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Localization of kinesin superfamily proteins to the connecting cilium of fish photoreceptors

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SUMMARY

Kinesin superfamily proteins (KIFs) are probable motors in vesicular and non-vesicular transport along microtubular tracks. Since a variety of KIFs have been recently identified in the motile flagella of *Chlamydomonas*, we sought to ascertain whether KIFs are also associated with the connecting cilia of vertebrate rod photoreceptors. As the only structural link between the rod inner segment and the photosensitive rod outer segment, the connecting cilium is thought to be the channel through which all material passes into and out of the outer segment from the rod cell body. We have performed immunological tests on isolated sunfish rod inner-outer segments (RIS-ROS) using two antibodies that recognize the conserved motor domain of numerous KIFs (anti-LAGSE, a peptide antibody, and anti-Klp1 head, generated against the N terminus of *Chlamydomonas* Klp1) as well as an antibody specific to a neuronal KIF, KIF3A. On immunoblots of RIS-ROS, LAGSE antibody detected a prominent band at ~117 kDa, which is likely to be kinesin heavy chain, and Klp1 head antibody detected a single band at ~170 kDa; KIF3A antibody detected a polypeptide at ~85 kDa which co-migrated with mammalian KIF3A and displayed ATP-dependent release from rod cytoskeletons. Immunofluorescence localizations with anti-LAGSE and anti-Klp1 head antibodies detected epitopes in the axoneme and ellipsoid, and immunoelectron microscopy with the LAGSE antibody showed that the connecting cilium region was particularly antigenic. Immunofluorescence with anti-KIF3A showed prominent labelling of the connecting cilium and the area surrounding its basal body; the outer segment axoneme and parts of the inner segment coincident with microtubules were also labelled. We propose that these putative kinesin superfamily proteins may be involved in the translocation of material between the rod inner and outer segments.

Key words: Connecting cilium, Kinesin, Microtubular transport, Motor protein, Photoreceptor, Rod

INTRODUCTION

Vertebrate rod photoreceptors are modified neurons that consist of a photosensory outer segment and an inner segment which contains the organelles required for protein synthesis and cell maintenance (Fig. 1a). The rod outer segment (ROS) is a specialized, non-motile cilium, the plasma membrane of which has expanded during early retinal development to envelop forming stacks of membranous disks that contain the photosensory pigment, rhodopsin. The ciliary axoneme lacks a central pair of microtubules and dynein arms but, like motile cilia and flagella, has a high content of acetylated and detyrosinated microtubules (Sale et al., 1988; Pagh-Roehl et al., 1991). The axoneme lies alongside the disks in the outer segment with its basal body situated in the adjacent (distal) end of the rod inner segment (RIS), called the ellipsoid (Fig. 1b). The portion of the axoneme traversing the boundary between the inner and outer segments is called the connecting cilium (Fig. 1c), because it is the only structural link between the RIS and the ROS (reviewed by Besharse and Horst, 1990).

The pigmented disks of the ROS are continually turned over: new disks are formed at the base of the ROS, adjacent to the connecting cilium, and the oldest disks are shed from the distal end of the ROS (Young, 1967; Young and Bok, 1969). The turnover of disks requires that opsin (and other disk proteins) be constantly transported from the myoid and ellipsoid portions of the RIS, where opsin integrates into membrane during its synthesis in the endoplasmic reticulum (Papermaster et al., 1975), to the forming disks at the base of the ROS. How this transport occurs is not known, though it seems likely that the plasma membrane of the connecting cilium, being the only membranous conduit between the inner and outer segments, would be the avenue of transport for an integral membrane protein (this and alternative models are reviewed by Besharse and Horst, 1990). Given that the axonemal microtubules underlie the ciliary plasma membrane, it is possible

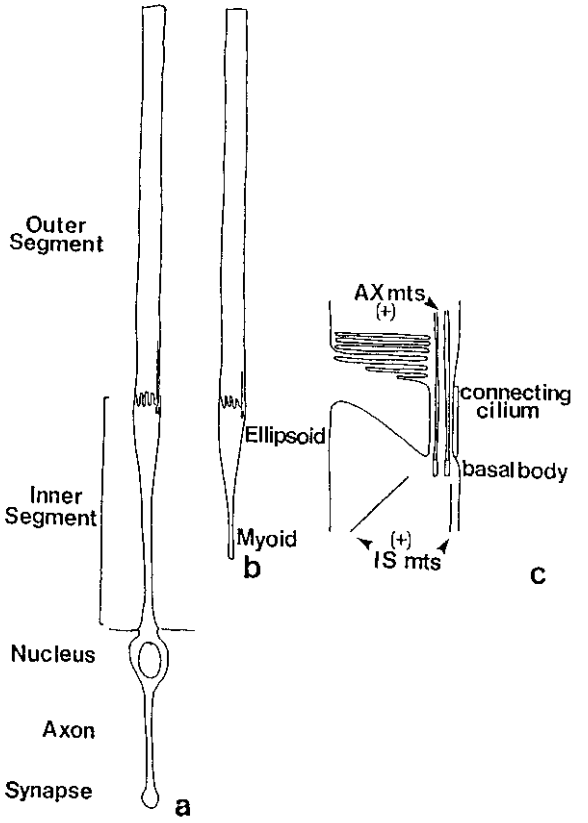

Fig. 1. Diagram of (a) an entire rod photoreceptor cell from the green sunfish, (b) the rod inner-outer segment (RIS-ROS) used in the experiments presented here, and (c) higher magnification of the region between the RIS and the ROS showing the connecting cilium and the plus (+) ends of the axonemal (AX mts) and inner segment (IS mts) microtubules. The minus ends of these two sets of microtubules are closest to the basal body. Modified from Pagh-Roehl and Burnside (1995).

that any transport of material along this membrane is dependent on microtubule-based mechanochemical motors.

One such group of motors is the kinesin superfamily of ATPases. Kinesin was first isolated from squid axoplasm as the probable motor for vesicular transport along neuronal microtubules (Brady, 1985; Vale et al., 1985). Many kinesin superfamily proteins (KIFs) have now been identified from different cell types where they have been implicated in various forms of microtubule-associated motility, in particular, the carriage of membrane-bound organelles along microtubules (reviewed by Brady, 1995; Goldstein, 1993; Goodson et al., 1994; Hirokawa, 1993; Walker and Sheetz, 1993). Several groups have recently shown that KIFs are also present in the flagella of the unicellular green alga, *Chlamydomonas reinhardtii* (Bernstein et al., 1994; Fox et al., 1994; Johnson et al., 1994; Kozminski et al., 1995; Walther et al., 1994). One study (Kozminski et al., 1995) has indicated that a flagellar KIF, the FLA10 gene product (KRP1; Walther et al., 1994), is important in the maintenance of flagella and the transport of material along the flagellar axoneme beneath the plasma membrane. In this report, we address the possibility that KIFs are also involved in microtubule-dependent transport in rod photoreceptors by determining if such proteins are associated with the connecting cilium.

