Increase in Actin Contents and Elongation of Apical Projections in Retinal Pigmented Epithelial Cells during Development of the Chicken Eye

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ABSTRACT The structural and biochemical changes of cytoskeletal components of retinal pigmented epithelial cells were studied during the development of chicken eyes. When the cytoskeletal components of the pigmented epithelial cells from various stages of development were examined by SDS PAGE, actin contents in the cells markedly increased between the 15-d-old and hatching stages. Immunofluorescence microscopy showed that chicken pigmented epithelial cells have two types of actin bundles. One is the circumferential bundle associated with the zonula adherens region as previously reported (Owaribe, K., and H. Masuda, 1982, *J. Cell Biol.*, 95:310–315). The other is the paracrystalline bundle forming the core of the apical projections.

The increase in actin contents after the 15-d-old stage is accompanied by the formation and elongation of core filaments of apical projections in the cells. During this period the apical projections extend into extracellular space among outer and inner segments of photoreceptor cells. Accompanying this change is an elongation of the paracrystalline bundles of actin filaments in the core of the projection. By electron microscopy, the bundles decorated with muscle heavy meromyosin showed unidirectional polarity, and had transverse striations with ~12-nm intervals, as determined by optical diffraction of electron micrographs. Since the shape of these bundles was not altered in the presence or absence of Ca^{2+}, they seemed not to have villin-like proteins. Unlike the circumferential bundles, the paracrystalline bundles did not contract when exposed to Mg-ATP. These observations indicate that the paracrystalline bundles are structurally and functionally different from the circumferential actin bundles.

Actin is a major component of the cytoskeletal and contractile elements in nonmuscle cells. It plays an essential role in cell motility and shape determination of the cell (7, 16, 20, 26, 31, 34). Depending on the physiological state of the cell, actin undergoes assembly and disassembly, and cellular actin levels are also regulated quantitatively in the cell. Actin content becomes a good marker for cell physiology and morphology in some specialized cells (15, 32, 35) and transformed cells (17, 38).

In the present studies, the changes in the cytoskeletal components of retinal pigmented epithelial cells were examined during the development of chicken eyes. Relative contents of a 42-kD protein of the cells from a 1-d-old chick were found to be much larger than those of an 11-d-old embryo when assayed by SDS gel electrophoresis. The aim of this study is to determine why there is an increase in a 42-kD protein in the cells and how the cells use this additional protein.

The increase in 42-kD protein is found to be closely related to the formation and elongation of apical projections that have paracrystalline core bundles in chicken epithelial cells.

MATERIALS AND METHODS

Preparation of Pigmented Epithelia: Retinal pigmented epithelia were prepared from chicks of various ages, ranging from 11-d-old embryos to 1-d-old chicks, and glycerinated as previously described (29, 30). Briefly, after removal of vitreous body and neural retina, the posterior halves of eyes were treated with a glycerol solution (50% glycerol, 0.1 M KCl, 5 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2) at 4°C for 24–48 h, and stored in the

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fresh glycerol solution at −20°C. The epithelia from embryos after the 17-d-old stage were glycerinated without removing neural retinas because adhesion between the pigmented epithelium and the neural retina in these materials was too tight. Treatment with the glycerol solution loosened most or all of the retina. Pigmented epithelia were then easily isolated after removal from the glycerinated specimen in 10 mM phosphate buffer (pH 7.2) containing 0.1 M KCl. Except where noted, all specimens were glycerinated. Any contaminating pieces of neural retinas were carefully removed.

Immunofluorescence Microscopy: Glycerinated pigmented epithelia were attached to polylysine-coated coverslips as described by Mazia et al. (23). The epithelia were fixed with 10% formalin in phosphate-buffered saline PBS for 15 min, and were washed with PBS and distilled water. The specimens were treated with acetone for 5 min at 0°C, air dried, and stained with actin antibody by indirect immunofluorescence as described previously (29). For staining with nitrobenzooxadiazol-Phallacidin (Molecular Probes, Inc., Junction City, OR), the epithelia were treated with a Triton solution (0.5% Triton X-100, 0.1 M KCl, 10 mM sodium phosphate buffer, pH 7.2) instead of fixation and acetone treatment, and were stained for 20 min at 25°C. Sometimes the epithelia were mounted on microscope coverslips to observe both sides of the specimen, because the cells are severely pigmented and only one side of the epithelium can be observed by epifluorescence microscopy.

