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Ciliary Transport of Opsin

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Abstract

As part of the renewal of photoreceptor outer segment disk membranes, membrane proteins are transported along the region of the cilium, connecting the inner and outer segments. Genetics studies have indicated the role of motor proteins in this transport. Direct analysis of live cells is needed to increase our understanding of the transport mechanisms further. Here, we show that transfection of hTERT-RPE1 cells with constructs encoding RHO-EGFP, but not RHO-mCherry, results in the distribution of fluorescently-tagged opsin in the plasma membrane. When the cells have differentiated and possess cilia, a portion of the RHO-EGFP was observed along the cilia. Due to the remarkable conservation of ciliary protein function, this system of Rho-Egfp transfected hTERT-RPE1 cells provides a valid model with which to study the ciliary transport of opsin directly in live cells.

21.1 Introduction

For the renewal of the disk membranes of photoreceptor outer segments, membrane proteins are delivered to the base of the outer segment from the inner segment (Young 1967). Early EM autoradiography studies demonstrated labeled protein in the connecting cilium (the region of the photoreceptor cilium between the basal body and the most basal disk membrane of the outer segment), indicating that the protein traveled along this conduit to the outer segment (Young 1968). However, subsequent immunocytochemistry analyses failed to detect opsin in this structure (e.g. Nir and Papermaster 1983; Besharse et al. 1985), suggesting the notion that delivery of opsin-containing membrane might follow an extraciliary route (Besharse and Wetzel 1995), as proposed originally by Richardson (1969). Later studies showed immunogold labeling of opsin in the connecting cilia of mice that were mutant for the molecular motor, myosin VIIa, thus not only supporting the ciliary route, but suggesting the involvement of a molecular motor in the transport of opsin along the cilium (Liu et al. 1999). Evidence for the participation in ciliary opsin transport by the microtubule motor, kinesin-2 (Marszalek et al. 2000), which is significantly faster than unconventional myosins, led to the realization that the rapid transport of opsin along the connecting cilium likely resulted in a relatively low concentration of the protein in this structure (Williams 2002). Only following extensive etching of sections, was significant immunogold labeling of opsin detected in the connecting cilia of wild-type retinas (Wolfrum and Schmitt 2000).

With the acceptance of the delivery of opsin (and other membrane proteins) to the outer segment occurring via the connecting cilium, attention has turned to understanding the mechanisms of its delivery. Molecular motors appear to be involved, based on genetics studies. While these in vivo studies indicate a physiological requirement for a given motor, it is not clear how direct

that role is, and the relative functions of different motors. In addition to kinesin-2 (Marszalek et al. 2000; Jimeno et al. 2006) and myosin VIIa (Liu et al. 1999), the requirement of another kinesin, based on a homodimer of KIF17, has been indicated from zebrafish studies (Insinna et al. 2008). Motor associated proteins, such as intraflagellar transport proteins (IFTs), are also required (Pazour et al. 2002). To gain a better understanding of the molecular mechanisms underlying opsin transport along the connecting cilium, the transport process needs to be studied directly, rather than inferred from endpoint studies. Imaging of live cells provides such an approach.

Since procedures to maintain photoreceptor cells in cell culture conditions are not well established, we have used lines of polarized ciliated cells as a starting point. Use of these cells is justified based on the extensive conservation of ciliary protein function. For example, from algae to mammals, kinesin-2 and intraflagellar transport (IFT) proteins function in the movement of proteins along the axonemes of cilia and flagella (Rosenbaum and Witman 2002). Conservation of ciliary organization is also manifest by several syndromic diseases that are based on ciliopathies (Fliegauf et al. 2007).

In the present paper, we describe our initial studies in establishing hTERT-RPE1 cells, expressing fluorescently-tagged opsin, and show that the tagged opsin is present in the cilia of these cells.

