

# Localization of Capping Protein in Chicken Epithelial Cells by Immunofluorescence and Biochemical Fractionation

Dorothy A. Schafer,\* Mark S. Mooseker,† and John A. Cooper\*

\*Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110; and

†Department of Biology, Yale University, New Haven, Connecticut 06511

**Abstract.** We have localized capping protein in epithelial cells of several chicken tissues using affinity-purified polyclonal antibodies and immunofluorescence. Capping protein has a distribution in each tissue coincident with proteins of the cell-cell junctional complex, which includes the zonula adherens, zonula occludens, and desmosome. "En face" views of the epithelial cells showed capping protein distributed in a polygonal pattern coincident with cell boundaries in intestinal epithelium, sensory epithelium of the cochlea, and the pigmented epithelium of the retina and at regions of cell-cell contact between chick embryo kidney cells in culture. "Edge-on" views obtained by confocal microscopy of intact single intestinal epithelial cells and of retinal pigmented epithelium showed that capping protein is located in the apical region of the epithelial cells coincident with the junctional complexes. These images do not resolve the individual types of junctions of the junctional complex. Immunolabeling of microvilli or stereocilia was faint or not detectable. Capping protein was also detected in the cytoplasm of

intact intestinal epithelial cells and in nuclei of cells in the pigmented retina and in the kidney cell cultures, but not in nuclei of cells of the intestinal epithelium or sensory epithelium.

Biochemical fractionation of isolated intestinal epithelial cells shows capping protein in the brush border fraction, which contains the junctional complexes, and in the soluble fraction. These results are consistent with the results of the immunolabeling experiments. Highly purified microvilli of the brush borders also contained capping protein; this result was unexpected based on the low intensity of immunofluorescence staining of microvilli and stereocilia. The microvilli were not contaminated with junctional complexes, as defined by the absence of several markers for cell junctions. The cause and significance of this discrepancy is not certain at this time. Since capping protein binds the barbed end of actin filaments *in vitro*, we hypothesize that capping protein is bound to the barbed ends of actin filaments associated with one or more of the junctions of the junctional complex.

CAPPING protein is an actin-binding protein that binds the barbed end of actin filaments, nucleates polymerization of actin filaments, does not sever filaments, and does not require  $Ca^{++}$  for activity (41). The physiologic role of capping protein of skeletal muscle (CapZ) is probably the stabilization of actin filaments of I-bands since capping protein binds to the barbed ends of actin filaments *in vitro* (7) and is located at the Z-disc (8, 24), the location of the barbed ends of actin filaments of the I-band. The physiologic role of capping protein in nonmuscle cells is less clear. In *Saccharomyces cerevisiae*, capping protein is colocalized with cortical patches of actin adjacent to the plasma membrane (1) and null mutations of capping protein lead to an altered actin cytoskeleton (2). In chicken, the same capping protein genes (two for alpha and one for beta) are expressed in muscle and nonmuscle tissues (6, 12), so it is likely that nonmuscle cells contain the same capping protein as muscle cells and that it serves to bind the barbed ends of actin filaments; however, a structural assembly analogous to the Z-disk of skeletal muscle has not been observed in any nonmuscle cells or tissues.

Epithelial cells possess an actin cytoskeleton that is organized differently from skeletal muscle. The best characterized example is the intestinal brush border cytoskeleton which includes at least two and possibly three distinct arrays of actin filaments that are associated with the plasma membrane (for reviews see 28, 32). Each microvillus contains a uniformly polarized bundle of actin filaments, the barbed ends of which are embedded in a dense matrix at the tip of the microvillus membrane. The junctional complex includes zonula adherens junctions, zonula occludens junctions, and, in some cells, desmosomes. A circumferential, contractile bundle of actin filaments of mixed polarity is associated with the zonula adherens. In enterocytes of some species, there is an additional network of actin filaments associated with the zonula occludens (29–31). Unlike the microvillus core filaments, the ultrastructure of the actin filaments of these junctional arrays with the membrane (i.e., end-on versus lateral) is not known.

Pigmented retinal epithelial cells and hair cells of the chick cochlea are organized in sheets of regularly arrayed polygonal cells and possess actin cytoskeletons that are or-

ganized similarly to those in intestinal epithelial cells. Actin filaments surround the cells at the level of the zonula adherens; a network of actin filaments is associated with the zonula occludens and projections (i.e., microvilli and stereocilia) containing uniformly polarized actin filaments are present on the apical surfaces (20, 22, 37, 40, 45). Stereocilia of hair cells are longer and wider than microvilli and the barbed ends of the actin filaments are situated at the distal tip of the projections (39, 42, 44).

Although a number of actin binding proteins of epithelial cells have been identified and characterized, the association of capping protein with the barbed end of actin filaments of these cells has not been investigated. We have determined the localization of capping protein in epithelial cells from the intestine of adult chickens, from the pigmented retina of chick embryos, from the chick cochlea, and in chick embryo kidney cells in culture by indirect immunofluorescence using antibodies to capping protein. Capping protein is colocalized with components of the junctional complexes in these cells. Capping protein is also present in the cytoplasmic compartment and, in some cells, in the nucleoplasm.

## Materials and Methods

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific Products (St. Louis, MO) unless stated otherwise. Chickens were purchased from Queen Esther Foods (St. Louis, MO) and fertile chicken eggs were from Spafas (Roanoke, IL). Immunological reagents were obtained from the following suppliers: DTAF-conjugated donkey anti-goat IgG and rhodamine-conjugated rabbit anti-mouse IgG (Chemicon, El Segundo, CA); rabbit anti-goat IgG and Texas red-conjugated rabbit anti-rat IgG (Cappel, Durham, NC); and mouse monoclonals specific for  $\alpha$ -actinin and vinculin (Sigma Chemical Co.). Rat monoclonal anti-ZO-1 protein was as described (3).

### Preparation of Proteins, Antibodies, and Tissue Extracts

Capping protein was purified from adult chicken pectoralis muscle as described (5). Goat polyclonal antibodies to capping protein were prepared by immunizing with 150–500  $\mu$ g of highly purified capping protein in Freund's adjuvant every 2 wk for 6 wk. Antibodies specific for the  $\alpha$ -subunit and  $\beta$ -subunit were affinity purified from antiserum (38) using either nitrocellulose strips or Sepharose columns containing glutathione-S-transferase fusion proteins expressed in *E. coli* transformed with plasmids containing the protein coding region of either the  $\alpha$ -subunit or  $\beta$ -subunit of chicken skeletal muscle capping protein (6, 12, 23). Antibodies were eluted from the nitrocellulose in 0.1 M glycine, pH 2.8, and the pH of the eluate was immediately neutralized by addition of 1 M Tris Cl, pH 8.0. Antibodies were eluted from the fusion protein-Sepharose columns in 4 M MgCl<sub>2</sub> in 50 mM Tris-Cl, pH 9.0. The affinity-purified antibodies were dialyzed in PBS (0.137 M NaCl, 2.6 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>PO<sub>4</sub>, 0.01% [wt/vol] NaN<sub>3</sub>) and stored at 4°C. Control immunoglobulins were purified by DEAE anion exchange chromatography from the 37% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction of preimmune serum from the same goat used for capping protein antibody production.

SDS-extracted samples of pectoral muscle, intestine, and retinal epithelium were prepared by quick-freezing a small piece of tissue in liquid N<sub>2</sub> immediately after dissection from the animal, pulverizing the frozen tissue in a mortar and pestle, and transferring the frozen powder to boiling SDS-sample buffer containing 1 mM PMSF, 4 mM diisopropylfluorophosphate, 0.4 mM EDTA, 1 mM benzamide, 1  $\mu$ g/ml pepstatin A, 1  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml aprotinin. The extracts were clarified by sedimentation for 15 min in a microfuge and aliquots were stored at -80°C until use.

Subcellular fractions were prepared from enterocytes isolated from the intestinal lining using a method that minimizes contamination from smooth muscle (17). Homogenates of the epithelial cells in low salt buffer (26) were subjected to sequential rounds of low-speed (10 min at 1,000 g), high-speed (20 min at 25,000 g), and ultra-speed (1 h at 100,000 g) sedimentation. Brush borders were obtained in the low speed pellet and further purified

on a sucrose gradient (34). Highly purified microvilli were prepared by homogenizing the purified brush borders in a tight fitting, stainless steel dounce (40–80 strokes) to shear microvilli. The microvilli were purified from the homogenate by sequential rounds of differential sedimentation (36). Aliquots of each fraction were prepared as an SDS gel sample containing equal cell equivalents with two exceptions. The entire low speed pellet fraction was used to purify the brush borders; the recovery of the brush borders from the sucrose gradient is not quantitative and the sample was prepared as a 10% (vol/vol) suspension that is 6–12-fold more concentrated than the original cell homogenate. Likewise, the isolation of microvilli is not quantitative and the microvilli pellet was prepared as a 10% (vol/vol) suspension.

