LOCALIZATION OF ACTIN AND MICROFILAMENT-ASSOCIATED PROTEINS IN THE MICROVILLI AND TERMINAL WEB OF THE INTESTINAL BRUSH BORDER BY IMMUNOFLUORESCENCE MICROSCOPY

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ABSTRACT

Indirect immunofluorescence microscopy was used to localize microfilament-associated proteins in the brush border of mouse intestinal epithelial cells. As expected, antibodies to actin decorated the microfilaments of the microvilli, giving rise to a very intense fluorescence. By contrast, antibodies to myosin, tropomyosin, filamin, and α-actinin did not decorate the microvilli. All these antibodies, however, decorated the terminal web region of the brush border. Myosin, tropomyosin, and α-actinin, although present throughout the terminal web, were found to be preferentially located around the periphery of the organelle. Therefore, two classes of microfilamentous structures can be documented in the brush border. First, the highly ordered microfilaments which make up the cores of the microvilli apparently lack the associated proteins. Second, seemingly less-ordered microfilaments are found in the terminal web, in which region the myosin, tropomyosin, filamin and α-actinin are located.

KEY WORDS microvilli · actin · myosin · tropomyosin · filamin · α-actinin

The apical end of the intestinal epithelial cell consists of a specialized structure known as the brush border. This structure contains about 1,000 microvilli which insert into the underlying region, called the terminal web, of each epithelial cell. Two classes of microfilamentous structures can be documented in the brush border by electron microscopy: first the compact bundle of ordered microfilaments which correspond to the core of each microvillus; and second, a netlike arrangement of seemingly less-ordered microfilaments found in the terminal web. Each microvillus core contains about 20 individual microfilaments (60 Å in diameter) running from the tip, down the microvillus, and finally inserting into the terminal web (2, 11, 12). Using decoration by the S_1 fragment of myosin, Mooseker and Tilney (11) showed that all core microfilaments have the same polarity with the typical arrowheads pointing towards the terminal web, i.e., the tip of the microvillus resembling the muscle Z line. Careful analysis of thin sections and freeze fractures has furthermore demonstrated the presence of regularly spaced cross-filaments which seem to connect the central core of microfilaments lengthwise to the inner side of the microvillus membrane (11, 12).

The terminal web region of the brush border does not show the simple and highly ordered organization of the microvillus proper. In thin sections, microfilaments and intermediate filaments have been documented (2, 11, 12). Mooseker and Tilney (11) also reported the presence of short myosin-like filaments which seem to be associated with the roots of the microvillus core microfilaments. The directionality of the microvillus core microfilaments suggests that they might interact with myosin filaments in the terminal web, allowing contraction to occur. Indeed, Mooseker (9) observed contraction of the microvilli into the terminal web when Ca^{++} and ATP were added to demembranated brush borders. Thus,

the terminal web not only supplies structural support for the microvilli but also interacts with them as a motile system.

To understand more about the organization and interaction between the microfilament cores of the microvilli and the terminal web, it is essential to know what proteins are associated with which ultrastructural component. Biochemical analysis has shown that brush borders contain actin, myosin, and tropomyosin (9, 10, 15). During the last four years, immunofluorescence microscopy has shown that myosin, tropomyosin, α-actinin, and filamin are associated with microfilament bundles in tissue culture cells (4, 5, 7, 8, 17). Using the same approach on intestinal epithelial cells, we show that microvilli do not contain myosin, tropomyosin, filamin, or α-actinin, whereas these proteins are present in the terminal web.

MATERIALS AND METHODS

Preparation of Intestinal Epithelial Cells

A mouse was sacrificed by cervical dislocation; the small intestine was immediately excised and its contents were removed with phosphate-buffered saline (PBS). After one end of the intestine was clamped, the lumen was filled with 3.7% formaldehyde in PBS, the other end was clamped, and the whole intestine immersed for 10 min at room temperature in the same fixative. The intestine was drained and cut open longitudinally. Epithelial cells were removed from the luminal surface by scraping, suspended in PBS, and single cells were prepared by passing the suspension several times through a 22-gauge syringe needle. The cells were filtered through a 100-μm mesh nylon cloth, collected at 300 *g* for 5 min, and finally resuspended in a small volume of PBS.