21.2 Methods

Cell culture

hTERT-RPE1 cells were grown in DMEM/F12 culture medium with 5% PBS and 1% penicillin/streptomycin, as suggested by ATCC. Cells were incubated at 37°C with 5% CO₂. To induce the growth of cilia in confluent cultures, the complete medium was replaced with the medium containing 0.5% FBS, one day after transfection. They were kept in the low serum condition for 48 h before imaging.

Transfection

Transfection was achieved using lipofectamine 2000 (Invitrogen). After 4–6 h, normal growth medium was replaced.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 min and subsequently washed with PBS. They were then blocked with 5% goat serum and incubated for 1 h in primary antibody. After subsequent washes with PBS, the cells were incubated with the secondary antibody, washed and mounted in mowiol. Acetylated tubulin (Cat# T7451, Sigma) and KAP3 (Cat# SC-8877, Santa Cruz) antibodies were used.

Microscopy

Both live and fixed cell confocal microscopy was performed using a spinning disk confocal microscope (Perkin Elmer). Images were processed using Volocity software.

21.3 Results

Opsin has been shown to be targeted to the apical surface of MDCK cells (Chuang and Sung 1998). Here, we have used another epithelial cell line, hTERT-RPE1 cells, to test whether, following transfection, opsin is targeted not only to the apical surface, but to the cilium protruding from the apical surface of each of these cells.

An exposed C-terminus is necessary for the correct localization of rhodopsin; the placement of a protein tag at the C terminus of opsin interferes with its ability to localize properly to the plasma membrane in cell culture as well as in vivo (e.g. Moritz et al. 2001). However, a fusion protein, containing RHO-EGFP, with the last 8 amino acids of opsin repeated after the EGFP, was found to function and localize normally in *Xenopus* (Moritz et al. 2001; Jin et al. 2003). We followed the same design, using bovine rod opsin cDNA, fused with either EGFP or mCherry (Fig. 21.1a). Both these constructs were used for transfection of hTERT-RPE1 cells. Although the RHO-EGFP localized to the plasma membrane in these cells, RHO-mCherry did not; it aggregated in inner compartments of the cells (Fig. 21.1b). This observation indicated that only the Rho-Egfp construct was useful for our studies.

In order to induce the hTERT-RPE1 cells to grow cilia, confluent cells growing in normal serum conditions were 'starved' of serum (0.5% serum). Under these conditions, they generated cilia within 48 h (Fig. 21.2). As expected, the cilia were found to contain KAP3, a component of the heterotrimeric kinesin-2 (Fig. 21.2b). To test whether opsin was located in the cilia, cells were cotransfected cells with Rho-Egfp and a construct that generated the protein, Smoothened (SMO), with a CFP tag. Smoothened, when overexpressed, can completely suppress Patched activity and is localized to the cilium (Taipale et al. 2000), and is thus an effective cilium marker. We observed that RHO-EGFP was present with SMO-CFP in the cilia (Fig. 21.3).

21.4 Discussion

In this preliminary study, we report that a portion of EGFP-tagged opsin (but not RHO-mCherry) is delivered to the cilia of transfected hTERT-RPE1 cells. Given the conservation among cilia, analysis of opsin transport in the hTERT-RPE1 cell cilia should provide insight into mechanisms underlying the transport of opsin from the inner segment to the outer segment.

The conservation of protein function in cilia is remarkable. Heterotrimeric kinesin-2 has been detected in photoreceptor cilia (Beech et al. 1996), and we have demonstrated its presence in the primary cilia of hTERT-RPE1, in the present study (Fig. 21.2b). However, much further afield, the FLA-10 protein of *Chlamydomonas* flagella was identified as a subunit of kinesin-2 (Kozminski et al. 1995). In some of the first studies on axonemal transport, FLA-10 was found to be required for the transport of 'rafts' of IFT protein complexes to the tip of the flagellum, where growth occurs (Cole et al. 1998). Similarly, KIN1 and KIN2, which are genes for motor subunits of kinesin-2 in *Tetrahymena*, are required for the assembly of new cilia and the maintenance of preexisting cilia (Brown 1999). In *C. elegans*, a kinesin-2 has been identified in immotile chemosensory cilia, together with the IFT proteins, *osm-1* and *osm-6*. GFP-tagged kinesin-2 and *osm-6* have been observed moving in a distal direction together along the sensory cilia of live worms (Orozco et al. 1999).