Electron Microscopy: Glycerinated or intact posterior halves of eyes from 1-d-old chicks were fixed wtih 3% glutaraldehyde for 3 h to overnight at 4°C, postfixed with 1% OsO_4 for 1.5 h at 4°C, and block stained with 1% uranyl acetate for 1 h. Specimens were dehydrated in a series of ethanol solutions, then with *n*-butyl glycidyl ether (QY-1), embedded in Epon, and sectioned with a glass knife.

For negative staining, the cells treated with glycerol and Triton X-100 were homogenized by passing through syringe needles of 25 and 27 gauge 3–5 times each as described in a previous report (30). A drop of the homogenate was put on a grid covered with carbon-coated collodion film. The specimen was negatively stained with 1% uranyl acetate, and examined with a JEM 100C electron microscope at 80 kV.

Others Methods and Materials: SDS PAGE was performed according to the method of Laemmli (22) on gels of 10% polyacrylamide with 5% stacking gels. The gels were stained with Coomassie Blue and scanned with a densitometer equipped with an integrator at a wavelength of 565 nm.

Heavy meromysin (HMM)[1] was prepared by the method of Weeds and Pope (37).

Optical diffraction was carried out according to the method described by Klug and Berger (19).

RESULTS

Actin Content Increases during Development of Pigmented Epithelium

To examine changes in the cytoskeletal components of pigmented epithelial cells during eye development, the cells from 11- to 21-d-old embryos or newly hatched chicks were analyzed by SDS PAGE. Fig. 1 shows the electrophoretic patterns of cytoskeletal components of the pigmented epithelial cells from various stages of development. The gels were loaded to constant myosin. Molecular masses of the major components were estimated to be 200, 55, and 42 kD.

All the patterns were quite similar except that of the 42-kD protein. The content of this protein increased markedly in embryonic pigmented epithelial cells between 15-d-old and 21-d-old stages (Fig. 1, *b–g*). Electrophoretic pattern of the cells from 1-d-old chicks (Fig. 1 *h*) was similar to that from 1-mo-old chicks (Fig. 1, *j* and *k*). In addition, the cytoskeletal components seemed not to change with or without glycerination (Fig. 1, *j* and *l*). By co-electrophoresis with muscle proteins, 200- and 42-kD proteins corresponded to myosin heavy chain and actin, respectively. The 42-kD protein of cytoskeletal components in the cells from an 11-d-old embryo was primarily actin (30). Since there was no difference between cytoskeletal 42-kD proteins from an 11-d-old embryo and 1-d-old chick in two-dimensional gel electrophoretic patterns, one can tentatively conclude that the increased 42-kD protein was actin. This conclusion seems to be supported by immunofluorescence and electron microscopic observations (see below).

[1] *Abbreviations used in this paper:* HMM, heavy meromyosin.

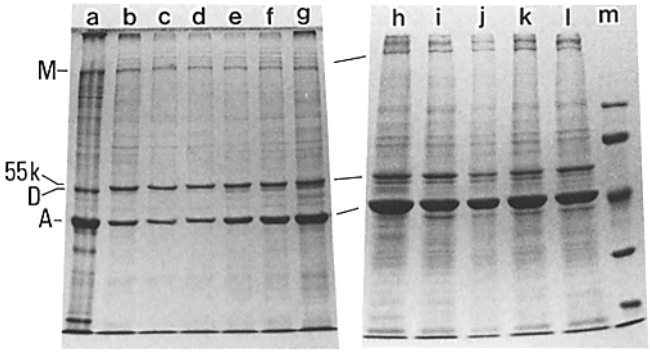


FIGURE 1 SDS gel electrophoretic patterns of pigmented epithelial cells at various stages of development. Cells were treated with glycerol and Triton X-100 except for those in lanes *a*, *i*, and *l*. (*a*) From chicken gizzard. From (*b*) 11, (*c*) 13, (*d*) 15, (*e*) 17, (*f*) 19, and (*g*) 21-d-old embryos. (*h*) From 1-d-old chicks. (*i*) From 1-d-old chicks treated with Triton X-100 without glycerination. The cells were prepared by incubating eye cups with Hank's solution for 45 min at 37°C. (*j* and *k*) 1-mo-old chicks. (*l*) Similar to i, but the incubation medium was PBS instead of Hank's soution. (*m*) Molecular weight markers: phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000). *M* (myosin heavy chain, 200,000), *D* (desmin, 52,000), and *A* (actin, 42,000).

