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Identification of Novel Molecular Components of the Photoreceptor Connecting Cilium by Immunoscreens

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The connecting cilium of photoreceptor cells is the only intracellular link between the morphologically, functionally and biochemically different compartments of the inner and outer segments. The non-motile modified cilium plays an important role in the organization and the function of photoreceptor cells, namely in delivery and turnover of enzymes and substrates of the visual transduction cascade, and the photosensitive membranes of the outer segment. The protein components of the cilium participate in the intracellular transport through the cilium, in the outer segment disk morphogenesis and in the maintenance of discrete membrane domains.

In order to identify yet unknown cytoskeletal components of the connecting cilium, a combined biochemical and molecular biological strategy was applied. For this purpose, antibodies were raised against proteins of photoreceptor cell axonemes. Using this AX-4-antiserum, a rat retina cDNA expression library was immunoscreened and clones encoding partial sequences of (i) already known photoreceptor specific proteins; (ii) ubiquitously expressed proteins; (iii) clones with homologies to retinal ESTs; and (iv) clones coding for cytoskeletal proteins were isolated. Further analysis revealed that these candidate clones have homologies to *Drosophila* flightless I, mouse APC-binding protein EB2, human microtubule associated protein 4 (MAP4), human centrin 3, human cytoplasmic dynein intermediate chain 2C, and human dynamitin.

The immunoscreening approach used here was successfully applied to isolate genes encoding yet unknown cytoskeletal proteins of photoreceptor cell axonemes. The obtained information will provide further insight into the role of the connecting cilium in photoreceptor cell function.

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Key words: retina; photoreceptors; cytoskeleton; cilium; connecting cilium; immunoscreen; flightless; EB-proteins.

1. Introduction

Vertebrate photoreceptor cells are highly polarized sensory neurons with distinct functional compartments: a photosensitive outer segment, a metabolically active inner segment, a cell body and a synaptic terminal (Fig. 1). The rod photoreceptor outer segment contains hundreds of membranous disks with the visual pigment opsin, and the other proteins of the visual transduction cascade. A thin cellular bridge, the so-called connecting cilium, serves to join the photoreceptor outer segment with the inner segment which contains the protein synthesis machinery. The cell body with the nucleus is connected to the synaptic terminal which is involved in neurotransmission.

The photosensitive outer segment membranes are continually turned over. New disks are formed at the base of the outer segment whereas the distal tips are shed and subsequently phagocytized by the cells of the retinal pigment epithelium (Young, 1976; Steinberg, Fischer and Anderson, 1980; Usukura and Obata, 1995). This turnover requires de novo synthesis of outer segment proteins in the proximal subcompartment of the metabolically active inner segment, followed by delivery of these proteins by intracellular transport through the connecting cilium to the base of the outer segment (Brown, Blazynski and Cohen, 1987; Philp, Chang and Long, 1987; Besharse and Horst, 1990; Fariss et al., 1997; Liu et al., 1999; Wolfrum and Schmitt, 1999, 2000; Marszalek et al., 2000). Furthermore, protein components of the connecting cilium participate in the outer segment disk morphogenesis (Arikawa and Williams, 1989; Williams, Hallett and Arikawa, 1992) and in the maintenance of discrete membrane domains (Horst, Forestner and Besharse, 1987; Besharse and Horst, 1990; Horst, Johnson and Besharse, 1990).

