26, 30). Biochemical analysis indicated the presence of myosin, tropomyosin, actin, and other associated proteins in the apical cytoplasm (3, 26, 28). Recently, these biochemical findings have been confirmed by the immunofluorescent localization of myosin, tropomyosin, and actin in the apical cytoplasm of mouse, rat, and chicken intestinal epithelial cells (4, 13, 14, 28).

Ultrastructurally, the microvilli of rat intestinal epithelium contain an orderly axial bundle of actin filaments in parallel alignment, which penetrates ~0.5–1 μm into the apical cytoplasm to form a rootlet, which terminates abruptly (5). As judged by heavy meromyosin binding, the actin filaments are attached to the apical membrane of the microvilli with the same polarity seen in actin filaments attached to the Z line of striated muscle (1, 23, 27). The apical cytoplasm contains a dense network of actin filaments (terminal web) and individual short myosin-like filaments, which appear to be associated with the rootlets of the microvillar filament bundles (27, 30). On the basis of these findings, it has been suggested that movement of the entire microvillar filament bundle could be effected by interaction of myosin with the rootlet filaments (26, 27, 30).

In the immunocytochemical study presented here, we combined light and electron microscopy to obtain more detailed information on the distribution of myosin, tropomyosin, and actin in the brush-border region of rat intestine.

MATERIALS AND METHODS
Preparation and Specificity of Antibodies

The antigens myosin, tropomyosin, and actin were extracted from the muscle layer of chicken gizzard and were purified as described elsewhere (8, 18, 19). An additional chromatography step of myosin on hydroxyapatite (29) has recently been added. Control antigens (myosin and tropomyosin from chicken striated pectoral muscle) were prepared in the same manner. Antisera to the above contractile proteins were raised in rabbits as previously described (8, 18, 19).
immunoglobulin fraction of the immune sera and pre-immune controls were prepared according to Harboe and Ingild (20). SDS polyacrylamide gel electrophoresis (10% gels) separated such fractions into IgG heavy and light chains and no impurities were noted.

Anti-gizzard myosin was shown to be specific for smooth muscle myosin by immunodiffusion, ATPase inhibition test, and immunofluorescence (10). It reacted also with nonmuscle myosin extracted from rat corneal epithelium (11). Anti-gizzard actin reacted with both smooth and striated muscle actin in immunodiffusion and immunofluorescence. It was shown to inhibit the actin-activated Mg\(^{2+}\) myosin ATPase activity (19). Anti-gizzard tropomyosin was shown to react in immunodiffusion with smooth and striated muscle tropomyosin but not with myosin (8). All three gizzard antibodies reacted in immunofluorescence also with various nonmuscle cells (14, 17). Their different affinities towards 3T3 cells, differentiated and dedifferentiated smooth muscle cells, fibroblasts, and endothelial cells were used as a tool to discriminate between these cell types in tissue culture (6-9, 17). Antibodies to striated muscle myosin and tropomyosin did not show any reaction with the corresponding smooth muscle and nonmuscle antigens, by immunodiffusion, immunofluorescence, and ATPase inhibition tests (17, 18), and were thus used as controls.

**Immunocytochemistry**

Immunocytochemical staining was performed before embedding (pre-embedding staining) using the unlabeled antibody enzyme technique according to Sternberger (for review, see reference 33). All attempts to localize myosin, actin, and tropomyosin on thin plastic sections (postembedding staining) of unfixed (freeze-substituted) and fixed (2% formaldehyde in phosphate-buffered saline) unfixed tissues were reported (2, 33). All attempts to localize myosin, actin, and tropomyosin on thin plastic sections (postembedding staining) of unfixed (freeze-substituted) and fixed (2% formaldehyde in phosphate-buffered saline) unfixed tissues were reported (2, 33). All attempts to localize myosin, actin, and tropomyosin on thin plastic sections (postembedding staining) of unfixed (freeze-substituted) and fixed (2% formaldehyde in phosphate-buffered saline) unfixed tissues were reported (2, 33).

**RESULTS**

**Effect of the Fixation Procedure and Detergent Treatment on the Ultrastructure and on the Immunocytochemical Staining Patterns**

Fixation, detergent treatment, and the immunocytochemical staining procedure did not alter the pattern of thin (5–8 nm) and intermediate (10 nm) filaments in the apical part of the intestinal epithelium. However, exposure to detergents led to considerable changes in the structure of the cytomembranes. Swelling of mitochondria and the formation of numerous small and large vacuoles were noted as first signs of detergent penetration. In the more progressive stages, the structure of the microvillar plasma membrane was affected, as noted by undulation of the membrane, appearance of demembranated microvillar segments, and irregularly curved microvilli.