Indirect Immunofluorescence Microscopy and Antibodies

10 μl of the cell suspension was spread on a cover slip and left at room temperature until nearly dry. Under these conditions, many cells become attached to the glass surface. The cover slips were then processed for immunofluorescence microscopy as described (18), by first making the cells permeable with cold methanol and acetone followed by air drying and incubation with the appropriate IgGs. Cells were viewed in a Zeiss microscope, using epifluorescence illumination and oil immersion objectives at 25, 40, 63, or 100 power.

The rabbit immune IgGs to chicken gizzard actin, chicken gizzard myosin, bovine brain tropomyosin, chicken gizzard filamin, and pig muscle α-actinin have been described in detail (19). Antibodies to chicken gizzard α-actinin were also elicited in rabbits. All antibodies were purified by affinity chromatography of their IgG fraction on their corresponding antigen covalently bound to Sepharose 4B (16) and used at 0.05 mg/ml in PBS. The mono-specific IgGs, which were all derived from sera giving a precipitin reaction in double diffusion with their corresponding antigens, stained nonmuscle cells in immunofluorescence microscopy to give the typical patterns documented in this and other laboratories (4, 5, 7, 8, 17). The fluorescein-labeled goat-anti-rabbit IgGs were purchased from Miles-Yeda (Israel). A 20-fold dilution (0.5 mg/ml) in PBS was preabsorbed twice for 1 h each on fixed intestinal epithelial cells before use in fluorescence microscopy.

RESULTS

Preliminary experiments on the preparation of intestinal epithelial cells suggested that rapid fixation was essential for good morphological preservation. Cells were therefore fixed with formaldehyde *in situ* immediately after removal of the intestine. Single cell preparations were attached to cover slips and then made permeable with organic solvents and processed for immunofluorescence microscopy. Two problems were encountered when such cells were studied by indirect immunofluorescence microscopy. First, only limited resolution of the immunofluorescent location of proteins could be expected as the cells are small, rather thick, and the brush border is a curved structure. Second, background fluorescence was observed, especially in the region of the brush border, even when low dilutions (1:32) of nonimmune sera were used. The latter problem was overcome by the exclusive use of antigen-affinity column purified immune IgGs ("mono-specific" IgGs) and preabsorption of the fluorescent second antibody on intestinal cells (see Materials and Methods). Under these conditions, immunofluorescence microscopy with nonimmune rabbit IgGs at a concentration fivefold higher (Fig. 1 *a* and *b*) than that used in subsequent experiments with immune IgGs shows very little fluorescence, particularly in the region of the brush border.

Immunofluorescence microscopy on fixed intestinal cells using monospecific IgGs to actin stains the microvilli of the brush border very intensely (Fig. 1 *c* and *d*). In favorable specimens, fluorescence emitted by the individual microvilli can be discerned microscopically, although we found that photographic documentation is extremely difficult. This result demonstrates that under our experimental conditions, the cores of the microvilli are accessible to antibodies. The presence of actin in the terminal web was not possible to