The non-motile photoreceptor cell connecting cilium is homologous to the transistion zone of motile cilia, a very short proximal compartment between the basal body and the axoneme (Röhlich, 1975; Besharse and Horst, 1990). In mammals, the connecting cilium measures about 1 μm in longitudinal extension and $< 0{\cdot}2$ μm in diameter. The microtubule-based axoneme of the connecting cilium has a $9\times2+0$-structure and lacks the central microtubule pair, dynein arms and accessory structures of

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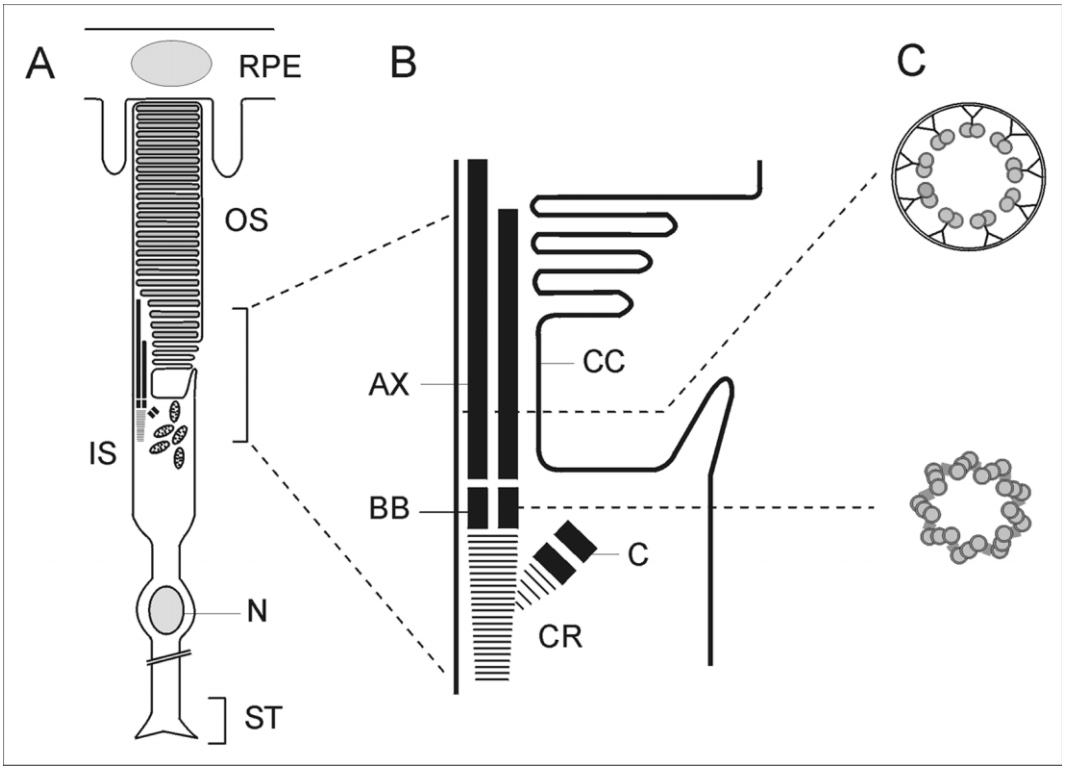

FIG. 1. Diagram illustration of a rod photoreceptor cell and its cilliary apparatus. (A) Rod photoreceptor cells consist of an outer segment (OS), linked to the inner segment via the connecting cilium (CC), the cell body containing the nucleus (N), the axon and synaptic terminal (ST). RPE: retinal pigment epithelium. (B) Detail of the ciliary apparatus of photoreceptor cells. The ciliary apparatus consists of the microtubule based axoneme (AX), and the basal body–centriole complex with a ciliary rootlet (CR) which projects into the inner segment. BB: basal body, C: centriole. (C) Scheme of a cross section of a basal body and the ciliary axoneme. The basal body shows the characteristic 9×3-structure, the modified cilium a $9\times2+0$-structure.

common motile cilia (Röhlich, 1975). Photoreceptor cilia arise from basal bodies which contain centrioles and act as microtubule organizing centers (MTOC) of the photoreceptor cell (Troutt and Burnside, 1988; Muresan, Joshi and Besharse, 1993; Wolfrum and Salisbury, in preparation). Together with the ciliary rootlets, these components comprise the ciliary apparatus of photoreceptor cells (Fig. 1).

