

# Components of the Cytoskeleton in the Retinal Pigmented Epithelium of the Chick

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The retinal pigmented epithelium (RPE) is a simple cuboidal epithelium with apical processes which, unlike many epithelia, do not extend freely into a lumen but rather interdigitate closely with the outer segments of the neural retina. To determine whether this close association was reflected in the cytoskeletal organization of the RPE, we studied the components of the cytoskeleton of the RPE and their localization in the body of the cell and in the apical processes. By relative mobility on SDS gels and by immunoblotting, we identified actin, vimentin, myosin, spectrin (240/235), and alpha-actinin as major components, and vinculin as a minor component. In addition, the RPE cytoskeleton contains polypeptides of  $M_r$  280,000 and 250,000; the latter co-electrophoreses with actin-binding protein. By immunofluorescence, the terminal web region appeared similar to the comparable region of the intestinal epithelium that consists of broad belts of microfilaments containing myosin, actin, spectrin, and alpha-actinin. However, the components of the apical processes were very different from those of intestinal microvilli. We observed staining along the processes for myosin, actin, spectrin, alpha-actinin, and vinculin. The presence in the apical processes of contractile proteins and also of proteins typically found at sites of cell attachments suggests that the RPE may actively adhere to, and exert tension on, the neural retina.

The surfaces of many cells are covered with cellular extensions whose functions are multifarious. For example, the apical surface of intestinal epithelial cells (IECs)<sup>1</sup> is characterized by highly organized microvillus processes whose major function is to increase the effective surface area for absorption. These microvilli contain a bundle of actin filaments held together by fimbrin and villin and linked to the membrane by a 110K protein (1, 2). Myosin is not found in the microvilli of the IECs, supporting other evidence that these processes are non-motile.

The cells of the retinal pigmented epithelium (RPE) are also highly polarized cells with apical filopodia. However, these processes differ from those of the IECs because they are longer and ensheath the outer segments of photoreceptor cells rather than project freely into a lumen. Retinal attachment depends on this close relationship, which also is required for retinal viability, probably because the RPE transports metabolites to and from the retina. Some evidence suggests that interdigitation may help to orient the photoreceptor cells (3).

In addition, the apical processes are responsible for phagocytosis of the spent ends of the outer segments (4, 5), a crucial process in maintaining vision.

In the present study we show that, just as the functions of the apical processes of the RPE differ from those of the microvilli of the IECs, so do the cytoskeletal components. The RPE processes contain myosin and a distinct set of actin binding proteins. Our results suggest that the apical processes are motile and that they may exert tension on the neural retina.

## MATERIALS AND METHODS

*Isolation and Demembration of RPE Cells:* Eyes from 15–21-d-old white leghorn chick embryos were dissected in physiological saline to remove the anterior segment, the vitreous, and the neural retina. The posterior eye cups were incubated for 15 min at 22°C in a phosphate-buffered saline (PBS) composed of 136 mM NaCl, 8.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, made 0.1 M in sucrose and 0.02 M in EDTA (6), and the RPE cells were then microdissected from the underlying tissue. Sheets of RPE cells were demembrated in a buffer containing 75 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM imidazole pH 7.2, 1% Triton X-100, 0.2 mM phenylmethyl sulfonyl fluoride, 0.4 mM benzamidine, and 0.05 mg/ml leupeptin for 30 min at 4°C. The demembrated cytoskeletons were collected by centrifugation at

<sup>1</sup> *Abbreviations used in this paper:* IEC, intestinal epithelial cell; RPE, retinal pigmented epithelium.

12,000 *g* in an Eppendorf centrifuge. The pellets were washed once in the same buffer at 4°C and then solubilized in SDS sample buffer.

