

## COMPARISON OF INTESTINAL BRUSH-BORDER 95-KDALTON POLYPEPTIDE AND ALPHA-ACTININS

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### ABSTRACT

To explore the suggestion that alpha-actinin cross-links actin filaments to the microvillar membrane (Mooseker and Tilney, 1975, *J. Cell Biol.* **67**:725-743; Mooseker, 1976, *J. Cell Biol.* **71**:417-433), we have assessed the possible relatedness of alpha-actinin and the brush-border 95-kdalton protein by four independent criteria: antigenicity, mobility on SDS gels, extractability in nonionic detergents, and peptide maps. We have found that anti-chicken gizzard alpha-actinin stains the junctional complex region of intact cells (Craig and Pardo, 1979, *J. Cell Biol.* **80**:203-210) but does not stain isolated brush borders even though these structures contain a 95-kdalton polypeptide. Lack of staining is not caused by failure of the antibody to penetrate, as antiactin stains both the terminal web and the microvilli of isolated brush borders. By the antibody SDS gel overlay technique, we have established that anti-gizzard alpha-actinin recognizes homologous molecules in chicken skeletal and cardiac muscles, as well as in intestinal epithelial cells, but fails to recognize the brush-border 95-kdalton polypeptide. Conversely, anti-95-kdalton polypeptide does not recognize gizzard alpha-actinin. On high-resolution SDS polyacrylamide gel electrophoresis, alpha-actinin and brush-border 95-kdalton protein exhibit distinct mobilities. The two proteins also differ in their ability to be extracted in nonionic detergent: epithelial cell immunoreactive alpha-actinin is soluble in NP-40, whereas 95-kdalton protein is insoluble. Finally, two-dimensional peptide mapping of iodinated tryptic peptides, as well as one-dimensional fingerprinting of partial tryptic, chymotryptic, papain, and *S. aureus* V8 protease digests, have revealed <5% homology between gizzard alpha-actinin and brush-border 95-kdalton polypeptide. The data suggest that there is no major structural homology between gizzard alpha-actinin and brush-border 95-kdalton protein. We conclude that it is unlikely that alpha-actinin cross-links actin filaments to the microvillar membrane.

**KEY WORDS** alpha-actinin · brush border · villin · microvilli · intestinal epithelium

Microvilli of intestinal epithelial cells provide a unique system for analysis of the molecules that mediate attachment of microfilaments to mem-

brane. Unlike most nonmuscle cell systems, in which the interaction of actin with membrane appears to be a dynamic event and is morphologically undefined, the interaction between the microvillar actin filaments (19, 28, 31, 41) and the microvillar membrane is relatively stable, al-

though not irreversible (40). By electron microscopy, it is actually possible to visualize cross bridges between the microfilaments and the membrane (18, 28, 29, 31). Identification and functional characterization of the molecules in these cross bridges has high priority for progress in studies of microfilament-membrane interactions, for although epithelial cells are unique in the density and stability of their microvilli, they are not unusual in terms of possessing such structures. All cell types appear to extend and retract microvillar structures. These organelles may be involved in mitochondrial transport (2), capping of cell surface proteins (12), and virus release (10). In addition, if microfilaments interact with intracellular membrane-bounded organelles such as secretory vesicles (32), lysosomes (26), and mitochondria (2), the microvillar cross-bridge molecules may have homologues that mediate these latter types of membrane-actin interactions.

Because of the potential importance of molecules that link microfilaments to membranes, great interest was generated by the suggestion that alpha-actinin might be the cross bridge between actin bundles and microvillar membrane (28, 29). At the time, several types of indirect evidence were mustered to support this hypothesis: the dimensions of the cross filaments are similar (28) to those of isolated alpha-actinin (39); isolated brush borders contain a polypeptide with a subunit molecular weight reportedly identical to that of alpha-actinin (100,000) (29); and it is known from *in vitro* studies that alpha-actinin can bind to F-actin and laterally cross-link the filaments (22, 34). A preliminary communication by Schollmeyer et al. (35) reported that alpha-actinin could be detected in microvilli by immunoelectron microscopy and immunofluorescence techniques, but these studies were not subsequently documented.

On the basis of the above data, it is now widely believed that alpha-actinin is a membrane anchor site for actin filaments. Because there is little evidence for this important concept, we decided to obtain further data. In a recent publication (8), we demonstrated that chicken intestinal epithelial cells do indeed have a molecule with a subunit mol wt = 100,000 which cross reacts with antibody to chicken gizzard alpha-actinin. We also showed that epithelial cell alpha-actinin is specifically localized in the junctional complex region, a result that has subsequently been confirmed by Geiger et al. (13). We were unable, at the time, to determine whether alpha-actinin was also present in

microvilli. Concurrently, Bretscher and Weber (3, 4) reported that alpha-actinin is localized in the terminal web region but is not detectable in microvilli of isolated intact mouse epithelial cells. Bretscher's result has been confirmed by Geiger et al. (13).

Because the failure of an antibody to cross react with a putative homologous antigen is not sufficient evidence to conclude that the test antigen is not homologous, we have compared gizzard alpha-actinin to the brush-border 95-kdalton<sup>1</sup> polypeptide by a number of independent biochemical criteria. In this paper, we report the results of these studies.

## MATERIALS AND METHODS

### *Preparation of Antibodies*

The preparation and characterization of rabbit anti-chicken gizzard alpha-actinin and rhodamine-labeled goat anti-rabbit IgG (Fc fragment) have been described in an earlier report (8).

**ANTI-ACTIN:** Chicken gizzard actin was prepared by the method of Sobieszek and Bremel (37). The resulting actin, ~70% homogeneous, was prepared for immunization by the method of Lazarides (21, and personal communication). Briefly, 1 ml of a 2 mg/ml solution of actin in 20 mM imidazole, 60 mM KCl, 1 mM cysteine, 1 mM MgCl<sub>2</sub>, 1% SDS, and 40 mM dithiothreitol (DTT), pH 6.8, was boiled for 3 min, precipitated with -20°C acetone, collected by centrifugation, and resuspended in 0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.5 (PBS). The solution was made 0.5% in aluminum chloride and the pH was adjusted to 7.0 with 15% Na<sub>3</sub>PO<sub>4</sub> · 12 H<sub>2</sub>O. The resulting floc was collected by centrifugation, resuspended in 1 ml of PBS, emulsified with 2 ml of complete Freund's adjuvant (CFA), and injected subcutaneously in multiple sites along the flanks of one rabbit. 3 wk later, the animal was boosted with 1 mg of crude actin emulsified in incomplete Freund's adjuvant (IFA). 2 wk after the boost, the rabbit was bled. The serum contained precipitating antibody against actin. Monospecific antiactin was prepared by affinity chromatography of the antiserum on Sepharose 4B covalently coupled to rabbit skeletal muscle G-actin (>98% homogeneous) by the CNBr procedure (33). The antibody was eluted with 0.05 M acetic acid, pH 4.0, neutralized immediately with 2 M Tris, pH 8.0, and then dialyzed against PBS. The purified antibody was stored at 4°C in 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

**ANTI-BRUSH-BORDER 95-KDALTON POLYPEPTIDE:** The 1-M Tris extract prepared from Tergitol-extracted brush borders (see Fig. 1) was electrophoresed on preparative SDS slab gels. The 95-kdalton polypeptide was eluted from a strip of the gel. A sample of the eluted protein was rerun on a mini-slab gel to check for homogeneity. The sample (2.4 mg) was precipitated with 10% TCA, resuspended in 50 mM Tris-HCl, pH 7.6, mixed

<sup>1</sup> The 95-kdalton brush-border protein that we discuss in this paper is the same protein first noticed by Mooseker (29) and mistakenly designated as 100K. It is also identical to the 95-kdalton brush-border protein studied by Geiger et al. (13), by Bretscher and Weber (3-5) who named it "villin," and by Matsudaira and Burgess (24).