Although many research efforts have been focused on the structural composition of the connecting cilium, very few components have been identified in recent years. The prominent microtubule cytoskeleton of the cilium is composed of conventional and post-translational tubulins including γ-tubulin at the basal body complex (Sale, Besharse and Piperno, 1987; Horst et al., 1990; Arikawa and Williams, 1993; Muresan et al., 1993). More recently, microtubule-associated motor proteins, e.g. kinesin II, have been identified and suggested to play a role in anterograde transport through the photoreceptor connecting cilium (Beech et al., 1996, Muresan, Lyass and Schnapp, 1999; Marszalek et al., 2000). Furthermore, a retinitis pigmentosa GTPase regulator (RPGR) and its binding partner (Hong et al., 2000, 2001), a nucleolin-related protein, and a casein kinase II (Hollander, Liang and Besharse, 1999) have been identified as ciliary components. In addition, two unconventional cytoskeletal proteins, namely centrin (Wolfrum, 1995; Wolfrum and Salisbury, 1998) and myocilin (Kubota et al., 1997), components of the actin cytoskeleton have been identified in the photoreceptor connecting cilium. Actin filaments are localized in the distal domain of the connecting cilium at the base of the outer segment (e.g. Chaitin et al., 1984; Chaitin and Bok, 1986; Arikawa and Williams, 1989; Chaitin and Burnside, 1989; Obata and Usukura, 1992; Usukura and Obata, 1995; Watanabe et al., 1999). In this ciliary domain, the actin filaments are linked via the actin-bundling protein α-actinin, and may interact with myosin II during the formation of new outer segment disks (Arikawa and Williams, 1989; Chaitin and Coelho, 1992; Williams et al., 1992). Furthermore, actin has been recently detected at the membrane of the entire connecting cilium (Wolfrum and Schmitt, 1999, 2000). This may provide the structural basis for the motility of the membrane-associated motor protein myosin VIIa (Liu et al., 1997), an unconventional class VII myosin which is the product of the Usher syndrome 1B gene (Weil et al., 1995). The Usher syndrome is the most common hereditary disorder of combined blindness and hearing loss (Usher, 1913/14) causing in some cases phenotypic structural changes in cilia (Arden and Fox, 1979; Hunter et al., 1986; Barrong et al., 1992). Recent results indicate that myosin VIIa participates in the active transport of the visual