Each gel pattern was traced by densitometry, and the amount of each component was measured. The relative amounts of actin to myosin heavy chain are plotted against age in Fig. 2 (*A/M*). The actin contents in the cells from 21-d-old embryos increased more than four times as compared with those from 11-d-old embryos. Plots of the relative amount of actin to total cytoskeletal protein instead of myosin gave the same result. However, the A/M value from 1-mo-old chicks was 2–3 times higher than that from 21-d-old embryos, although the relative amount of actin to total cytoskeletal protein was similar in both materials. It seems that the relative amount of myosin to total cytoskeletal protein becomes lower with the growth of the animal.

Actin Immunofluorescence

The pigmented epithelial cells from various stages of embryogenesis were stained with actin antibody. An apical view of the epithelial cell of an 11-d-old embryo showed clearly the circumferential actin bundle associated with the zonula adherens region as a bright polygon (Fig. 3 *a*). In basal view (Fig. 3 *b*), only a few, thin actin bundles were found in several cells in the epithelium.

Fluorescent images of the cells from various stages of chick embryos show the process of formation and elongation of the new actin bundles that are different from circumferential bundles (Fig. 4). At first many short actin bundles were found in the apical sides of the cells; then they grew and extruded into extracellular space during embryogenesis (Fig. 4, *a–e*). The new actin bundles in each cell tended to gather with their elongation and seemed to become rigid. In the early stages of elongation of the actin bundles, the circumferential bundles were well recognized in the cell peripheries. However, concomitant with their elongation of the new bundles, they

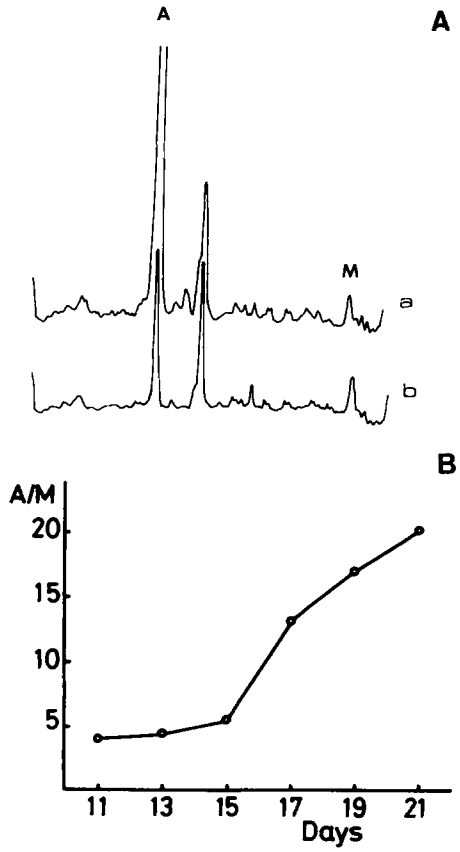


FIGURE 2 (*A*) Examples of densitometric tracings of Fig. 1, (a) *g* pattern and (*b*) *b* patterns. In addition to actin, the amounts of several components including the 55-kD protein increased in *a* as compared with *b*. (*B*) Increase in cytoskeletal actin contents of pigmented epithelial cells during development. The ordinate shows relative values of actin to myosin heavy chain. The abscissa shows incubation time of the eggs. The actin content increased more than four times between 15-d-old and hatching stages. The experiment was repeated three times with similar results.