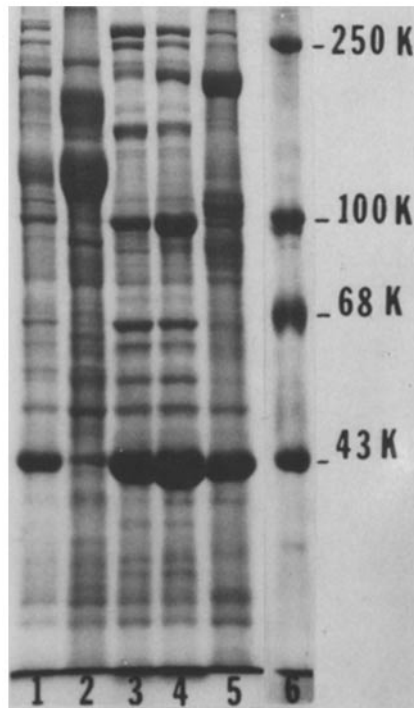


FIGURE 1 SDS polyacrylamide slab gel analysis of purified brush borders and of soluble and insoluble fractions prepared from these structures. (Lane 1) Purified brush borders. (Lane 2) Brush-border proteins soluble in 1% Tergitol, 2 mM EDTA, 2 mM EGTA, 2 mM Tris, pH 7.0. (Lanes 3 and 4) Proteins soluble in 1 M Tris, pH 7.4. Two extractions of the same material are made to quantitatively solubilize the 95-kdalton protein. (Lane 5) Proteins insoluble in either 1% Tergitol or 1 M Tris. (Lane 6) Molecular weight standards; filamin, 250,000; alpha-actinin, 100,000; bovine serum albumin, 68,000; actin, 43,000. Gels were 7.5% acrylamide and the Laemmli buffer system was used.

with an equal volume of CFA, emulsified, and injected subcutaneously into a rabbit. Booster injections in IFA were given at 2 wk (500  $\mu$ g) and at 3 mo (142  $\mu$ g). Nonprecipitating antibody to 95-kdalton protein appeared after the second boost; precipitating antibody was evident after the third immunization.

#### Immunolocalization of Proteins on SDS Gels

Protein A iodinated by the Chloramine T procedure (15) was used to localize antigen-antibody complexes in mini SDS gels (23) by the technique of Burrige (6) as modified by Adair et al. (1) for use with Protein A.

#### Isolation of Brush Borders

Brush borders were isolated from chicken intestinal epithelium by modification and recombination of the procedures of Mooseker et al. (27) and Mooseker and Tilney (28) for chicken, of Harrison and Webster (16) for rat, and of Miller and Crane (25)

for hamster. Briefly, the small intestines from two to four young laying hens were flushed with ice-cold 0.01 M  $\text{PO}_4$  buffer containing 0.15 M NaCl, 0.02%  $\text{NaN}_3$ , 10 U/ml Trasylol (FDA Pharmaceuticals, New York), pH 7.5. Washed segments were clamped at one end, filled with an ice-cold solution of 0.2 M sucrose, 0.076 M  $\text{Na}_2\text{HPO}_4$ , 0.019 M  $\text{KH}_2\text{PO}_4$ , 0.012 M EDTA, 0.02%  $\text{Na}_3$ , 10 U/ml Trasylol, pH 7.0 (sucrose solution), and incubated for 20 min at 4°C (28). Loosened cells were removed from the gut segment by gently rubbing the walls of the filled segments together, collecting the cells, refilling with the sucrose solution, and repeating the rubbing procedure until the walls of the gut appeared translucent. Cells were collected by centrifugation at 250 g for 10 min and washed once in the sucrose solution. Cell pellets were resuspended in ice-cold 2.5 mM EDTA, pH 7.0, 10 U/ml Trasylol (10 ml of solution/ml of pelleted cells) and incubated for 10 min at 0°C (25). The suspension of swollen cells was homogenized in a J. W. Greer Mini-Mill (Sellers Process Equipment Co., King of Prussia, Pa.) for 1 min with a gap of 25 and a rheostat setting of 30. This was sufficient to reduce the cells to small fragments, with the exception of the nuclei and brush borders which were left intact. Centrifugation of the homogenate at 375 g for 10 min pelleted the brush borders and many of the nuclei. Pellets were washed once in 2.5 mM EDTA, 10 U/ml Trasylol, pH 7.0. The pelleted organelles were resuspended in an equal volume of 5 mM EDTA, 10 U/ml Trasylol, pH 7.0, and layered onto a premoistened pad of glass wool (~2.5 g in a 10-cm-diameter glass funnel) for 5 min at room temperature (16). Brush borders were eluted from the glass wool with 6–10 vol of the 50mM EDTA solution. For optimal recovery of brush borders, the glass wool must be fluffed up with a stirring rod during the elution procedure. Approximately 90% of the nuclei remain adherent to the glass wool. Brush borders and contaminating nuclei were collected by centrifugation at 550 g for 10 min. Pellets were resuspended in cold MT4 solution (75 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 10 mM imidazole, 10 U/ml Trasylol, pH 7.3) and pelleted at 550 g for 10 min. The few remaining nuclei were removed by use of the sucrose gradient described by Mooseker et al. (27). Nuclei pellet to the bottom of the tube, whereas brush borders can be collected from the 40–45% and 45–50% interfaces. The brush borders from each gradient were diluted with 8 vol of MT-4 solution and pelleted at 550 g, 15 min. Brush borders were washed twice with MT-4. In general, this modified method produces ~190 mg of brush border from 30 g (wet weight) of pelleted intestinal mucosa. The brush borders are entirely free of nuclei. The modifications responsible for the good yield and total removal of nuclei are the method of breaking the cells and the use of glass wool to remove the bulk of the nuclei before the brush borders are loaded onto the sucrose gradient.