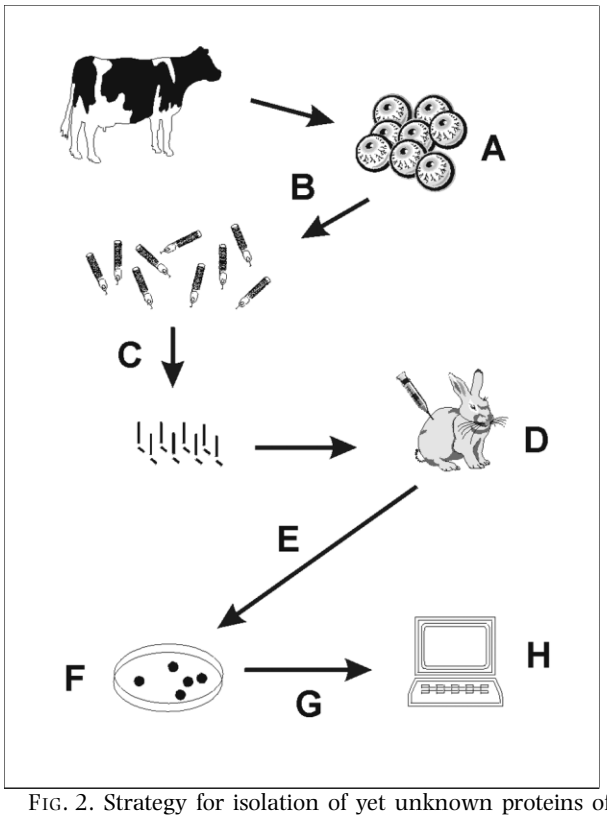

FIG. 2. Strategy for isolation of yet unknown proteins of the connecting cilium of photoreceptors. After isolation of the retinas out of bovine eyes (A), photoreceptor cell inner segments–outer segments (RIS–ROS) were purified using sucrose density gradient centrifugations (B). For cytoskeleton extraction, RIS–ROS were resuspended in cytoskeleton extraction buffer and purified using discontinuous sucrose step gradient centrifugations (C). Isolated photoreceptor cell axonemes were used for immunization of a rabbit (D). The obtained polyclonal AX-4 antiserum was purified on Protein A agarose (E) and applied for immunoscreening a rat retina cDNA expression library (F). The 5′ and 3′ termini of the isolated clones were sequenced (G) and analyzed using BLAST and FASTA.

pigment rhodopsin through the connecting cilium, and defective ciliary rhodopsin transport may cause the retinal degeneration found in Usher patients (Liu et al., 1999; Wolfrum and Schmitt, 1999, 2000).

In view of the missing information on the molecular composition of the ciliary apparatus of photoreceptor cells, a combined biochemical and molecular biological method was applied. For this purpose, bovine photoreceptor cell axonemes were isolated and this cytoskeletal fraction was used for immunization of a rabbit. The obtained antiserum with antibodies specific for axonemal proteins was used to screen a rat retina expression library (Fig. 2). This resulted in the isolation of several candidate clones potentially encoding yet unknown proteins.

2. Materials and Methods

Animals and Tissue Preparation

All experiments described herein conform to the statement by the Association for Research in Vision and Ophthalmology as to care and use of animals in research. Adult Sprague–Dawley albino rats and C57BL76 mice were maintained on a 12/12 L/D cycle, food and food and water ad libidum. After the animals were killed with CO_2, retinas were removed through a slit in the cornea, fixed, and embedded for cryosection. Fresh bovine eyes were obtained from the local slaughterhouse.

Isolation of Retinal Rod Outer Segments and Extraction of the Ciliary Apparatus

Bovine eyes were dark adapted and photoreceptor inner segment–outer segments (RIS–ROS) purified from isolated retinas using sucrose density gradients in combination with cytoskeleton extraction at 4°C under dim red light (Fleischmann et al., 1980; Horst et al., 1987; Pagh-Roehl and Burnside, 1995). Bovine retinas were isolated and suspended in HERT buffer (4 mM $NaHCO_3$, 0·18 mM sucrose, 2·1 mM HEPES, 0·1 mM ascorbic acid, 0·5 mM taurine, 1× Earles balanced salt solution (EBSS, Sigma, Deisenhofen, Germany) pH 7·2, with 0·1 mM phenylmethylsulfonyl fluoride (PMSF) containing 50 % sucrose (w/v). RIS–ROS were broken off by gentle shaking for 1 min, filtered and enriched by centrifugation (1 hr at 31 000 ***g***, Sorvall ultracentrifuge RPE) on a 50 % sucrose cushion in HERT buffer (w/v). RIS–ROS were collected from the top, injected at the bottom of a 25–50 % continuous sucrose gradient (w/v) and centrifuged (2 hr at 31 000 ***g***). RIS–ROS were collected from the top half of the gradient and enriched on a 50 % sucrose cushion in HERT buffer (w/v) and sedimented by decreasing the sucrose concentration. The purification procedure of RIS–ROS was monitored by counting the RIS–ROS and the other retinal cells seen with each probe. 4 × 10 μl from each of the four samples were embedded for microscopic analysis and the mean number of RIS–ROS and other retinal cells of 500 lM^2 were determined. For cytoskeleton extraction, pelleted RIS–ROS were resuspended in cytoskeleton extraction buffer (100 mM Hepes, 10 mM $MgSO_4$, 10 mM EGTA, 100 mM KCl, 5 % dimethylsulfoxide (DMSO), 20 mM DTT, 0·2 mM GTP, 25 μg phalloidin, 0·04 mM taxol, 2 % Triton X-100 adjusted to pH 7·5, containing 0·24 μM leupeptin, 0·42 μM pepstatin A, 0·2 μM aprotinin) and extracted for 1 hr. Detergent soluble material and axonema were separated by centrifugation (3 hr at 31 000 ***g***) on a discontinuous sucrose step gradient composed of 5 ml each 40, 50 and 60 % (w/v) sucrose in CMOD buffer (100 mM Hepes, 10 mM $MgSO_4$, 10 mM EGTA and 100 mM KCl, pH 7·5). The axoneme-enriched fraction was collected at the 50 %/60 % sucrose interface and fractions containing axonemes were identified by optic microscopic analysis using indirect anti-centrin immunofluorescence labeling and by Western blot analysis.