### Electrophoresis and Immunoblotting

Proteins were subjected to electrophoresis in polyacrylamide gels (27) and transferred to nitrocellulose (46). Blots were blocked in either 5% (wt/vol) non-fat milk and 10% heat-inactivated newborn calf serum in TTBS (0.3 M NaCl, 20 mM Tris/Cl, pH 7.8, 0.1% (vol/vol) Tween-20 and 0.01% NaN<sub>3</sub>) or Super-block blocking buffer (Pierce Chemical Co., Rockford, IL) and incubated overnight at 4°C with the primary antibodies diluted in 1% (wt/vol) non-fat milk and 2% heat-inactivated newborn calf serum in TTBS. Blots were washed in TTBS and bound antibodies were detected using either alkaline phosphatase-conjugated secondary antibody (Tago, Burlingame, CA) or using a double sandwich of rabbit anti-goat IgG at 0.1  $\mu$ g/ml followed by <sup>125</sup>I-labeled goat anti-rabbit Ig (Amersham Corp., Arlington Heights, IL) at 0.1  $\mu$ Cu/ml. Blots were washed with TTBS after each incubation and developed to yield the alkaline phosphatase reaction product or exposed to XAR-5 film using an intensifier screen. Immunoblot analysis of the brush border fractions for the presence of brush border myosin I and myosin II was performed as described (33). Rainbow molecular weight markers (Amersham Corp.) were used and migrate in SDS gels slightly slower than predicted from the size of the proteins in the absence of bound dyes.

### Immunofluorescence Staining and Microscopy

General procedures for indirect immunofluorescence experiments were as follows unless stated otherwise. Paraformaldehyde (Polysciences EM grade, Warrington, PA) used as fixative was freshly prepared as a 10% (wt/vol) stock solution and used at a concentration of 1–4%. Excess fixative was quenched by a 15-min incubation in 10 mM ethanolamine in PBS. Blocking solution was 3% BSA, 5% heat-inactivated newborn calf serum in TTBS. Goat polyclonal antibodies were used at 5  $\mu$ g/ml diluted in blocking solution. Incubations in primary antibodies were overnight at 4°C; secondary antibodies were incubated with specimens at room temperature for 1–2 h. Labeled specimens were overlaid with a mounting medium consisting of freshly prepared 1% (wt/vol) *n*-propylgallate, 50% (vol/vol) glycerol in 10 mM Tris Cl, pH 8.0, and a number 1.5 thickness coverslip was sealed in place using clear nail polish.

Immunofluorescence microscopy was performed using either a Zeiss 100 $\times$  (1.3 n.a.) Neofluor objective lens or a Zeiss 63 $\times$  (1.4 n.a.) planapochromat objective lens. Filters for epifluorescence excluded cross-over between fluorescein and rhodamine. Confocal microscopy was performed using an MRC-500 scanning laser confocal microscope (Bio-Rad Laboratories, Cambridge, MA). Photomicrographs were recorded on Kodak T-max 400 ASA film; photographs of images from video monitors were recorded on T-max 100 ASA film.

**Cryosections.** 1-cm pieces of chicken small intestine were quick frozen in melting isopentane chilled in liquid N<sub>2</sub>. 4- $\mu$ m cryosections were cut and transferred to gelatin-coated glass slides, fixed in 2% paraformaldehyde in PBS for 15 min at room temperature, blocked, and incubated in anti-capping protein antibodies or preimmune IgG. Goat anti-capping protein was detected using DTAF-conjugated donkey anti-goat IgG at 10  $\mu$ g/ml. For double labeling with anti-capping protein and anti- $\alpha$ -actinin, the primary antibodies were mixed and incubated with the cryosections overnight. Anti-capping protein  $\beta$ -subunit was detected using DTAF-conjugated donkey anti-goat IgG and anti- $\alpha$ -actinin was detected using rhodamine-conjugated rabbit anti-mouse IgG. After each antibody incubation, sections were washed three times for 15 min in TTBS.

**Intact Intestinal Epithelial Cells.** The proximal 12 in of the intestine of an adult chicken was removed and flushed with cold saline. The intestine was filled with 4% paraformaldehyde in 50 mM potassium phosphate, pH 7.0, containing 50 mM EGTA, clamped at each end, and immersed in the fixative for 40 min on ice. The intestine was slit along its length and the cells lining the intestine were scraped out using a rounded scoop. The

scraped tissue was a mixture of aggregates of enterocytes that were still joined by intercellular junctions, intact crypts, and intact villi. The tissue was resuspended in 10 mM ethanolamine in PBS for 20 min, washed twice in PBS, and several small pieces ( $\sim 0.5 \times 2$  mm) were selected using a forceps; the tissue pieces were permeabilized by incubation in 1% Triton X-100, 0.6 M KCl in TBS (10 mM Tris-Cl, pH 7.4, 0.15 M NaCl) for 10 min on ice and blocked in 3% BSA, 5% heat-inactivated newborn calf serum in TTBS for 30 min, incubated in anti-capping protein  $\alpha$ -subunit, anti- $\beta$ -subunit or preimmune IgG and then incubated in rabbit anti-goat IgG at 1 mg/ml followed by 10 mg/ml DTAF-donkey anti-rabbit IgG. Samples were observed using confocal microscopy.

**Brush Borders.** Brush borders were isolated from chicken intestine by standard methods (33) except that care was taken to limit homogenization of the isolated sheets of intestinal epithelium to produce fragments containing 3–10 brush borders still attached by intercellular junctions. Brush borders were fixed in 2% paraformaldehyde in BBSB (75 mM KCl, 5 mM  $MgSO_4$ , 1 mM EGTA, 4 mM  $NaN_3$ , 0.1 mM DTT, 0.1 M PMSF) for 15 min at room temperature. Fixed brush borders were pelleted by centrifugation at 1,500 g for 5 min and resuspended in BBSB containing 10 mM ethanolamine for 15 min. Calf serum was added to 10% (vol/vol) and the suspension was gently rocked for 30 min at 4°C. The brush borders were pelleted again and resuspended in an equal volume of BBSB containing 10% calf serum. An aliquot of the suspension was added to each primary antibody at 5  $\mu$ g/ml and the mixture was incubated overnight at 4°C on a rotator. The brush borders were pelleted and washed three times in BBSB containing 10% calf serum and then incubated in DTAF-conjugated donkey anti-goat IgG at 10  $\mu$ g/ml.

**Retinal Pigmented Epithelium.** Retinal pigmented epithelium (RPE) was isolated by a modification of the protocol described by Turksen and Kalnins (48). Eyes were excised from 14- or 17-d chick embryos and the anterior segments and vitreous were gently removed. The eye cup was slit several times to flatten the structure and the neural retina was removed. The pigmented epithelium with its supporting choroid was cut into small pieces ( $\sim 6$  mm<sup>2</sup>) which were fixed in 2% paraformaldehyde in 80 mM PIPES, pH 6.8, containing 5 mM EGTA, 2 mM  $MgCl_2$ , 4% (wt/vol) polyethylene glycol (PEG 8000, USB, Cleveland, OH) and 0.1% (vol/vol) Triton X-100. Samples were quenched in 10 mM ethanolamine in PBS and blocked in 3% BSA, 5% heat-inactivated calf serum in TTBS for 30 min at room temperature. Anti-capping protein antibodies or preimmune Igs at 5  $\mu$ g/ml were applied overnight at 4°C. The tissue was washed three times in TTBS and incubated for 1–2 h in DTAF-conjugated donkey anti-goat IgG at 10  $\mu$ g/ml. To prepare RPE double stained with anti-capping protein  $\beta$ -subunit and anti-ZO-1 protein, a 1:100 dilution of rat ascites fluid specific for the ZO-1 protein of mouse hepatocytes (3) was included in the anti-capping protein  $\beta$ -subunit incubation. After washing in TTBS, samples were incubated in DTAF-conjugated donkey anti-goat IgG at 10  $\mu$ g/ml for 1 h, washed in TTBS, and finally incubated for 1 h in Texas red-conjugated rabbit anti-rat IgG at 10  $\mu$ g/ml. The RPE pieces were carefully mounted choroid-side down on glass slides for observation by confocal microscopy.