#### Differential Extraction of Brush-Border Proteins

Isolated brush borders (160 mg of protein as estimated by the Lowry method) were mixed with 8 ml of 1% Tergitol (Sigma Chemical Co., St. Louis, Mo.; NP-40 works as well), 2 mM EDTA, 2 mM EGTA, 2 mM Tris, pH 7.0. In addition, all solutions used throughout this procedure contained a protease inhibitor cocktail consisting of 250 U/ml Trasylol, 1  $\mu$ g/ml antipain, 1  $\mu$ g/ml pepstatin, and 15.7  $\mu$ g/ml benzamidin. The mixture was rocked gently at 24°C for 1 h and then centrifuged at 127,000  $g_{\text{max}}$  for 30 min at 4°C. The supernate was saved. The pellet was resuspended in 4 ml of the Tergitol buffer, rocked for another 30 min at 24°C, and centrifuged as described above. The

second supernate was combined with the first and is referred to as the Tergitol extract. The pellet was resuspended in 6 ml of 1 M Tris, pH 7.4, rocked for 1 h at 24°C, and then centrifuged at 127,000  $g_{max}$  for 30 min. The supernate is referred to as the Tris extract. The pellet was resuspended in 4 ml of H<sub>2</sub>O and is called the Tergitol-Tris insoluble material. All samples were frozen in small aliquots at -70°C. Both the use of protease inhibitors throughout the procedure and the storage of the samples at -70°C were essential to prevent rapid degradation of the proteins. Even with these precautions, some degradation occurs.

### Immunofluorescence

Isolated brush borders were fixed for 10 min at 24°C in 2% paraformaldehyde, 75 mM NaCl, 75 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10 mM imidazole, pH 7.0. The samples were washed twice in the same buffer minus the paraformaldehyde by centrifugation at 550  $g$  for 10 min. The brush borders were pelleted onto glass microscope slides using a Shandon cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.) at 550 rpm for 5 min. The specimens were prevented from drying out by placing them in a humidified box.

Indirect immunofluorescence was used to localize antigens. The unlabeled antibodies were diluted in 0.05 M Tris, containing 0.15 M NaCl, 0.05% NP-40, 0.1% bovine serum albumin (BSA), 0.02% NaN<sub>3</sub>, pH 8.0. The inclusion of 0.05% NP-40 allows the antibody to penetrate the fixed structure. Alternatively, the NP-40 was omitted and the specimens were made permeable by freeze-thawing. Identical localization results were achieved with both methods of permeabilization. The specimens were overlaid with 50  $\mu$ l of affinity-purified antiactin (30  $\mu$ g/ml), affinity-purified anti- $\alpha$ -actinin (125  $\mu$ g/ml), or normal rabbit IgG (250  $\mu$ g/ml), and incubated for 30 min in a moist chamber at room temperature. Unbound antibody was removed by two 8-min washes in 50 ml of PBS. The slides were then overlaid with 50  $\mu$ l of rhodamine-labeled goat anti-rabbit IgG (1 mg/ml in PBS). The staining and washing protocol was identical to that used with the first antibody. The preparations were mounted in a mixture of one part glycerol to nine parts PBS. Specimens were examined with a Leitz Ortholux II equipped with a Ploem vertical illuminator, a planapo  $\times 40$  oil immersion objective (NA = 1.0) and a phaco  $\times 63$  oil immersion objective (NA = 1.3). Photographs were taken on Ektachrome 400 color film or Tri X black and white film (ASA 400). Exposure times were 15–20 s.

### Preparation of Proteins for Two-Dimensional Peptide Analysis

**GIZZARD ALPHA-ACTININ:** 1 ml of purified gizzard alpha-actinin (1.8 mg/ml) was precipitated by addition of cold 100% TCA to a final concentration of 20% TCA. Protein was collected by centrifugation at 2,230  $g$  for 10 min. The pellet was taken up in 200  $\mu$ l of 0.1% SDS (Pierce Sequanal grade) in 0.25 M NaPO<sub>4</sub> buffer, pH 7.4, containing 100 U/ml Trasylol. To this sample was added 20  $\mu$ l of <sup>125</sup>I (1 mCi/10  $\mu$ l) and 20  $\mu$ l of Chloramine T at 5 mg/ml in 0.25 M NaPO<sub>4</sub> buffer, pH 7.4. The reaction was incubated at 24°C for 30 min, then quenched with 20  $\mu$ l of sodium metabisulfite at 10 mg/ml in 0.25 M NaPO<sub>4</sub>, pH 7.4. 1 ml of SDS gel sample (20) was added. The sample was boiled for 3 min and then loaded onto a preparative slab gel (7.5  $\times$  16.25  $\times$  3 cm) and electrophoresed at 60 V for 6–8 h. The gel was exposed to Kodak XR-5 X-ray film for 2–15 min. Using the autoradiograph to locate alpha-actinin, strips of gel containing the protein were cut out and forced through a piece of 80-mesh

stainless steel screen (Small Parts, Inc., Miami, Fla.) placed at the bottom of a 10-ml plastic syringe barrel. The fragmented gel was collected into a 20-ml glass scintillation vial. 3–5 ml of 0.05 M NH<sub>4</sub>HCO<sub>3</sub> containing 100 U/ml Trasylol was added and the vial was shaken for 1 h at 37°C in an Eberbach water bath (Eberbach Corp., Ann Arbor, Mich.) set at No. 6 shaking speed. The gel slurry was pipetted into a plastic cone having a pinhole opening at the bottom (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) which was plugged with glass wool. The cone was placed into a 40-ml plastic tube and centrifuged at 67  $g$  for 15 min. The eluate contained 70–80% of the radioactivity in the gel slice. Small aliquots of the eluate were TCA precipitated and electrophoresed on SDS mini-gels along with appropriate standards. Gels were autoradiographed for 2 h using Kodak XR-5 and a Cronex intensifying screen (DuPont Instruments, Wilmington, Del.). This procedure allowed us to verify that the right band had been cut out of the preparative gel and that no breakdown had occurred during preparation of the sample.

**BRUSH BORDER 95-KDALTON POLYPEPTIDE:** The 1-M Tris-soluble brush-border proteins (see Fig. 1) were used as the source of 95-kdalton polypeptide. 1 ml of the Tris extract, containing ~8 mg of protein was precipitated by addition of 100% cold TCA to a final concentration of 20%. The precipitated protein was collected by centrifugation, solubilized in SDS gel sample buffer, and loaded onto a 7.5% polyacrylamide preparative slab gel. The gel was constructed with a mini-slot in the middle into which was loaded a proportional amount of sample containing 2  $\times$  10<sup>6</sup> cpm of iodinated phosphorylase (phosphorylase was found to have the same mobility as the brush-border protein which has been referred to as the 95-kdalton polypeptide). After electrophoresis, the gel was exposed to Kodak XR-5 X-ray film for 2–15 min. The autoradiograph was used to cut out the 95-kdalton region of the gel, care being taken to exclude the region containing the radioactive marker. The protein was eluted from the gel, TCA precipitated, and iodinated as described for alpha-actinin. The iodinated sample was electrophoresed on a preparative slab gel and the 95-kdalton protein was re-isolated.

