Actin Filament Polarity at the Site of Rod Outer Segment Disk Morphogenesis

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The rod outer segment (ROS) is attached to the visual cell body by a connecting cilium. Axonemal components of this cilium extend into the ROS, an organelle which undergoes continuous renewal throughout life. New membranous disks are added at the ROS base, while older ones are shed from the tip. The formation of new disks is believed to result from plasma membrane evaginations at the distal end of the connecting cilium, but the mechanism responsible for disk morphogenesis is not yet understood. Within the ciliary axoneme, at the base of the ROS, an actin-rich domain has been localized with immunoelectron microscopy, and filamentous actin has been detected with fluorescent phalloidin. However, actin filaments have not previously been observed in electron micrographs of this region. We now report that a meshwork of decorated actin filaments was observed within the center of the ciliary axoneme, at the base of the ROS, after visual cells were permeabilized with saponin and incubated with myosin subfragment-1 (S-1). Furthermore, individual filaments were seen to extend from the center of the axoneme into the base of the ROS disk stack by passing between pairs of ciliary microtubule doublets. Arrowheads on these filaments uniformly pointed toward the cilium, while the barbed (or fast-growing) ends were oriented in the direction of disk expansion and were often associated with the ROS plasma membrane. In control retinas, undecorated filaments were observed. Thus, S-1 binding did not induce filament formation. These results suggest that an actin filament network may provide cytoskeletal support and guidance for the growing ROS disks. Invest Ophthalmol Vis Sci 30:2461–2469, 1989

The rod outer segment (ROS) is a light-sensitive organelle composed of a plasma-membrane-enclosed stack of disks (Fig. 1). It is attached to the visual cell body by a connecting cilium that is analogous in structure to the transitional zone of a motile cilium.1,2 Axonemal components of the connecting cilium extend into the ROS and can be observed to run along the side of the ROS disk stack. Continuous membrane renewal allows the ROS to maintain a relatively constant length throughout life. This is made possible by an equilibrium between disk morphogenesis at the ROS base3,4 and disk shedding from the tip.5 These two processes have been shown to follow a diurnal rhythm;6–8 however, the mechanisms responsible for them remain unclear.

Immunoelectron microscopy has been used to identify an actin-rich domain in the distal end of the connecting cilium9 and in the ciliary axoneme at the base of the outer segment.9–11 Since this region is also believed to be the site of new disk formation from ciliary plasma membrane evaginations,4 it has been suggested that an actin-mediated contractile mechanism or cytoskeletal network may regulate some aspect of ROS disk morphogenesis.9,11 Recently, a rhodamine-phalloidin binding study has demonstrated that at least a portion of this ciliary actin is in the filamentous form.12 However, actin filaments have not previously been observed in electron micrographs of this region.

In the present study, isolated neural retinas from the pinfish Lagodon rhomboides and from the Fischer rat were permeabilized with saponin and incubated with myosin subfragment-1 (S-1). Since the specific binding of myosin fragments to actin filaments forms a characteristic arrowhead complex that is visible at the electron microscope level,13,14 this technique allowed us to demonstrate the distribution and polarity of actin filaments at the site of ROS disk morphogenesis.
ROD OUTER SEGMENT

Actin-Rich
Domain

Developing Disk

Microtubules

Connecting Cilium

ROD INNER SEGMENT

Fig. 1. Schematic of the rod connecting cilium and adjoining portions of the rod inner and outer segments. Rod outer segment disks are believed to develop from plasma membrane evaginations at the distal end of the connecting cilium. These lamellae expand in size to the full diameter of the organelle and then separate from the plasma membrane to form isolated disks. An actin-rich domain, which contains filamentous actin, has been localized to the ciliary axoneme at the base of the rod outer segment.

Materials and Methods

Animals

Pinfish (*Lagodon rhomboides*) were maintained in 75-gal sea-water tanks in a constant-temperature (22°C) room with a 12-hr light: 12 hr dark cycle. Adult Fischer rats were selected from a colony maintained on a 12-hr light-12-hr dark cycle at room temperature. All animals were sacrificed during the morning hours. Pinfish were sacrificed by spinal section and brain pithing. Rats were sacrificed with chloroform. Eyes were then enucleated in the light and the neural retinas dissected out. This investigation adhered to the ARVO Resolution on the Use of Animals in Research.