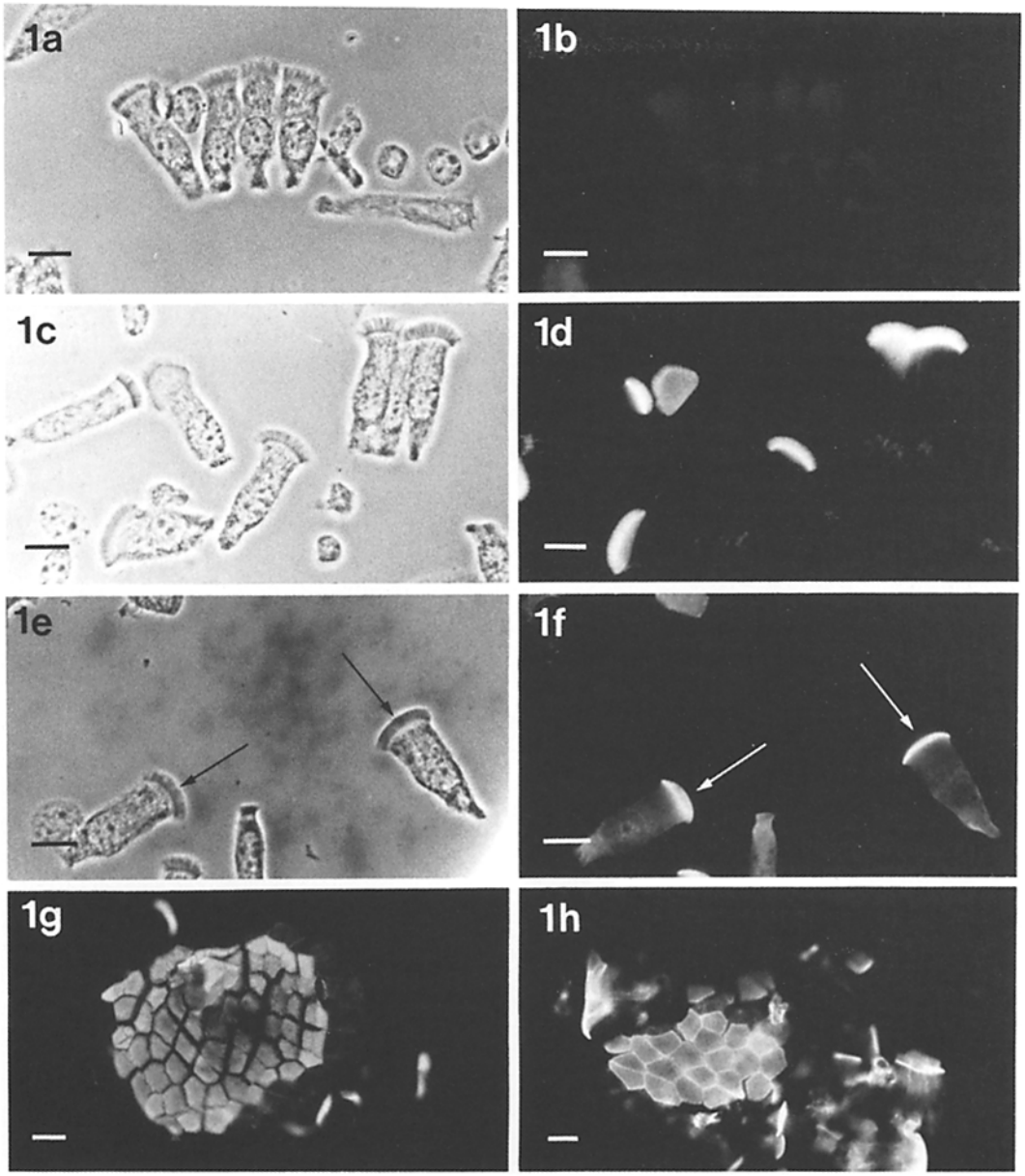