We have used two differerent antibodies to the conserved motor domain of KIFs, and antibodies to KIF3A (Kondo et al., 1994), a neuronal KIF from mouse that has strong sequence homology to Fla10 (Walther et al., 1994), to show that polypeptides with biochemical and immunological characteristics of KIFs are present in rod photoreceptor cells. The putative KIFs are localized to areas where microtubules are found: the connecting cilium and adjoining basal body region, the axoneme of the outer segment, and in the ellipsoid and myoid portions of the RIS. It is possible that one or more KIFs are involved in the translocation of material between rod inner and outer segments.

MATERIALS AND METHODS

Preparation of photoreceptor inner-outer segments

Rods and cones were isolated from green sunfish, *Lepomis cyanellus*, obtained from Fender Fish Hatchery (Baltik, OH) and maintained on a 14 hour light/10 hour dark cycle in indoor aquaria.

For immunofluorescence studies, fish were dark-adapted for one hour in the afternoon and retinas were isolated under infrared illumination using an image converter as described previously (Pagh-Roehl et al., 1993). Dark-adaptation of fish was necessary to facilitate separation of retinas from the underlying retinal pigmented epithelia. To prepare suspensions of rod inner-outer segments (RIS-ROS), isolated retinas were shaken in 1 ml of a Hepes-based Earle's Ringer solution (Pagh-Roehl et al., 1993) without taurine (HER).

To prepare RIS-ROS (Fig. 1b) for biochemical studies, fish were dark-adapted for one hour in the morning, and retinas were isolated in dim room light. This strategy ensured a RIS-ROS preparation with minimal cone contamination because cones are more contracted in the morning and in the light and therefore do not break off easily from the retinal surface. To release RIS-ROS, isolated retinas were shaken in 0.75 ml of HER with 5 mM taurine (HERT). RIS-ROS suspensions were filtered through a 100 μm mesh and applied to a 33%/50% step gradient of Percoll (Sigma) as described previously (Pagh-Roehl et al., 1993). Purified RIS-ROS were recovered from the 50% interface, diluted 1:1 with HERT, and centrifuged at 1,000 ***g*** for 20 minutes at 4°C. Pelleted RIS-ROS were resuspended in SDS-PAGE sample buffer (Laemmli, 1970) at 100 μl of buffer per retina equivalents of RIS-ROS, or prepared as follows for cytoskeleton studies.

Cytoskeletons of RIS-ROS were prepared by resuspending RIS-ROS in ice-cold CB buffer plus 1% Triton X-100 and 1 mM AMP-PNP at 100 μl buffer per retina's worth of RIS-ROS. The CB buffer contained 50 mM Hepes, pH 7.5, 10 mM $MgSO_4$, 5 mM EGTA, 50 mM KCl, 1 mM DTT, 20 μM taxol (Calbiochem, San Diego, CA), 10% DMSO, and a cocktail of protease inhibitors (as described by Pagh-Roehl et al., 1993). RIS-ROS were extracted at 4°C for 10 minutes and centrifuged for 5 minutes at 10,000 ***g***. The cytoskeletal pellets were resuspended in CB containing 10 mM Mg-ATP or 1 mM AMP-PNP at 10 μl buffer per retina equivalent of RIS-ROS, then incubated for 20 minutes at room temperature. Samples were recentrifuged at 10,000 ***g*** for 5 minutes. Supernatants were mixed with a 3× concentrated solution of SDS-PAGE sample buffer and the pellets were resuspended in the same volume of a 1× sample buffer solution.

Antibodies

Monoclonal anti-β-tubulin antibody, WA3, was provided by Dr Ursula Euteneuer, University of Munich, Germany; the peptide antibodies, anti-LAGSE (L1) and anti-HIPYR (Sawin et al., 1992), were provided by Drs Ken Sawin and Timothy Mitchison; H2, a monoclonal antibody to kinesin heavy chain (Pfister et al., 1989) was provided by Dr George Bloom, and K2.4, a monoclonal antibody to the 85 kDa subunit of sea urchin KRP (85/95) was provided by Dr

John Scholey. Affinity purified, polyclonal KIF3A antibody was raised against murine neuronal kinesin KIF3A as described (Kondo et al., 1994). Klp1 head antibody (Klp1Hd) was generated against the N-terminal motor domain region of *Chlamydomonas* Klp1 (Bernstein et al., 1994) expressed in *Escherichia coli* as a His_6-Klp1 head fusion protein as follows: full length KLP1 cDNA in the His_6 fusion vector pQE32 (Qiagen Inc., Chatsworth, CA; Bernstein et al., 1994) was digested with Bal 31 nuclease from a *Not*I site at nucleotide 1,561. Plasmids were digested with *Kpn*I, filled in with T4 DNA polymerase, and then recircularized. Plasmids were transformed into *E. coli* strain M15, isolated and sequenced by the dideoxy method using Sequenase T7 DNA polymerase (USB, Cleveland, OH). A clone encoding the first 323 amino acids of Klp1 was used for the purification of recombinant protein as described (Bernstein et al., 1994). Polyacrylamide gel slices containing the 40 kDa recombinant protein were used to immunize 2 rabbits. Immunizations and bleeds were performed by Yale Veterinary Services following the protocol of Bernstein et al. (1994). Affinity purification of the Klp1Hd-specific antibody was performed on nitrocellulose strips following the method of Olmsted (1981) with the modifications of Snyder (1989).

To deplete the Klp1Hd antibody, the affinity purified antibody was incubated with the original antigen, immobilized on nitrocellulose strips (containing only the 40 kDa fusion protein), for 24 hours at 4°C. The adsorbed Klp1Hd antibody was tested on western blots to show that depletion had occurred before use as a control for immunofluorescence. The KIF3A antibody was depleted after adsorption with KIF3A proteins expressed in *E. coli* as described by Kondo et al. (1994).