Myosin S-1

For the study of pinfish retinas, S-1 prepared from rabbit skeletal muscle was obtained from Dr. Roger Cooke (University of California, San Francisco, CA). It was used as a 10.75 mg/ml solution in 10 mM TES (N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid), 150 mM KCl, and 0.2 mM dithiothreitol (DTT). A 100-μl aliquot was added to the tissue for each incubation, as described below.

During our study of rat retinas, the S-1 preparation described above was no longer available. In its place, we used S-1 obtained from Sigma Chemical Co. (St. Louis, MO). This S-1 was supplied as a 4.1 mg/ml solution in 25 mM potassium phosphate, pH 6.2, 500 mM KCl, and 50% glycerol. It was diluted 1:1 with the appropriate standard salt solution (SSS) and used in 250-μl aliquots for the tissue incubations.

Myosin S-1 Binding and Tissue Processing

Neural retinas from the pinfish were cut into pieces and permeabilized in 0.05% saponin (Sigma) in SSS (10 mM sodium phosphate, pH 7.0, 150 mM KCl, 15 mM MgCl₂, and 0.2 mM DTT) for 4 min on ice. After two rinses of 2 min each in cold SSS and one rinse for 2 min at room temperature, the tissue was incubated in the S-1 solution for 30 min at room temperature. This was followed by two rinses of 2 min each in 100 mM sodium phosphate, pH 7.0 at room temperature and fixation in 1% glutaraldehyde, 0.2% tannic acid, and 0.10 M sodium phosphate, pH 7.0 for 30 min at room temperature. The tissue was then rinsed twice for 10 min each in 0.1 M sodium phosphate, pH 7.0 and postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate, pH 6.0 on ice for 30 min. Four rinses in cold distilled water for 2 min each and one 2 min rinse at room temperature were followed by en bloc staining in 1% aqueous uranyl acetate for 45 min at room temperature. The tissue was then dehydrated in a graded ethanol series and embedded in Epon. Silver-gold tissue sections were cut, collected onto grids, and stained with uranyl acetate and lead citrate before being viewed with an electron microscope.

Isolated neural retinas from rat were initially treated as described above; however, results improved by using the following modifications. The SSS was 10 mM sodium phosphate, pH 7.0, 150 mM KCl, 5 mM MgCl₂, 0.1 mM EGTA, and 0.2 mM DTT, and the retina pieces were incubated in saponin for 30 min and in S-1 for 60 min. Fixation was in 2% glutaraldehyde, 0.2% tannic acid, and 100 mM sodium phosphate, pH 7.0 for 30 min at room temperature, and samples were embedded in Araldite. All micrographs of rat retina presented in this report were taken from tissue treated in this manner, except for that in Figure 2C, which was prepared with the original procedure.

Controls

Control incubations were carried out on samples of rat retina only. In place of S-1, tissue pieces were incubated in S-1 plus 9 mM ATP (Sigma Chemical Co.), or in SSS alone.
Results

Retinal photoreceptors were found to retain their basic morphology after saponin treatment and subsequent incubation and tissue processing. The ROS displayed regions of normal disk stacking, interspersed with areas of vesiculated disks. These vesiculated membranes could be observed at any level of the ROS, from the base to the distal end. The structures of the connecting cilium were well preserved. Although the plasma membranes appeared intact, saponin treatment did permeabilize these membranes, as evidenced by the ability of S-1 to enter the cells and bind to actin filaments. These observations were in agreement with those of earlier studies, in which saponin was used either before or during tissue fixation to permeabilize cell membranes while still retaining good cellular morphology. In pinfish rods, inner segment filaments which displayed the characteristic arrowhead configuration were observed. These filaments have previously been described and extend from the calycal processes, which are microvilluslike extensions of the inner segment. Similar filaments rarely were observed in rat rods. Calycal processes reportedly are absent in rats except for two or three short processes that sometimes are observed at the base of the rod cilium. As others have noted, there was no binding of S-1 to the filaments of the striated rootlet.