**Sensory Epithelium.** The sensory epithelium was obtained from cochlea of 19-d chick embryos as described (45) except that the sensory epithelium was separated from the tectorial membrane by manual dissection. The pieces of sensory epithelium were fixed in 2% paraformaldehyde in 50 mM potassium phosphate, pH 6.3, on ice for 1 h, permeabilized in 80 mM Pipes, pH 6.8, containing 5 mM EGTA, 2 mM  $MgCl_2$ , 0.1% Triton X-100, and 1% BSA for 30 min on ice, and incubated in anti-capping protein  $\alpha$ -subunit, anti-capping protein  $\beta$ -subunit or preimmune IgG. After washing in TTBS, bound antibodies were detected using rabbit anti-goat IgG at 1  $\mu$ g/ml and DTAF-conjugated donkey anti-goat IgG at 10  $\mu$ g/ml. Immunofluorescence was visualized using a confocal microscope.

**Kidney Epithelial Cells.** Primary cultures of kidney epithelial cells were prepared by trypsin digestion of the kidneys from 15-d chick embryos (9). Cells were cultured on glass coverslips in DME containing 10% FCS for 7 d. The cultures were monitored by phase-contrast microscopy to observe the formation of epithelial monolayers in some regions of the cultures. Cells on coverslips were fixed for 15 min in 2% paraformaldehyde in PBS, treated with 10 mM ethanolamine in PBS permeabilized in 0.5% Triton X-100 in PBS (15 min), and processed for double immunolabeling with anti-capping protein  $\beta$ -subunit and anti-ZO-1 protein as described above for RPE.

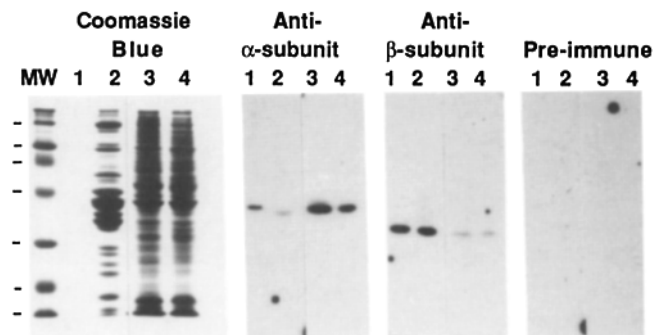
## Results

### Specificity of Antibodies to Chicken Capping Protein

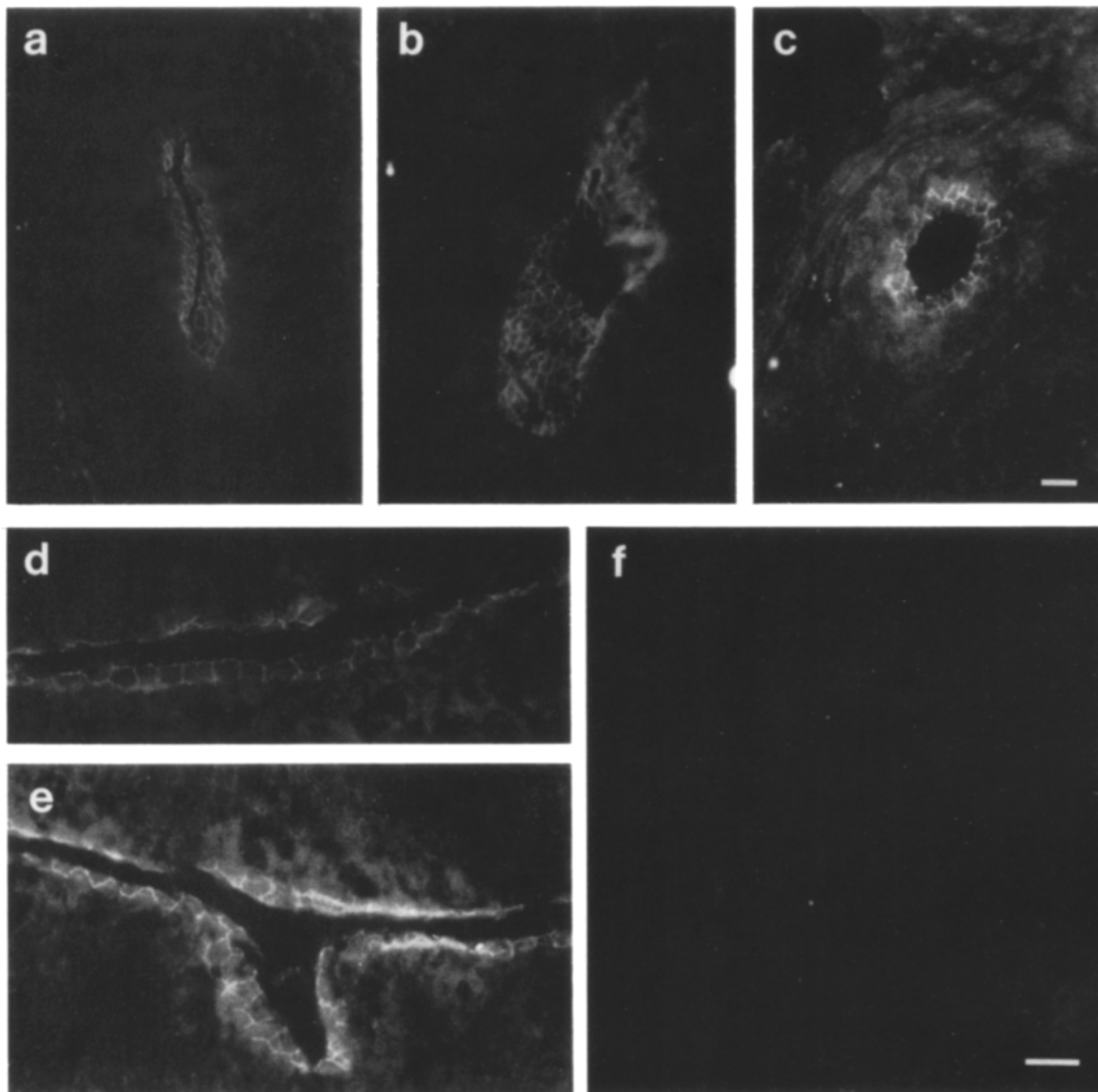
Affinity-purified goat polyclonal antibodies specific for the

$\alpha$ - and  $\beta$ - subunits of chicken skeletal muscle capping protein (CapZ) recognized the 36-kD  $\alpha$ -subunit and the 32-kD  $\beta$ -subunit of CapZ on immunoblots (Fig. 1, lane 1). The antibodies reacted with the capping protein subunits present in SDS-extracts of chicken pectoral muscle, retinal epithelium, and brush border epithelial cells (Fig. 1, lanes 2–4). (The migration of the  $\alpha$ -subunit of CapZ in SDS-extracts of skeletal muscle appears anomalously fast because of the high amount of actin migrating just behind it in the gel.) Antibodies specific for the individual subunits did not cross-react with each other; this property also was observed for antibodies to capping protein from *Acanthamoeba* (11) and chicken (7). As expected (8, 24), staining of myofibrils from chicken pectoral muscle by the  $\alpha$ -subunit and  $\beta$ -subunit antibodies was exclusively at the Z-line (Schafer, D. A., J. A. Waddle, and J. A. Cooper, manuscript submitted for publication).

Two differences in the reactivity on immunoblots of the affinity-purified antibodies with capping protein from muscle and nonmuscle tissues were observed. First, the electrophoretic mobility of the capping protein  $\beta$ -subunit from intestine and pigmented retinal epithelia was slightly faster than that of the  $\beta$ -subunit of skeletal muscle. Second, whereas the intensities of the bands on the Western blot corresponding to the  $\alpha$ - and  $\beta$ -subunits of capping protein of skeletal muscle were approximately equal, the intensity of the band corresponding to the nonmuscle  $\beta$ -subunit was considerably less than the band corresponding to the nonmuscle  $\alpha$ -subunit. This result may be due to differences in the reac-



**Figure 1.** Characterization of the affinity-purified anti-capping protein antibodies and detection of capping protein in SDS-extracts of chicken pectoral muscle, chick embryo retinal epithelium, and brush border epithelial cells. Immunoblot analysis of purified chicken skeletal muscle capping protein (CapZ) (lane 1), SDS-extract of chicken pectoral muscle (lane 2), SDS-extract of 14-d chick embryo retinal pigmented epithelium (lane 3), and SDS-extract of intestinal epithelial cells (lane 4). Multiple blots were prepared from gels containing the proteins shown in the Coomassie blue-stained gel and probed with affinity-purified goat anti-capping protein  $\alpha$ -subunit, anti-capping protein  $\beta$ -subunit or preimmune IgG. Approximately 100  $\mu$ g of protein was loaded in lanes 2–4. The purified capping protein is not detected in lane 1 of the Coomassie blue-stained gel because the amount loaded (20 ng) was too little to detect using this dye but was sufficient for detection on the immunoblot. The mobility of capping protein  $\alpha$ -subunit of skeletal muscle (lane 2) is altered by the high amount of actin migrating in that region of the gel. Molecular mass marker proteins are indicated on the left and include (from high to low molecular weight): myosin, 200 kD; phosphorylase b, 97.4 kD; BSA, 69 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD; trypsin inhibitor, 21.5 kD; and lysozyme, 14.3 kD.