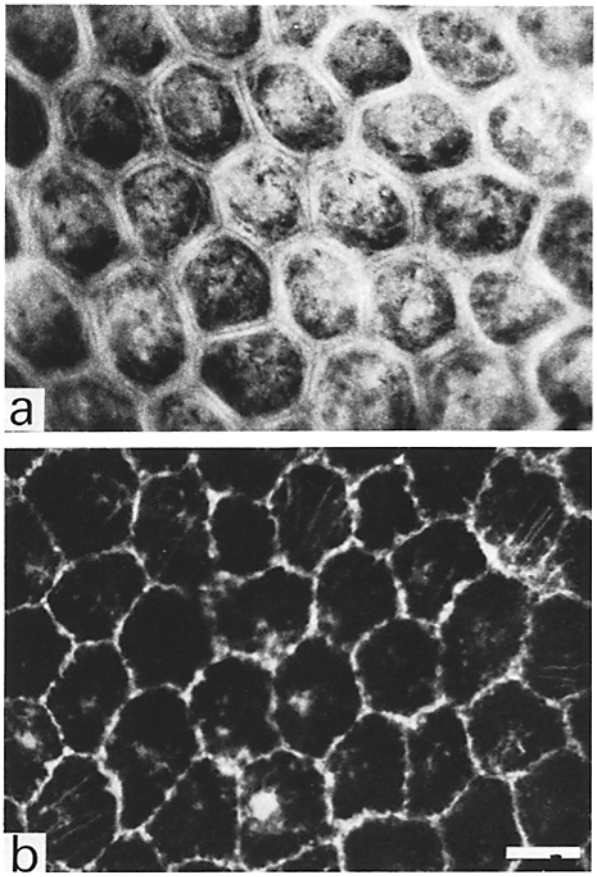


FIGURE 3 Actin immunofluorescence of pigmented epithelial cells from an 11-d-old embryo. (*a*) Apical view. (*b*) Basal view. Bar, 10 μm. × 980.

became hard to visualize and disappeared from the apical view. The circumferential bundles seemed near the basal side (Fig. 4*f*; see also Figs. 5.18 and 5.19 of reference 21). The elongation extent of actin bundles was closely correlated to the increase in cytoskeletal actin contents observed in the gel electrophoretic pattern.

Ultrastructure of Isolated Actin Bundles

The pigmented epithelial cells treated with glycerol and Triton X-100 from 1-d-old chicks were homogenized by passing through syringe needles. When the epithelia were gently homogenized, some single cells were isolated (Fig. 5*a*). Bundles, 20–30 μm in length, were clearly observed. By appropriate shearing, two types of microfilament bundles were found in the homogenate with an electron microscope. One is the circumferential microfilament bundle described previously (29, 30). The other, which is more abundant, is a more densely packed paracrystalline bundle of microfilaments. The length of the isolated paracrystalline bundles was usually 20–30 μm, but the length depended on the shearing force of homogenization. By gentle shearing, complexes of the bundles were often isolated.

Fig. 5*b* shows an example of a single bundle in which the characteristic transverse striations are easily seen. In a homogenate of the cells from a 15-d-old embryo, thin short bundles were observed but their paracrystalline structures were not so clear. The appearance of the bundles is not altered in the presence or absence of Ca^{2+}, Mg-ATP, or 0.2 M NaCl.

Paracrystalline bundles were decorated with HMM, and negatively stained. The bundles tended to fray into single filaments when decorated with HMM. From the frayed ends, the polarities of the actin filaments could be observed. In every decorated bundle, all actin filaments had the same polarity. Fig. 5*c* shows one example of such a bundle. From the observation of a whole cell decorated with HMM, it was found that the arrowhead pointed toward cell body.

Paracrystalline bundles of microfilaments were not found in the homogenate of pigmented epithelial cells from rabbits and guinea pigs. In these two cases, only fragments of the circumferential bundles were observed (data not shown).

Optical Diffraction

Fig. 6 shows an optical diffraction pattern of a bundle similar to that in Fig. 5*b*. The first layer-line which occurs at ~1/36 nm^{-1} and sixth layer-line which occurs at ~1/5.9 nm^{-1}, are usually present in the diffraction patterns of actin bundles. Besides these layer-lines, a meridional reflection was observed at ~1/12 nm^{-1}. The meridional reflection suggests the presence of another component(s) which is probably a cross-linking protein(s). Optical diffraction pattern of this image showed ratios of Z coordinate of layer-lines of 1:2.92:6.05. This result indicates that an actin filament in the bundle has