More severe changes of the microvillar structure, such as fragmentation and final loss of the microvilli were numerous in response to exposure to 0.1% Triton X-100, but were considerably reduced or missing in tissue treated with 0.05% Triton X-100 or Nonidet P 40.

In fixed tissue not exposed to detergents, immunoreactivity was only seen in a few cells scattered along the surface of the section. The number of immunoreactive cells was very much increased by exposure of the tissue to detergents. The majority of immunoreactive cells showed more or less pronounced detergent-induced changes of the brush border (demembranated microvillar segments and irregular curving of microvilli). Only few of the immunoreactive cells possessed rather well-preserved microvilli.

**Control Experiments**

In tissue samples treated as described above, no immunostaining was observed when the various control antibodies were applied, such as anti-striated muscle myosin or tropomyosin, pre-immune Ig, or antibody adsorbed to the homologous antigen. This is documented in Fig. 1.

**Light Microscopy**

In 1-μm Araldite sections of tissue slices incubated with anti-smooth muscle myosin, specific immunoperoxidase staining was confined to a narrow band in the apical cytoplasm located beneath the brush border. At higher magnification, this immunoreactive zone was seen to be composed of intensely stained dots and streaks separated by less reactive spacings (Fig. 2). In addition, a thin immunoreactive band was regularly noted at the base of the epithelial cell, presumably representing the basal subplasmalemmal web (Fig. 2). Anti-actin stained both the microvilli of the brush border and a narrow band in the apical cytoplasm (Fig. 3), as well as the basal subplasmalemmal web region. The staining pattern of anti-smooth muscle tropomyosin (Fig. 4) was similar to that of anti-myosin, i.e., a dotted line within the terminal web region, a narrow band at the base of the epithelial cell, and no detectable staining of the microvilli.

**Electron Microscopy**

**ANTI-MYOSIN:** The interrupted staining pattern seen in light microscopy corresponds to a tight packing of PAP complexes along the rootlets of microvillar filament bundles (Figs. 5 and 6). In most preparations, the staining was too dense to distinguish individual PAP complexes; in less intensely stained rootlets, however, the immunocomplexes were seen to be arranged along the periphery of the filament bundles (Fig. 6, inset). The label terminated abruptly at the base of the microvilli, and there was no association of myosin immunoreactivity with core filaments, neither in completely demembranated microvilli nor in microvilli with an ultrastructurally intact plasma membrane. Within the terminal web, the myosin-specific label was less densely packed.

**ANTI-ACTIN:** Anti-actin stained the entire microvillar fila-
FIGURE 1 Electron micrographs (no heavy metal counterstain) of the apex of intestinal epithelium incubated with the following control Igs using the unlabeled antibody peroxidase method: (a) anti-striated muscle tropomyosin, (b) anti-gizzard myosin previously absorbed with the antigen, (c) pre-immune IgG. Tissue was exposed to Triton X-100 (0.05-0.1% for 5 min) before fixation and immunocytochemical staining procedure. No immunoperoxidase label is seen. MV, microvilli; R, rootlet of microvillar filament bundles; D, spot desmosome. Bar, 0.5 μm. (a) x 46,000; (b and c) x 37,500.

FIGURES 2-4 Light micrographs of unstained 1-μm-thick Araldite sections of intestinal epithelium incubated with anti-gizzard myosin (Fig. 2), anti-actin (Fig. 3), and anti-tropomyosin (Fig. 4) before embedding (exposure to 0.1% Triton X-100 for 5 min before fixation and immunostaining). Bar, 10 μm. x 1,600.

FIGURE 2  The myosin-specific label is concentrated in a small band within the apical cytoplasm below the microvilli and in a small zone along the base of the epithelial cells (arrowheads). Arrow points to interruptions of the apical immunoreactive zone.

FIGURE 3  Anti-actin stains microvilli as well as a narrow zone within the apical cytoplasm.

FIGURE 4  The distribution of tropomyosin-specific stain is identical to that seen with anti-myosin.

ment bundles from the tip down to the rootlets, and also the terminal web (Figs. 7-9). There was a considerable variation in the staining intensity of both rootlets and terminal web, independent of the intensity observed in the microvilli of the same preparation. These findings will be discussed below.

ANTI-TROPOMYOSIN: Anti-tropomyosin stained the rootlet filaments and the terminal web (Figs. 10 and 11) in a pattern similar to that described for anti-myosin. Generally, the microvillar core was devoid of PAP label when the tissue was pretreated with 0.05% TritonX-100 (Fig. 10) or 0.05% Nonidet P 40 (not shown). However, when tissue had been exposed to 0.1% Triton X-100 for 5 min, the microvilli of many cells showed a rather weak, irregular staining along their entire length (Fig. 11). It appears that the more rigorous treatment
FIGURE 5  Electron micrograph showing the distribution of gizzard myosin immunoreactivity in a cell located at the cut edge of the Vibratome section (no detergent treatment). Immunoperoxidase label is confined to the microvillar rootlets (appearing as dots and streaks in this oblique section) and the terminal web (arrows). Note the absence of myosin label in the remaining cytoplasm. Bar, 0.5 μm. X 10,200.