Iodinated proteins were prepared for tryptic digestion according to the procedure of Gibson (14). The protein concentration of the sample was adjusted, if necessary, to 50  $\mu$ g/ml by addition of BSA, Cohn Fraction II. The samples, in 15-ml Corex tubes (Corning Glass Works, Corning, N. Y.), were precipitated at 0°C by addition of cold 100% TCA to a final concentration of 20%. After 2 h at 0°C, the precipitated proteins were pelleted at 12,000  $g$  for 30 min. Pellets were washed twice with cold 20% TCA, once with 0.1 N HCl in acetone at -20°C, and once with -20°C spectral-grade acetone. The pellet was dried under a gentle stream of nitrogen. Samples were then subjected to performate oxidation by the method of Hirs (17). The pellets were soaked in 200  $\mu$ l of methanol:98% formic acid (1:5) at -8°C while the performate was made. To generate performate, 1.9 ml of 98% formic acid was mixed with 0.1 ml of 30% H<sub>2</sub>O<sub>2</sub> and allowed to stand at room temperature for 90 min. Before use, the performate was cooled to -8°C in a rock-salt/ice-water bath. To oxidize the proteins, 50  $\mu$ l of the performate was added to each 200- $\mu$ l sample. Samples were kept at -8°C for 2–6 h after which time the reaction was diluted by addition of 10 ml of distilled water. Samples were frozen and lyophilized. The samples were re-lyophilized twice from 5 ml of H<sub>2</sub>O to remove traces of formic acid.

The oxidized proteins were digested with trypsin as follows: Samples (100–200  $\mu$ g protein) were suspended in 0.5 ml of 0.05 M NH<sub>4</sub>HCO<sub>3</sub>. 20  $\mu$ l of L-(tosylamido 2-phenyl)ethyl chloromethyl ketone (TPCK) trypsin (Worthington Biochemical Corp.,

Freehold, N. J., 180–220 U/mg) at 1 mg/ml was added. Proteins were digested at 23°C for 12 h and then another 20 µl of trypsin was added. After 36 h, the samples were diluted 1:5 with H<sub>2</sub>O and lyophilized. Samples were re-lyophilized once from 1 ml of distilled water.

**TWO-DIMENSIONAL PEPTIDE ANALYSIS:** Digested proteins were taken up in 20–50 µl of butanol:pyridine:acetic acid: H<sub>2</sub>O, pH 4.7, vol/vol (2:1:1:36). Sample volumes of 1–2 µl were spotted on 20 × 20 cm × 0.01 mm cellulose thin-layer plates (EM Laboratories, Elmsford, New York). Plates were dampened with buffer and electrophoresed at 800 V for 1.5 h in a water-cooled apparatus. After electrophoresis, the plates were air-dried and further developed by ascending chromatography in butanol:pyridine:acetic acid:H<sub>2</sub>O, vol/vol, pH 5.3 (87:75:15:60) at 23°C for ~6 h. Plates were air-dried and exposed to Kodak XR-5 X-ray film with a Cronex intensifying screen at –80°C for 2 h to 2 d.

**ONE-DIMENSIONAL PEPTIDE ANALYSIS:** Samples of gizzard alpha-actinin and brush-border 95-kdalton polypeptide were eluted from preparative SDS gels and prepared for one-dimensional peptide mapping (7). TCA-precipitated samples were dissolved at 1.0 mg/ml in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 10% sucrose, 0.5% SDS, 10 µg/ml Pyronin Y. Proteolytic digestion was carried out at 37°C for 15 min by addition of given amounts of various proteases as indicated in the appropriate figure legend. Trypsin, papain, chymotrypsin (Worthington Biochemical Corp.) and *S. aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) were used as probes of primary structure. All enzymes were made up to 1 mg/ml in 10 mM Tris, pH 7.4. The papain stock solution also contained 1 mM EDTA, 5 mM cysteine, and 6 × 10<sup>-6</sup> M mercaptoethanol. Digestion was halted by addition of an equal volume of sample buffer containing, in addition to the previously listed components, 2% SDS and 80 mM DTT. Samples were boiled for 3 min; 20 µg of each sample were loaded onto 10 and 15% polyacrylamide slab gels (7.5 cm × 16.25 cm × 1 mm). Samples were electrophoresed at 100 V for 5 h using the Laemmli (20) buffer system.

## RESULTS

### *Differential Extractability of Epithelial Cell Alpha-Actinin and Brush-Border 95-kdalton Polypeptide*

In a recent paper (8) we demonstrated that there is an epithelial cell alpha-actinin which is soluble in 0.5% NP-40. In contrast, the 95-kdalton polypeptide observed in isolated brush borders is not extracted by vigorous treatment with as much as 1% nonionic detergent (either NP-40, Tergitol, or Triton X-100), (Fig. 1, lane 2). The brush-border 95-kdalton protein is, however, extracted in 1 M Tris, pH 7.4 (Fig. 1, lanes 3 and 4).

### *Immunofluorescent Localization of Alpha-Actinin in Isolated Brush Borders and in Intact Epithelial Cells*

Affinity-purified antibody to chicken gizzard alpha-actinin stains the junctional complex region

of intact epithelial cells (8) but does not stain either the microvilli or the terminal web of isolated brush borders (Fig. 2*d*). This lack of staining is not caused by inability of the antibody to penetrate the isolated structure, as both the terminal web and the microvilli of the brush borders stain brilliantly with antiactin (Fig. 2*b*). The specificity of the affinity-purified anti-actin is documented in Fig. 3. When tested against total protein of brush borders, the antiactin recognizes only actin. Because alpha-actinin is known to dissociate from actin in low ionic strength buffers (39) and in the presence of chelators, it is assumed that the junctional alpha-actinin is lost from the terminal web region during isolation of the brush borders. This point is considered in greater detail in the Discussion. Notice, however, that the brush-border 95-kdalton polypeptide, which has been postulated to be homologous to alpha-actinin (29), is retained in the isolated structure (Fig. 1, lane 1).