**Figure 2.** Localization of capping protein in 4- $\mu$ m cryosections of chicken intestine. Immunolabeling by anti-capping protein  $\alpha$ -subunit and anti-capping protein  $\beta$ -subunit of structures in a polygonal pattern was observed in favorably cut regions along the apical surface of the epithelium. (a) Anti-capping protein  $\alpha$ -subunit; (b-e) anti-capping protein  $\beta$ -subunit; (f) preimmune IgG. Bars, 10  $\mu$ m (in c for a-c and in f for d-f).

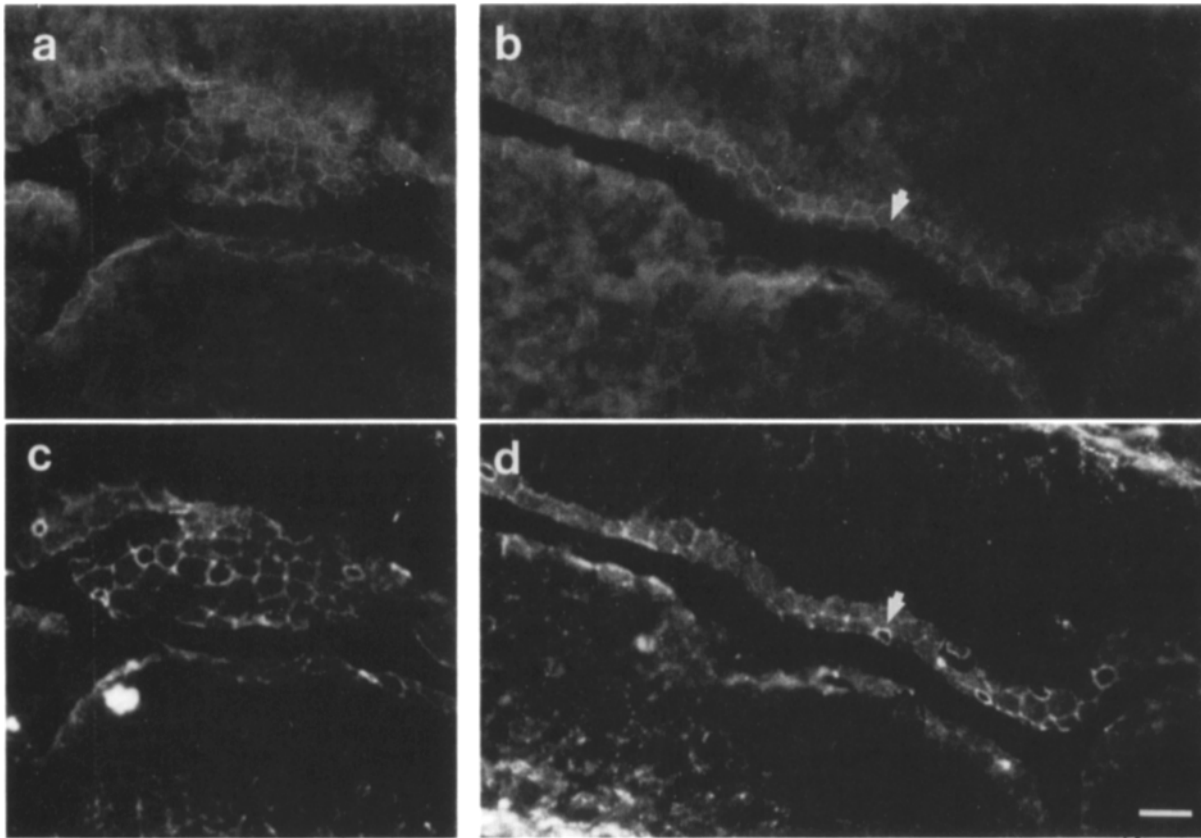
tivity of the anti- $\beta$ -subunit antibodies with  $\beta$ -subunits of skeletal muscle and nonmuscle tissue or to differences in relative amounts of the two capping protein subunits in muscle and nonmuscle tissues. Evidence against the latter possibility is that single subunits are unstable in yeast (Amatruda, J. F., D. J. Gattermeir, and J. A. Cooper, manuscript submitted for publication).

The anti- $\alpha$ -subunit and anti- $\beta$ -subunit antibodies usually gave identical staining patterns in the immunofluorescence localization studies. However, the intensity of immunolabeling obtained using the anti- $\alpha$ -subunit antibodies was considerably less than that obtained using the anti- $\beta$ -subunit antibodies and we sometimes observed little or no reactivity of the anti- $\alpha$ -subunit antibodies in immunolabeling experiments. Poor reactivity of anticapping protein  $\alpha$ -subunit in

immunofluorescence labeling of skeletal muscle has been encountered by other investigators using a different antiserum (8).

#### **Capping Protein Is Colocalized with Components of Cell Junctions in Intestinal Epithelium**

**Intact Intestinal Epithelial Cells.** When cryosections of chicken intestine were reacted with anti-capping protein  $\alpha$ -subunit or  $\beta$ -subunit, the predominant pattern of staining was an array of irregular polygons located along the luminal surfaces of villi and crypts (Fig. 2). The size of the individual polygons and their distribution in a closely packed array suggested that the pattern corresponded to staining around the periphery of individual cells in the epithelial layer. Areas of each section displaying the polygonal staining pattern were



**Figure 3.** Colocalization of capping protein  $\beta$ -subunit and  $\alpha$ -actinin in 4- $\mu\text{m}$  cryosections of chicken intestine. Capping protein (a and b) and  $\alpha$ -actinin (c and d) were detected in identical polygonal patterns along the apical surface of the epithelium. Occasional round cells that are most likely goblet cells (arrows in b and d) stained brightly with anti- $\alpha$ -actinin but did not stain with anti-capping protein unless a tertiary antibody was used to amplify the immunofluorescence signal (not shown). Bar, 10  $\mu\text{m}$ .

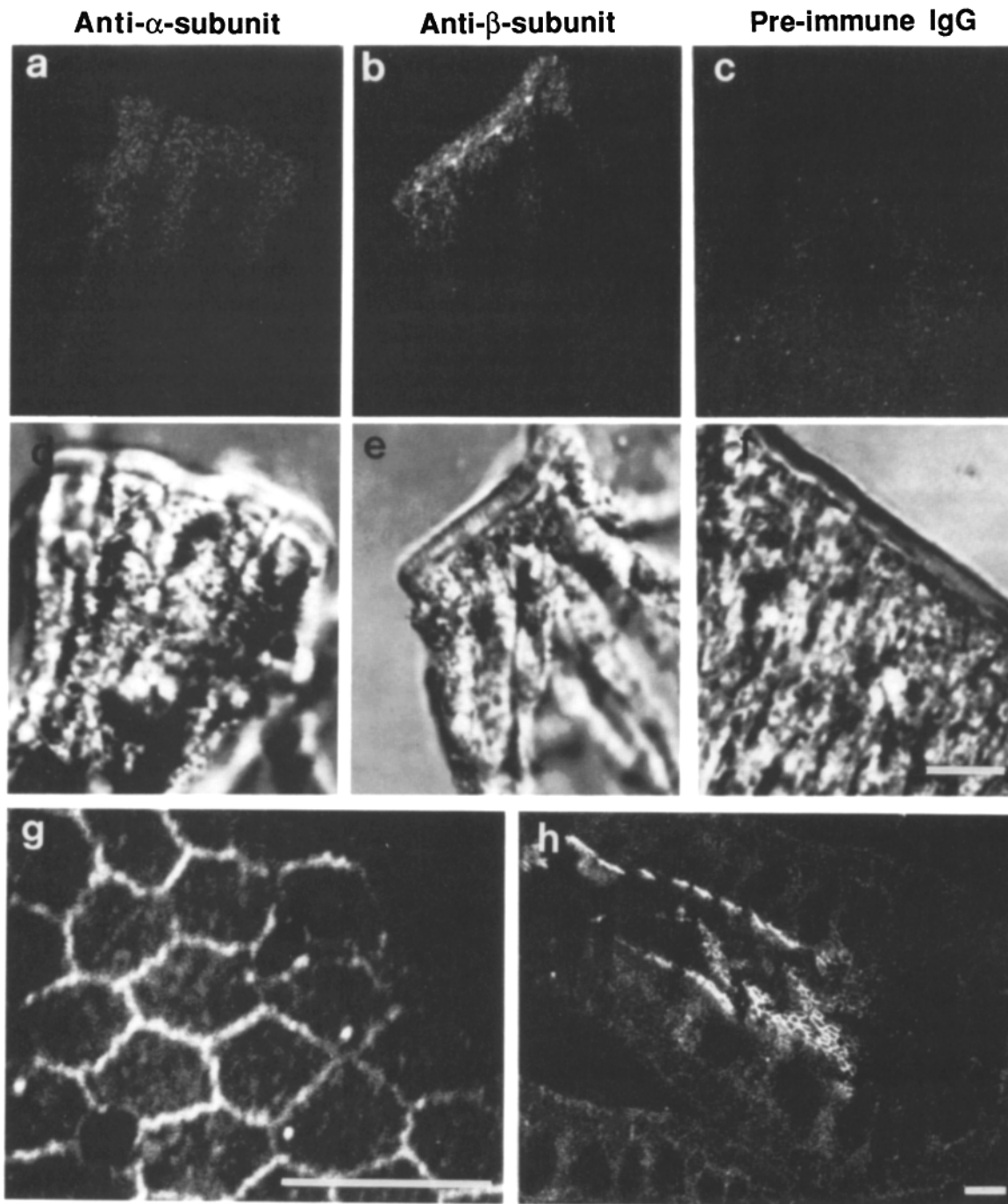
rare and likely reflect favorably cut regions that sectioned the epithelial cells in a plane perpendicular to their long axis. Less intense, diffuse staining by the capping protein antibodies was observed in regions corresponding to the cytoplasm. Controls using IgG prepared from preimmune serum (Fig. 2f) or omission of the primary antibody (not shown) did not label the tissue.