FIGURE 6  Ultrastructural distribution of gizzard myosin-specific immunoreactivity in the brush-border region (exposure to 0.1% Triton X-100 for 5 min before fixation and staining). The PAP complexes (small arrows) are located along the rootlets (R) and in the terminal web (TW). Inset shows a cross-sectioned rootlet which is surrounded by individual PAP complexes (arrows) and aggregates of PAP complexes (asterisks). Bar, 0.5 μm. X 45,000. Inset: Bar, 0.1 μm. X 125,000.

with a higher concentration of detergent leads to a displacement of tropomyosin from its site in the terminal web or rootlets to the microvilli.

Desmosomal Zone of the Apical Filament Web

The desmosomal or basal zone of the apical filament web (5, 22) contains mainly intermediate filaments with only a few thin filaments showing. Thus, none or very few immunocomplexes specific for actin, myosin, or tropomyosin were seen in this zone.

Cytomembranes

Cytomembranes adjacent to the immunoreactive apical cytoplasm (membranes of vacuoles, mitochondria, and the
plasma membrane) usually displayed an irregular electrondense staining (Figs. 6, 8–11), although the cytomembranes were virtually devoid of attached immunocomplexes (PAP). This strongly indicates that the staining of the cytomembranes is caused by a nonspecific binding of the lipophilic and osmiophilic peroxidase reaction product (a phenazine polymer), originating from adjacent filament-bound PAP complexes.

**Basal Subplasmalemmal Web**

A delicate network of thin filaments (4–8 nm in diameter) was seen in the basal cytoplasm of the intestinal epithelium. It was located underneath the basal plasmalemma and was occasionally intermingled with some intermediate filaments (Figs. 12 and 13). This web of thin filaments was clearly distinguishable from the intermediate filament bundles traversing the basal cytoplasm or running parallel to the plasmalemma. In the basal cytoplasm, the antibodies to contractile proteins were mainly associated with this subplasmalemmal web (Figs. 7 and 14, see also Fig. 2), but not with the intermediate filament bundles.

**DISCUSSION**

The microfilament organization in the brush border of intestinal epithelial cells has previously been characterized by immunofluorescence microscopy using antibodies to various contractile proteins (4, 13, 28). The limited resolution of light microscopy, however, demanded the use of the electron micro-

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**Figure 8** Anti-actin label is concentrated along the core of the microvilli and their rootlets. The dotlike PAP complexes are clearly visible. In this preparation (0.05% Nonidet P 40 before fixation and staining), the terminal web (TW) is only weakly labeled. Bar, 0.5 μm. X 37,000.

**Figure 9** Portion of the apical cytoplasm illustrating the actin-specific label within the terminal web (same processing as in Fig. 8). Arrows point to PAP complexes, some of which exhibit a ringlike substructure. Bar, 0.5 μm. X 46,000.

**Figure 10** Distribution of gizzard tropomyosin immunoperoxidase label. The immunocytochemical staining pattern is similar to that obtained with anti-gizzard myosin (labeling of rootlets and the terminal web). Tissue was exposed to 0.05% Triton X-100 before fixation and staining. Bar, 0.5 μm. X 46,000.

**Figure 11** Irregular anti-tropomyosin staining of microvilli in demembranated brush border exposed to 0.1% Triton X-100 for 5 min before fixation and staining. Bar, 0.5 μm. X 35,000.
scope in conjunction with highly specific antibodies. This technique requires considerable compromises in the handling of the tissue to be examined, in order to guarantee both optimal penetration of antibody and maximum preservation of the antigenic structure.

In the study presented here, the unlabeled antibody enzyme method was chosen as the most satisfactory technique. Only immunological bonds were used here to attach the detector molecule (horseradish peroxidase) to the antigenic site in the tissue section. Covalent labeling of the antibodies, which may significantly impair their specificity, is thus avoided (for review, see reference 33). In addition, the PAP method exceeds the labeled antibody techniques in sensitivity by several orders of magnitude (34). Lastly, the cyclic structure of the PAP complex confers high stability to the detector molecule and allows the direct visualization of the 20- to 30-nm diameter rings or dots in the electron microscope. These characteristic structures are easily distinguishable from the nonspecific electron-dense peroxidase reaction products often deposited near the site of reaction; and they were never observed when the first incubation step was performed with Ig fractions derived from pre-immune sera, from specific antibodies exhaustively absorbed to the homologous antigen, or from heterologous non-cross-reacting antibodies (e.g. anti-striated muscle myosin and tropomyosin). With the specific antibodies to smooth muscle myosin, actin, and tropomyosin the immunoreactivity was consistently confined to the apical and basal cytoplasm, irrespective whether tissue sections or fragmented epithelial cells were used, and the patterns closely resembled those seen in immunofluorescence (4, 13).