Electrophoresis and immunoblotting

Samples of RIS-ROS dissolved in SDS-PAGE buffer were separated on 8% mini-gels according to the method of Laemmli (1970). Polypeptides were transferred to nitrocellulose membranes in Tris-glycine buffer containing 20% methanol at 50 V for 3 hours at room temperature. Membranes were stained with Ponceau S before blocking in blotto (5% non-fat dry milk powder, 0.05% Tween-20 and 0.02% NaN_3 in Tris-buffered saline (TBS)) for 1 hour. Primary antibodies were diluted in blotto (anti-KIF3A at 1:5,000; anti-LAGSE at 1:5,000; Klp1Hd at 1:250) and filters were incubated overnight at 4°C. Filters were rinsed twice in TBS + 0.05% Tween-20 (TBST) and then incubated in alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) at 1:10,000 dilution in TBST for 1 hour at room temperature. Filters were washed in TBST and then developed in 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate according to the method of Harlow and Lane (1988).

Immunofluorescence microscopy

Purified RIS-ROS were fixed with either paraformaldehyde or cold methanol. For paraformaldehyde preparations, RIS-ROS suspensions in HER were mixed 1:1 with 8% paraformaldehyde in 2× PHEM (100 mM K_2Pipes, 50 mM Hepes, 16 mM EGTA, 4 mM $MgSO_4$, pH 7) containing 4% sucrose, and stored at 4°C for up to 48 hours. Fixed cells were settled for 15 minutes onto coverslips coated with poly-L-lysine (0.1%, w/v; Sigma) and permeabilized in 1% Triton X-100 in PHEM buffer for 60 minutes. For methanol preparations, RIS-ROS suspensions were settled for 5 minutes onto poly-L-lysine-coated coverslips, rinsed once with HER, then immersed for 2 minutes in methanol maintained on dry ice. Thereafter, treatment of aldehyde and methanol fixed samples was essentially the same except as noted. Coverslips were rinsed four times in wash buffer (PBS + 0.05% Tween-20) and, for the paraformaldehyde fixed samples, blocked in blocking buffer (wash buffer + 1% BSA (A-7906, Sigma) + 0.02% NaN_3) for 30 minutes before incubation overnight at 4°C in primary antibodies diluted in blocking buffer (anti-KIF3A and immunodepleted KIF3A at 1:500 or, for double labelling experiments, at 1:250 mixed 1:1 with culture supernantant containing the anti-β-tubulin antibody; anti-LAGSE at 1:500; anti-Klp1Hd and immunodepleted anti-Klp1Hd at 1:25). Coverslips were rinsed four or five times in wash buffer and then incubated for two hours at room temperature in FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:30 in blocking buffer (or, for double labelling, mixed 1:1 with Texas Red-conjugated anti-mouse IgG; Cappel, Organon Teknika Corp., Durham, NC). Coverslips were rinsed five times in wash buffer and once in PBS before mounting in Prolong anti-fade medium (Molecular Probes) or in PBS (pH 9.0) containing 70% glycerol and 0.5% *n*-propyl gallate. Slides were viewed on a Zeiss Universal microscope equipped with epifluorescence and photographed using Kodak TMAX 400 (developed in Kodak TMAX developer) or Kodak PlusX film (developed in Microphen to an ASA of 1450).

For negative controls, the primary antibodies were substituted with normal rabbit IgG at 10 μg/ml, or with an affinity purified Klp1 tail antibody (Bernstein et al., 1994) that does not react with RIS/ROS on western blots (data not shown), or with depleted Klp1Hd and KIF3A antibodies (described above). To control for bleed-through in the KIF3A/β-tubulin double labelling experiment, each of the primary antibodies was also used individually.

Immunoelectron microscopy

RIS-ROS were fixed in 0.5% glutaraldehyde in 2× PHEM buffer + 2% sucrose for 60 minutes at 4°C, rinsed in 2× PHEM buffer with 2% sucrose, and then dehydrated to 80% ethanol; all subsequent steps were performed at –20°C. Cells were embedded in LR Gold (London Resin Company, Hampshire, UK) and polymerized at –20°C for 36 hours under fluorescent light with benzil (0.2%) as the catalyst. Gold sections were collected on Formvar-coated nickel grids and then inverted onto blocking buffer + 0.1% cold water fish gelatin (BBG) for 15 minutes (all steps at room temperature). After blocking, grids were incubated for two hours at room temperature in anti-KIF3A diluted 1:500 in BBG, anti-LAGSE at 1:500, and anti-Klp1Hd at 1:25. Grids were washed in wash buffer (4× 5 minutes) and incubated in 12 nm colloidal gold conjugated to goat anti-rabbit IgG (Jackson Immunologicals, West Grove, PA), diluted 1:100 in BBG, for 1-2 hours. Grids were rinsed in wash buffer (3× 5 minutes), PBS (2× 5 minutes), water (2× 5 minutes) and then stained with 2% aqueous uranyl acetate before a final rinse in water. Sections were viewed on a Zeiss EM10 transmission electron microscope at 80 kV.

Negative controls were performed by substituting the LAGSE antibody with either normal rabbit IgG or with Klp1 tail antibody as for the immunofluorescence experiments.

For quantification of gold labelling, micrographs were enlarged to 60,000× and the areas of three different regions (connecting cilium and axoneme of the ROS, the RIS, and the ROS excluding the axoneme) were measured for longitudinal sections through 14 RIS-ROS, and the number of gold particles in each of the three regions was counted. The density of labelling for each region was calculated and we thereby determined the mean and standard deviation (σ_{n-1}) of labelling density for each region (n=14).

RESULTS

Motor domain antibodies detect potential KIFs in RIS-ROS

To detect KIFs that might be present in RIS-ROS, we first used two antibodies that recognize portions of the conserved motor domain (encompassing the microtubule- and ATP-binding regions) of kinesin superfamily members. Anti-LAGSE was raised against the synthetic decapeptide, LNLVDLAGSE (Sawin et al., 1992). Anti-Klp1 head (Klp1Hd) was raised against amino acids 1-323 of *Chlamydomonas* Klp1 and detects known KIFs in *Chlamydomonas* flagella (P.L.B., unpublished observations). Antibodies to the KIF motor

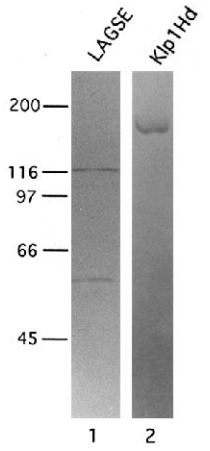

Fig. 2. Immunoblot analysis of green sunfish rod inner-outer segments (2.4×10^6 RIS-ROS per lane) with anti-LAGSE (lane 1) and anti-Klp1 head (lane 2) antibodies. A ~117 kDa and a ~55 kDa polypeptide were detected by anti-LAGSE antibody; a ~170 kDa polypeptide was detected by the anti-Klp1Hd antibody. Molecular mass markers (in kilodaltons) are shown at left.

domain have been used successfully to detect a range of KIFs in various cell types (Cole et al., 1992; Fox et al., 1994; Johnson et al., 1994; Sawin et al., 1992).