Capping protein is colocalized with  $\alpha$ -actinin, a protein known to be associated with the bundle of actin filaments encircling each epithelial cell at the level of the zonula adherens (13, 19). Double immunofluorescence staining of cryosections of intestine showed that capping protein, detected using the anti- $\beta$ -subunit antibody, and  $\alpha$ -actinin were distributed in similar polygonal arrays (Fig. 3). One difference in the distribution of these two antigens was the more prominent detection of  $\alpha$ -actinin around the periphery of round cells which probably correspond to the mucus-secreting goblet cells interspersed among the enterocytes (Fig. 3, arrows); these cells were lightly stained by anti-capping protein when a tertiary antibody layer was used to amplify the signal (data not shown) and, thus, these cells may contain less capping protein in the vicinity of the junctional complex than enterocytes.

The distribution of capping protein in intestinal epithelial cells was further investigated by immunolabeling intact epithelial cells that were fixed in situ before removal from the intestine (Fig. 4). This sample contained aggregates of intact

epithelial cells ranging in size from three to four cells to large numbers of cells that remained attached at intercellular junctions; intact crypt structures that were lined with epithelial cells were also observed. Confocal microscopy was used to obtain images of the cells. Capping protein was detected in the cytoplasm using the anti- $\alpha$ -subunit and anti- $\beta$ -subunit antibodies (Fig. 4, a and b). Cell junctions were detected using the anti- $\beta$ -subunit antibodies as seen in "edge-on" views (Fig. 4 b) and in "en face" views (Fig. 4 g). Capping protein  $\beta$ -subunit also was detected at junctional regions of epithelial cells lining crypts (Fig. 4 h). Microvilli were faintly stained by anti- $\beta$ -subunit antibodies throughout the microvilli fringe. Capping protein  $\alpha$ -subunit antibodies stained the intact cell preparation weakly; no immunolabeling at cell junctions was detected but weak immunolabeling was detected in the cytoplasm. The poor reactivity of the anti- $\alpha$ -subunit antibodies in intact cells may be due to poor accessibility to antigen in these intact cell preparations.

**Brush Border Fragments.** Anti-capping protein stained brush border fragments in a polygonal pattern that colocalized with cell-cell junctions observed using differential interference contrast microscopy (Fig. 5, a and b). This pattern was best observed in "sheets" containing clusters of several brush borders. The immunolabeling was continuous around the periphery of most "cells" in the fragment but was usually absent from cell junctions at the margin of the fragment. When brush borders were prepared using more vigorous

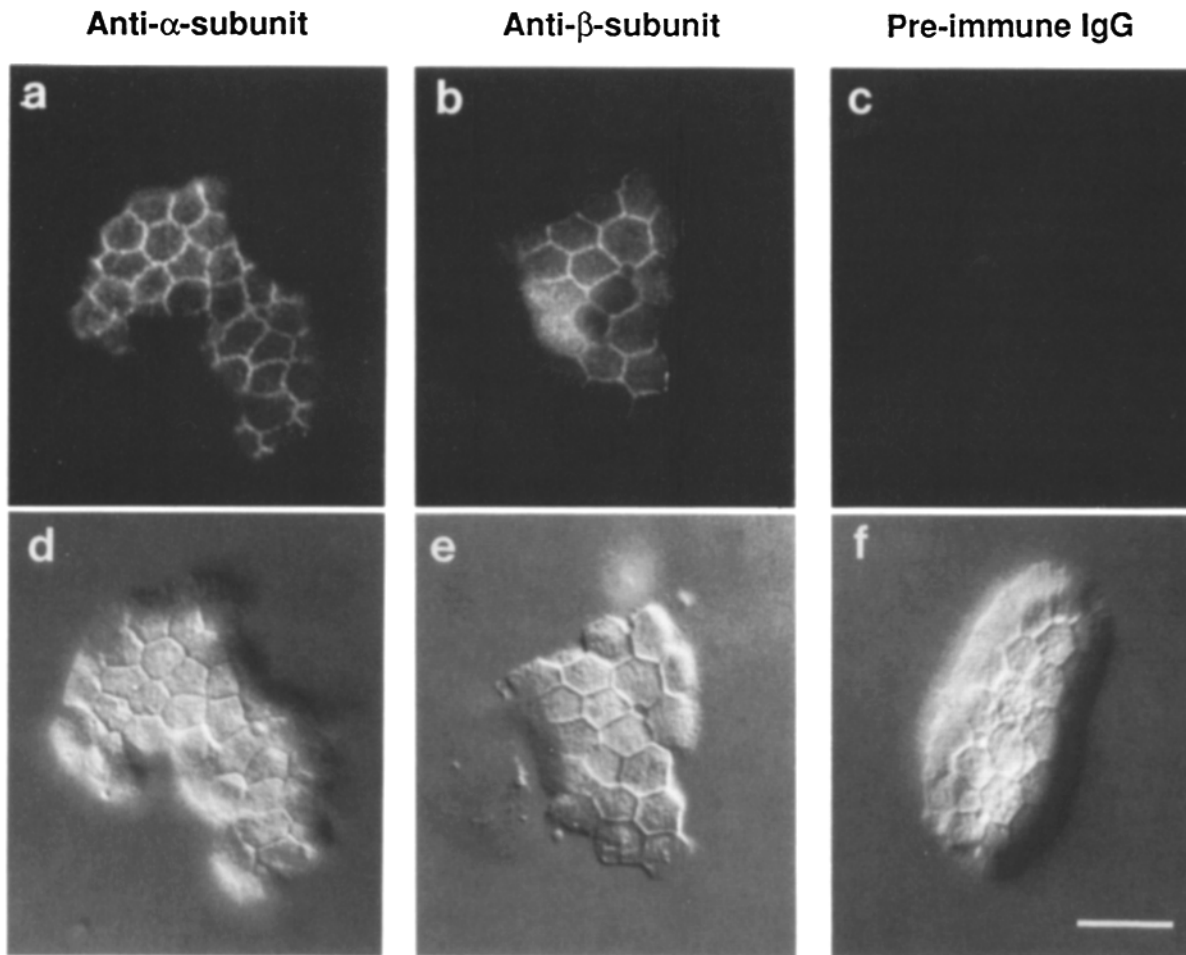


**Figure 4.** Localization of capping protein in intact intestinal epithelial cells using confocal microscopy. Capping protein  $\alpha$ - (*a* and *d*) and  $\beta$ - (*b* and *e*) subunits were detected in a diffuse distribution in the cytoplasm of intact enterocytes. The optical section shown in the fluorescence image in *b* does not include the entire cell. Anti-capping protein  $\beta$ -subunit also labeled cell-cell junctions of intact cells (*b* and *g*), including those of epithelial cells lining crypts (*h*) and faint, punctate immunolabeling by anti- $\beta$ -subunit was observed in microvilli (*b*). Anti- $\alpha$ -subunit did not stain the intact cells intensely and cell junctions were not detected. Differential interference contrast micrographs corresponding to the confocal micrographs in *a-c* are shown in *d-f*, respectively. Preimmune IgG stained the cytoplasm very weakly (*c*). Bars, 10  $\mu\text{m}$  (in *f* for *a-f*).

homogenization that dissociated the epithelial sheets into brush borders derived from 1 or 2 cells, capping protein immunolabeling at cell junctions was significantly diminished and detected as weak, diffuse staining within the terminal web (data not shown). Immunolabeling in microvilli of the brush border fragments was not detected using these conditions which did not include detergent extraction.