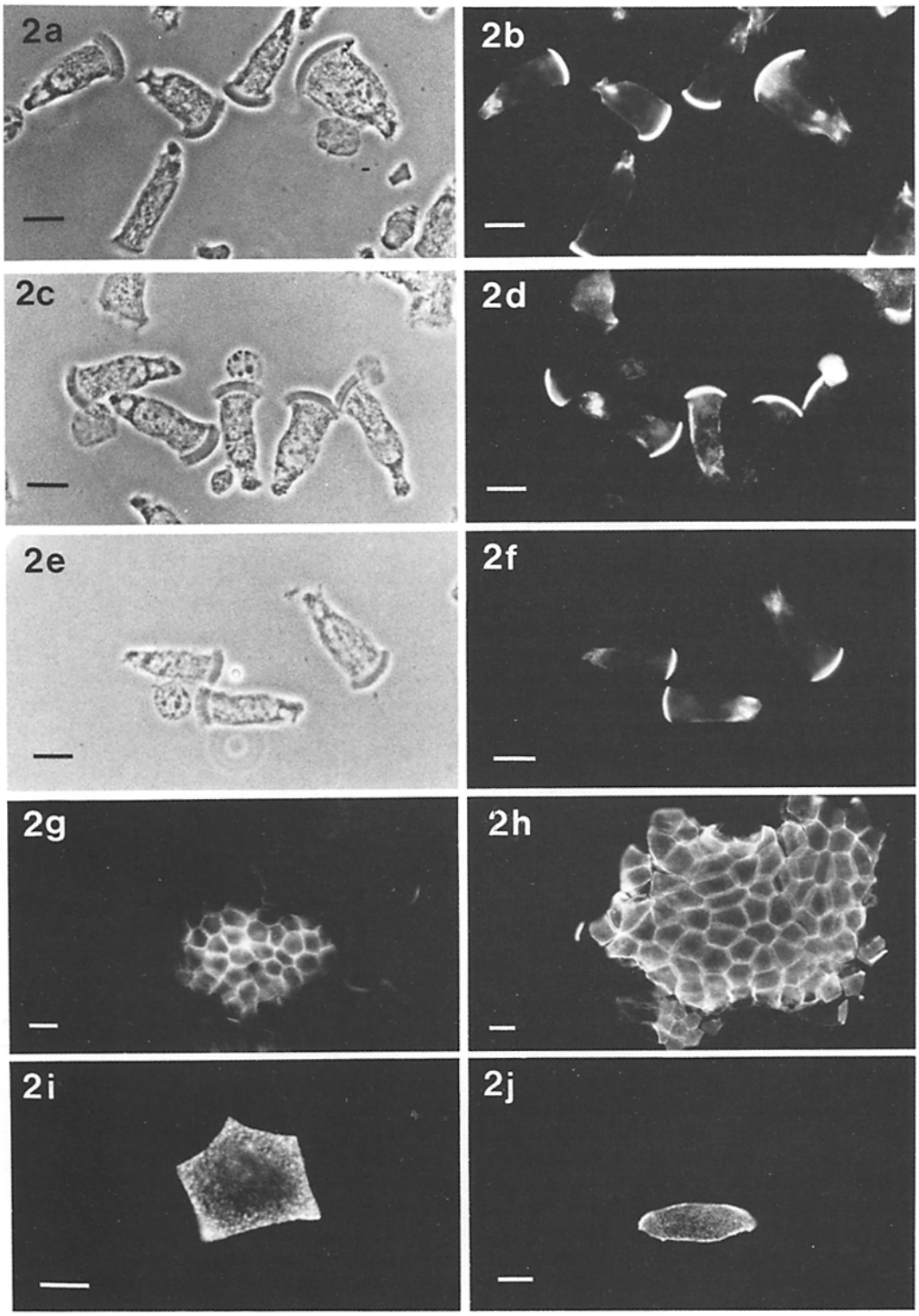