Antibodies

The following antibodies against centrin were used as molecular markers for the connecting cilium (Wolfrum, 1995; Liu et al., 1997; Wolfrum and Salisbury, 1998). Mouse monoclonal antibody (MAb) clone 20H5 (e.g. Wolfrum and Salisbury, 1998) and the polyclonal antibody raised against mouse centrin 1 (Schmitt et al., in preparation). MAbs against calmodulin (clone 6D4), α-tubulin (clone DM 1A), and acetylated α-tubulin (clone 6-11B-1) were purchased from Sigma (Deisenhofen, Germany). MAb anti-arrestin antibodies (clone 3D1.2) were used, previously characterized by Nork et al. (1993). MAbs against bovine rod opsin were B6-30a1, K16-155, R2-15 which were previously described by Adamus et al. (1988). Polyclonal antibodies against γ-tubulin were kindly provided by Dr Joshi, Atlanta, U.S.A. PAb 2·2 anti-myosin VIIa were generated and purified as described by Liu et al. (1997). The MAb against chicken gizzard actin (clone C4) has been previously characterized (Lessard, 1988, Wolfrum and Schmitt, 2000). The MAb against an epitope conserved in EB1 and EB2 was purchased from Transduction Laboratories, (Santa Cruz, CA, U.S.A.). The generation of the polyclonal antibody raised against recombinant expressed rat flightless will be described elsewhere (Schmitt and Wolfrum, in preparation).

AX-4-antibody Generation

The polyclonal antiserum AX-4 against ciliary proteins was generated by injecting a rabbit four times with 250 μg protein of isolated photoreceptor cell axonemes taken from about 200 bovine retinas according to standard immunization protocols (Harlow and Lane, 1988). The obtained antiserum was purified using protein A agarose columns (Biorad Life Technologies, Munich, Germany) as described by Harlow and Lane (1988). The pre-immunoserum and the antiserum were tested by immunocytochemistry and Western blot analysis.

Immunoscreening

The screening of an oligo(dT)-primed rat retina γZAP II cDNA expression library with antibodies against ciliary proteins was performed according to Sambrook, Fritsch and Maniatis (1989). Expression of recombinant proteins was induced by applying nitrocellulose filters pre-incubated with 1 mM-β-D-thiogalactopyranoside (IPTG, AGS, Heidelberg, Germany) 4–5 hr after plating the phages. After additional incubation for 4–6 hr at 37°C, the filters were carefully removed from the plates, washed in Tris buffered saline (TBS, 20 mM Tris–HCl pH 7·5, 50 mM NaCl), blocked for 2 hr in 3 % bovine serum albumin (Sigma) in TBS, and incubated overnight in the same blocking solution containing 1/500 volume AX-4 antiserum. Binding of primary antibodies was detected by using alkaline phosphatase conjugated anti-rabbit antibodies (Sigma), and nitroblue tetrazolium-X-phosphate as a chromogene (Sigma). Positive clones were re-screened and plasmid DNA obtained by in vivo excision. The 5′- and 3′-terminal regions of the isolated cDNA clones were sequenced using the Cycle Sequencing Thermosequenase Kit and the automatic DNA sequencer Alfexpress (Amersham Pharmacia Biotech, Munich, Germany). Obtained cDNA-sequences were analyzed using BLAST (Altschul et al., 1997) and FASTA (Pearson and Lipman, 1988). cDNA inserts of clones encoding already known proteins were used to prepare in vitro transcribed digoxigenin labeled cDNA probes with the DIG DNA Labelling Mix (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Immunocytochemistry