**Localization of Capping Protein at Junctional Complexes Formed by Pigmented Retinal Epithelial Cells, Sensory Epithelial Cells of the Cochlea, and by Chick Kidney Epithelial Cells in Culture**

**Pigmented Retinal Epithelium.** Capping protein  $\alpha$ -subunit and  $\beta$ -subunit was localized in the vicinity of cell junctions



**Figure 5.** Localization of capping protein at the junctional complexes in brush borders from chicken intestine. Capping protein was detected around the periphery of individual cells in sheets of brush borders. (a) Anti-capping protein  $\alpha$ -subunit; (b) anti-capping protein  $\beta$ -subunit; (c) preimmune IgG; (d-f) corresponding differential interference contrast images of a-c, respectively. Bar, 10  $\mu$ m.

of pigmented retinal epithelial cells from chick embryos (Fig. 6). Faint, diffuse staining at the apical surface also was detected. Staining of nuclei of the retinal epithelial cells was observed when the appropriate focal plane was selected. The detection of capping protein in the cytoplasm of the retinal epithelium was difficult to assess because the dark pigment granules prevented the excitation light from penetrating uniformly into the sample.

We confirmed the localization of capping protein to a region near the cell junctions of retinal epithelial cells by visualizing the distributions of capping protein and ZO-1 protein, a 225-kD protein that is localized exclusively at the zonula occludens of mouse hepatocytes (3), in double-stained preparations (Fig. 7). Confocal microscopy was used to obtain "en face" views (Fig. 7, a and b) and "edge-on" views (Fig. 7, c and d) of the double-stained epithelium. In the "edge-on" views, the tight junctions were identified with the anti-ZO-1 antibody as bright spots near the apical surface at regions of cell-cell contact. Capping protein  $\beta$ -subunit immunolabeling was prominent at these same sites of cell-cell contact. The resolution of the confocal microscope is not sufficient to assign a specific location for capping protein within the junctional complex.

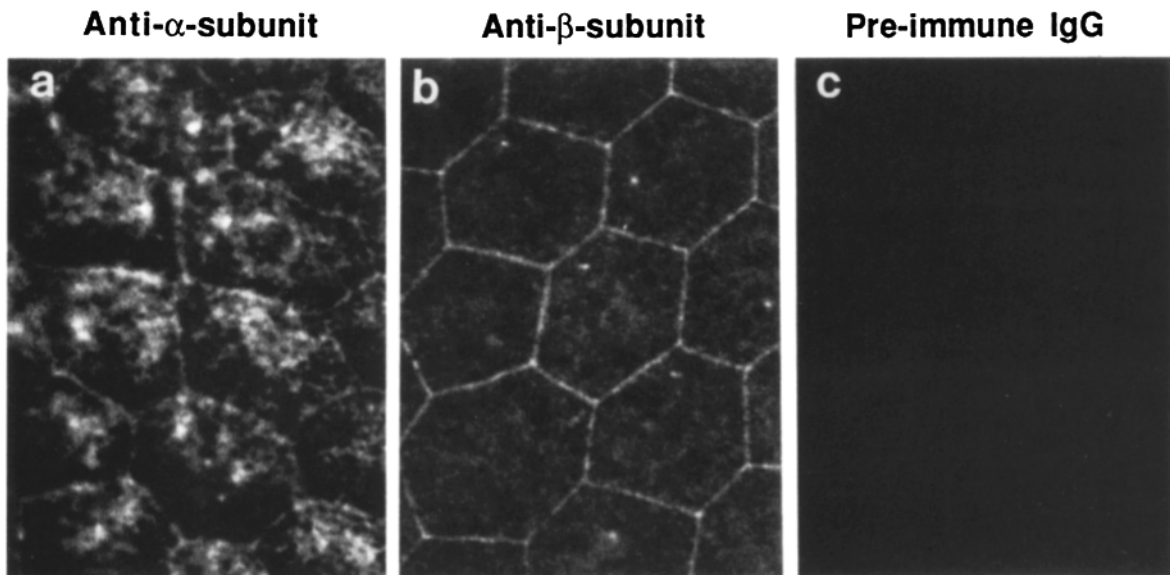
**Sensory Epithelium.** Individual hair cells are surrounded by supporting cells to which they are tightly associated via

cell junctions near the apical surface. Actin filaments encircle the apical surface of hair cells and supporting cells (15, 21). Capping protein  $\beta$ -subunit was detected around the periphery of hair cells and supporting cells in a band at the apical surfaces of these cells (Fig. 8). No prominent staining was observed in the cuticular plate. Weak immunolabeling was detected in stereocilia of hair bundles but additional experiments are required to definitively assign a location for capping protein in stereocilia. Anti- $\alpha$ -subunit antibodies stained the sensory epithelium weakly and distinct cell junctions were not observed (data not shown).

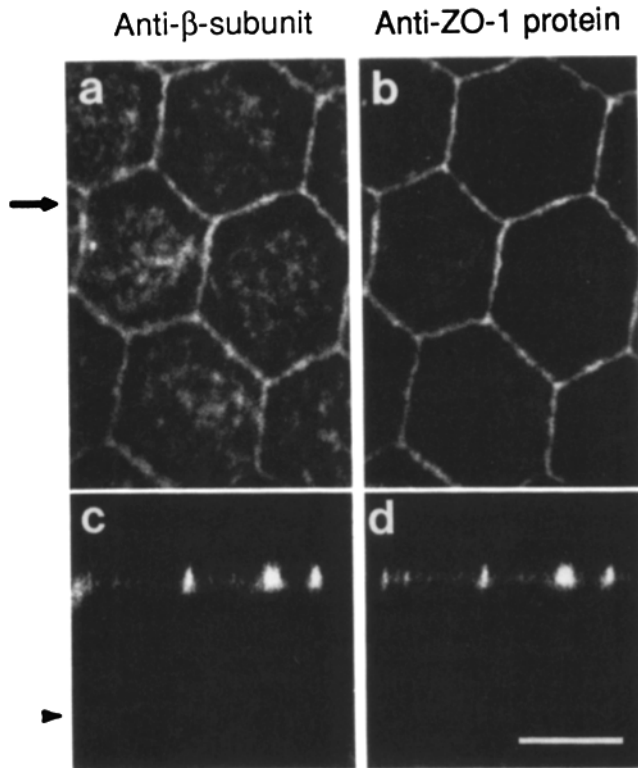
**Kidney Epithelial Cells in Culture.** Epithelial cells in primary cultures of cells derived from 15-d chick embryo kidney formed junctions at regions of contact between individual epithelial cells. Capping protein  $\beta$ -subunit and ZO-1 protein were colocalized in a distinct linear pattern along regions of cell-cell contact (Fig. 9). Capping protein  $\beta$ -subunit also was detected in nuclei, specifically in the nucleoplasm, and in a diffuse distribution in the cytoplasm of cells in the cultures. Anti- $\alpha$ -subunit antibodies were not tested on the kidney epithelial cells.

#### **Biochemical Fractionation of Capping Protein in Intestinal Epithelial Cells**

To characterize the distribution of capping protein further,



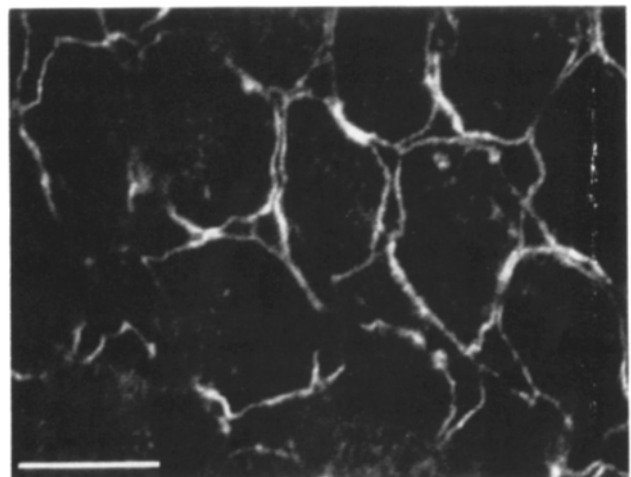
**Figure 6.** Localization of capping protein in retinal pigmented epithelia of 14-d chick embryos using confocal microscopy. Capping protein  $\alpha$ -subunit (a) and  $\beta$ -subunit (b) was detected around the periphery of each cell in the epithelial sheet. Some staining throughout the apical region was observed and may correspond to capping protein in microvilli or in the apical cytoplasm. Preimmune IgG (c) did not stain the RPE. Bar, 10  $\mu$ m.



