Actin filaments in apical projections of the primate pigmented epithelial cell. 

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A highly-ordered array of filaments is found within the apical processes of retinal pigmented epithelial cells in monkeys and humans. These filaments, approximately 100 A in diameter and 250 A apart, line the cytoplasmic face of the plasma membrane, in parallel with the long axis of the apical processes.

Since these filaments bind rabbit myosin subfragment-1 to form arrowhead complexes, we conclude that they contain actin. Such membrane-bound actin filaments could have any of several different functions: they could stabilize the apical projections and by so doing play a cytoskeletal role, and/or they could take part in the phagocytosis of shed outer segment discs.

Recently, it has become clear that virtually all eukaryotic cells contain actin filaments within their cytoplasm. This view is based not only on biochemical identification of actin extracted from numerous types of cells but also on a powerful technique which allows us to specifically identify actin filaments in situ. This procedure depends upon the specific binding of myosin molecules to actin filaments. When actin-binding parts of myosin molecules are incubated with actin filaments, they attach to form a characteristic arrowhead configuration which is visible with the electron microscope. These arrowhead complexes are considered specific indicators for actin. Arrowhead complexes may be formed with isolated actin filaments, or in situ in cells which have been glycerinated to make their membranes permeable to the large myosin fragment. Fragments of myosin, either subfragment-1 or heavy meromyosin, are necessary since the intact myosin molecules are insoluble at physiological ionic strengths and would thus not penetrate the cells.

In the eye this technique has already been applied to the study of melanosome motility in the pigment epithelial cells of amphibia and of fish. In mammals, melanosomes are, as well as anyone knows, stationary. However, there remain the motility problems of ingestion and translocation of phagosomes in the retinal pigment epithelial cell. To this end, we have studied the distribution of actin filaments in the pigment epithelial cells of the squirrel monkey, Saimiri sciureus using myosin subfragment-1 binding to identify actin.

Methods and materials.

Subjects. Under deep barbiturate anesthesia (35 mg. Nembutal per kilogram intravenously) the eyes of an adult squirrel monkey (S. sciureus) were enucleated, rapidly dissected in cold standard salt solution (SSS) and then immersed in an appropriate solution as described below. A human retina, from surgical enucleation for orbital tumor, was prepared in the same manner.

Electron microscopy. Retinas were immersion-fixed in 3 per cent glutaraldehyde (Fisher, reagent grade) in SSS consisting of 0.1 M KCl, 5 mM MgCl2, 6 mM Na-phosphate buffer at pH 7.0, to which 1 M dithiothreitol (DTT) had been added. After fixation for two to three hours at room temperature, retinas were washed in SSS,
Fig. 1. Paraxial filaments in apical projections of monkey pigmented epithelial cells decorate with rabbit myosin subfragment-1 to form arrowhead complexes typical of actin. A, filaments in apical projection which most clearly illustrate arrowhead configuration (arrows). Filaments extend into apical cell body at proximal ends of projections. (×86,000.) B, distribution of decorated filaments in apical projections. En face views of membranes in this fairly thick section show that filaments are predominantly paraxially aligned near the plasma membrane of the projections (arrows). In some cases, however, especially in projections which have ballooned in the glycerination process, the filaments are detached from membranes (double arrow). pg—pigment granule. (×86,000.)
Fig. 2. Membrane-associated filaments in apical projections of monkey retinas fixed in glutaraldehyde in SSS. A, en face view of a leaf-like projection surrounding a rod outer segment (ROS) tip. Note the rows of filaments visible in the plane of the membranes (arrows). (×46,000.) B, another en face view of filament rows (arrows). (×46,000.) C, section cut perpendicular to the long axis of the apical projections reveals membrane-associated filaments cut in cross-section (arrows). Rows of filaments in a leaf-like projection which ensheaths a rod outer segment (ROS) are indicated with double arrows. pg—pigment granule, srs—subretinal space. (×53,000.) D, higher magnification of membrane-associated filaments in cross-section (arrows). Note that the filaments follow the membrane contours whether sandwiched between two membranes (in leaf-like projections) or when associated with only one membrane. Such views suggest that the filaments are in fact attached to the plasma membrane. Note also that the spacing between filaments resembles that between decorated filaments in Fig. 1. B. (×86,000.) E, longitudinal section of a filament in a leaf-like projection (arrows). This fortuitous section captures a small segment of a single filament sandwiched between the two apposed membranes of a leaf-like projection. Note the regular membrane-to-filament distance. (×76,000.)

then in 0.1 M Na-cacodylate buffer, pH 7.5, postfixed for two hours in cold 1 per cent osmium tetroxide in 0.1 M Na-cacodylate buffer, pH 7.5, dehydrated in graded ethanols, and embedded in epon. Blocks were cut on a Porter-Blum Mt-2 ultramicrotome; grey to silver sections were collected on naked copper grids, stained with uranyl acetate (one part aqueous saturated uranyl acetate; one part methanol; one part 70 per cent ethanol) and lead citrate, and examined on a JEOL 100B electron microscope.