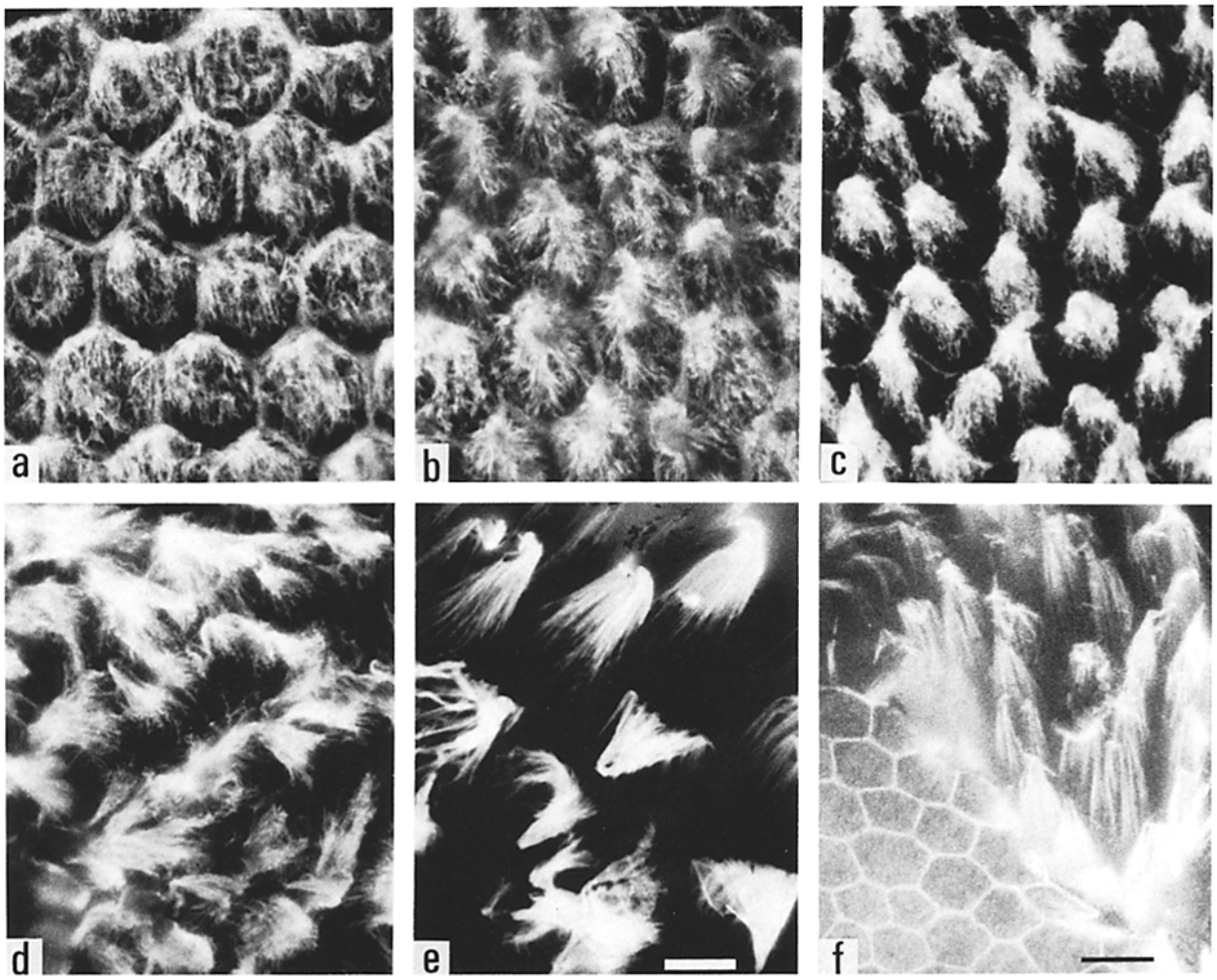


FIGURE 4 An example of the elongation process of the actin bundles in pigmented epithelial cells. Cells were treated with glycerol and stained with actin antibody (*a–e*) or NBD-Phallacidin (*f*). Epithelium from a (*a*) 15, (*b* and *c*) 17, and (*d*) 19-d-old embryo. (*e* and *f*) Epithelium from a 1-d-old chick. *f* shows both sides of epithelium in the same field because a part of the epithelium is folded and turned over. The circumferential actin bundles that disappeared in the apical view are found on the basal side, but they are not found in the basal side of every epithelium. (*a–e*) Bar, 10 μm. × 980. (*f*) Bar, 20 μm. × 532.

13 subunits in 6 turns of the genetic left-handed helix, and the interval of the transverse striations is ~1/3 of the half-pitch of the actin double helix. From the row-lines in the diffraction pattern, the spacing between actin filaments in the paracrystalline bundle was found to be ~8.8 nm.