**Figure 7.** Colocalization of capping protein and ZO-1 protein in the junctional region of retinal pigmented epithelium. En-face views (a and b) of pigmented retinal epithelium from 17-d chick embryos showed colocalization of capping protein  $\beta$ -subunit (a) and ZO-1 protein (b) at the periphery of each cell. Edge-on views (c and d) taken at the position marked by the arrow beside a show colocalization of capping protein and ZO-1 protein in the same region near the apical surface at sites of cell-cell contact. The images were obtained using a laser scanning confocal microscope. The edge-on views were collected by scanning in a plane perpendicular to the plane of the epithelial sheet at the position indicated by the arrow; the arrowhead marks the position of the basolateral surface of the RPE cells. Bar, 10  $\mu$ m.

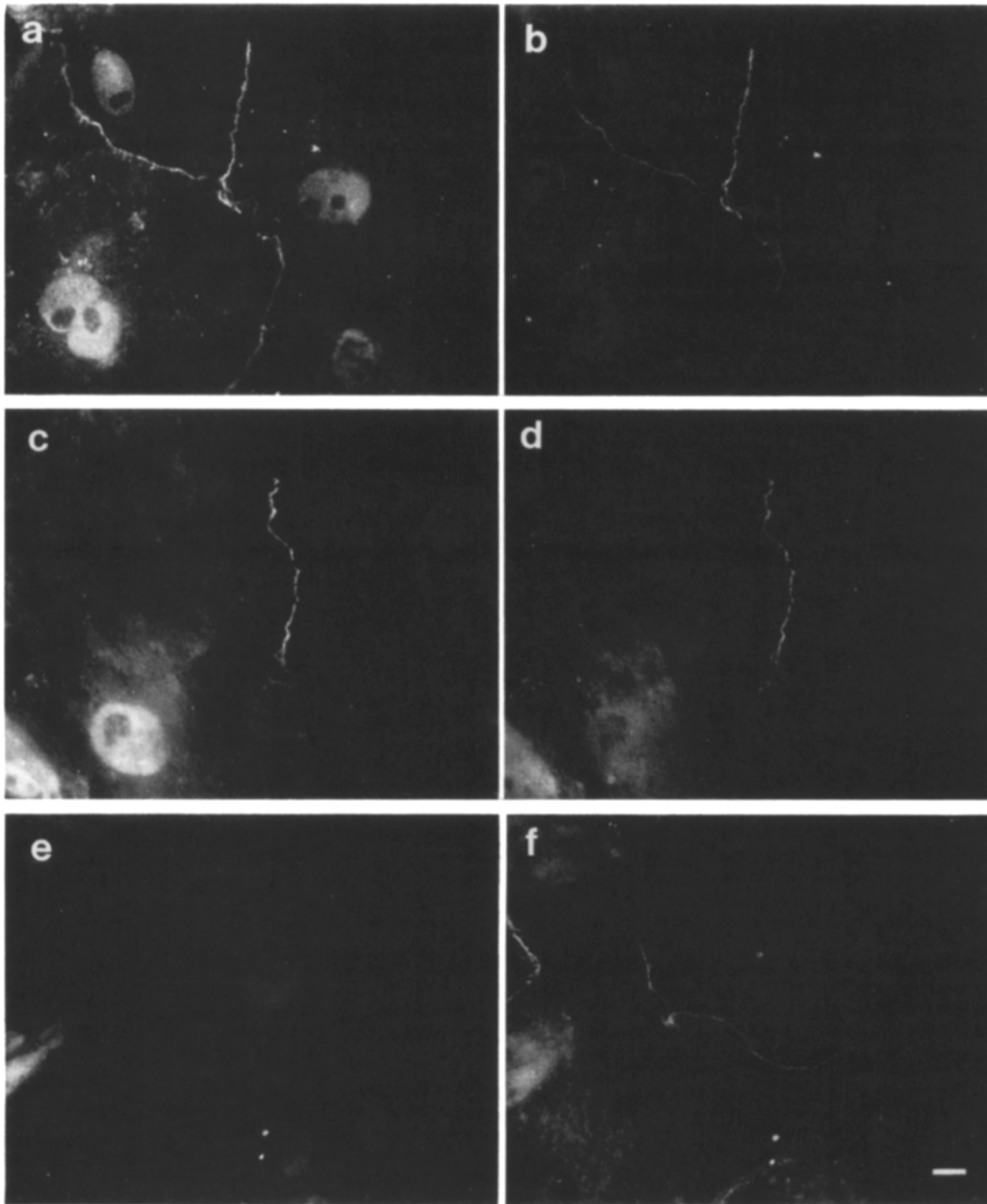
intestinal epithelial cells were fractionated by homogenization and differential centrifugation. Whole cells were prepared from chicken intestine and homogenized under mild conditions to obtain brush borders. The brush borders were isolated by a low-speed centrifugation of the whole cell homogenate and purified by sedimentation on a sucrose step gradient. The supernatant fraction obtained from the low-speed centrifugation was then subjected to high-speed and ultra-speed centrifugations. Microvilli were prepared from the purified brush borders by vigorous homogenization to release some, but not all, microvilli. Sequential rounds of low and high speed centrifugation, checked at each step by phase microscopy, resulted in highly purified microvilli.

Samples of each subcellular fraction were subjected to immunoblot analysis with anti-capping protein  $\alpha$ -subunit (Fig. 10 d), anti- $\beta$ -subunit (data not shown), and with antibodies specific for brush border myosin I heavy chain (Fig. 10 c)



**Figure 8.** Localization of capping protein at cell junctions in sensory epithelium of chick cochlea. Capping protein  $\beta$ -subunit was detected at cell junctions using confocal microscopy. Bar, 10  $\mu$ m.

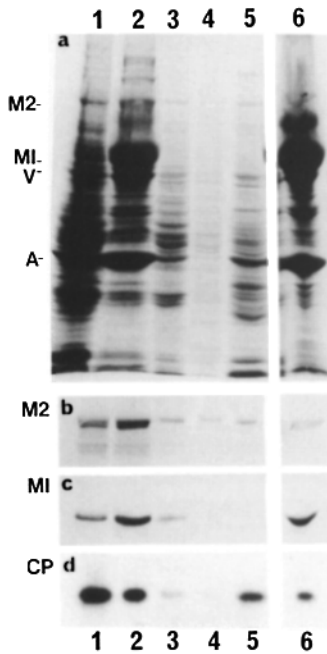




**Figure 9.** Colocalization of capping protein  $\beta$ -subunit and ZO-1 protein at junctional complexes formed by chick kidney epithelial cells in culture. Double immunofluorescence staining with anti-capping protein  $\beta$ -subunit (a and c) and anti-ZO-1 protein (b, d, f). Capping protein and ZO-1 protein were localized along regions of cell-cell contact. Capping protein was also detected in the nuclei and diffuse cytoplasmic staining also was observed. Preimmune IgG (e) did not stain the cells. Bar, 10  $\mu$ m.

and myosin II heavy chain (Fig. 10 b). Lanes 1-5 contain equal cell equivalents related to the whole cell homogenate with exception of the purified brush border fraction (lane 2) which is loaded at 6-12-fold excess compared to the samples in lanes 1 and 3-5. Capping protein was detected in the whole cell homogenate (Fig. 10 d, lane 1), in the brush border fraction (Fig. 10 d, lane 2), and in the ultra-speed supernatant fraction (Fig. 10 d, lane 5), consistent with the im-

munofluorescence staining observed at cell junctions and in cytoplasm of intact intestinal epithelial cells. A small amount of capping protein was detected in the high-speed pellet fraction (Fig. 10 d, lane 3) and is likely derived from small brush border fragments that did not sediment during the initial low-speed centrifugation. Capping protein was also detected in the highly purified microvilli fraction (Fig. 10 d, lane 6); this result was not expected since the immunofluorescence stain-