While the presence of kinesin-2 appears to be of particular importance with respect to opsin transport, as shown by genetics studies (Marszalek et al. 2000; Pazour et al. 2002; Jimeno et al. 2006), a number of other ciliary proteins are linked to syndromic ciliopathies that include photoreceptor degeneration, thus adding further support to the conservation of protein function in cilia. Examples include NPHP5, which underlies a form of Senior-Loken syndrome (Otto et al. 2005), CEP290 (or NPHP6), which has been linked to Joubert syndrome (Sayer et al. 2006), and the Bardet-Biedl syndrome (BBS) proteins (Blacque and Leroux 2006).

Together, the conservation of protein function in cilia, and the delivery of opsin along the cilia of hTERT-RPE1 cells, indicate that polarized epithelial cells, such as hTERT-RPE1 cells, that are transfected with tagged opsin provide a valid model system in which to study the ciliary transport of opsin.

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References

- Beech PL, Pagh Roehl K, Noda Y, et al. Localization of kinesin superfamily proteins to the connecting cilium of fish photoreceptors. *J Cell Sci* 1996;109:889–897. [PubMed: 8718680]
- Besharse JC, Forestner DM, Defoe DM. Membrane assembly in retinal photoreceptors. III. Distinct membrane domains of the connecting cilium of developing rods. *J Neurosci* 1985;5:1035–1048. [PubMed: 3156973]
- Besharse JC, Wetzel MG. Immunocytochemical localization of opsin in rod photoreceptors during periods of rapid disc assembly. *J Neurocytol* 1995;24:371–388. [PubMed: 7544404]
- Blacque OE, Leroux MR. Bardet-Biedl syndrome: an emerging pathomechanism of intracellular transport. *Cell Mol Life Sci* 2006;63:2145–2161. [PubMed: 16909204]
- Brown SS. Cooperation between microtubule- and actin-based motor proteins. *Ann Rev Cell Dev Biol* 1999;8:1751–1755.
- Chuang JZ, Sung CH. The cytoplasmic tail of rhodopsin acts as a novel apical sorting signal in polarized MDCK cells. *J Cell Biol* 1998;142:1245–1256. [PubMed: 9732285]
- Cole DG, Diener DR, Himmelblau AL, et al. Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons. *J Cell Biol* 1998;141:993–1008. [PubMed: 9585417]
- Fliegeauf M, Benzing T, Omran H. When cilia go bad: cilia defects and ciliopathies. *Nat Rev Mol Cell Biol* 2007;8:880–893. [PubMed: 17955020]
- Insinna C, Pathak N, Perkins B, et al. The homodimeric kinesin, Kif17, is essential for vertebrate photoreceptor sensory outer segment development. *Dev Biol* 2008;316:160–170. [PubMed: 18304522]
- Jimeno D, Feiner L, Lillo C, et al. Analysis of kinesin-2 function in photoreceptor cells using synchronous Cre-loxP knockout of Kif3a with RHO-Cre. *Invest Ophthalmol Vis Sci* 2006;47:5039–5046. [PubMed: 17065525]
- Jin S, McKee TD, Oprian DD. An improved rhodopsin/EGFP fusion protein for use in the generation of transgenic *Xenopus laevis*. *FEBS Lett* 2003;542:142–146. [PubMed: 12729914]
- Kozminski KG, Beech PL, Rosenbaum JL. The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. *J Cell Biol* 1995;131:1517–1527. [PubMed: 8522608]
- Liu X, Udovichenko IP, Brown SDM, et al. Myosin VIIa participates in opsin transport through the photoreceptor cilium. *J Neurosci* 1999;19:6267–6274. [PubMed: 10414956]
- Marszalek JR, Liu X, Roberts EA, et al. Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors. *Cell* 2000;102:175–187. [PubMed: 10943838]
- Moritz OL, Tam BM, Papermaster DS, et al. A functional rhodopsin-green fluorescent protein fusion protein localizes correctly in transgenic *Xenopus laevis* retinal rods and is expressed in a time-dependent pattern. *J Biol Chem* 2001;276:28242–28251. [PubMed: 11350960]
- Nir I, Papermaster DS. Differential distribution of opsin in the plasma membrane of frog photoreceptors: an immunocytochemical study. *Invest Ophthalmol Vis Sci* 1983;24:868–878. [PubMed: 6223003]
- Orozco JT, Wedaman KP, Signor D, et al. Movement of motor and cargo along cilia [letter]. *Nature* 1999;398:674. [PubMed: 10227290]
- Otto EA, Loeys B, Khanna H, et al. Nephrocystin-5, a ciliary IQ domain protein, is mutated in Senior-Loken syndrome and interacts with RPGR and calmodulin. *Nat Genet* 2005;37:282–288. [PubMed: 15723066]
- Pazour GJ, Baker SA, Deane JA, et al. The intraflagellar transport protein, IFT88, is essential for vertebrate photoreceptor assembly and maintenance. *J Cell Biol* 2002;157:103–113. [PubMed: 11916979]