Ultrastructures of Intact and Glycerinated Pigmented Epithelial Cells

Thin section electron microscopy showed that intact pigmented epithelial cells of a 1-d-old chick have many long apical projections >30 μm in length and 0.2 μm in diameter (Fig. 7). The apical projections extend into the extracellular space between outer and inner segments of photoreceptor cells, and may extend as far as the outer limiting membrane. Pigmented granules often found in the projections are closely associated with microfilaments.

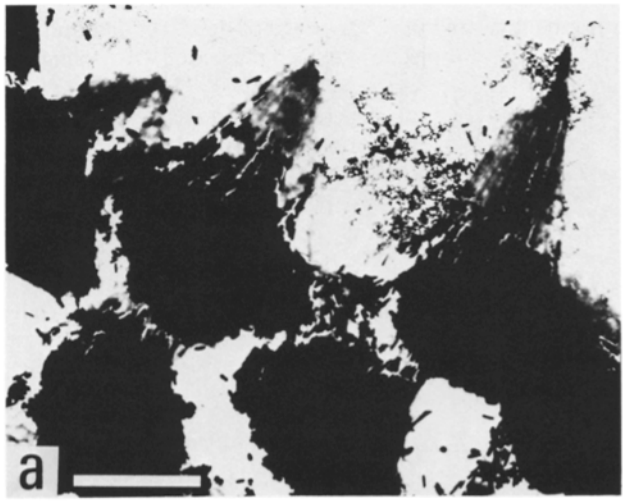
In the case of glycerinated cells from a 1-d-old chick, the apical projections in most of the cells fused to produce a cone-like protrusion containing many microfilament bundles (see Fig. 4*e* and 5*a*). The apical projections tend to fuse to one another as the pigmented epithelial cell is separated from the neural retina even when the cells are not glycerinated.

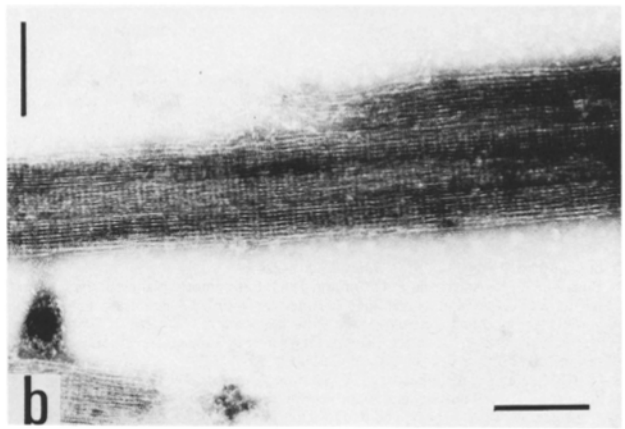
DISCUSSION

The actin content may be regulated according to the physiological needs of the particular cell type. Although the actin content in nonmuscle cells is considerably less than that in muscle or myoepithelial cells, actin still comprises 5–10% of the total protein (7, 17).

In chicken pigmented epithelial cells, the amount of cytoskeletal actin increased markedly between near the 15th day of incubation and the hatching stage of development. During actin accumulation in the cells, paracrystalline bundles of microfilaments were formed, and protruded to the apical side. A rough estimation from the densitometries of the SDS PAGE patterns revealed that the actin accounted for >60% of the cytoskeletal protein in the epithelial cells from a newly hatched chick. There is a possibility that glycerination induced polymerization of actin (1). However, by electrophoresis, actin content and other cytoskeletal components of the cells treated with Triton X-100 after glycerination showed almost the same pattern as that of the cells treated with Triton X-100 without glycerination. Presumably, glycerination had little effect on cytoskeletal composition in this case.