In western blot analyses of whole RIS-ROS, two bands at ~117 kDa and ~55 kDa were detected by anti-LAGSE (Fig. 2, lane 1). We have several reasons to believe that the 117 kDa band represents kinesin heavy chain: the same LAGSE antibody is known to recognize kinesin heavy chain in bovine brain fractions at ~120 kDa (Sawin et al., 1992), and a single polypeptide that co-migrated with the 117 kDa anti-LAGSE immunoreactive band was detected on western blots of RIS-ROS by both a peptide antibody to the KIF motor domain, anti-HIPYR, which is known to detect kinesin heavy chain (Sawin et al., 1992), and by monoclonal antibody H2, which is specific to kinesin heavy chain (Pfister et al., 1989) (K.P.-R. unpublished observations). The 55 kDa band recognized by anti-LAGSE in RIS-ROS labelled to varying degrees, but was always less prominent than the 117 kDa band. We note that in diatom spindles, the LAGSE antibody recognized a polypeptide of 55-60 kDa that did not display the properties of a kinesin superfamily protein (Hogan et al., 1993). When higher concentrations of RIS-ROS were probed with the LAGSE antibody, additional, faint bands were detected between 95 and 130 kDa (data not shown), suggesting that the LAGSE antibody may recognize numerous KIFs in RIS-ROS. The Klp1Hd antibody detected a single polypeptide at ~170 kDa in RIS-ROS (Fig. 2, lane 2).

We performed immunolocalization experiments on whole RIS-ROS with each of the motor domain antibodies. Light microscopic localization of anti-LAGSE (Fig. 3a,b) showed pronounced antigenicity of the outer segment axoneme, with the strongest labelling occurring in the ~10 μm closest to the inner segment; the inner segment was labelled at the periphery of the ellipsoid and also in the myoid, although, in contrast to the anti-KIF3A labelling (see below), no microtubule-like staining patterns were observed. Electron microscopic localization of anti-LAGSE on thin-sections of RIS-ROS fixed and embedded in LR Gold resin are shown in Fig. 3c,d. Gold label was associated predominantly with microtubules of the connecting cilium, and the core of the connecting cilium was devoid of label (Fig. 3c). As expected from our immunofluorescence experiments with anti-LAGSE (Fig. 3a), gold label was found in the inner segment where it appeared to localize to the periphery of the RIS; some localization with microtubules was also apparent (Fig. 3c,d). Measurements of the densities of the anti-LAGSE gold labelling on 14 longitudinal sections of RIS-ROS revealed that the connecting cilium and axoneme showed a mean of 76.74 particles/μm^2 (σ_{n-1} = 35.71), the ROS 2.26 particles/μm^2 (σ_{n-1} = 2.29), and the RIS 21.08 particles/μm^2 (σ_{n-1} = 10.09). These quantifications of the gold labelling reveal that the LAGSE antigen is concentrated in the connecting cilium and axoneme by at least three times the level of the labelling in the RIS, and that the ROS shows only minimal (background level) labelling.

In immunofluorescence experiments on RIS-ROS using the Klp1Hd antibody (Fig. 4a-f), labelling was typically observed only in RIS-ROS that had the myoid detached from the ellipsoid of the inner segment, indicating cell damage. In these cells, punctate labelling was evident along the axoneme of the outer segment and, to a lesser extent, in the inner segment (Fig. 4a-d); the connecting cilium showed no distinct labelling. Negative controls, performed with either Klp1Hd antibody immuno-depleted with the original antigen (Fig. 4e,f), or with normal rabbit IgG (data not shown), showed no labelling of damaged or intact RIS-ROS. Attempts to localize the Klp1Hd antigen at the electron microscope level resulted in only very weak labelling of the outer segment axoneme and the RIS (data not shown).

A KIF3A-like polypeptide is present in RIS-ROS

Given that experiments with the LAGSE and Klp1Hd antibodies indicated that KIFs are present in RIS-ROS, we then probed for specific KIFs that might be involved in transport between the rod inner and outer segments. We have recently shown that in *Chlamydomonas* the KIF Fla10 is located between the axonemal outer doublet microtubules and flagellar membrane, and is therefore a good candidate to be a motor driving intraflagellar transport (Kozminski et al., 1995), the bidirectional movement of particles beneath the flagellar membrane. We reasoned that a similar process of flagellar or ciliary transport might be associated with the connecting cilium; however, antibodies to *Chlamydomonas* Fla10 did not detect any polypeptides on western blots of sunfish RIS-ROS (P.L.B. and K.P.-R., unpublished observations). We thus used antibodies to KIF3A, a neuronal KIF that was first isolated from mouse brain (Kondo et al., 1994) and which has the greatest known sequence homology to Fla10 (Walther et al., 1994). In whole RIS-ROS, anti-KIF3A recognized a single polypeptide of ~85 kDa (Fig. 5, lane 2). When extracts of mouse brain were probed with the anti-KIF3A antibody, an 80/85 kDa doublet was detected (as described by Kondo et al., 1994), the upper band of which had a similar molecular mass to that of the anti-KIF3A immunoreactive band from RIS-ROS (compare lanes 1 and 2 in Fig. 5). The 85 kDa putative KIF3A polypeptide was not detected by anti-LAGSE in RIS-ROS, probably because the ten amino acid stretch corresponding to the potential anti-LAGSE epitope in KIF3A is LHLVDLAGSE (Aizawa et al., 1992), that is, there is an amino acid substitution of an asparagine to a histidine in comparison to the

