ALPHA-ACTININ LOCALIZATION IN THE JUNCTIONAL COMPLEX OF
INTESTINAL EPITHELIAL CELLS

SUSAN W. CRAIG and JOSÉ V. PARDO. From the Department of Physiological Chemistry, Johns
Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

We have used antibody to chicken gizzard alpha-actinin to identify and localize
this molecule in chicken intestinal epithelium. The antibody binds only to alpha-
actinin when tested against a crude extract of chicken gizzard. Extracts of purified
epithelial cells contain a molecule which has a subunit molecular weight of
100,000 on sodium dodecyl sulphate gels and which is able to inhibit the
interaction of alpha-actinin antibody and ¹²⁵I-labeled chicken gizzard alpha-
actinin. By indirect immunofluorescence, alpha-actinin is localized in the apical
portion of chicken intestinal epithelial cells. Ethanol-fixed cryostat sections of
intestine taken through the apical portion of the epithelial cells and in a plane
perpendicular to the long axis of the cells show that alpha-actinin is organized in
a polygonal pattern which corresponds to the outlines of the polygonally packed
epithelial cells. We interpret the data as indicating that alpha-actinin is a
component of the tight junction (zonula occludens) and/or the belt desmosome
(zonula adherens), both of which are membrane structures known to encircle the
cell and to be confined to its apical portion.

KEY WORDS alpha-actinin • junctional
complex • immunofluorescence • brush
border • epithelium

Alpha-actinin is one of the proteins found in the
Z line of skeletal muscle (17, 21). In vitro, the
purified molecule is able to cross-link F-actin (9,
15) and to potentiate actin-activated myosin ATP-
ase (1). Its physiological role is unknown, but
localization studies performed with alpha-actinin
antibody in skeletal muscle (17), smooth muscle
(18), fibroblasts (8, 18), and dividing chick em-

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results and hypotheses have important implications with respect to the possible function of alpha-actinin as a membrane anchor site for actin filaments.

Since the immunochemical localization studies of Schollmeyer et al. (19) were not subsequently documented, the localization of alpha-actinin in the tight junction and in microvillar cross bridges and dense tips has been uncertain. This paper demonstrates by two criteria that intestinal epithelial cells contain an alpha-actinin-like molecule. First, a well-characterized antibody to chicken gizzard alpha-actinin stains the brush border region of chicken intestinal epithelial cells. Second, extracts of isolated epithelial cells contain a molecule of subunit molecular weight 100,000 which competes with 125I-labeled chicken gizzard alpha-actinin for binding to alpha-actinin antibody. In addition, we provide evidence that alpha-actinin is localized in the junctional complex in association with the tight junction and/or the belt desmosome.

We have not yet been able to determine whether or not alpha-actinin is also present in microvilli.

MATERIALS AND METHODS

Preparation of Alpha-Actinin

Chicken gizzards packed in glycerol and stored at –20°C were obtained from Pel-Freez Farms, Inc., Rogers, Ark. Alpha-actinin was purified by modifications of the procedure used for cardiac alpha-actinin (20), (S. W. Craig, J. A. Cooper, and C. L. Lancashire, unpublished results). Purified alpha-actinin was labeled with 125I by the Chloramine T method (5).

Preparation of Alpha-Actinin Antibody

Alpha-actinin antibody was raised in rabbits and isolated by affinity chromatography of the antiserum on Sepharose 2B covalently coupled by the cyanogen bromide method (13) to chicken gizzard alpha-actinin. The antibody was eluted at 4°C with 0.05 M acetic acid, pH 4.0, neutralized with 2M Tris HCl, pH 8, and then dialyzed against 0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.0. The antibody was stored at 4°C in 0.02% NaN3.

Preparation of Rhodamine-Labeled Goat Anti-Rabbit IgG (Fc Fragment)

Goat anti-rabbit IgG (Fc fragment) was a gift of Dr. John Cebra, The Johns Hopkins University, Baltimore, Md. Purified immune IgG was labeled with tetramethyl rhodamine isothiocyanate (N. L. Cappel Laboratories, Cochransville, Pa.) and subsequently fractionated as described by Cebra and Goldstein (2).