Since the myosin content did not increase significantly, the





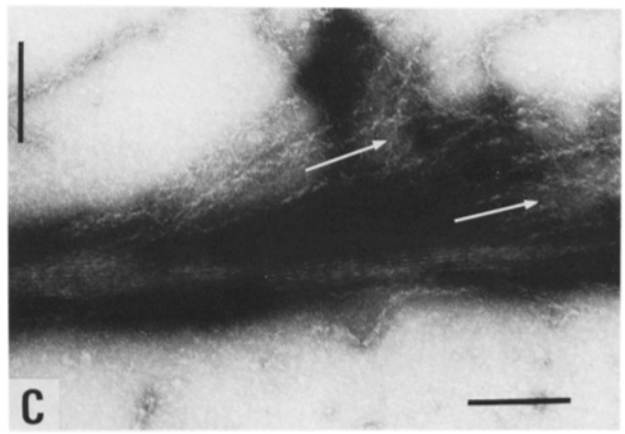


FIGURE 5 Electron micrographs of negatively stained actin bundles from apical projections. (*a*) Overall views of isolated pigmented epithelial cells. Long apical projections are clearly observed. The projections of each cell are probably fused during glycerination. Bar, 10 μm. × 1,680. (*b*) Isolated bundles of microfilaments by homogenization. Bar, 0.2 μm. × 63,200. (*c*) Bundle decorated with HMM. Arrows show the polarity of the arrowheads. Bar, 0.2 μm. × 66,750.

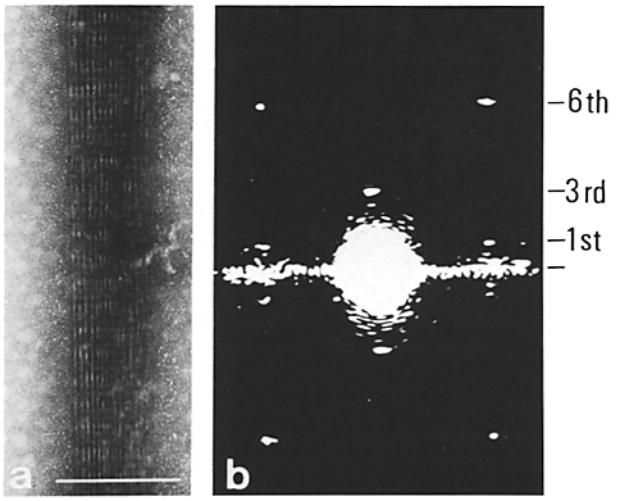


FIGURE 6 (*a*) Electron micrograph of a negatively stained actin bundle from pigmented epithelial cells of a 1-d-old chick. Bar, 0.2 μm. × 83,250. (*b*) Optical diffraction pattern of the bundles in *a*.

paracrystalline bundles would appear to have no myosin. However, further studies are needed because myosin has been detected in apical processes of rat and frog pigmented epithelial cells by immunofluorescence microscopy (12).

The optical diffraction pattern from the bundle described here suggests that it is composed of hexagonally packed actin filaments (10). The transverse striations and filament spacing of the bundle are closely similar to that induced by fascin, a 58-kD actin cross-linking protein (9, 10, 27, 33). Bryan and Kane (4) isolated the 58-kD protein and showed that this protein binds to actin filaments by maximum weight ratio to actin of 1:4.5 and induces paracrystalline actin bundles with a distinct 12-nm banding pattern.

The paracrystalline bundles of microfilaments from pigmented epithelial cells showed no distinct band corresponding to ~58 kD on SDS PAGE and were stable in a buffer solution that contained 0.25 M NaCl (13). Since the bundles were not affected by Ca^{2+}, they probably have no villin-like protein which is a major component of microvilli in intestinal epithelial cells and which can sever or bundle actin filaments in a Ca^{2+}-dependent manner (3, 24). On the other hand, several components showed a significant increase accompanied by increase in actin content. Especially one of the 50–55-kD components could be a protein relevant to the cross-linker. However, since the glycerol-Triton cytoskeleton contains many intermediate filaments (30), one of these components at least seems to be a subunit of the filaments (11). At present the protein cannot be identified because the paracrystalline bundles cannot as yet be isolated from circumferential bundles.

The pigmented epithelial cells from rabbits and guinea pigs, which have finger-like processes in the apical side, did not have a large amount of actin contents, nor did they show paracrystalline actin bundles. It seems that the cells of mammalian eyes have apical projections that contain no core filaments (6). In apical projections of frog pigmented epithelial cells, Murray and Dubin (25) have shown the presence of bundles of actin filaments by HMM decoration. The bundle from frog cells has not been fully characterized to date.