In the electron micrographs, however, we sometimes ob-

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\[\text{FIGURE 12} \quad \text{Basal of intestinal epithelium incubated with pre-immune Ig (B, basal lamina). Asterisks indicate the subplasmalemmal layer of interwoven thin filaments measuring 4-8 nm in diameter. Small arrows point to cross-sectional profiles of individual thin filaments; larger arrows point to bundles of intermediate filaments (8-10 nm). Uranyl-acetate and lead citrate counterstain. Bar, 0.2 \mu m. × 83,000.}\]

\[\text{FIGURE 13} \quad \text{Oblique section of the basis of intestinal epithelium incubated with pre-immune Ig (B, basal lamina). The subplasmalemmal web of thin filaments extends into a tangentially sectioned basal process (B, basal lamina). Arrows point to individual intermediate filaments projecting into the subplasmalemmal web. Uranyl acetate and lead citrate counterstain. Bar, 0.2 \mu m. × 83,000.}\]
intermediate filaments (IF) are not stained. Bar, 0.5 μm. x 46,000.

staining of the basal subplasmalemmal web (arrows). Bundles of to 0.05% Triton X-100 before fixation and staining). Note immuno-
thelial cell incubated with anti-gizzard tropomyosin (5 minexposure

small myosin aggregates with both the terminal web filaments
and the rootlet filaments. The microfilaments of the terminal web might serve as an anchoring system for the myosin units, preventing them from sliding up the rootlet filaments. The terminal microvillus web, in turn, is thought to be restrained from moving towards the cell surface by loops of intermediate filament bundles attached to the spot desmosomes (5, 22).

Further stability might be conferred to the terminal web by interconnections of the thin filaments with α-actinin. This protein, which is known to be present in the Z lines of skeletal muscle, has recently been shown by immunofluorescent and immunoferritin labeling to be scattered throughout the terminal web (10, 16). Cross links between α-actinin and the terminal web filaments would permit changes in filament polarity and this might provide a feltwork of interconnected actin filaments with changing polarity.

An isometric tension arising within the terminal web might be strong enough to overcome the rigidity of the microvillus membrane (to which the core filaments are attached), and movement of the microvilli would follow. In the model presented by us, the terminal web attached to the intercellular junctions represents the punctum fixum, and the punctum mobile is represented by the microvillus filament-membrane complex. This hypothetical contractile mechanism might also explain the ATP-induced microvillus retraction observed in isolated completely demembranated brush-border regions (26): the terminal web, detached from the junctional membrane, might shrink during contraction until it becomes attached to its most adjacent row of microvillar rootlets, which will then serve as a secondary or auxiliary punctum fixum for the detached web; the rigidity of which might then be strong enough to allow microvillus sliding. The centripetal force generated in the terminal web would cause the rootlets to approach each other and the tips of the microvilli to spread. As a matter of fact, this is exactly the picture that is regularly seen in the isolated contracted brush border (26, 30). This contraction and spreading of microvilli is less readily explained by the interaction of splayed rootlet filaments of adjacent microvilli, as originally
Lieberkühn to the tip of the intestinal villi (25). Of epithelial cells from their original sites in the crypt of contractile elements may participate in the upward movement beneath the basal plasma membrane. It is our suggestion that these bind to a very delicate network of 4- to 8-nm filaments just shown that antibodies to myosin, actin, and tropomyosin will references 4 and 13. In the study presented here, we were able to found in the brush-border region might not only serve in microvillar contraction, but may also be involved in other dynamic events, such as endo- or exocytosis. As it has been recently shown, contractile proteins are also concentrated in the terminal web region of acinar and duct cells of various exocrine glands and in liver cells (12, 14, 15, 21).

Contractile elements are not confined to the brush-border region, as recently they have also been localized in the basal cytoplasm by immunofluorescence (see figures shown in references 4 and 13). In the study presented here, we were able to show that antibodies to myosin, actin, and tropomyosin will bind to a very delicate network of 4- to 8-nm filaments just beneath the basal plasmalemma. It is our suggestion that these contractile elements may participate in the upward movement of epithelial cells from their original sites in the crypt of Lieberkühn to the tip of the intestinal villi (25).

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