Polyacrylamide Gel Electrophoresis

Cylindrical gels containing 4% acrylamide monomer and 0.15% bis-acrylamide were run in Tris acetate buffer, pH 7.4 with 1% sodium dodecyl sulphate (3).

Isolation of Chicken Epithelial Cells

Chicken intestinal epithelial cells were isolated by combination and modification of the procedures described by Weiser (23) for rat intestinal epithelial cells and by Ziomek and Edidin (24) for mouse intestinal epithelial cells. The duodenum from a fasted adult hen was rinsed thoroughly with 0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.5, everted, and tied at both ends. The gut segment was incubated for 10 min at 37°C in 40 ml of Solution A (96 mM NaCl, 1.5 mM KCl, 27 mM Na citrate, 8 mM KH2PO4, 5.6 mM Na2HPO4, 2 mM ADP, 2% Dextran 500, and 10 μg/ml gentamycin, pH 7.3). The gut segment was then placed in 100 ml of Solution B (150 mM NaCl, 1.5 mM EDTA, 8.3 mM Na2HPO4, 1.7 mM NaH2PO4·H2O, 2 mM ADP, 10 μg/ml gentamycin, 2% Dextran 500, pH 7.3) and agitated for 15 min at 37°C in an Eberbach waterbath shaker (Eberbach Corp., Ann Arbor, Mich.), setting no. 4. This procedure released sheets and strips of epithelial cells which were pelleted at 900 g for 15 min at 24°C. The pellets were resuspended in medium (Joklik's Minimal Essential Medium, containing 1 mM ADP, 2% Dextran 500, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), pH 7.3) with 2.5 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, Mo.), 510 U/mg and incubated for 1 h at 37°C with gentle shaking. The suspension was then repeatedly pipetted with a Pasteur pipette. Microscopic observation was used to optimize the number of single cells and minimize cell damage. The cells were pelleted, resuspended in medium, and passed through silk screen to remove clumps and cell debris.

Histochemical Stains for Alkaline Phosphatase and DNA

Cytocentrifuge preparations of epithelial cells fixed in cold, isotonic, PO4-buffered, 4% paraformaldehyde for 10 min were stained for alkaline phosphatase by the Gomori calcium-cobalt method (14) with a 4-h reaction time at 27°C. Nuclei were stained by incubating the slides in a 5 μg/ml aqueous solution of ethidium bromide for 10 min at 24°C.

Preparation of Cell Lysates and Indirect Immunoprecipitation

Packed epithelial cells were lysed by incubation in an equal volume of 4 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)N,N',N″,N‴-tetraacetate (EGTA), 10 mM imidazole, 10 U/ml of Transylol (Mibay Chemical Corp., N. Y.; N. Y.) and 0.5% Nonidet
P-40 (Gallard-Schlesinger, Carle Place, N. Y.) Cell disruption was facilitated by a few strokes in a Dounce homogenizer (Kontes Co., Vineland, N. J.) during the incubation period. The lysates were centrifuged at 100,000 g for 30 min at 4°C. The supernates were used in immunoprecipitation studies. Indirect precipitation of antigen-antibody complexes with formalin-fixed Staphylococcus aureus was done according to Kessler's procedure (7).

**Immuno fluorescence**

4 μm thick cryostat sections were prepared from chicken duodenum which had been quick frozen in 2-methyl butane at ~70°C. The sections were fixed in ethanol for 20 min at 24°C and rehydrated in Dulbecco's phosphate-buffered saline (D-PBS) for 5 min before staining. Sections were overlaid with 50 μl of affinity purified alpha-actinin antibody (140 μg/ml), or normal rabbit IgG (140 μg/ml), and incubated for 30 min in a moist chamber. Unbound antibody was removed by 2 eight-min incubations in 50 ml of D-PBS. The sections were briefly air dried before being overlaid with 50 μl of rhodamine goat anti-rabbit IgG (1 mg/ml). The staining and washing protocol was identical to that used with the first antibody. Stained sections were mounted in a mixture of nine parts glycerol to one part 0.01 M NaPO4 buffer containing 0.15 M NaCl, pH 7.5. The preparations were examined with a Leitz Ortholux II (E. Leitz, Inc., Rockleigh, N. J.) equipped with a Ploem vertical illuminator and a plan apo 40 x (numerical aperture = 1.0) oil immersion objective. Photographs were taken on Ektachrome 200 at 400 ASA. Exposure times were 15-20 s.