Pigmented epithelial cells of chicks have two types of microfilament bundles. One is the circumferential bundle, which has random polarity and loose packing. These bundles associate with the zonula adherens region in the cells. The bundles of this type contain actin, myosin, and other actin-associated proteins (2, 36), and have contractility (29, 30). These bundles are necessary to maintain the epithelial structure in general (8, 14, 18, 28). The other type is paracrystalline bundle, which has unidirectional polarity as described here. The bundles of

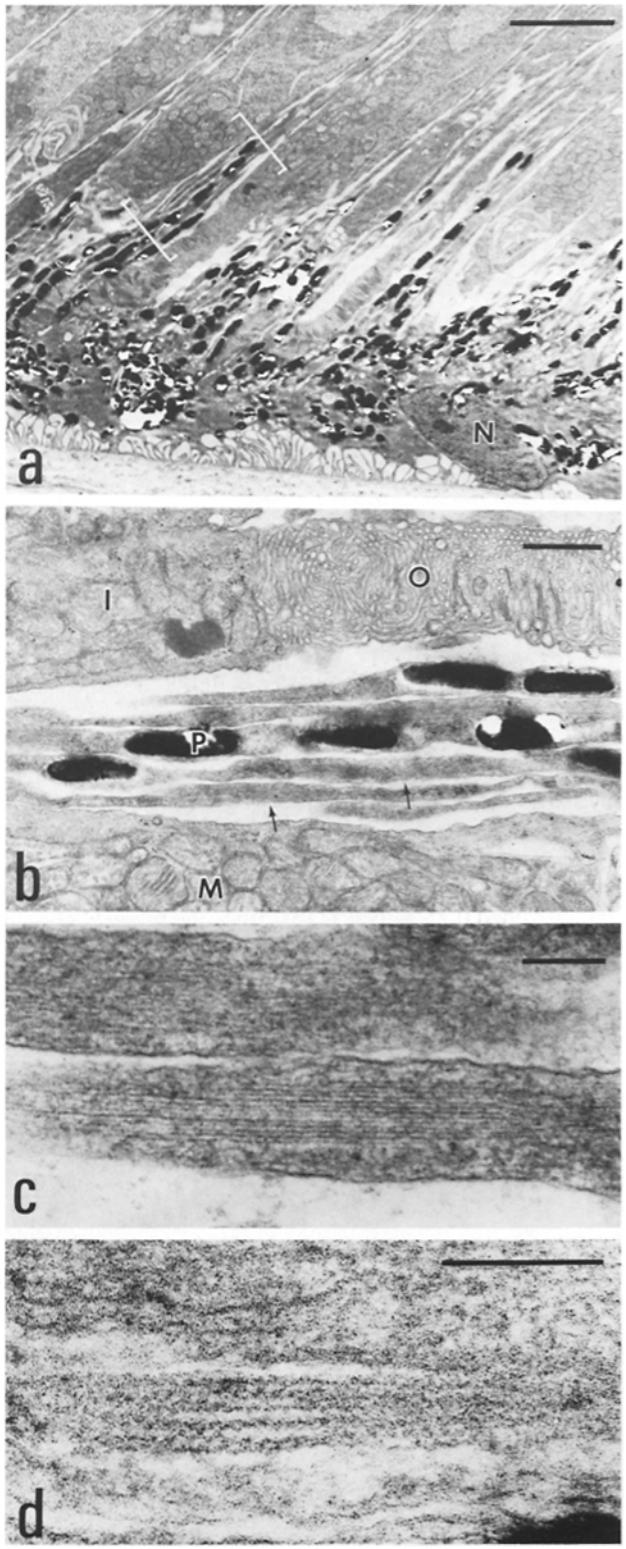


FIGURE 7 Electron micrographs of the intact pigmented epithelial cells from a 1-d-old chick. (a) Low magnification photograph showing long apical projections extending along outer and inner segments of photoreceptor cells. *N*, nucleus. Bar, 5 μm. × 2,730. (*b*) Enlargement of the bracketed part in *a*. *I*, inner segment; *O*, outer segment; *P*, pigmented granule; *M*, mitochondria in inner segment. Arrows show apical projections. Bar, 1 μm. × 10,500. (*c*) High-magnification micrograph of apical projections, in which the bundle of microfilaments is clearly seen. Bar, 0.2 μm. × 53,200. (*d*) Characteristic 36-nm repeat of actin filaments can be seen in some regions. Bar, 0.2 μm. × 106,860.

this type appeared in a later stage of development, and were found in apical projections, which may contribute to pigment migration (5, 25).

Further studies on structural components of the apical projections are needed to characterize their functions. In addition, the chicken pigmented epithelial cell provides a good system to study how paracrystalline actin bundles are formed and elongated in the cell.

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