### *Immunological Cross Reactivity of Alpha-Actinin and Brush-Border 95-kdalton Polypeptide*

To assess the antigenic relatedness between

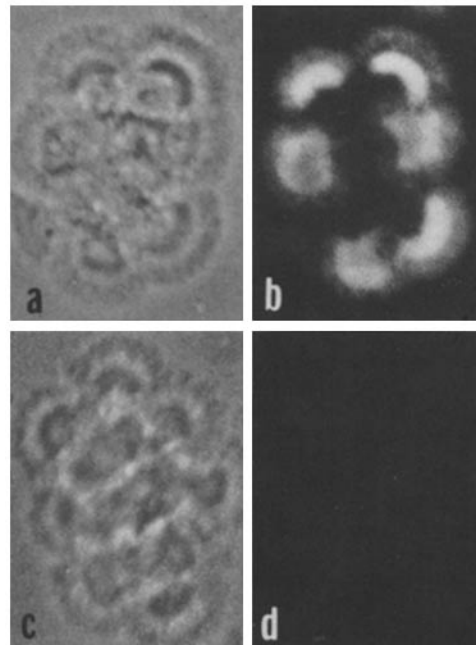


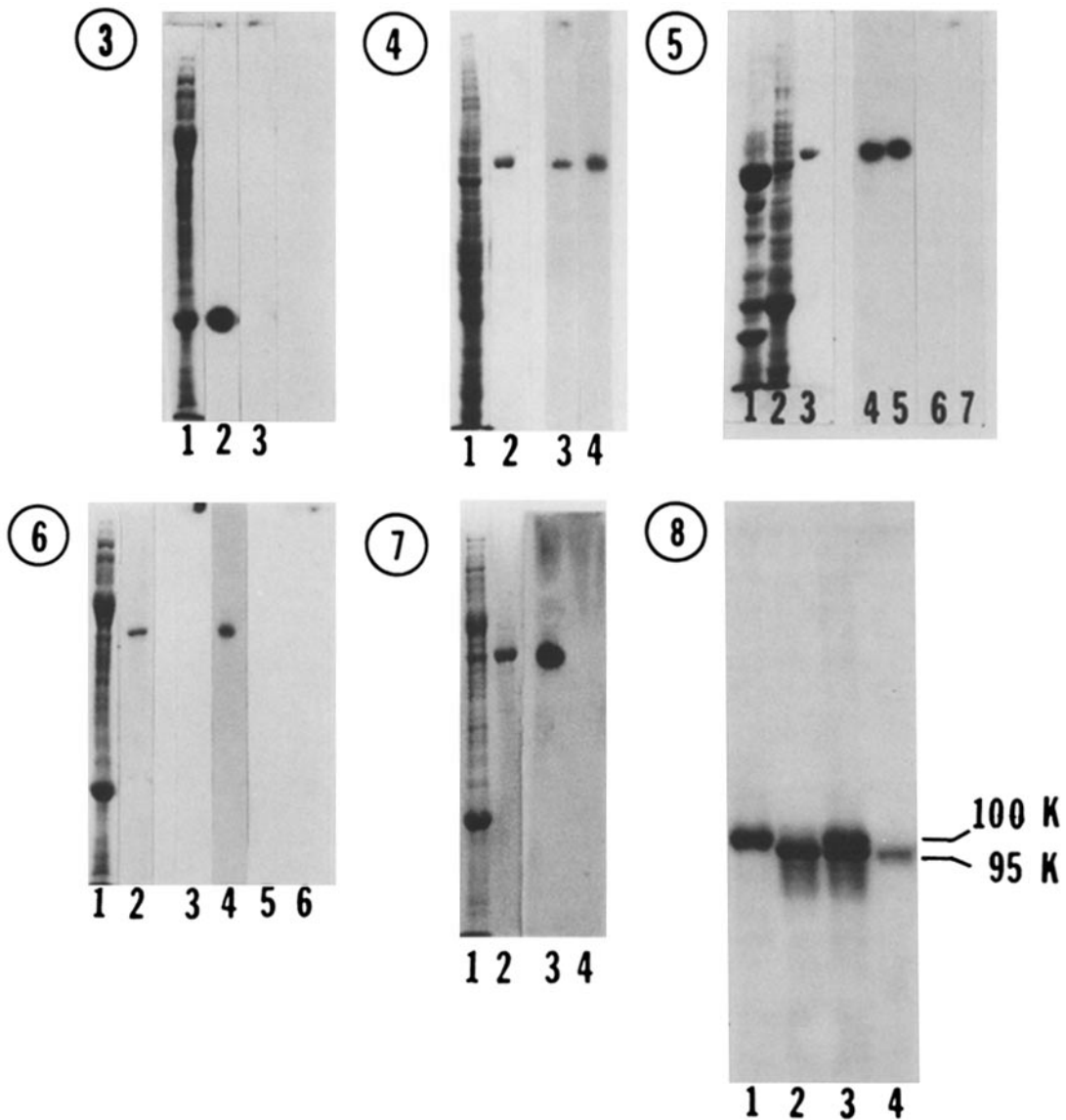
FIGURE 2 Indirect immunofluorescence staining of isolated brush borders with anti-alpha-actinin (*d*) or antiactin (*b*). *c* and *a* are phase micrographs of the same fields shown in *d* and *b*, respectively.

known alpha-actinins and brush-border 95-kdalton protein, we used a technique in which SDS polyacrylamide slab gels are overlaid with a specific antibody, washed, and then treated with  $^{125}\text{I}$ -Protein A to detect the bound antibody (1). This method is not only extremely sensitive (<1 ng of a particular antigen can be detected), but it also detects nonprecipitating, as well as precipitating, antibodies. Using this method, we found that rabbit anti-gizzard alpha-actinin recognizes homologous molecules in crude extracts of cardiac and skeletal muscle (Fig. 5), as well as in extracts of

isolated intestinal epithelial cells (Fig. 4). However, the antibody does not recognize the 95-kdalton polypeptide present in isolated brush borders (Fig. 6). Conversely, antibody raised to the 95-kdalton polypeptide recognizes this polypeptide in SDS gels of isolated brush borders, but does not recognize gizzard alpha-actinin (Fig. 7).

*Mobility of Gizzard Alpha-Actinin and Brush-Border 100-kdalton Polypeptide on SDS Gels*

During the course of these experiments, it was noticed that gizzard alpha-actinin did not always



electrophoresis to exactly the same position as brush-border 95-kdalton polypeptide on 7.5% acrylamide Laemmli SDS gels. To explore this observation, the proteins were electrophoresed on a 5–7.5% gradient SDS slab gel. Molecular weights were estimated by comparison to the mobilities of standards electrophoresed at the same time. The results (Fig. 8) show that brush-border 95-kdalton protein has a slightly faster mobility than gizzard alpha-actinin (100,000). The brush-border protein has the same mobility as rabbit muscle phosphorylase which is reported to be 95 kdaltons.

#### *Two-Dimensional Maps of Iodinated Tryptic Peptides Derived from Gizzard Alpha-Actinin and Brush-Border 95-kdalton Polypeptide*

Because the antigenic analysis provided no evidence for homology between alpha-actinin and the brush-border 95-kdalton protein, we decided to further test for structural similarity by comparing peptide maps of the proteins. Two types of

mapping were employed. First, we compared maps of  $^{125}\text{I}$ -labeled tryptic peptides made by separating the peptides in two dimensions. The results (Fig. 9) confirm the immunological studies in that there appears to be no homology between gizzard alpha-actinin and the brush-border 95-kdalton protein.

On the chance that observation of iodinated peptides might have been too narrow a probe, we compared gizzard alpha-actinin and the 95-kdalton protein by one-dimensional maps which were made by performing incomplete proteolytic digestions on SDS denatured proteins, followed by display of the partial digests on 10% acrylamide SDS gels (28). Four enzymes having distinct cleavage sites were employed as probes: trypsin, chymotrypsin, papain, and *S. aureus* V8 protease, which cleaves at the COOH terminus of glutamic acid residues. Again, the maps fail to reveal any homology between the two proteins (Fig. 10).

#### DISCUSSION

The results of our analysis indicate that brush-

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FIGURE 3 Characterization of affinity-purified anti-actin by the antibody SDS gel overlay technique. Proteins were separated on 7.5% polyacrylamide SDS mini-slab gels. (Lane 1) Brush borders/Coomassie Blue. (Lane 2) Brush borders/antiactin/ $^{125}\text{I}$ -Protein A. (Lane 3) Brush borders/normal rabbit IgG/ $^{125}\text{I}$ -Protein A.

FIGURE 4 Cross reactivity of anti-gizzard alpha-actinin with a homologous protein in chicken epithelial cells. Polypeptides were separated on 7.5% acrylamide SDS mini-slab gels. (Lane 1) Chicken intestinal epithelial cells/Coomassie Blue. (Lane 2) Chicken gizzard alpha-actinin/Coomassie Blue. (Lanes 3 and 4) Anti-alpha-actinin/ $^{125}\text{I}$ -Protein A of lanes 1 and 2, respectively.