- Richardson TM. Cytoplasmic and ciliary connections between the inner and outer segments of mammalian visual receptors. *Vision Res* 1969;9:727–731. [PubMed: 4979023]
- Rosenbaum JL, Witman GB. Intraflagellar transport. *Nat Rev Mol Cell Biol* 2002;3:813–825. [PubMed: 12415299]
- Sayer JA, Otto EA, O'Toole JF, et al. The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. *Nat Genet* 2006;38:674–681. [PubMed: 16682973]
- Taipale J, Chen JK, Cooper MK, et al. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* 2000;406:1005–1009. [PubMed: 10984056]
- Williams DS. Transport to the photoreceptor outer segment by myosin VIIa and kinesin II. *Vision Res* 2002;42:455–462. [PubMed: 11853761]
- Wolfrum U, Schmitt A. Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells. *Cell Motil Cytoskeleton* 2000;46:95–107. [PubMed: 10891855]
- Young RW. The renewal of photoreceptor cell outer segments. *J Cell Biol* 1967;33:61–72. [PubMed: 6033942]
- Young RW. Passage of newly formed protein through the connecting cilium of retina rods in the frog. *J Ultrastruct Res* 1968;23:462–473. [PubMed: 5692302]

Fig. 21.1.

Rho-Egfp and Rho-mCherry constructs. **a**, Schematic of Rho-Egfp and Rho-mCherry constructs. Bovine Rho cDNA was placed under the CMV promoter and tagged with either Egfp or mCherry at the C terminus. The 3' 24 base pairs of Rho were added to the 3' of the Egfp or mCherry tag. **b**, Comparison of the localization of RHO-EGFP and RHO-mCherry, following cotransfection of the same cell. RHO-EGFP (*left*) localizes to the plasma membrane while RHO-mCherry (*right*) forms internal aggregates

Fig. 21.2.

Cilia of hTERT-RPE1 cells. **a**, Serum starvation for 48 h leads to cell differentiation and the production of cilia. Cells are labeled with acetylated tubulin antibodies to indicate the cilia. **b**, KAP3 antibody localizes to the cilia. *Left panel*: DAPI staining of the cells to indicate the nuclei. *Middle panel*: Acetylated tubulin antibody labeling, showing the cilia of the cells. *Right panel*: KAP3 antibody labeling shows localization in the cilia (*arrowheads*)

Fig. 21.3. RHO-EGFP localization in cilia, as indicated by the presence of SMO-CFP, in hTERT-RPE1 cells, transfected with both Rho-Egfp and Smo-Cfp. **a**, Overexpressed SMO-CFP localized to cilia. **b**, RHO-EGFP in the same cell