**SDS PAGE:** Proteins were analyzed on microslab gels as described by Matsudaira and Burgess (7) using the buffer system of Laemmli (8). Gels were run at a constant voltage of 150 V then fixed and stained in 0.25% Coomassie Brilliant Blue R in 50% methanol and 10% acetic acid and destained by diffusion in 5% methanol, 10% acetic acid. Gels were scanned on a Canalco model K densitometer (Rockville, MD) and peak areas integrated using a Numonics integrator (Numonics Corp., Lansdale, PA).

**Immunological Procedures:** Cytoskeletal proteins separated on SDS polyacrylamide gels were electrophoretically transferred to nitrocellulose paper at 30 V and 0.1 A overnight using a TransBlot apparatus (Bio-Rad Laboratories, Inc., Richmond, CA). The nitrocellulose paper was washed for 1 h in buffer containing 50 mM Tris pH 7.8, 0.5% Nonidet P-40, 0.15 M NaCl, and 3% bovine serum albumin, and then incubated in the same buffer with the primary antibodies (10 µg/ml) for 1 h. After thorough washing a secondary antibody of peroxidase-labeled goat anti-rabbit IgG (Cappel Laboratories, West Chester, PA) was applied. Bands with adsorbed antibody were visualized with diaminobenzidine, 0.5 mg/ml in 0.05 M Tris, pH 7.5, containing 0.0015% hydrogen peroxide.

Cytoskeletal proteins were localized in RPE cells using indirect immunofluorescence on frozen sectioned tissue, sheets of RPE, and dissociated cells. All of the antibodies used in the studies were previously characterized. Antibodies to alpha-actinin and vinculin were from Dr. S. Craig (Johns Hopkins University, Baltimore, MD); to spectrin, TW260, and villin from Dr. J. R. Glenney (Salk Institute, La Jolla, CA); to vimentin from Dr. G. S. Bennett (University of Pennsylvania, Philadelphia, PA); and the antimyosin was prepared in our laboratory (9).

Sheets of RPE cells prepared as described were fixed in 1% paraformaldehyde in PBS for 15 min at 22°C. The sheets of cells were collected by centrifugation, washed, and resuspended in a small volume of PBS. A drop of the cell suspension was placed on a coverslip and left at room temperature for a few minutes so that the sheets of RPE adhered to the coverslip. To obtain isolated RPE cells we dissociated sheets of RPE cells in Coon's enzyme solution containing collagenase 6 U/ml, trypsin 0.1%, chick serum 2%, and EDTA 4 mM for 10 min at room temperature. The reaction was stopped with a large volume of PBS containing 5% BSA, and the cells were collected by centrifugation at 500 *g* for 5 min. The cells were washed in the same buffer, resuspended in PBS containing 1% paraformaldehyde for 5 min at 22°C, washed, and then resuspended in a small volume of PBS. The posterior eyecups were sometimes lightly fixed before the RPE cells were dissociated. This was done as a control to ensure that reorganization of the cytoskeleton did not occur during the isolation procedures. Cells were washed once in PBS then permeabilized with cold methanol at -20°C for 5 min. After several washes with PBS the primary antibodies were applied in 100 µl of PBS and cells were incubated for 1 h in a moist chamber at room temperature. After thorough washing, fluorescein-conjugated goat anti-rabbit Fab<sub>2</sub> (Cappel Laboratories) was applied. Specimens were examined on a Zeiss Research microscope equipped with Zeiss planapochromatic objectives. Photographs were taken on Kodak Tri-X film and developed to yield an ASA of 1000.

**Light and Electron Microscopy:** Eyes from 3-d-old chicks were fixed in 2.5% glutaraldehyde with 2% tannic acid in 100 mM cacodylate buffer, pH 7.0, for 1 h at room temperature and then at 4°C overnight. The tissue was washed then treated for 1 h in 1% OsO<sub>4</sub> in the same buffer. The tissue was dehydrated in a graded series of ethanol and embedded in Spurr resin. Both the thin and thick sections were cut on a Sorvall MT2 microtome (DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE). Electron micrographs of thin sections were taken with a Phillips 200 microscope and light micrographs were taken of thick sections stained with methylene blue.