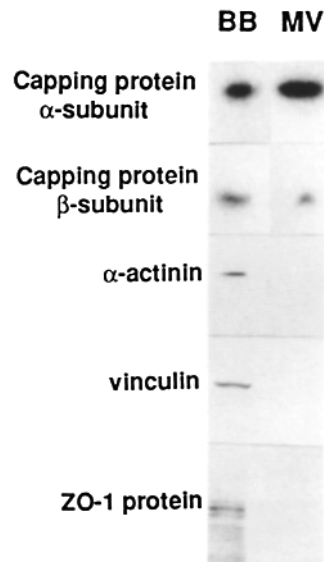
**Figure 10.** Immunoblot analysis of subcellular fractions of isolated intestinal epithelial cells. (a) Chicken intestinal epithelial cells were homogenized and subcellular fractions were prepared by differential sedimentation as described in Materials and Methods. Lanes 1-5 are samples containing equal cell equivalents except that the purified brush border fraction (lane 2) contains 6-12-fold greater the amount as in lanes 1 and 3-5. (lane 1) Whole cell homogenate; (lane 2) purified brush border fraction; (lane 3) high-speed pellet fraction; (lane 4) ultra-speed pellet fraction; (lane 5) ultra-speed supernatant fraction. The brush border fraction was further fractionated to yield highly purified microvilli (lane 6);

the microvilli gel sample was an aliquot of a 10% suspension of the microvilli pellet and is not stoichiometric with the samples in lanes 1-5. The migration positions of brush border myosin II heavy chain (M2), brush border myosin I (MI), villin (V), and actin (A) are indicated. (b) Immunoblot analysis of the subcellular fractions using antimyosin II heavy chain (M2). Note that this terminal web protein is found in only trace amounts in the microvilli fraction (lane 6), demonstrating that the microvilli preparation is relatively free of contamination with intact brush borders or terminal web fragments. (c) Immunoblot analysis of the subcellular fractions using anti-brush border myosin I heavy chain (MI). As expected, this microvilli core protein is present in both brush border (lane 2) and microvilli (lane 6) fractions. (d) Immunoblot analysis of the subcellular fractions with anti-capping protein  $\alpha$ -subunit. Capping protein (CP) was detected in the whole cell homogenate (lane 1), in the purified brush borders (lane 2), in the soluble fraction (lane 5), and in the microvilli fraction (lane 6); a small amount of capping protein was detected in the high speed pellet fraction (lane 3). The distribution of capping protein  $\beta$ -subunit in these fractions (not shown) was identical to that of the  $\alpha$ -subunit.

ing of microvilli was low or not detectable. To document the purity of the microvilli, we determined that the preparation contains the specific marker brush border myosin I (Fig. 10 c, lane 6) but relatively small amounts of the terminal web marker myosin II (Fig. 10 b, lane 6). In addition, immunoblotting analysis with anti- $\alpha$ -actinin and anti-vinculin, two components of the zonula adherens, and with anti-ZO-1 protein, a component of the zonula occludens (3), were not detected in the microvilli fraction which indicates the absence of contamination with cell junctions (Fig. 11).

## Discussion

We report the localization of capping protein in epithelial cells by immunofluorescence microscopy of several chicken tissues, including the intestine, the pigmented retinal epithelium, the sensory epithelium of the cochlea, and kidney cells in culture. Biochemical fractionation was performed on the



**Figure 11.** Immunoblot analysis of purified brush borders (BB) and purified microvilli (MV) demonstrating that the microvilli fraction is free of contamination by intercellular junctional components. Identical blots were probed with anti-capping protein  $\alpha$ -subunit,  $\beta$ -subunit, and with mAbs specific for  $\alpha$ -actinin, vinculin, and ZO-1 protein. None of these intercellular junctional proteins were detected in the purified microvilli fraction, indicating that this preparation is free of contamination by fragments of the intercellular junctions. Samples for blots of anti-capping protein were electrophoresed on a 10% gel; 8% gels

were used for  $\alpha$ -actinin and vinculin and a 6% gel was used for ZO-1 protein. Blots probed with mAbs were incubated with the appropriate alkaline phosphatase-conjugated secondary antibody; blots probed with goat anti-capping proteins were incubated with rabbit anti-goat IgG followed by  $^{125}\text{I}$ -labeled goat anti-rabbit IgG. Only the region of each blot containing the protein of interest is shown.

intestinal epithelial cells as an independent method of characterizing the cellular locations for capping protein.

## Capping Protein at Junctional Complexes

Immunofluorescence labeling for capping protein in all epithelia examined included a pattern corresponding to that of cell-cell junctional complexes. En face views showed a polygonal pattern corresponding to the boundaries of cells. Double immunofluorescence labeling showed staining coincident with known components of cell junctions, which included  $\alpha$ -actinin in intestine, and ZO-1 in retina and kidney. Capping protein was enriched in the apical region of the cells as shown by edge-on views of intestine and retina. Biochemical fractionation of isolated intestinal epithelial cells revealed that some capping protein was associated with brush borders, which contain the junctional complexes, terminal web, and microvilli.

Junctional complexes contain several elements, including the zonula occludens, zonula adherens, desmosomes, and gap junctions. Zonula occludens and zonula adherens, but not the other two junctions, have actin filaments associated with the junctions that encircle the apical membrane in a network or bundle (18, 43). The actin filaments of the zonula adherens are relatively long and have mixed polarity (20). The positions of their ends and the nature of their interaction with the cell membrane is not known. Since capping protein binds barbed ends of actin filaments *in vitro* and colocalizes with barbed ends at the Z-discs of striated muscle, capping protein may bind the actin filaments associated with one or both of these two junctions. The ends of these filaments are functionally capped, based on the observation that actin subunits add to actin filaments of microvilli but not to those associated with cell junctions (35). The possibility that capping protein is associated with desmosomes or gap junctions

is less likely since these junctions do not have prominent actin filament arrays associated with them and retinal epithelial cells lack desmosomes (14). Radixin, an 82-kD protein proposed to cap barbed ends of actin filaments, also has been localized to adherens junctions of liver and intestine (47). Perhaps radixin and capping protein bind to the barbed ends of distinct subsets of actin filaments of the junctional complex. The experiments reported here do not distinguish the exact junctional location of capping protein within the junctional complex; our experiments to localize capping protein using immuno-electron microscopy have not shown specific labeling at junctions of intestinal epithelial cells.

### Other Cellular Pools of Capping Protein

The immunofluorescence distribution of capping protein in epithelial cells also includes a prominent diffuse cytoplasmic distribution. A substantial portion of the capping protein appears in the ultra-speed supernatant during biochemical fractionation of intestinal epithelial cells suggesting that soluble capping protein is abundant in the cytoplasm. In *Acanthamoeba castellanii*, approximately two-thirds of capping protein in the crude cell extract was soluble and had the same Stokes' radius as pure capping protein (11). Immunofluorescence localization of capping protein of *Saccharomyces cerevisiae* revealed that some of the capping protein is diffusely distributed in the cytoplasm and a large fraction of the capping protein partitions with the soluble fraction after ultra-speed centrifugation (2). Together these results suggest that cells contain a substantial amount of capping protein that is not bound to actin filaments. Since capping protein binds actin filaments with high affinity under physiologic conditions we speculate that exposed barbed ends are rapidly capped by capping protein in vivo.

We also observed capping protein in the nuclei of retinal epithelial cells and kidney epithelial cells in culture, but not epithelial cells of the intestine or cochlea. In other experiments, we have observed staining in nuclei of several types of cells in culture, including chicken fibroblasts, skeletal myotubes and neuronal cells, and MDCK cells, as well as nuclei of chicken erythrocytes. These observations are consistent with previous observations localizing capping protein  $\alpha$ -subunit in nuclei of *Xenopus laevis* oocytes and bovine lens epithelial cells in culture by cell fractionation or immunofluorescence (4). In contrast, in other experiments, we found that nuclei in cryosections of several chicken tissues including liver, skeletal muscle, cardiac muscle, brain, and gizzard were not immunolabeled by anti-capping protein. In addition, a nuclear location for capping protein was not observed by immunofluorescence in *Acanthamoeba castellanii* (11) or *Saccharomyces cerevisiae* (11). While these observations are reproducible, the significance of the variability in a nuclear localization for capping protein among different cells and the function of capping protein in the nucleus is unclear. Actin has been detected in nuclei (10, 25) and has been suggested to play a role in expression of some genes (16).

The tip of the microvillus may be functionally or structurally related to Z-discs since barbed ends of actin filaments are situated there. In the experiments described in this report, the detection of capping protein in stereocilia and microvilli by indirect immunofluorescence was weak or non-existent. On the other hand, biochemical fractionation of intestinal epithelial cells showed that capping protein was pres-

ent in the microvilli fraction, and that the microvilli were highly purified and free of contamination with cell junction components. Two possible explanations for this discrepancy exist. Capping protein may be a component of the microvillus and not be labeled in the immunofluorescence experiments because its epitopes are not accessible. Alternatively, capping protein may not be a component of the microvillus but is re-distributed there during cell fractionation. The soluble capping protein of the cytoplasm may add to the barbed ends at the tip of microvilli when cells are homogenized or fixed. Additional experiments will be required to distinguish between these possibilities.

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