FIGURE 5 Cross reactivity of anti-gizzard alpha-actinin with homologous proteins in chicken cardiac and skeletal muscle. Polypeptides were separated on 7.5% acrylamide SDS gels. (Lanes 1 and 2) Polypeptides present in 1 mM  $\text{KHCO}_3$  extracts of chicken skeletal and cardiac muscles, respectively/Coomassie Blue. (Lane 3) Gizzard alpha-actinin/Coomassie Blue. (Lanes 4 and 5) Anti-gizzard alpha-actinin/ $^{125}\text{I}$ -Protein A overlay of lanes 1 and 2. (Lanes 6 and 7) normal rabbit IgG/ $^{125}\text{I}$ -Protein A overlay of samples similar to those shown in lanes 1 and 2.

FIGURE 6 Analysis of cross reactivity between anti-chicken gizzard alpha-actinin and brush-border 95-kdalton polypeptide. Proteins were separated on 7.5% acrylamide SDS gels. (Lanes 1 and 2) polypeptides of isolated brush borders and gizzard alpha-actinin, respectively/Coomassie Blue. (Lanes 3 and 4) anti-alpha-actinin/ $^{125}\text{I}$ -Protein A overlay of lanes 1 and 2, respectively. (Lanes 5 and 6) Normal rabbit IgG/ $^{125}\text{I}$ -Protein A overlay of samples similar to those shown in lanes 1 and 2.

FIGURE 7 Analysis of cross reactivity between anti-brush-border 95-kdalton protein and gizzard alpha-actinin. Proteins were separated on 7.5% acrylamide SDS gels. (Lanes 1 and 2) Polypeptides of isolated brush borders and gizzard alpha-actinin, respectively/Coomassie Blue. (Lanes 3 and 4) Anti-95-kdalton protein/ $^{125}\text{I}$ -Protein A overlay of lanes 1 and 2.

FIGURE 8 Mobility of chicken gizzard alpha-actinin and brush-border 95-kdalton protein on linear 5–7.5% gradient polyacrylamide gels. (1) Gizzard alpha-actinin purified by conventional means. (2) Brush-border 95-kdalton protein purified by elution from a preparative SDS gel. (3) Co-electrophoresis of gizzard alpha-actinin and brush-border 95-kdalton protein. (4) Rabbit muscle phosphorylase (95 kdaltons).

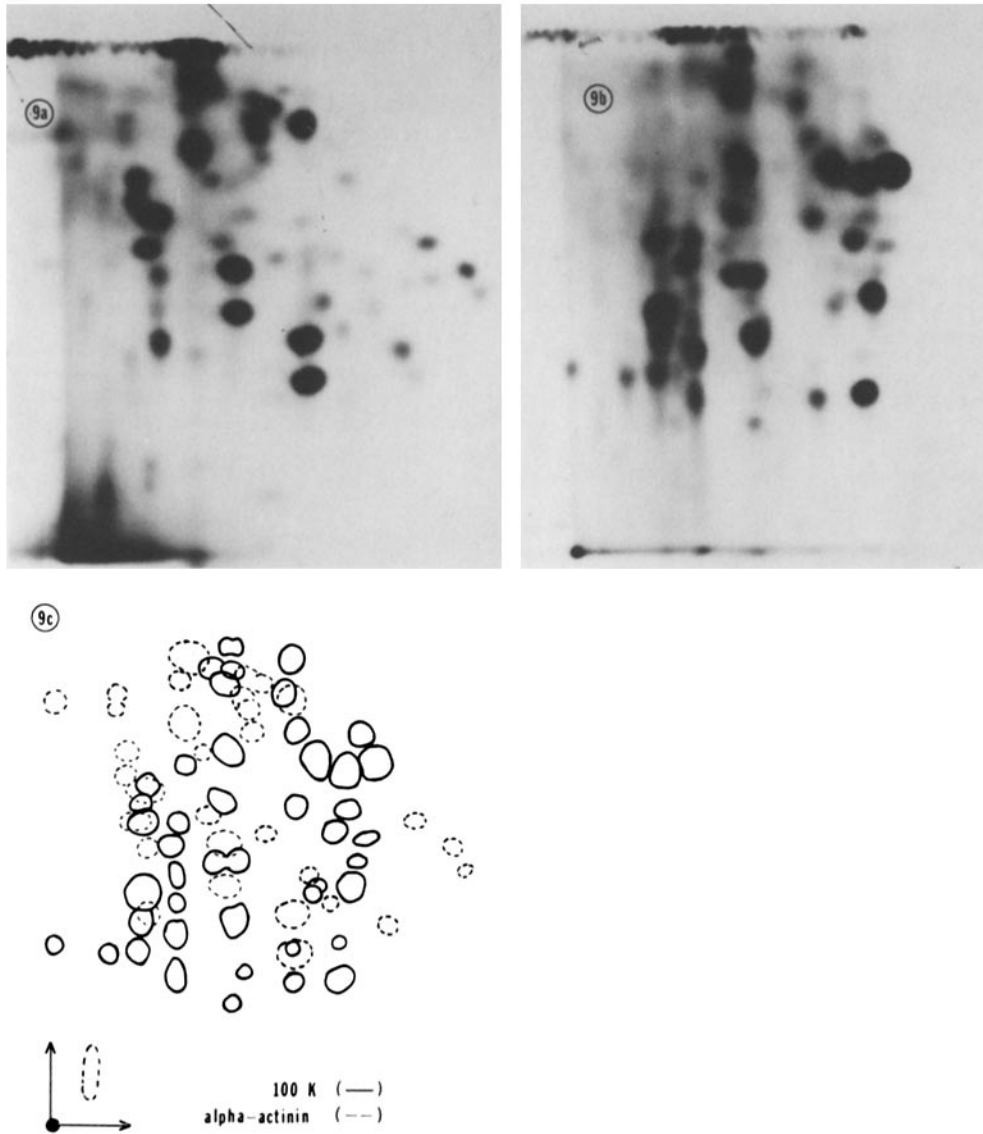


FIGURE 9 Peptide maps of iodinated tryptic fragments derived from gizzard alpha-actinin (*a*) and brush-border 95-kdalton protein (*b*). The sample origin is in the lower left corner of each map. Electrophoresis was carried out in the horizontal direction; chromatography in the vertical direction. (*c*) Diagrammatic composite of individual peptide maps shown in *a* and *b*. All spots on autoradiographs were traced onto transparent sheets. The sheets were overlaid and photographed to obtain the composite maps.

border 95-kdalton polypeptide and gizzard alpha-actinin are unrelated molecules. In addition to differing by the four independent criteria of mobility on SDS gels, antigenicity, extractability in nonionic detergent, and peptide maps, the native proteins also differ in Stokes radius as recent studies in our laboratory have shown: that of alpha-actinin is 77 Å (39) and that of the 95-

kdalton protein is 40 Å (S. W. Craig, manuscript in preparation). Whereas any two homologous proteins might appear unrelated by any one of the criteria we have used, we think it improbable that they would appear unrelated by all five parameters. The data strongly indicate, but do not rigorously prove, that there is no major structural homology between gizzard alpha-actinin and brush-