In the present study, we observed actin filaments in a region of the ROS where these filaments have not previously been found. A meshwork of S-1–decorated filaments was detected within the center of the ciliary axoneme at the base of the ROS. This was most clearly seen in rat retina (Figs. 2A, B). In some cells, the decoration on individual filaments could be distinguished and, in these instances, the arrowheads always pointed distally (Fig. 2A). In other cells, however, the filament meshwork appeared dense and the polarity of individual filaments was difficult to resolve (Fig. 2B).

In longitudinal section, S-1–decorated filaments were observed to radiate out of the ciliary axoneme and into the base of the ROS disk stack (Figs. 2A, B, 3A–C, 4). In all cases, the polarity of these filaments was such that their arrowheads uniformly pointed toward the cilium. This is most clearly demonstrated in Figures 3A–C and 4. At their barbed ends, the filaments appeared to be associated with either the ROS plasma membrane or with the membranes of vesicular structures. These vesicles were probably an artifact attributable to detergent treatment or tissue processing. No structures which could be positively identified as new membrane evaginations were observed.

In cross section, the filament polarity was clearly visible. Individual filaments extended from the center of the ciliary axoneme and into the base of the ROS disk stack by passing between pairs of microtubule doublets on the ROS side of the cilium (Fig. 5A, C). In all instances, the arrowheads on these filaments pointed toward the cilium. Filaments did not pass between the outer microtubule doublets on the side of the cilium opposite the ROS disk stack.

When cilia were cross-sectioned at a point between the inner and outer segments, decorated filaments were found not to be associated with the axonemes, and the cilia were completely surrounded by their plasma membrane (Fig. 5B, D). The Y-shaped cross-linkers which connect the microtubule doublets to the ciliary plasma membrane could be seen clearly, as could the nexin links between the doublets (for review see ref. 24).

In the S-1-incubated pinfish retina we observed a photoreceptor in which the ciliary filaments were preserved but not decorated with S-1. In this instance, the filaments were seen to radiate out of the ciliary axoneme and to associate with the basal ROS plasma membrane (Fig. 6).

In rat retina controls, inclusion of ATP in the incubation medium blocked S-1 binding to the filaments, as a result of competition between the ATP and the actin filaments for the myosin ATPase. However, filaments were still preserved and could be detected as a meshwork within the ciliary axoneme at the base of the ROS (Fig. 2C). Similar results were obtained when S-1 was omitted from the incubation and only buffer was used.

We also noted that in longitudinal sections, the diameter of the ciliary axoneme appeared to increase slightly at the base of the ROS (Figs. 2, 4, 6). This is the same portion of the axoneme that contains the actin filament meshwork.

Discussion

The present study provides the first ultrastructural demonstration of an actin filament meshwork within the ciliary axoneme at the base of the ROS. Permeabilization of visual cell membranes with saponin allowed S-1 to enter the cells for filament decoration. However, ciliary filaments could also be observed in saponin-treated tissue in the absence of S-1 binding. It is likely that some cytoplasmic components were extracted from the cells during saponin treatment. In previous studies, in which retinas were prepared using classical fixation and processing methods for electron microscopy, these components may have obscured visualization of the ciliary actin filaments.

Individual actin filaments were also observed to
Fig. 2. Longitudinal sections of saponin-treated rat rods. (A, B) Myosin S1 incubation results in a meshwork of decorated actin filaments (straight arrows) within the ciliary axoneme at the base of the rod outer segment (ROS). Some of the filaments in (A) display arrowheads which point toward the distal end of the ROS (one example is at the tip of the straight arrow). In (B), the filament meshwork is so dense that the polarity of decorated filaments cannot be resolved. Individual filaments in (A) and (B) extend from the axoneme into the base of the ROS disk stack (curved arrows) and associate at their barbed ends with the ROS plasma membrane or with the membranes of vesicular structures. Arrowheads on these filaments are difficult to resolve, although in other cells (see Figs. 3, 4) they are found to point uniformly toward the cilium. The vesicles are probably artifacts which result from saponin treatment or tissue processing. (C) Control retina in which the tissue was incubated with myosin S1 plus ATP. The filament meshwork in the ciliary axoneme was preserved (straight arrow); however, decoration of the filaments was blocked. In (A–C), note the slight widening of the ciliary axoneme in the base of the ROS. Original magnifications: (A) ×57,000; (B) ×45,600; (C) ×51,300.