Myosin subfragment-1 binding. Rhesus monkey retinas were immersed directly in cold 50 per cent glycerol: 50 per cent SSS with DTT and maintained on ice for 5 or 24 hours, transferred to 25 per cent glycerol in SSS with DTT for five hours, then to 5 per cent glycerol for 1.5 hours. Retinas were then washed three times with SSS plus DTT, placed in subfragment-1 (S-1) solution (5.3 mg. per milliliter in SSS plus DTT), or in SSS plus DTT alone, and left for 24 hours in the cold. The retinas were then washed once with SSS plus DTT and fixed as described above.

Identical procedures were also performed on human retina obtained from a surgical enucleation, except that an additional control retinal fragment was exposed to S-1 and 5 mM adenosine triphosphate (ATP) for 24 hours.

Myosin subfragment-1, prepared by the method of Lowey and co-workers (kindly supplied by L. G. Tilney) was used in all histochemical procedures.

Measurements. Pigment cell processes containing filaments were photographed in cross section at 30,000 × and printed at a total magnification of 390,000 ×. Diameters of 30 filaments were measured, and their actual diameter calculated by comparison to a calibrated diffraction grating photographed and printed in the same fashion.
Filament to filament and membrane to filament distances for 50 filaments were also determined by the same procedure.

**Results.** After reaction with myosin subfragment-1, numerous decorated filaments are clearly visible in the apical area of the pigmented epithelial cells (Fig. 1). Decorated filaments are largely confined to the cell's villous and leaf-like apical projections, many of which cup the distal ends of the photoreceptor outer segments. Within the projections the decorated filaments have a noticeable degree of order. In some regions they appear to lie in rows parallel to the long axis of the projection (Fig. 1), in spite of the disruption of morphology produced by the glycerination process. When incubated with S-1 in the presence of 5 mM ATP no decoration is observed, thus the binding appears to be specific for actin.

When sections of untreated eyes (fixed directly in glutaraldehyde and SSS) are examined closely for comparable parallel rows of filaments, we find an almost paracrystalline array of filaments lining the cytoplasmic faces of the plasma membranes of the apical projections (Fig. 2). These filaments are approximately 100 Å in diameter, and lie approximately 180 Å from the plasma membrane in rows approximately 235 Å apart (Fig. 2). Cross, longitudinal, and tangential views of the filament arrays are illustrated in Figs. 2 and 3.

Decorated filaments in parallel rows were also observed in the apical projections of human pigmented epithelial cells in specimens obtained at surgery (usually for malignant melanoma).

**Discussion.** Our observations indicate that the apical projections of the pigmented epithelial cells contain numerous actin filaments which are attached to the plasma membranes of the apical projections in parallel axial rows. The orderly arrangement of the filaments in these projections is illustrated schematically in Fig. 3.

The function of the membrane-associated actin filaments in pigment cells is not clear. In frog
Fig. 3. Schematic drawing of monkey pigmented epithelial cell apical projections illustrating (A) their relationship to the pigment cell body and the rod outer segments, (B) the orientation of membrane-associated filaments in the three morphological types of projections, and (C) the association of the three types of projections with the rod outer segment tip. Together leaf-like projections (1), villous-like projections (2), and pigment granule-containing projections (3) ensheath the distal four microns of the rod outer segment. Rows of 100 Å diameter filaments line the cytoplasmic faces of the plasma membranes of all three types of apical projections.

Retina. Murray and Dubin have proposed that axially oriented, actin-containing filaments play a role in the movement of pigment granules. It has long been known that in frogs and in several other poikilotherms, pigment granules migrate in response to light, acting as a variable and partial shield to the rod outer segments. In the monkey, however (as far as is known), the pigment granules are fixed; thus the actin filaments must do something else.