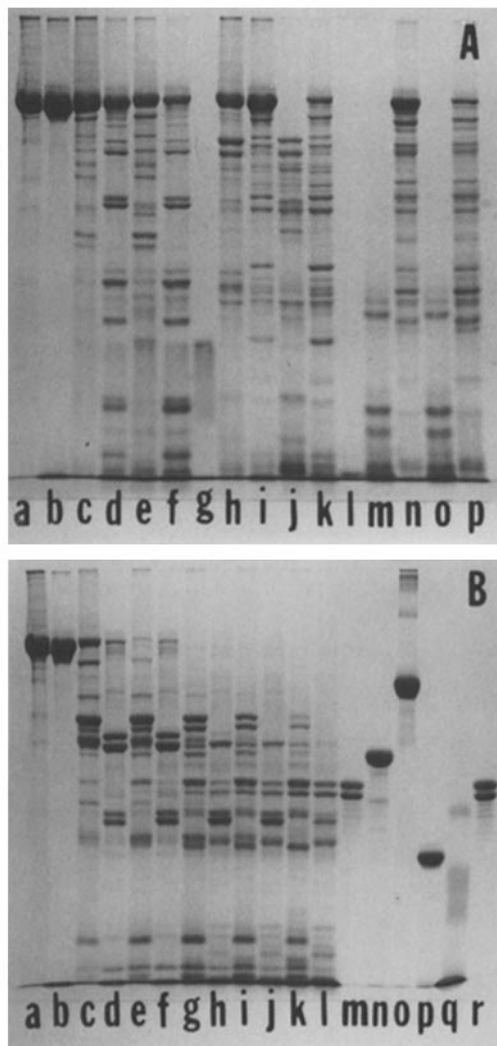


FIGURE 10 One-dimensional peptide fingerprints of gizzard alpha-actinin and brush-border 95-kdalton protein. For digestion, the proteins were solubilized at a concentration of 1 mg/ml in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 10% sucrose, 0.5% SDS, 10 µg/ml pyronin Y. (A) Digestion of alpha-actinin (a, c, e, h, j, m, and o) and brush-border 95-kdalton protein (b, d, f, i, k, n, and p) with different concentrations of TPCK-trypsin, chymotrypsin, and papain. (a and b) No protease. (c and d) 0.2 mg/ml TPCK-trypsin. (e and f) 0.4 mg/ml TPCK-trypsin. (g) Digestion products of 16 µg TPCK-trypsin. (h and i) 0.05 mg/ml chymotrypsin. (j and k) 0.15 mg/ml chymotrypsin. (l) Digestion products of 6 µg chymotrypsin. (m and n) 0.01 mg/ml papain. (o and p) 0.02 mg/ml papain. Digests were analyzed on 10% acrylamide slab gels. (B) Digestion of 40 µg of alpha-actinin (a, c, e, g, i, and k) and 40 µg of brush-border 95-kdalton protein (b, d, f, h, j, and l) with different concentrations of *S.*

border 95-kdalton polypeptide. We conclude that it is unlikely that alpha-actinin is a component of the cross filament that links the microvillar actin bundle to the membrane.

Recently, similar conclusions have been published by two other laboratories (5, 13). To facilitate evaluation of the data, we will review the findings of all three laboratories with respect to the hypothesized role of alpha-actinin in linking the actin bundle to the microvillar membrane (28, 29). The thrust of the research has been twofold: first, to determine the localization of alpha-actinin in epithelial cells using immunological techniques; and second, to biochemically assess the degree of homology between alpha-actinin and the polypeptide of ~100 kdaltons which is found in isolated brush borders.

#### Immunocytochemical Localization of Alpha-Actinin

By immunofluorescence staining of isolated intact mouse intestinal epithelial cells, Bretscher and Weber (3) found that anti-chicken gizzard alpha-actinin stained the terminal web region but not the microvilli of brush borders. Little or no staining was evident in other parts of the cell. Lack of staining in microvilli was not caused by inaccessibility of these structures to the antibody because antiactin stained both the terminal web and the brush border. A drawback to this report was that the reactivity of the anti-alpha-actinin to total brush-border components was not assessed, so some doubt would have remained concerning the molecular identity of the reactive molecules in the brush border.

Concurrently, we reported (8) that affinity-purified anti-chicken gizzard alpha-actinin was able to selectively precipitate a polypeptide of 100 kdaltons from an extract of isolated epithelial cells. In addition, we showed that this epithelial cell alpha-actinin could compete with iodinated gizzard alpha-actinin for binding to the antibody. Thus, it was demonstrated that epithelial cells do indeed contain a molecule which, by antigenicity and molecular size, is related to smooth muscle alpha-

*aureus* V8 protease. (a and b) no protease. (c and d) 0.025 mg/ml. (e and f) 0.05 mg/ml. (g and h) 0.1 mg/ml. (i and j) 0.2 mg/ml. (k and l) 0.4 mg/ml. (m) Digestion products of 32 µg *S. aureus* V8 protease. (n-r) Molecular weight standards; actin, BSA, chymotrypsin, cytochrome c, and *S. aureus* V8 protease.

actinin. In addition, our antibody recognized only this one polypeptide out of all the proteins present in the epithelial cell. By immunofluorescence staining of cryostat sections obtained in planes parallel and perpendicular to the long axis of cell, we were able to deduce that alpha-actinin is preferentially localized in the region of the junctional complex which is at the level of the terminal web. In these 2- to 4- $\mu$ m cryostat sections, we were not able to determine whether alpha-actinin was present in microvilli.

Subsequently, Geiger et al. (13) confirmed our finding that alpha-actinin is preferentially localized in the junctional complex region of the terminal web. In addition, they extended the observation by immunocytochemistry and electron microscopy to show that alpha-actinin is preferentially concentrated near the zonula adherens (intermediate or belt desmosome). Lighter ferritin labeling was observed along the roots of the core microfilaments, and very little or no labeling was observed in the microvilli. These ultrastructural results were obtained by immunoferritin staining of ultrathin cryostat sections of tissue fixed in 3% paraformaldehyde and 0.2% glutaraldehyde. The authors believe that the microvilli were accessible to the antibodies, but they did not demonstrate this by the use of antiactin, for example. This point is important because the authors found that if cryostat sections were treated with 0.1% Triton before application of anti-alpha-actinin, ferritin labeling of the microvillar actin filaments was detected by immunofluorescence analysis, while labeling of the terminal web was apparently reduced. In the absence of any further experimental data, the authors' interpretation of these results is that Triton acted to solubilize the junctional alpha-actinin and that this solubilized material rebinds to the microvilli. They conclude that alpha-actinin is present in the zonula adherens and in the roots of the microvillar core filaments, but is not normally present in microvilli. It is pertinent to note that although we demonstrated (8) that epithelial cell alpha-actinin is soluble in nonionic detergent, no investigator has presented evidence to show that exogenous alpha-actinin can bind to Triton-treated microvilli.

Although there may be slight reservations about some aspects of the experimental data from each of the three laboratories, the reservations are different in each case and, therefore, the general conclusion that alpha-actinin is present in terminal webs (3, 8, 13), is preferentially localized in the

zonula adherens (13) of the junctional complex (8, 13), and is not present in microvilli (3, 13) of intact epithelial cells is most probably accurate.