FIGURE 1 Corresponding pahse contrast (*a*, *c*, and *e*) and fluorescence (*b*, *d*, and *f*) micrographs (× 620) of intestinal epithelial cells decorated for immunofluorescence microscopy. Cells decorated with nonimmune IgGs (*a* and *b*) show very little fluorescence. Cells decorated with antiactin IgGs (*c* and *d*) show intense fluorescence from the microvilli of the brush border. Decoration with antimyosin IgGs (*e* and *f*), however, shows fluorescence emanating from the terminal web but not from the microvilli (arrows indicate the same positions in the two micrographs). Bars, 10 μm. Groups of cells decorated either with antiactin IgGs (*g*) or antimyosin IgGs (*h*) and viewed from the apical end (× 390). These micrographs were taken with the microscope focussed on the most fluorescent part of the cell, i.e., on the microvilli (*g*) and on the terminal web (*h*). Bars, 10 μm.

demonstrate independently because this region is obscured by the intense fluorescence emitted by the microvilli. Fig. 1 *d* also shows that the majority of the actin in intestinal epithelial cells is found in the brush border. Fluorescence from the distribution of actin in the cell body was also seen, but


2a
2b
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2g
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does not show on the micrograph because of the intense fluorescence emitted by the brush border.

Antimyosin IgGs displayed this protein in the terminal web but not in the microvilli (Fig. 1 *e* and *f*). The thin crescent-shaped fluorescent pattern seen with antimyosin, as compared to the brush shape seen when the brush border is stained with antiactin, is typical of a protein located in the terminal web and not in the microvilli. A careful examination of the phase contrast and fluorescence micrographs of the same cells shows that no fluorescence originates from the microvilli. The location of actin and myosin was also observed in the plane of the brush border by examining groups of cells viewed from the apical end. Using antiactin IgGs, the extent of each brush border is delineated by the fluorescence emitted from the microvilli (Fig. 1 *g*). Because microvilli do not contain myosin, the localization of this protein in the terminal web was possible. Thus, myosin is present throughout the terminal web, although it appears to be slightly more concentrated around the periphery of the brush border where it joins adjacent cells (Fig. 1 *h*).

The microvilli of the brush border were not decorated by antibodies to either tropomyosin (Fig. 2 *a* and *b*), two different antibodies against α-actinin (Fig. 2 *c* and *d*), or antibodies to filamin (Fig. 2 *e* and *f*), although these antibodies stained the terminal web region. The distribution of tropomyosin and α-actinin in the terminal web (Fig. 2 *g* and *h*) was similar to that seen for myosin, where the protein seems to be preferentially located around the edges of the organelle. Higher magnification of the antitropomyosin and anti-α-actinin decorated cells suggested a "dotted" distribution of these proteins in the terminal web (Fig. 2 *i* and *j*) which is very difficult to document photographically. A dotted fluorescence distribution in the terminal web was also seen with antimyosin (not shown). The intensity of fluorescence using anti-filamin antibodies was relatively weak, making it difficult to determine the precise distribution of this protein in the terminal web.

DISCUSSION

We have characterized the microfilament organization in the brush border of the intestinal epithelial cell by immunofluorescence microscopy using affinity column purified antibodies against actin, myosin, tropomyosin, filamin, and α-actinin. The results clearly differentiate the organization of the terminal web microfilament system from that of the microvillus microfilaments.

The highly ordered microfilament bundles of the microvilli cores show a very simple organization. Immunofluorescence microscopy reveals the virtual absence of myosin, tropomyosin, filamin, and α-actinin from the microvilli, but the presence of actin. Antiactin antibodies have been used previously to visualize microvilli in amphibian oocytes (3). That intestinal epithelial cell microvilli do not contain myosin, tropomyosin, or filamin is in agreement with our previous biochemical analysis of microvilli (1), but the absence of α-actinin is unexpected and will be discussed below. The absence of tropomyosin and filamin is important. Tropomyosin has been suggested to be selectively associated with semi-permanent or permanent microfilaments (6). Its absence from the microvilli microfilaments argues that this proposal is not universally true and may indicate that tropomyosin could act also in nonmuscle cells as part of a Ca^{++}-mediated control of contraction. If tropomyosin plays such a regulatory role, its absence from the microvilli, and its presence in the terminal web in the same locations as myosin, is to be expected. Filamin has up to now been considered to be a microfilament-associated protein following the intracellular distribution of ac-

FIGURE 2 Corresponding phase contrast (*a*, *c*, and *e*) and fluorescence (*b*, *d*, and *f*) micrographs (× 620) of cells decorated with either antitropomyosin (*a* and *b*), anti-pig muscle α-actinin (*c* and *d*), or antifilamin (*e* and *f*) antibodies. Note the fluorescence emitted from the terminal web region (*b*, *d*, and *f*) and the absence of fluorescence from the microvilli. Decoration of intestinal epithelial cells with anti-gizzard α-actinin gave identical results to the decoration seen with anti-pig α-actinin shown in Fig. 2 *d*; Bars, 10 μm. The distribution of tropomyosin (*g*) and α-actinin (*h*) in groups of cells viewed from the apical end. These two micrographs (× 390) were taken with the microscope focussed slightly below the plane of the terminal web to show the location of these proteins near the zonula adherens. Bars, 10 μm. Higher power visualization of the distribution of tropomyosin (*i*, × 1,500) and α-actinin (*j*, × 950) in the terminal web region of the brush border. Note the dotted distribution of these proteins throughout the terminal web and their preferential location towards the edges of the organelle. Bars, 5 μm.

tin, even in those cases, like the membrane ruffle, where myosin and tropomyosin seem to be absent (4). The absence of filamin from the microvillus seems to be the first exception to this hypothesis. However, we cannot exclude the unlikely possibility that some associated proteins were lost from our microvilli during biochemical fractionation (1) and that these proteins were also lost or not detected during immunofluorescence microscopy.