One alternative is that the filaments are membrane skeletons. Since the actin filaments are indeed membrane bound, they are ideally placed to play a cytoskeletal role, a role both in the development and in the maintenance of the pigment cell apical projections. Since most pigment cell processes lack microtubules but nevertheless remain extended into the extracellular space (without any obvious attachment), and since many processes contain no visible structural elements other than the membrane-bound actin filaments, we suggest that the filaments provide the support necessary to maintain the elongate shape of the processes.

In turn, the processes can then support and protect the frail distal portions of the photoreceptor outer segments. Structural stabilization of the processes by the membrane-bound filaments coupled with adhesion between processes and outer segments may serve to maintain attachment between pigmented epithelium and neural retina. Thus actin filaments may be important in preventing retinal detachment.

Last, that the membrane-bound filaments might take part in phagocytosis of effete photoreceptor outer segments is an interesting possibility. Young has proposed that the tips of growing rod outer segments pinch off autonomously, the shed discs being then enveloped by the pigmented epithelial cell processes. Subsequently, the surrounded fragment is withdrawn into the pigment cell body. The direction of movement of the ingested fragment (phagosome) is roughly parallel to the orientation of the actin filaments in the...
In recognition of this possibility, we are currently studying a series of animals affected with hereditary retinal degeneration to see if in any of these a derangement of actin filaments within pigment epithelial cells can be discovered.

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REFERENCES


Sulfated glycolipids in ciliary body epithelium. J. Peter Bentley, Lynette Feeney, Albert N. Hanson, and Robert N. Mixon.

The sulfated material which locates in the basal membrane of ciliary body epithelium when immature rat eyes are incubated with Na$^{35}$SO$_4$ was studied. The basis of its chromatographic behavior compared with standard preparations, we conclude that the material consists of sulfatides.

Feeney and Mixon reported$^1$ that when immature rat eyes were incubated in a medium containing radioactively labeled sulfate, there was a rapid uptake of the label into a material which located at the basal surface of the unpigmented epithelium of the ciliary body. Initially, the label was localized in the apical cytoplasm but with longer periods of incubation in nonradioactive medium it moved to the basal surface of the cell. Electron microscope autoradiography showed that it was associated with the infolded plasma membrane of the unpigmented epithelial cells and that it appeared to remain in this site with even longer chase periods. In this paper, we report on the nature of this sulfated material.

Methods. Ciliary body rings were dissected from the eyes of 10- to 12-day-old rats. Sixteen eyes yielded less than 80 mg. of fresh tissue. The tissue was incubated as previously described$^1$ in Eagle’s minimum essential medium to which were added 800 $\mu$Ci of Na$^{35}$SO$_4$ per milliliter (700 to 900 mc. per mmole).

Following incubation for a “chase” period in nonradioactive medium as previously described,$^1$ the tissue specimens were blotted dry and placed in a dialysis sac with 0.5 ml. of water. This was dialyzed against frequent changes of distilled water at 4°C in order to remove free sulfate. Inhibitors of endogenous degradative enzymes were added to the dialysis medium (N-ethylmaleimide 1 mM., EDTA 10 mM., di-isopropyl fluorophosphate 1 mM.). After two changes of water, the dialysis sac was placed in two successive solutions of 0.1M sodium sulfate for a total of 24 hours, then returned to water. The release of radioactivity was monitored and after no further radioactivity was released into the dialysate (six changes), the tissue was removed and homogenized in a glass/glass homogenizer in chloroform methanol (2:1). The homogenate was added to the dialysis medium (N-ethylmaleimide 1 mM., EDTA 10 mM., di-isopropyl fluorophosphate 1 mM.). After two changes of water, the dialysis sac was placed in two successive solutions of 0.1M sodium sulfate for a total of 24 hours, then returned to water. The release of radioactivity was monitored and after no further radioactivity was released into the dialysate (six changes), the tissue was removed and homogenized in a glass/glass homogenizer in chloroform methanol (2:1). The homogenate was extracted with chloroform methanol (2:1) for three 24 hour periods and the extracts were pooled. The tissue residue following this extraction procedure was digested with pronase at pH 7.6 for 24 hours at 50°C. A preliminary experiment showed that no further radioactivity could be extracted with chloroform methanol from the pronase digestion mixture.

The chloroform methanol extract was concentrated under reduced pressure to a small volume and subjected to column chromatography on silicic acid and subsequently on DEAE-cellulose.$^2$ Commercially obtained sulfatide (bovine brain, P. L. Biochemicals, Inc.) was added as a carrier. The specific conditions for chromatography are shown in the legends to Figs. 1 and 2. Fractions were collected and 0.2 ml. aliquots transferred di-