## RESULTS

Fig. 1 *a* is a light micrograph of a longitudinal section through the posterior eye cup of a 3-d-old chick. The RPE is a simple epithelium of hexagonally packed cuboidal cells located between the choriocapillaris (basal) and the outer segments of the photoreceptor cells (apical). The bodies of the RPE cells are 5–6 µm high and the apical processes are 12–13 µm long. These processes interdigitate with the rod and cone outer segments and can be seen more clearly when the outer segments have been removed (Fig. 1 *b*). These processes contain regularly spaced microfilaments in bundles (Fig. 1 *c*).

The detergent-insoluble fraction of the RPE cells contains

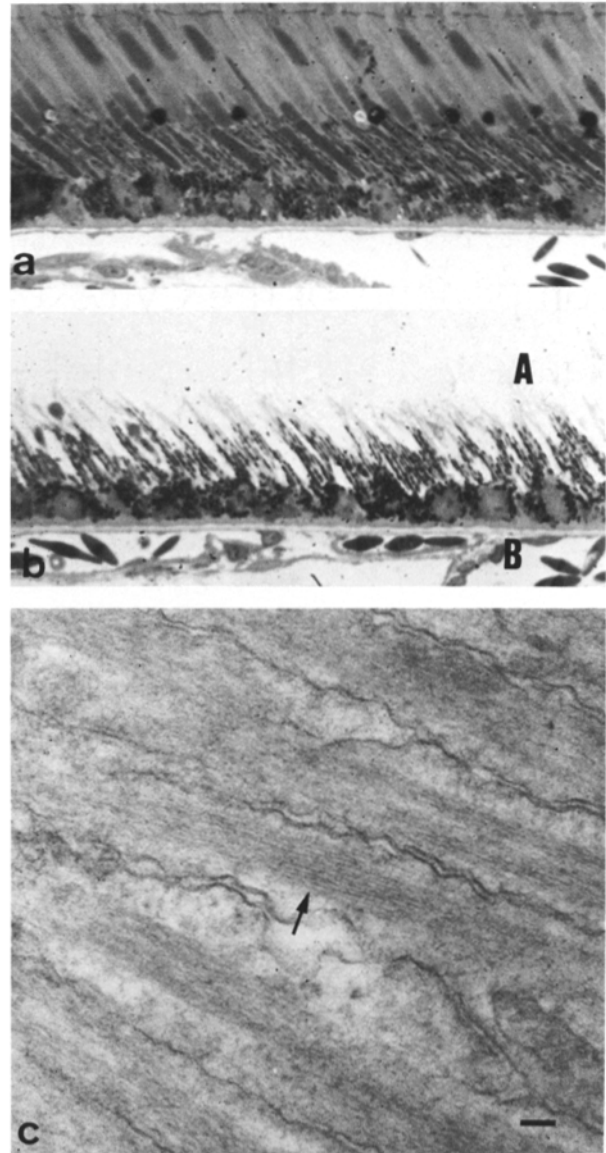


FIGURE 1 (*a* and *b*) Light micrographs of longitudinal sections of the retinal pigmented epithelium from a 1-d-old chick. In *a* the neural retina is present, showing the interdigitations of the apical processes of the RPE with the outer segments of the photoreceptor cells. In *b* the neural retina has been removed, illustrating more clearly the length of the apical processes. *A* and *B* indicate the apical and basal surfaces of the RPE.  $\times 1,000$ . *c* is an electron micrograph of a longitudinal section through the apical processes of the RPE cells. Regularly spaced actin filaments are evident in the processes and are seen most clearly at the arrow. Bar, 0.1 µm.  $\times 48,000$ .