**RESULTS**

**Homogeneity of Alpha-Actinin Immunogen**

The alpha-actinin used as immunogen was at least 95% homogeneous as judged by polyacrylamide gel electrophoresis. When 10 μg of alpha-actinin were electrophoresed on gels which were stained and destained in a manner which will detect 0.5 μg of albumin, only one band was observed (Fig. 1). Isolated chicken gizzard alpha-actinin was identified by its subunit molecular weight of 100,000 on sodium dodecyl sulphate polyacrylamide gels and by its ability to cross-link F-actin at 0°C, resulting in the formation of a gel (9) which sediments at 2,000 g in 10 min (data not shown).

**Characterization of Alpha-Actinin Antibody**

Affinity purified alpha-actinin antibody was tested for its ability to bind to proteins present in the crude extract of chicken gizzard used for the isolation of alpha-actinin. Immune complexes formed between proteins in the extract and the purified antibody were precipitated with S. aureus (7). When the precipitated proteins were analysed on sodium dodecyl sulphate polyacrylamide gels, it was found that the antibody reacted solely with alpha-actinin (Fig. 2). Since this method detects precipitating as well as nonprecipitating antigen-antibody complexes, it is a reliable way to characterize the reactivity of antisera which are to be used for immunofluorescence studies.

**Immuno fluorescence Localization of Alpha-Actinin in Cryostat Sections of Chicken Duodenum**

When cryostat sections of chicken intestine were stained for alpha-actinin, two patterns of localization in the epithelial cells of the villi were noted. In some sections, the alpha-actinin stain appeared as a thin uneven band at the apical surface of the cells (Fig. 3a, b) while in others (Fig. 3c) the stain occurred in the form of irregular polygons. In both patterns, however, the stain-
Figure 2 Polyacrylamide gel analysis of immune complexes formed between alpha-actinin antibody and proteins in a crude extract of chicken gizzard. The complexes were precipitated with paraformaldehyde-fixed *S. aureus*, washed, and eluted from the bacteria by incubation in gel sample buffer (reference 4) for 15 min at 37°C. (gel 1) Crude gizzard extract. (gel 2) Gizzard extract proteins precipitated by alpha-actinin antibody and *S. aureus*. (gel 3) Gizzard extracts proteins precipitated by *S. aureus*. (gel 4) Gizzard extract proteins precipitated by normal rabbit IgG and *S. aureus*. The positions of heavy and light chains of IgG are marked by *H* and *L*, respectively. (gel 5) Purified alpha-actinin (*A*).

The polygonal pattern of alpha-actinin staining was predominantly restricted to the apical portion of the cells. Occasional sections showed both patterns of staining on the same villus. For example, in Fig. 3d, the thin band of alpha-actinin stain gradually splays out into the polygonal pattern. This observation indicates that a single structure is being observed in two different planes of section.

Scanning electron micrographs of rat intestinal villi show that the epithelial cells are packed in a polygonal array (16). Examination of formaldehyde-fixed, toluidine blue-stained sections of human duodenum, which we obtained from the Johns Hopkins Anatomy Slide Collection, showed that epithelial cells in cross section have polygonal shapes which are nearly identical in size and form to the fluorescent polygons observed when chicken intestinal sections are stained with alpha-actinin antibody. Therefore, we conclude that alpha-actinin is localized at the apical surface of the cell in a band that corresponds to the outline of the cell in cross section. We interpret these results to indicate that alpha-actinin is probably a component of the tight junction and/or the belt desmosome, both of which are membrane-associated structures known to encircle the cell and to be confined to its apical surface.

The polygonal pattern of alpha-actinin staining would be apparent whenever an epithelial cell is sectioned in a plane perpendicular to its long axis (as long as the section contains the apical portion of the cell). Fig. 3e is a dramatic illustration of the polygonal pattern of alpha-actinin staining. Such large areas of polygonal pattern were rare and probably represent a cross section through a sheet of epithelial cells. The round (*r*) outlines in Fig. 3e probably represent goblet cells which would be round in cross section and which are joined to neighboring epithelial cells by junctional complexes.