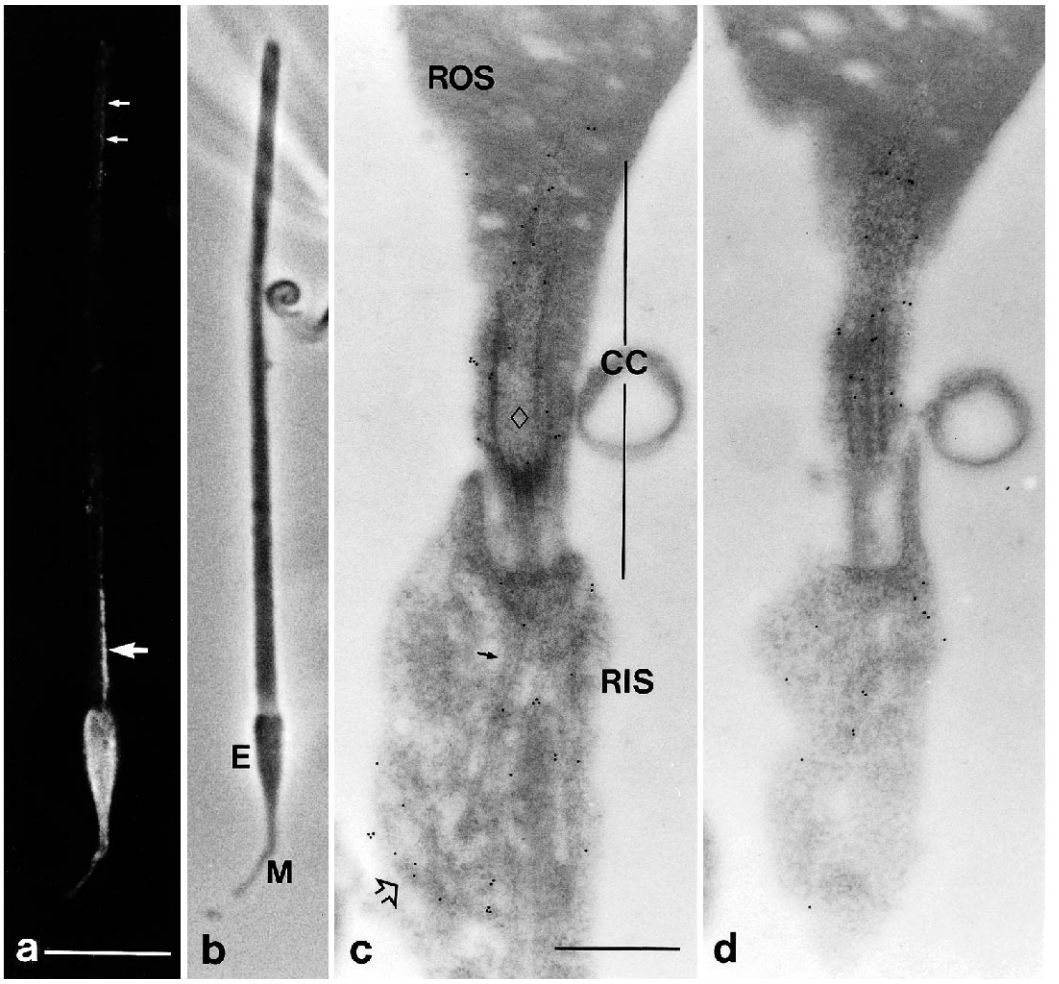

Fig. 3. Immunolocalization of anti-LAGSE antibody to green sunfish rod inner-outer segments. (a) Indirect immunofluorescence microscopy of formaldehyde fixed RIS-ROS; (b) corresponding phase contrast image. Anti-LAGSE labelled the axonemal region of the entire outer segment (small arrows in (a) indicate faint label at the distal end of the outer segment), but antigenicity was strongest at the base of the outer segment (large arrow: a). The periphery of the ellipsoid (E) of the inner segment and the myoid (M) were also antigenic. (c,d) Consecutive longitudinal sections of a rod inner-outer segment (RIS-ROS), fixed in 0.5% glutaraldehyde and embedded in LR Gold, labelled with anti-LAGSE antibody and 12 nm gold particles conjugated to goat anti-rabbit IgG. Gold label was prominent in the microtubular region of the connecting cilium (CC) and absent from the center of the ciliary axoneme (◇); labelling also occurred in the inner segment where it appeared to associate with microtubules (small arrow) and the periphery of the RIS (open arrow). Bars: 10 μm (a); 0.5 μm (c).

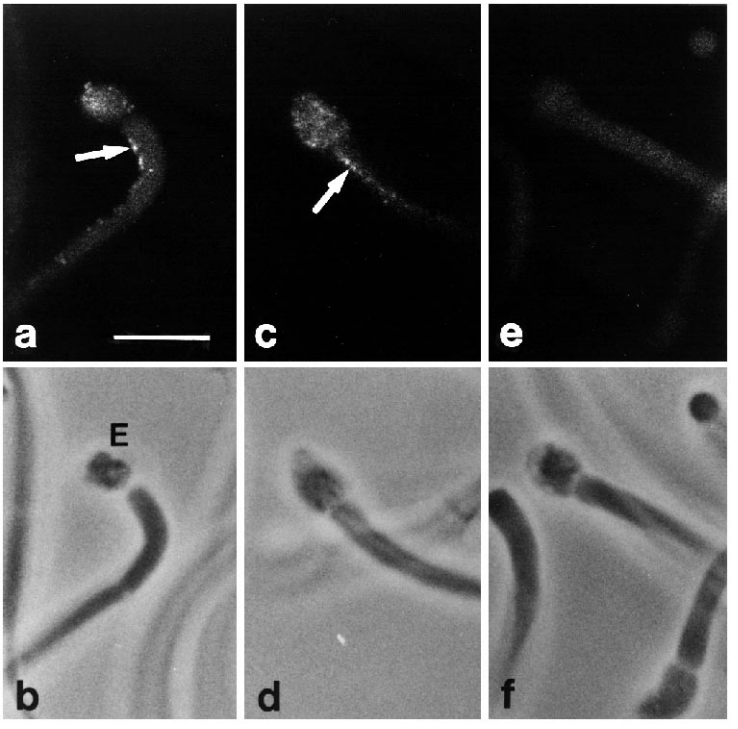

Fig. 4. Indirect immunofluorescence microscopy of green sunfish rod inner-outer segments fixed in formaldehyde and labelled with anti-Klp1Hd (a,c) or immuno-depleted anti-Klp1Hd (e). (b,d,f) Corresponding phase contrast images. Only RIS-ROS that had the myoid broken off from the ellipsoid (E) of the inner segment reacted to the Klp1Hd antibody. Punctate labelling of the outer segment axoneme (arrows) and the ellipsoid was evident in these broken cells (a,c); no labelling was evident in broken cells after the antibody was depleted of Klp1Hd reactivity (e). Bar, 5 μm.