a group of five bands with reduced molecular weights of 280,000, 250,000, 240,000, 235,000, and 200,000 in the relative proportions (as determined by dye binding) of 0.4:0.5:1.0:0.75:0.90 (Fig. 2). Major bands below this region were a doublet at 100K and very intense bands at 58K and 45K. The 58K band is vimentin (Fig. 3). Unless the cytoskeletons were made in the presence of the calcium protease inhibitor leupeptin, the vimentin band appeared as a doublet even if other protease inhibitors were included (data not shown). The 240K band is a subunit of brain spectrin, the 200K band is myosin, and both bands in the 100K doublet

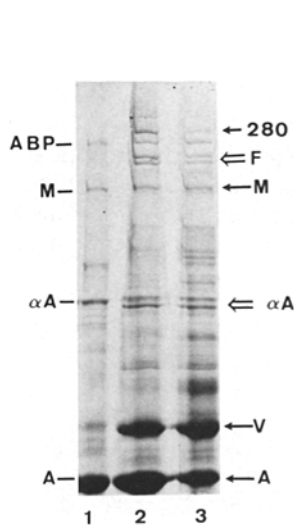


FIGURE 2 Triton-insoluble proteins from chick RPE cells electrophoresed on a 7.5% SDS polyacrylamide gel and stained with Coomassie Blue. Lane 2 is from the RPE of 17-d-old chick embryos and lane 3 is RPE from 10-d-old chick embryos. Cytoskeletal proteins from human platelets are shown in lane 1. A, actin;  $\alpha A$ , alpha-actinin; ABP, actin-binding protein; M, myosin; V, vimentin; and F, the 240/235 subunits of brain spectrin. The most obvious change that occurs as a function of development is an increase in the amount of actin associated with the cytoskeletons. Also observed are an increase in the 280K protein and a decrease in a second, broad band that migrates above vimentin.

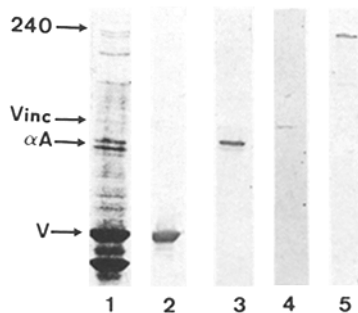


FIGURE 3 Immunoblot analysis of cytoskeletal components of RPE cells. Proteins run on 7.5% SDS polyacrylamide gels were electrophoretically transferred to nitrocellulose paper. The strips were reacted with antibodies to vimentin (V; lane 2), alpha-actinin ( $\alpha A$ ; lane 3), vinculin (Vinc; lane 4), and the 240K subunit of spectrin (lane 5). Lane 1 shows the Coomassie Blue-staining pattern of the RPE cytoskeletons used for the immunoblots.

reacted with the antibody to alpha-actinin. We observed this doublet under all conditions of preparation. Although a band at 130K was barely discernable, we obtained a reaction at this position by immunoblotting with an antibody to vinculin. The 250K band comigrated with actin binding protein from platelet cytoskeletons. The 235K polypeptide coprecipitated with the 240K subunit when *Staphylococcus aureus* bound antibody to the 240K subunit was used (data not shown). The RPE cells do not express the spectrinlike protein TW260, which is found in the terminal web region of the intestinal epithelial cells (10). This was shown by immunocytochemistry on both isolated cells and immunoblots of isolated cytoskeletons (manuscript in preparation). The identity of the 280K band is not known at this time although we have shown that this protein is phosphorylated in intact cells and in isolated cytoskeletons. As shown in Fig. 2, this band also increases in intensity with the age of the embryo.

To localize these proteins within the cells we used immunofluorescence. Identical results were obtained with frozen sections of tissue and with isolated cells.

Electron micrographs indicated the presence of microfilaments both in the region under the zonula adherens (11, 12) and in the apical processes (Fig. 1 c). Rhodalloidin (Fig. 4)

and antibodies to myosin (Fig. 4), alpha-actinin (Fig. 4), and the 240K subunit of spectrin (not shown) all gave a hexagonal pattern of staining near the lateral plasma membrane. Staining for vimentin was seen at the periphery of the cell but apparently not as close to the membrane as the staining for alpha-actinin (Fig. 4 d).