Controls for the immunofluorescent staining included staining sections with rhodamine-labeled goat anti-rabbit IgG without any first antibody; substitution of normal rabbit IgG for anti-alpha actinin IgG in the first step; and substitution of alpha-actinin antibody pretreated with excess alpha-actinin in the first step. All three controls resulted in staining similar to that shown in Fig. 3f.

**Inhibition of ¹²⁵I-Labeled Alpha-Actinin Binding to Alpha-Actinin Antibody by Lysates of Purified Intestinal Epithelial Cells**

To characterize the epithelial cell protein which reacts with alpha-actinin antibody in the immunofluorescence studies, Nonidet P40 extracts of purified intestinal epithelial cells were assayed for their ability to inhibit the binding of ¹²⁵I-labeled chicken gizzard alpha-actinin to alpha-actinin antibody. In addition, the subunit molecular weight of the epithelial cell protein recognized by alpha-actinin antibody was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of dithiothreitol.

Preparations of purified epithelial cells were characterized by a histochemical stain for alkaline phosphatase (14) to distinguish epithelial cells...
Figure 3: Indirect immunofluorescence localization of alpha-actinin in ethanol-fixed cryostat sections of chicken duodenum. (a–e) Sections stained with alpha-actinin antibody and rhodamine-labeled goat anti-rabbit IgG. (a) Longitudinal section through a villus. The alpha-actinin stain in the epithelial cells appears as an interrupted thin band (B) and is confined to the apical surface of the cells. The villus core (C) also contains stained structures some of which are smooth muscle cells. (b) Cross-section through a crypt of Lieberkühn. The brush border region of the epithelial cells (B) is brightly stained as well as some fine fibrils in the crypt epithelial cells. Also stained are the smooth muscle cells of the muscula ris mucosae (M). (c) Cross-section through villus epithelial cells. The alpha-actinin stain in the epithelial cell region is in the form of irregular polygons (P). (d) Mixed plane of section through villus epithelial cells. On the same villus, the thin band of alpha-actinin stain at the rim of the epithelial cells (B) gradually splays out into the polygonal (P) pattern. (e) Large area of cross-sectioned epithelial cells demonstrating the polygonal pattern of alpha-actinin staining. The bright, round outlines (r) are most likely cross-sections of the intervening goblet cells. (f) Section stained with rhodamine-labeled goat anti-rabbit IgG. Bar, 10 μm. × 400.
Figure 4 Chicken intestinal epithelial cells stained for alkaline phosphatase and DNA. (a) Epithelial cell preparation heated at 90°C for 3 min before carrying out the histochemical reaction for alkaline phosphatase. (b) Epithelial cell preparation simultaneously stained for alkaline phosphatase (black reaction product) and DNA (red fluorescence emitted by ethidium bromide when it intercalates the DNA helix). The preparation was viewed by combined epi-fluorescence and bright-field optics, allowing simultaneous visualization of both stains. Bar, 10 μm × 630.

from nonepithelial cells, and by ethidium bromide staining to distinguish nucleated cells from cell fragments. A 4-h reaction time for alkaline phosphatase staining was chosen to detect epithelial cells with low amounts of enzyme. Control slides were heated at 90°C for 3 min before staining for alkaline phosphatase (Fig. 4a). A typical epithelial cell preparation stained for alkaline phosphatase and for DNA is shown in Fig. 4b. Although there are occasional free nuclei, cell fragments, and alkaline phosphatase-negative cells, the vast majority (94–96%) of the population consists of nucleated, alkaline phosphatase-positive epithelial cells. The alkaline phosphatase-negative cells are small and round and therefore quite distinct from smooth muscle cells which would be elongated or spindle shaped. Chicken intestinal smooth muscle cells and lymphocytes are alkaline phosphatase negative (data not shown).