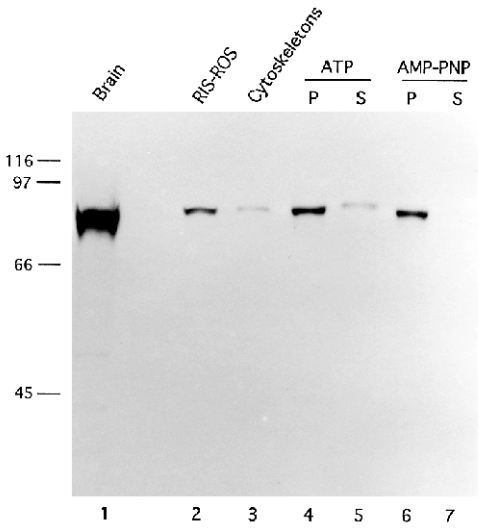

Fig. 5. Immunoblot analysis, using anti-KIF3A antibody, of mouse brain (lane 1; ~20 μg of brain homogenate), whole green sunfish rod inner-outer segments (lane 2; 2×10^6 RIS-ROS), RIS-ROS cytoskeletons (lane 3; Triton X-100 insoluble pellet from 2×10^6 RIS-ROS, plus 1 mM AMP-PNP), and nucleotide-treated RIS-ROS cytoskeletons (lanes 4-7; cytoskeletons from 10^7 RIS-ROS/lane). KIF3A ran as an 80/85 kDa doublet in mouse brain (lane 1). An ~85 kDa polypeptide was recognized in whole RIS/ROS (lane 2) and ~10% of the antigen was associated with the detergent insoluble cytoskeletons (lane 3). When RIS-ROS cytoskeletons were treated with 10 mM ATP, the KIF3A antigen was partially released to the supernatant (S; lane 5), whereas all of the antigen remained associated with the pellet (P) when cytoskeletons were alternatively treated with 1 mM AMP-PNP (lanes 6, 7). Molecular mass markers (in kilodaltons) are shown at left.

LAGSE antigen. To further confirm the identity of the 85 kDa KIF3A immunoreactive polypeptide, we probed western blots with K2.4, a monoclonal antibody specific to the 85 kDa subunit of KRP(85/95) the sea urchin homologue of KIF3A (Cole et al., 1993; Yamazaki et al., 1995): a single polypeptide of 85 kDa was detected by K2.4 on western blots of sunfish RIS-ROS (K.P.-R., unpublished observations), further indicating that an 85 kDa homologue of KIF3A is present in sunfish RIS-ROS.

To determine the subcellular distribution of the KIF3A antigen, we performed detergent extractions on whole RIS-ROS in the presence of 1 mM AMP-PNP. A characteristic of many KIFs is that they bind to microtubules in the absence of ATP or in the presence of non-hydrolyzable ATP analogs such as AMP-PNP, and that they are released in the presence of exogenous ATP (Brady, 1985; Vale et al., 1985). With our extraction procedure, any KIF3A antigen that is in direct association with microtubules of the RIS-ROS would be expected to pellet, in the presence of AMP-PNP, with the detergent insoluble cytoskeletons; these cytoskeletons contain microtubules of both the inner and outer segments (K.P.-R. and L. Lanier, unpublished observations; see also Pagh-Roehl et al., 1992). Only about 10% of the KIF3A antigen that was present in whole RIS-ROS was recovered in the cytoskeletal fraction (compare lanes 2 and 3 of Fig. 5), and the majority of the antigen was released to the supernatant (data not shown), indicating that most of the antigen is either soluble or bound to membrane and not directly associated with the cytoskeleton. The results of this experiment are in good agreement with data from other neuronal KIFs (e.g. see Hollenbeck, 1989; Pfister et al., 1989; Hirokawa et al., 1991), including KIF3A from mouse (Kondo et al., 1994), that have shown KIFs to be soluble or bound to membrane and only weakly associated with endogenous microtubules.

The nucleotide-dependent cytoskeletal-binding properties of the KIF3A antigen were tested further. The addition of 10 mM MgATP to RIS-ROS cytoskeletons released a low percentage of the 85 kDa polypeptide to the supernatant, whereas in the presence of AMP-PNP none of the antigen was released (Fig. 5, lanes 4-7). This ATP-dependent, partial release of the KIF3A antigen from RIS-ROS cytoskeletons (repeatable in three independent experiments) indicates that at least some of the antigen shares biochemical properties with KIFs and, since the 85 kDa polypeptide also has antigenic epitopes in common with and co-migrates with murine KIF3A, we believe it to be a homologue or close relative of KIF3A.

To determine the in situ distribution of the KIF3A antigen, whole RIS-ROS were processed for indirect immunofluorescence microscopy. RIS-ROS were fixed in either methanol or formaldehyde; KIF3A antigenicity was observed only in the methanol fixed material. Fig. 6 shows two examples of double labelling of RIS-ROS with anti-KIF3A (Fig. 6a,d) and anti-tubulin (Fig. 6b,e) antibodies. Anti-KIF3A labelling was strongest in the region of the axonemal basal bodies (large arrows, Fig. 6a,d). Distinctive labelling was also apparent in the part of the ciliary axoneme that is closest to the inner segment, that is, in the region of the connecting cilium (small arrows, Fig. 6a,d). The anti-KIF3A signal overlapped with the positions of microtubular bundles along the outer segment axoneme and in the basal portion of the ellipsoid and in the myoid (compare Fig. 6a with b). KIF3A labelling of the axonemes was discontinuous and usually stopped halfway along the outer segment, while tubulin staining continued to almost the distal tip. In some cases, connecting cilium staining was brighter than the adjacent (proximal) portions of the axoneme (Fig. 6a,d). Single labelling experiments with anti-KIF3A and with anti-β-tubulin resulted in the same patterns of antigenicity as those observed in the double labelling experiments (data not shown). After adsorption with KIF3A protein, the affinity purified antibody no longer stained the connecting cilium, axoneme and inner segment microtubular bundles; distinctive labelling of the basal body region was no longer apparent above background staining of the ellipsoid (data not shown).

We attempted to visualize KIF3A antigenicity at the electron microscope level by labelling thin-sections of RIS-ROS fixed in 0.5% glutaraldehyde and embedded in LR Gold resin; no labelling was observed (data not shown). Given that we also observed no reaction to anti-KIF3A, at the light microscope level, in RIS-ROS fixed in formaldehyde, we conclude that aldehydes mask the KIF3A epitopes in RIS-ROS.