To see the components of the apical processes, we examined isolated cells that adhered laterally to the coverslip. With rhodalloidin, long strands of fiberlike extensions were observed running deep into the cell bodies from the apical processes (Fig. 5). More surprising was that myosin, spectrin, alpha-actinin, and vinculin were all localized in the apical processes as well as in the terminal web region of the cell (Figs. 4 and 5). Not all of the antibodies gave the same localization. With anti-alpha-actinin we found a distinct punctate staining in the region of the zonula adherens. This was most marked in the frozen sections but was maintained as a distinct spot in the isolated cells (Fig. 4 f). Vimentin, unlike the other proteins, was excluded from the apical processes but was present as a filamentous meshwork throughout the body of the cells (Fig. 4 h). In a few cases, in both the frozen sections and in the isolated cells an occasional wisp of vimentin staining appeared in the processes. Although staining with anti-fodrin extended to the tips of the apical processes, anti-myosin staining did not (compare Fig. 5, c with d and e with f). Antibodies to villin were negative by immu-

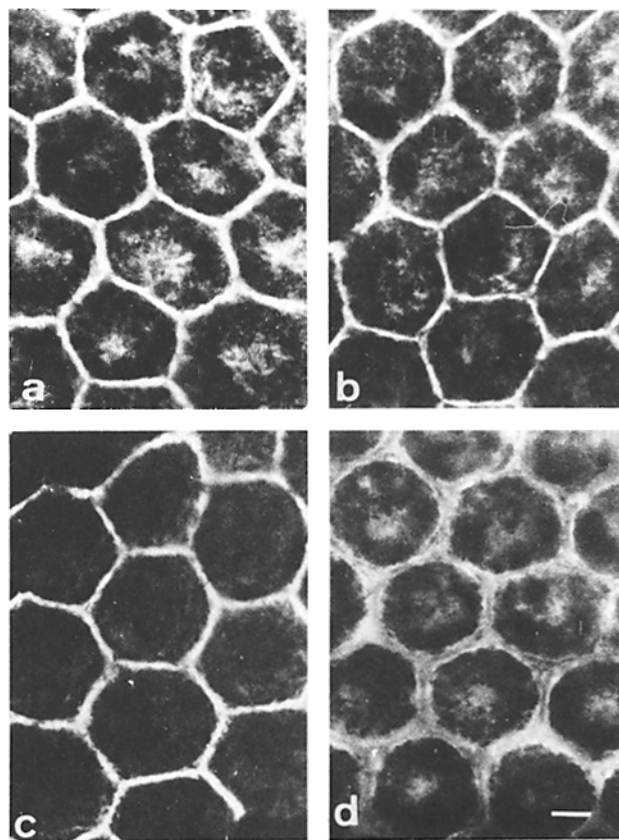


FIGURE 4 Indirect immunofluorescence on isolated sheets of RPE cells demonstrates that the circumferential bundles of filaments contain actin (a), myosin (b), alpha-actinin (c), and vimentin (d). The cells were stained with rhodalloidin for actin filaments. Controls using preimmune serum or secondary antibody alone here as elsewhere showed little or no fluorescence.  $\times 1,000$ .