In contrast to the agreement which exists concerning the presence and localization of alpha-actinin in the brush borders of intact epithelial cells, an apparent discrepancy between the three laboratories exists concerning the amount of alpha-actinin in isolated brush borders. Geiger et al. (13) are able to detect alpha-actinin by immunofluorescence staining in isolated brush borders. Similarly, by immunoradiography of SDS gels, Bretscher and Weber can detect a very small amount (i.e., not visible as a Coomassie Blue-stained band) of alpha-actinin in isolated brush borders (5). On the other hand, our preparations of isolated brush borders do not contain detectable alpha-actinin, either by immunofluorescence (Fig. 2) or by solid phase radioimmunoassay in SDS gels (Fig. 6). The latter method readily detects the alpha-actinin present in intact epithelial cells (Fig. 4). Isolated brush borders prepared by Mooseker's laboratory also do not contain any detectable immunoreactive alpha-actinin (M. Mooseker, personal communication). As alpha-actinin is known to dissociate from actin in low ionic strength and in the presence of chelators, we think that the brush-border alpha-actinin is lost from the junctional complex and other elements in the terminal web region during isolation of the brush borders. The differences in results between laboratories might be explained by differences in the length of exposure of the organelles to low ionic strength conditions.

#### *Biochemical Comparison of Alpha-Actinin and Brush-Border 95-kdalton Polypeptide*

Although our isolated brush borders contain no immunoreactive alpha-actinin, they do contain a major Coomassie Blue-stained band of ~100 kdaltons (95,000) (Fig. 1). Because the subunit mol wt of alpha-actinin is 100,000 (39), it was necessary to consider the possibility of a microvillar alpha-actinin which was not cross reactive with the three independently raised antibodies to gizzard alpha-actinin (5, 8, 13). Therefore, detailed biochemical comparisons have been made between brush-border 95-kdalton polypeptide and alpha-actinin.

The differences in mobility on SDS gels we found between alpha-actinin and the brush-border polypeptide and the demonstration of antigenic uniqueness confirm the observations of Bretscher

and Weber (5). In addition, we extended the significance of the antigenic analysis by showing that antibody to gizzard alpha-actinin can recognize the homologous but not identical alpha-actinins found in skeletal and cardiac muscle, as well as in epithelial cells. Because all known alpha-actinins can be detected by the antibody, the conclusion based on antigenic criteria that the brush-border protein is not alpha-actinin is greatly strengthened.

Various types of peptide maps of alpha-actinin and the brush-border 95-kdalton protein have been reported (13, 30). Geiger et al. (13) presented two-dimensional tryptic maps of iodinated proteins and concluded that the two proteins have no significant homology. However, the resolution in the map of the brush-border 95-kdalton protein does not seem adequate to allow one to make this statement unequivocally. Mooseker and Stephens (30) reported, but did not subsequently document, that two-dimensional maps of total tryptic peptides showed that the two proteins showed at least 45% homology. However, subsequent analysis and evaluation has apparently indicated that these proteins are nonhomologous (M. Mooseker, personal communication). Obviously, it will be necessary to await publication of Mooseker's extensive and thorough analysis of homology between various brush-border proteins and alpha-actinins before the data can be evaluated. Because of their resolution, our two-dimensional tryptic maps of iodinated proteins and one-dimensional maps of tryptic, chymotryptic, *S. aureus* V8 protease, and papain digests clearly demonstrate for the first time that brush-border 95-kdalton polypeptide and gizzard alpha-actinin are nonhomologous.

With respect to peptide maps, it should be noted that interpretation is not always straightforward. For example, troponin C and calmodulin share 50% sequence homology (11), yet they have only one tryptic peptide in common (38). In general, peptide-mapping techniques tend to overestimate differences in actual protein structure as a single amino acid change can alter the chromatographic behavior of peptides containing many conserved amino acids.

Finally, we have documented a difference between epithelial cell alpha-actinin and the brush-border 95-kdalton polypeptide in terms of their extractability in nonionic detergent. In sum, the biochemical and cytochemical data of three laboratories lead to the conclusion that alpha-actinin is not present in microvilli and that, therefore, it cannot be a component of the cross filament be-

tween the core microfilaments and the microvillar membrane.

### *What is the Molecular Nature of the Actin-Membrane Linkage?*

With alpha-actinin seemingly eliminated as a candidate for the cross filament, attention is focused anew on the molecular identity of this structure. On the basis of the finding that purified microvillar actin cores, prepared by treating microvilli with Triton, contain only actin and the brush-border 95-kdalton polypeptide, Bretscher and Weber have proposed that the 95-kdalton protein (which they name "villin") is the cross-filament molecule (5). Their hypothesis is supported by electron micrographs of negatively stained microvillar cores which are decorated with knoblike structures. These knobs were interpreted to be the cross filaments, and antivillin was shown to decorate the actin cores in a periodic fashion. Although these data are intriguing, caution is required in making the deduction that villin is the *in vivo* cross bridge. It is known that detergent treatment can disrupt normal interactions and create new associations (36). In addition, Bretscher's preparations were made with chelators as the only protease inhibitors. It is therefore possible that the two proteins remaining in the demembrated microvillar core represent protease-resistant components. In terms of function, there could be at least three different actin-binding proteins present in microvilli: one to cross-link individual filaments to each other, one to tether the actin bundle laterally to the membrane, and one to bind the ends of the filament bundles to the tip of the microvillus.

In this light, it is interesting to note that experiments similar to those of Bretscher and Weber have recently been reported by Matsudaira and Burgess (24). However, the results give rise to a totally different picture. Demembrated microvilli isolated in the presence of protease inhibitors exhibit knoblike structures arranged in a helical fashion about the actin filament core. In contrast to Bretscher and Weber's results, these cores contain four major large polypeptides (110, 68, 95, and 42 kdaltons), five major small polypeptides between 12.5 and 16.5 kdaltons, and several minor components. When the cores are treated with  $Mg^{2+}$ -ATP, the knobs disappear and the structures are depleted in 110 and 16.5 kdaltons, as well as in some of the minor polypeptides. Villin (95

kdaltons), in contrast, is not depleted by this treatment. When the cores are treated with deoxycholate, the cytoskeletons are found to be composed of a loose bundle of filaments to which knoblike structures remain attached. These preparations retain the 110 kdaltons but are depleted in 95 (villin), 68, and 16.5 kdaltons, as well as in some of the minor components. Matsudaira and Burgess propose that the 110-kdalton protein is a component of the cross filament (which they call lateral arms) and that the 68- and 95-kdalton (villin) proteins are involved in bundling the actin filaments. It is interesting to note that both the association of villin (95 kdaltons) with actin filaments (5) and the association of the 110- and 16.5-kdalton protein with actin (24) can be correlated with cytoskeletal structures which appear to have the lateral arms. It would seem, then, that identification of lateral arms by negative staining is an inadequate assay for this structure.

Clearly, the molecular composition of the cross filament between actin and the microvillar membrane is an unsolved problem. Proof of the involvement of any protein in actin-membrane linkage will ultimately require isolation and functional characterization of the native molecule.

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