DISCUSSION

Our observations indicate that several kinesin superfamily proteins (KIFs) are present in rod photoreceptor cells of the vertebrate retina. We detected putative KIFs in sunfish rod inner-outer segments using antibodies to the kinesin motor

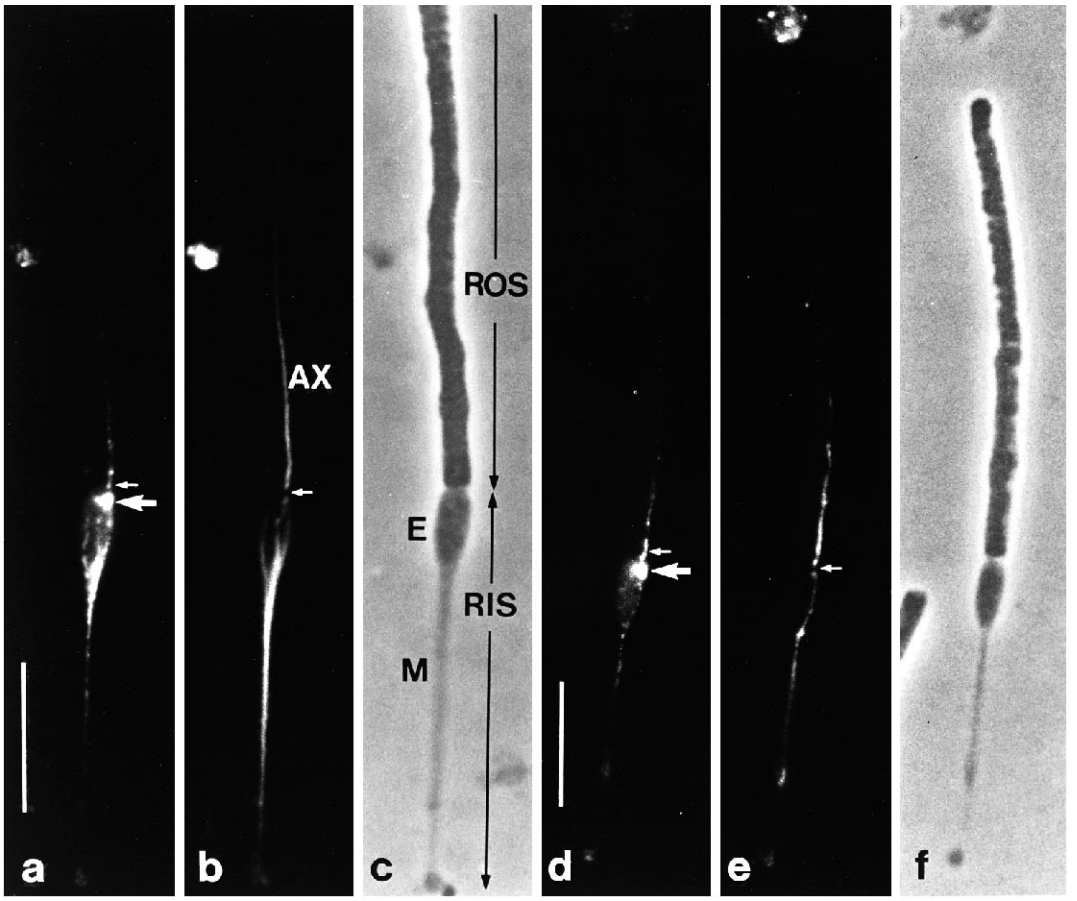

Fig. 6. Indirect immunofluorescence microscopy of green sunfish rod inner-outer segments fixed in methanol and double labelled with anti-KIF3A (a,d) and anti-β-tubulin (b,e) antibodies; (c,f) corresponding phase-contrast images. Anti-KIF3A reacted strongly with the regions of the basal bodies (large arrows: a,d) and connecting cilium (small arrows: a,d). KIF3A labelling was also visible along the more distal portions of the axoneme (AX) of the rod outer segment (ROS), and it extended from the base of the ellipsoid (E) into the myoid (M) of the rod inner segment (RIS). Note the overlap of the KIF3A and anti-β-tubulin signals in the ellipsoid and myoid. The small arrows in b and e mark the absence of labelling of the basal bodies with anti-β-tubulin, which is typical for this antibody (Pagh-Roehl et al., 1991). Bars: 10 μm.

domain: anti-LAGSE (Sawin et al., 1992) recognized a major polypeptide of ~117 kDa (which is likely to be kinesin heavy chain); anti-Klp1Hd, made to the conserved motor domain of *Chlamydomonas* flagellar Klp1 (Bernstein et al., 1994), detected a single band of ~170 kDa. Furthermore, an antibody specific to the neuronal, murine KIF, KIF3A (Kondo et al., 1994), recognized an 85 kDa polypeptide in sunfish RIS-ROS. The 85 kDa rod polypeptide is likely to be a homologue of KIF3A because, in addition to sharing antigenic epitopes with murine KIF3A, the polypeptide co-migrated with the upper band of the KIF3A doublet detected in brain and, like many KIFs, was partially extracted from the microtubular cytoskeleton by ATP, but not by AMP-PNP. We also note that an 85 kDa polypeptide, which co-migrated with the KIF3A immunoreactive band, is recognized by antibodies to the 85 kDa subunit of KRP(85/95), the sea urchin homologue of KIF3A.

Immunolocalization experiments indicate that the LAGSE, Klp1Hd and KIF3A antigens are found in microtubule-rich regions of RIS-ROS, and also that each of the antibodies give distinctive labelling patterns. The LAGSE antibody detected epitopes in the connecting cilium, which we showed by immunoelectron microscopy to be associated with the axonemal microtubular doublets, but, unlike that with anti-KIF3A (see below), labelling extended to the end of the outer segment and no localization around the basal bodies was observed. Both anti-LAGSE and anti-Klp1Hd antibodies labelled portions of the ROS axoneme but Klp1Hd antigenicity was not detected in the connecting cilium. The KIF3A antibody strongly labelled the basal body area and the connecting cilium; labelling was also observed along portions of the microtubular axoneme of the outer segment and along microtubular bundles in the ellipsoid of the inner segment. Detergent extractions of RIS-ROS in the presence of AMP-PNP suggested, however, that only a portion of the KIF3A antigen is bound to the RIS-ROS microtubules at any one time.