Filaments entering the base of the ROS disk stack exhibited a uniform polarity when decorated with myosin S1. All of the arrowheads pointed toward the cilium, while the barbed ends were oriented in the direction of disk expansion and often appeared to associate with the ROS plasma membrane. This polarity was similar to that observed on membrane-associated actin filaments in other systems. These include the filaments in the pseudopodia of human blood platelets, the growth cone of cultured neuroblastoma cells, and the leading edge of cultured fibroblasts. The filament polarity was similar also to the bundle of filaments within microvilli of the intestinal brush border and of the sea urchin egg as well as to the filament bundle within sea urchin coelomocytes, the stereocilia of the inner ear, and
Fig. 3. Longitudinal sections of saponin-treated and myosin S-1-incubated rat rods. Myosin S-1 decorated filaments (arrows) extend from the ciliary axoneme at the base of the rod outer segment (ROS) into the bottom of the disk stack. The arrowheads on these filaments uniformly point toward the cilium. The barbed ends are oriented away from the cilium and are associated with the ROS plasma membrane or with membranes of vesicular structures. These vesicles are probably artifacts which result from saponin treatment or tissue processing. Original magnifications: (A) ×85,500; (B) ×68,900; (C) ×123,250.

the acrosomal process of sperm cells. In these examples, the arrowheads always pointed away from the membrane and toward the cell body. It is likely that actin filaments in the ROS have a cytoskeletal or contractile function similar to filaments in some of these other systems.

In the present study, it was noted that the ciliary axoneme at the base of the ROS had a slightly enlarged diameter. This larger axonemal space may be necessary to accommodate the actin filament meshwork. However, it might also be an artifact produced by differential shrinkage and contraction of the axoneme during tissue fixation and processing.

In our study, every rod cilium in the rat retina was found to contain a meshwork of actin filaments. In the pinfish retina, however, the meshworks were often not as large, and they were difficult to detect in many of the cells. The reason for this is unclear, and it may be due to a species difference. The presence of glycerol in the S-1 preparation used for rat retina incubations may have made a difference in the results; we will investigate this possibility further. In the past, cell biologists have used glycerol for cell permeabilization when conducting S-1 incubation studies. In our study, however, the visual cells had already been permeabilized with saponin prior to the introduction of glycerol.

In a recent in vitro study in which cytochalasin D was used to disrupt the actin filament network in photoreceptors, it was found that the initiation of new ciliary membrane evaginations was inhibited. However, outer segment disks which had already...
started to form continued to expand to larger than normal dimensions. These oversized disks lay along the outside of the inner or outer segments and, in some instances, extended from the opposite side of the cilium. In a follow-up study it was shown that when cytochalasin D was given as an intraocular injection, the effect on photoreceptors was the same:36 oversized disks formed, although once the pulse of drug wore off, new evaginations were initiated and normal sized disks were produced. The oversized disks were retained and pushed higher up into the disk stack.

These observations suggest that an actin filament network within the evaginations is not required for the addition of new membrane to the growing disks, although filaments do appear to be required for the formation of new evaginations. The actin filaments in the basal ROS may participate in disk initiation and growth by providing stability to the emerging structures or by guiding their growth in the proper direction. The actin filament polarity demonstrated in the present report is consistent with the results of the cytochalasin D study, since the barbed end of each filament is oriented in the direction of disk expansion. The barbed end is the site of preferential actin polymerization37–39 and is also the site where cytochalasin D is believed to block the polymerization of actin filaments.40 In other cell types, actin filaments have been shown to elongate from their barbed, membrane associated ends.31–43