Analysis of the immune complexes formed between 125I-labeled alpha-actinin and alpha-actinin antiserum in the presence and absence of epithelial cell lysate showed that the lysate contained a 100,000-dalton polypeptide chain (Fig. 5A, gel 1) which effectively competed with the 125I chicken gizzard alpha-actinin for binding to alpha-actinin antibody (Fig. 5B). Omission of the cell lysate, or substitution of normal rabbit serum for immune serum, resulted in the loss of the 100,000-dalton Coomassie blue-stained polypeptide in the gel (Fig. 5A, gels 2 and 3). These results demonstrate that the alpha-actinin antibody reacts selectively with an alpha-actinin-like protein present in epithelial cells. It is also significant that no other lysate protein was recognized by the alpha-actinin antibody even though whole immune serum was used for these studies rather than affinity purified alpha-actinin IgG.

DISCUSSION

Despite the excitement over the possibility that alpha-actinin might mediate the interaction of actin filaments with cell membranes in the brush border of epithelial cells, there has been no documentation either of the existence of alpha-actinin in epithelial cells or of its subcellular localization. This paper provides evidence for both of the latter points.

Our demonstration that epithelial cells contain an alpha-actinin-like molecule and that alpha-actinin is localized in the brush border region is consistent with the recent report that peptide maps of chicken gizzard alpha-actinin and a brush border 100,000-dalton polypeptide show that 45% of the spots are coincident (10), indicating a degree of homology between the two proteins. Our data are also consistent with the preliminary report of Schollmeyer et al. (19) concerning the presence of alpha-actinin in tight junctions. However, immuno-ultrastructural localization will be required to locate alpha-actinin with respect to the zonula occludens and the zonula adherens. Electron microscopy is also expected to yield the answer to whether or not alpha-actinin is localized in microvilli and specifically whether it is associated with the lateral cross filaments and the dense tips.
FIGURE 5 Inhibition of $^{125}$I-labeled alpha-actinin binding to alpha-actinin antiserum by Nonidet P-40 lysates of chicken intestinal epithelial cells. (A) Sodium dodecyl sulphate polyacrylamide gel analysis of immune complexes formed between $^{125}$I-labeled chicken gizzard alpha-actinin and alpha-actinin antiserum in the presence (gel 1) and absence (gel 2) of epithelial cell lysate. The assay was done by incubating 20 $\mu$l of alpha-actinin antiserum with or without 450 $\mu$l of epithelial cell extract and 100,000 cpm of $^{125}$I-labeled alpha-actinin (2 ng) in a total reaction volume of 500 $\mu$l at 0°C for 10 min. The immune complexes were precipitated by addition of 200 $\mu$l of a 10% suspension of paraformaldehyde-fixed *S. aureus* followed by another 10 min incubation at 0°C. After three washes, the immune complexes were eluted from the bacteria by incubation in gel sample buffer containing 2 mM dithiothreitol for 15 min at 37°C. The arrow next to gel 1 indicates a lightly stained band which is the alpha-actinin precipitated from the epithelial cell lysate. The 2 ng of $^{125}$I-labeled chicken gizzard alpha-actinin are not enough to give a Coomassie blue-stained band (gel 2). (gel 3) The incubation mixture for gel 3 was identical to that of gel 1 except that normal rabbit serum was substituted for immune serum. (gel 4) Purified alpha-actinin (A). (gel 5) Rabbit IgG heavy chain (H) and light (L) chain. (B) Radioactivity profiles of gels 1 (O O) and 2 (O O O O O O) from Fig. 5A. The gels were sliced and each 1-mm slice was counted in a gamma counter. The individual slices of gel 3 (Fig. 5A) did not have radioactivity above background.

Although localization of alpha-actinin at sites where bundles of actin filaments seem to attach to membranes is consistent with the proposed role of alpha-actinin as a membrane anchor site for actin, it is not proof of this function. It is only on the basis of in vitro studies which show that alpha-actinin can cross-link actin filaments laterally (9, 15) that it is not unreasonable to suggest that in vivo alpha-actinin might organize parallel arrays of microfilaments and play a role in the lateral association of these actin filament bundles with cell membranes. It is important to note that such interactions between actin filaments, alpha-actinin, and cell membranes have not yet been demonstrated either in vivo or in vitro.

Lastly, it should not be overlooked that the identification of alpha-actinin in the junctional complex is a first step toward the molecular analysis of the components of this important membrane specialization.

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A preliminary account of the work presented in this paper has appeared previously (25).

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