The presence of KIFs in rod photoreceptor cells is not surprising, since KIFs have been found in a wide variety of cell types, including neurons. Rods themselves are modified neurons, and KIF3A was first described as a neuronal KIF. However, our immunolocalizations of the KIF3A and LAGSE antigens to the region of the connecting cilium are provocative with respect to the possible mechanisms by which opsin, and other proteins, might be delivered to the ROS. Several authors have proposed that the connecting cilium is involved in the transport of opsin from its site of synthesis, in the RIS, to the ROS where it is incorporated into the photosensitive disks (e.g. Matsusaka, 1974; Rohlich, 1975; Besharse and Pfenninger, 1980). Opsin-containing vesicles accumulate at the distal end of the RIS near the base of the connecting cilium (Besharse and Pfenninger, 1980; Peters at al., 1983; Papermaster et al., 1985; Deretic and Papermaster, 1991) but reports of weak opsin immunoreactivity in the plasma membrane of the connecting cilium (Nir and Papermaster, 1983; Nir et al., 1984; Besharse et al., 1985) have been considered a major obstacle to accepting that opsin moves from the RIS to the ROS via the plasma membrane of the connecting cilium (Besharse, 1986; Besharse and Horst, 1990). However, strong opsin labelling of the con-

necting cilium is, in fact, apparent in recent confocal studies (Matsumoto and Hale, 1993). Further data consistent with opsin transport along the membrane of the connecting cilium have come from rapid-freeze, deep-etch studies on rat photoreceptors; these studies indicated that the ciliary plasma membrane has a density of opsin-like particles similar to that found on the plasma membrane of the ROS (Miyaguchi and Hashimoto, 1992). If opsin is translocated along the plasma membrane of the connecting cilium by one or more KIFs, then the microtubular doublets of the axoneme, which are the only microtubules in the connecting cilium (Besharse and Horst, 1990), would serve as tracks along which these molecular motors must move. In amphibian rod cells, each axonemal doublet microtubule is adjacent to a groove in the ciliary plasma membrane: this periciliary ridge complex is proposed to act as a channel through which opsin is delivered to the ROS (Peters et al., 1983). Miyaguchi and Hashimoto (1992) have noted similar infoldings which overlie microtubules in rod cells from rat. Our localizations of the KIF3A and LAGSE antigens to the region of the connecting cilium are consistent with a model in which one or more KIFs act to translocate membrane-bound opsin from the distal end of the RIS along the plasma membrane of the connecting cilium. Newly transported membrane-bound opsin would be incorporated into disks at the distal end of the connecting cilium, where disk morphogenesis takes place (Steinberg et al., 1980).

The reported anterograde motor activity (i.e. the movement of cargo toward the plus-end of microtubules) of KIF3A (Kondo et al., 1994), and the strong localization patterns of the KIF3A antigen reported here in rods, makes a homologue of this motor an especially good candidate for axonemal-associated transport from the basal body, or periciliary region, of the RIS to the ROS. The polarity of microtubules in the green sunfish axoneme and connecting cilium is such that, as in other cilia, the microtubule plus-ends are distal to the basal body (Troutt and Burnside, 1988; Fig. 1c). The strong labelling of the KIF3A antigen around rod basal bodies (i.e. at the minus end of the axonemal microtubules) may indicate that this is a site for the accumulation of motor protein in the RIS until it is needed for use in axonemal/ciliary transport. In this respect, it is worth noting that antibodies to Fla10, a KIF that is present in *Chlamydomonas* flagella and which has strong sequence homology to KIF3A (Walther et al., 1994, see below), also strongly label the area surrounding basal bodies in *Chlamydomonas* (P.L.B., unpublished observations).

Our localization of the KIF3A and LAGSE antigens to the ellipsoid and myoid suggest a function for KIFs in transport within the RIS. In these regions of the RIS, however, microtubule-plus ends are directed away from the basal bodies (Troutt and Burnside, 1988; Fig. 1c), suggesting that any microtubule-dependent translocation of material through the RIS, toward the base of connecting cilium, could not be due to an anterograde microtubule motor such as KIF3A or conventional kinesin. Consistent with this interpretation, transport of opsin through the ellipsoid to the periciliary region of the frog RIS is insensitive to microtubule depolymerizing agents (Vaughan et al., 1989). Therefore, localization of KIF3A and LAGSE antigens to the ellipsoid and myoid of the RIS may indicate a KIF-driven, microtubule-associated translocation of material away from the ROS, towards the rod synaptic terminal; such a role would be similar to the presumed role of KIF3A and kinesin in axonal transport (Kondo et al., 1994; Hirokawa et al., 1991; Vale et al., 1985).

Though kinesin superfamily proteins are well documented transporters of vesicles or membrane-bound organelles along microtubules, the present study has indicated that KIFs are associated with a part of the cell that is conspicuously devoid of vesicles, that is, with the connecting cilium, where KIFs could be utilizing axonemal microtubules for the transport of material along the ciliary plasma membrane. Other support for the hypothesis that KIFs power ciliary/flagellar membrane motilities comes from recent studies on *Chlamydomonas*, which have shown that intraflagellar transport, the bi-directional movement of granule-like particles beneath the flagellar membrane (Kozminski et al., 1993), is dependent on a flagellar KIF, the FLA10 gene product, which localizes between the axonemal outer doublet microtubules and the flagellar membrane (Kozminski et al., 1995). KIF3A has strong sequence homology with Fla10: the proteins are 64% identical in their motor domains and 38% identical over half their stalks (Walther et al., 1994). KIF3A and Fla10 are members of a group of KIFs that include heterotrimeric and heterodimeric kinesins (Cole and Scholey, 1995; Yamazaki et al., 1995); indeed, KIF3A and Fla10 may be representatives of a subgroup of KIFs that act in the translocation of particles or molecules along the flagellar or ciliary membrane. In this regard, it is intriguing that another relative of KIF3A, osm-3 (Tabish et al., 1995), has been shown to be critical to the function of sensory cilia in the nematode, *Caenorhabditis elegans*. Mutants defective for the osm-3 gene display aberrant chemosensory behaviour and lack the distal portion of the cilia of the lateral sensilla (Perkins at al., 1986). Thus, there is precedence from nematodes for the postulated role for KIF3A relatives in the morphogenesis and maintenance of the sensory cilia of photoreceptors.

In summary, we have shown by immunological methods that kinesin superfamily proteins are found in the rod inner-outer segments of fish photoreceptors and, in particular, that there are prominent localizations of KIF3A and LAGSE antigens to the connecting cilium, suggesting a role for these KIFs in transport between the RIS and the ROS. Characterization of the function of KIFs in rod photoreceptors awaits the isolation of genes or probes specific for the motors in these cell types, and the identification of the cargo carried by these motors.

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