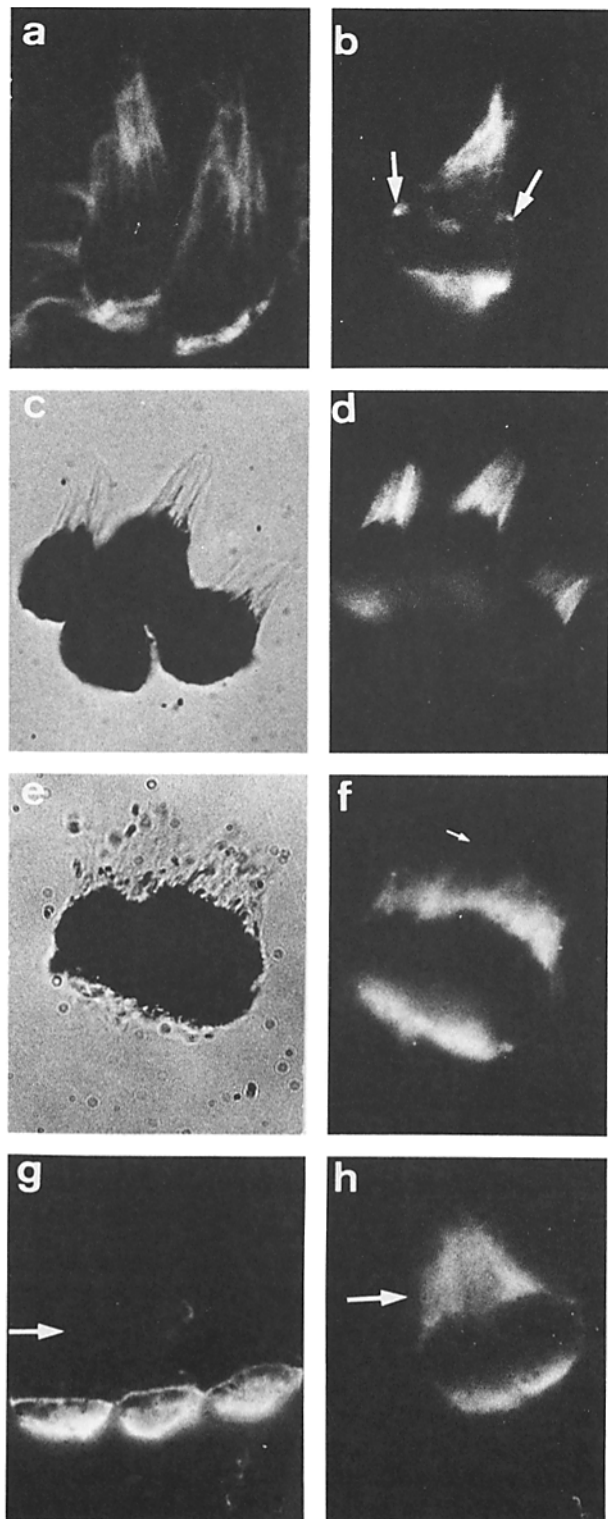


FIGURE 5 Localization of cytoskeletal proteins in isolated RPE cells with rhodalloidin as a stain for actin (a), and indirect immunofluorescence with antibodies to alpha-actinin (b), the 240K subunit of spectrin (d), myosin (f), vimentin (g), and vinculin (h). Brightfield pairs are shown for spectrin (c) and myosin (e). The punctate staining observed with antibody to alpha-actinin that is seen in cryosections (not shown) is retained in the isolated cells (b, arrows). Staining of the apical processes is seen with antibodies to alpha-actinin, the 240K subunit of spectrin, myosin, and vinculin. Myosin does not appear to extend all the way to the tip of the apical processes, as indicated by the small arrow (f). Staining with antibody to vimentin

nofluorescence although staining was observed in intestinal epithelial cells under the same conditions.

## DISCUSSION

The RPE contains two distinct groupings of microfilament bundles orthogonal to each other (references 11 and 12 and Figs. 1 and 4). One is the circumferential band of microfilaments at the level of the zonula adherens; the other is made up of the regularly arranged longitudinal filaments in the apical processes.

In this study we show by indirect immunofluorescence that actin, myosin, alpha-actinin, and vimentin are localized in the region of the zonula adherens. These same proteins have been shown to be components of this region in IECs (13-16) as well as of RPE cells grown in culture (17; and Philp, N. J., and V. T. Nachmias, unpublished observations). This suggests that the components of the zonula adherens may be relatively constant in all cuboidal or columnar epithelial cells.