The absence from the microvillus of a protein cross-reacting with α-actinin is unexpected. Schollmeyer et al. (14) reported in an abstract that anti-α-actinin antibody decorated not only the terminal web but also the microvilli. Our results demonstrate α-actinin as part of the terminal web, but not of the microvilli. Currently, it is difficult to reconcile these different data. Although one could invoke species specificity of our α-actinin antibodies, our finding of α-actinin in the terminal web argues against it. In addition our anti-α-actinin antibodies decorate tissue culture cells of various species including mouse, giving rise to the same pattern as that described by Lazarides and Burridge (7). The possibility remains, however, that there exists a protein in the microvillus immunologically related to α-actinin but that our antibodies do not reveal this relation. Mooseker and Tilney (11) have argued that the cross-filaments connecting the core microfilaments to the microvillus membrane are indeed α-actinin. Their reasons were not only the immunofluorescence results discussed above (14), but, in addition, they observed that the cross-filaments have dimensions very similar to those of α-actinin and that brush borders contain a protein of a polypeptide molecular weight similar to that of α-actinin (100,000 daltons). Our studies on pure microfilament cores of microvilli (1) showed that they contain only two major proteins: actin and a 95,000-dalton polypeptide, present in a molar ratio of approx. 10 to 1. Thus, the 95,000-dalton protein is indeed the candidate for the cross-filament protein. We have therefore begun to isolate this protein to compare it with α-actinin. Our present results suggest that it is different from α-actinin, but further protein-chemical and immunological data on the purified, 95,000-dalton protein are needed for conclusive proof.

Immunofluorescence microscopy shows that myosin, tropomyosin, filamin, and α-actinin are associated with the microfilaments in the terminal web. This finding agrees with biochemical analyses of intestinal brush borders which revealed the presence of actin (15), myosin (10), and tropomyosin (9). Indeed, the Abstract (10) reported that myosin was exclusively located in the terminal web. Furthermore, it provides independent evidence which substantiates our recent data on the protein compositions of pure microvilli and pure brush borders, where myosin, tropomyosin, and filamin were found to be present in the terminal web but not in the microvilli (1). The finding of myosin exclusively in the terminal web, and somewhat concentrated where adjacent cells join, is interesting. It supports the electron microscope result showing the occasional short myosin-like filaments associated with the roots of the microvillus microfilament bundles where they insert into the terminal web (11). This location of myosin also agrees with the Ca^{++}-dependent contraction of the microvilli into the terminal web observed by Mooseker (9). The result that myosin is preferentially located near areas of cell contact indicates that it may be present in the region of the zonula adherens, which is known to be rich in microfilaments (13). The rounding up of intact brush borders upon addition of Mg^{++} and ATP noticed by Rodewald et al. (13) could be explained by a myosin-mediated contraction between the zonula adherens of opposite sides of the organelle. Although the resolution of the light microscope was limiting, we were able to detect some regular arrangement or striations in the organization of myosin, tropomyosin, and α-actinin in the plane of the terminal web which suggested a repeated macromolecular arrangement as previously shown in tissue culture cells for these proteins (5, 7, 17). Because the terminal web supplies structural support for the microvillus cores as well as interacting with them to cause contraction, elucidation of its fine structure should provide generally applicable information about microfilament organization and function in nonmuscle cells.

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Note added in proof: We have recently found that antibodies to α-actinin do not cross-react with the microvillus 95,000-dalton protein in vitro and antibodies to the 95,000-dalton protein do not cross-react with α-actinin. We conclude that the 95,000-dalton protein is not identical with α-actinin.

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