In a recent report in which the diurnal rhythm of ROS disk formation was studied,44 it was found that near the time of light onset, disordered membranes accumulated at the ROS base, sometimes extending along the ROS or indenting into the apical rod inner segment. These membrane structures were similar to those observed in the cytochalasin D study mentioned above, and they were not found later in the day. The authors of this study44 proposed that the ordering of the accumulated membrane into a well-aligned stack of disks was a process separate from the actual assembly of disk membrane. They suggested that the membrane may become ordered into disks by a contractile mechanism as part of the process to place new disks in register. Decorated actin filaments are known to slide in the direction of their arrowheads when interacting with anchored myosin molecules.29 Given the polarity of the filaments in the basal ROS, an ideal location for myosin in any acto-myosin interaction would be within the ciliary axoneme from which the actin filaments radiate. However, attempts to localize myosin in this domain by immunocytochemistry have been inconclusive. One well-characterized anti-myosin was found to label the
Fig. 5. Cross sections of saponin-treated and myosin S-1-incubated rod cilia from pinfish (A, B) and rat (C, D) retinas. In (A) and (C), the sections are through the ciliary axoneme at the base of the rod outer segment (ROS). Decorated actin filaments (arrows) radiate out of a meshwork of filaments within the axoneme and extend into the ROS by passing between pairs of microtubule doublets. This occurs only on the ROS side of the cilium. Filaments do not pass between microtubule doublets on the opposite side of the cilium. In all cases, the arrowheads on these filaments point toward the cilium. In (B) and (D), the cross sections are at a level between the ROS and the rod inner segment (RIS). Note that the axonemes are completely surrounded by a plasma membrane and that no decorated filaments are found within or extending from them. Y-shaped crosslinkers connect the microtubule doublets to the ciliary plasma membrane, and nexin links connect the doublets. In (B), the decorated actin filaments in the left and bottom portions of the micrograph are found within the RIS. They are part of the filament bundles which extend from calycal processes and which lie subjacent to the RIS plasma membrane. They bend around the dense packing of mitochondria in the apical portion of the RIS and are often sectioned at an oblique angle, as in this micrograph. The filaments in the bundles have splayed out somewhat, thus allowing S-1 decoration. In many instances, as in Figures 4 and 6, the cells have been sectioned between the bundles. Original magnifications: (A) ×79,200; (B) ×82,650; (C) ×131,950; (D) ×124,700.

entire ciliary backbone of the ROS, while several other anti-myosins tested by one of the current authors gave inconsistent results. Recently, other investigators have reported that both myosin and α-actinin can be localized to the ciliary axoneme at the base of the ROS. It is also noteworthy that a spectrin-like protein has been localized along the periphery of the ROS in the region where disk rims come into close contact with both the plasma membrane and the ciliary backbone.

Our preliminary report of actin filament polarity in pinfish photoreceptors represented the first ultrastructural demonstration of individual actin filaments that are associated with a ciliary axoneme. Subsequently, we presented preliminary results of our work on ciliary actin filaments in rat rods. In the present paper, we document our work for both pinfish and rat visual cells. Recently, another group has also demonstrated actin filaments associated with cilia in rat photoreceptors.

Studies using immunocytochemical techniques have indicated that actin is contained within the motile cilia from ovipositor and tracheal epithelial cells and within sperm flagella. Additionally, there is biochemical evidence for actin in axonemes from Chlamydomonas flagella. However, studies with NBD-phallacidin have found no binding to ovipositor cilia, Chlamydomonas flagella, or sperm flagella suggesting that the actin in these structures is in a nonfilamentous form. Additional work is needed in this area.

Although we were able to preserve and decorate actin filaments within the photoreceptor cilium and outer segment, it is possible that a portion of the
whether their ends associate with the plasma membrane along the rims of the evaginations and whether the filaments associate laterally with the surfaces of the nascent disks. This information, along with the identification of actin-binding proteins and a knowledge of any diurnal changes in the network, should allow us to determine the mechanism of actin function during ROS disk morphogenesis.

Key words: photoreceptor, rod outer segment, disk morphogenesis, actin, filament polarity

Acknowledgments

The authors wish to thank Mr. Richard Carlsen and Ms. Juana Alvarez for their excellent technical assistance, Dr. Roger Cooke for supplying myosin S-1, and Drs. David Burgess, Melanie Pratt, Kathryn Pagh-Roehl, and Robert Knighton for helpful discussions and advice.

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