This circumferential band of filaments associated with the zonula adherens has been shown to contract when ATP is added to glycerinated sheets of RPE and IECs (11, 12, 18, 19). Curved structures result, with the concavity at the apical surface due to contraction of the zonula adherens. We often observed marked indentations at the level of the zonula adherens in isolated RPE cells, which suggests contraction of this region (data not shown). This would be expected when cells are released from an epithelial sheet so that tension is no longer counteracted by adjacent cells. Continuous tension by the zonula adherens may act to keep the epithelial sheet taut and the cells in close apposition. Burgess has shown that contraction of the circumferential ring in intestinal epithelial cells causes splaying out of microvilli (19). He made the interesting suggestion that a cycle of such contractions interspersed with relaxation could cause gentle stirring of the luminal contents by the IECs. A similar effect exerted by the processes of the RPE cells on the interphotoreceptor matrix could facilitate the continuous exchange of metabolites that occurs between the RPE cells and the neural retina.

The apical processes constitute a second cytoskeletal domain in the RPE. This region of the RPE cells has been relatively unexplored since most of the work on RPE cells has been done on tissue culture cells that do not elaborate the long processes seen *in vivo* (17, 20-22). Unlike the short processes of the IEC, the apical processes of RPE cells *in vivo* extend into a space that is filled with the outer segments of the photoreceptor cells. Our studies show that the cytoskeletal components of the RPE cell processes differ from the ordered actin bundles present in the intestinal microvilli (1, 2, 13). The RPE processes contain myosin, vinculin, alpha-actinin, and fodrin. The localization is probably not artifactual since the immunofluorescent staining with fixed and frozen sections is identical to that with the isolated cells (see also reference 23). Vimentin was localized only to the cell bodies. This is consistent with the results of Takeuchi and Takeuchi, who observed the presence of intermediate filaments in the cell body of goldfish RPE cells but not in the processes (24). Two of the major components of the intestinal microvilli, villin

is confined to the cell body (g). Vimentin does not extend out into the apical processes indicated by the arrows (compare g and h).  $\times 1,000$ .

and the 110K protein, were not detected in RPE cells.

During development, the apical processes of the RPE cells increase in length at the time of elongation of the photoreceptor cell outer segments. We examined the cytoskeletal components of the RPE cells at embryonic day 10, before the outer segments appear, and embryonic day 17, after the outer segments elongate. The major change observed is that cytoskeletal actin increases during development whereas vimentin remains relatively unchanged (Fig. 3). The only other component of the cytoskeleton that clearly increases during development is the 280K band, an unidentified phosphorylated protein.

The presence of myosin in the apical processes suggests that they are capable of active contraction. The apical processes phagocytose the shed rod outer segments (5, 26). This probably requires the interaction of actin and myosin for the movements involved in surrounding and engulfing the particles. Normally, the neural retina adheres to the RPE. The presence of alpha-actinin and vinculin, which are both associated with regions of cell-substrate and cell-cell adhesions, may be related to this function. The adherence of the neural retina to the RPE as well as the alignment of the outer segments (3, 27) may be maintained by tension on the neural retina exerted by the apical processes. In primate retina, the polarity of the actin in the apical processes has been determined to be appropriate for the exertion of inwardly directed tension (27). We have observed (unpublished observation) that RPE myosin is phosphorylated *in vitro*; phosphorylation of light chains has been correlated with myosin activation and tension production (28).

In summary, the present study reinforces the idea that cytoskeletal components in the region of junctional complexes may be basically the same in all epithelial cell sheets. However, the apical processes of the RPE cells are constructed from quite different cytoskeletal elements that are the microvilli of the IECs. These differences correlate with differences in function. The apical processes of the RPE cells interdigitate with, and may exert tension on, the outer segments of the photoreceptor cells, have evolved from cells in which pigment migration occurred, and are involved in circadian phagocytic activity.

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