Cryosections of isolated retinas of Sprague–Dawley rats were prepared as described previously (Wolfrum, 1995) and incubated with 0·01 % Tween 20 in phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7·2), washed in PBS and blocked in PBS with 0·5 % cold water fish gelatin (Sigma) plus 0·1 % ovalbumin (Sigma, blocking buffer). The sections were incubated in the primary antibody overnight at 4°C, washed in PBS and incubated with secondary antibodies conjugated to fluorescein or rhodamine (Cappel Oreganon Teknika Corp., Durham, NC, U.S.A.) diluted in blocking buffer for 1 hr in the dark. After washing, sections were mounted in Mowiol 4·88 (Farbwerke Hoechst, Frankfurt, Germany) containing 2 % *n*-propyl-gallate (Sigma).

SDS–PAGE and Western Blot Analysis

Isolated bovine RIS–ROS, bovine photoreceptor cell axonemes, and rat retinas were homogenized and placed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (62·5 mM Tris–HCl pH 6·8, 10 % glycerol, 2 % SDS, 5 % β-mercaptoethanol, 1 mM EDTA, 0·025 % bromphenol blue). Proteins were separated by SDS–PAGE according to Laemmli (1970) on 8–20 % gradient gels, electrophoretically transferred with a semi-dry blotter (Biorad Life Technologies, Munich, Germany) to a polyvinylidene fluoride (PVDF) membrane (Biorad Life Technologies, Munich, Germany), and probed with primary and secondary antibodies. The latter were conjugated to alkaline phosphatase, so that labeling was detected by the formation of the insoluble product of 5-bromo-4-chloroindoyl phosphate hydrolysis.

3. Results

Purification of Photoreceptor Cell Axonemes

Bovine photoreceptor RIS–ROS were isolated using sucrose density gradient centrifugations according to Fleischmann et al. (1980) and Pagh-Roehl and Burnside (1995). The contents of photoreceptor cell RIS–ROS in all four isolation steps were monitored via optical microscopy [Fig. 3(A)]. Fig. 3(B) demonstrates the purification of RIS–ROS with respect to other retinal cells: the number of isolated RIS–ROS were counted in the four samples obtained during the purification process with respect to other retinal cells [Fig. 3(B)]. The applied method shows a significant enrichment of RIS–ROS, but in spite of several purification steps, small amounts of retinal contaminants were present in the final probe (Papermaster and Dreyer, 1974; Pagh-Roehl and Burnside, 1995).

The RIS–ROS were resuspended in cytoskeleton extraction buffer and loaded on a sucrose density step gradient to separate the axonemal fraction from soluble material (Fleischmann et al., 1980; Horst et al., 1987; Pagh-Roehl and Burnside, 1995). The photoreceptor cell axoneme containing fractions were identified by indirect immunofluorescence and Western blot analysis using antibodies against centrin (Fig. 4), a well accepted molecular marker for the connecting cilium of vertebrate photoreceptor cells (Wolfrum, 1995; Liu et al., 1997; Wolfrum and Salisbury, 1998). Western blot analysis shows, that centrin was present in nearly all fractions but significantly enriched in the fractions 3–5 and 15–17 [Fig. 4(A)]. Immunocytochemistry revealed the accumulation of centrioles in the gradient fractions 3–5, and of photoreceptor cell axonemes in the fractions 15–17 [Fig. 4(A) and (B)]. As expected from previous observations by Paoletti et al. (1996) centrin was also found as a soluble protein in other fractions of the gradient.

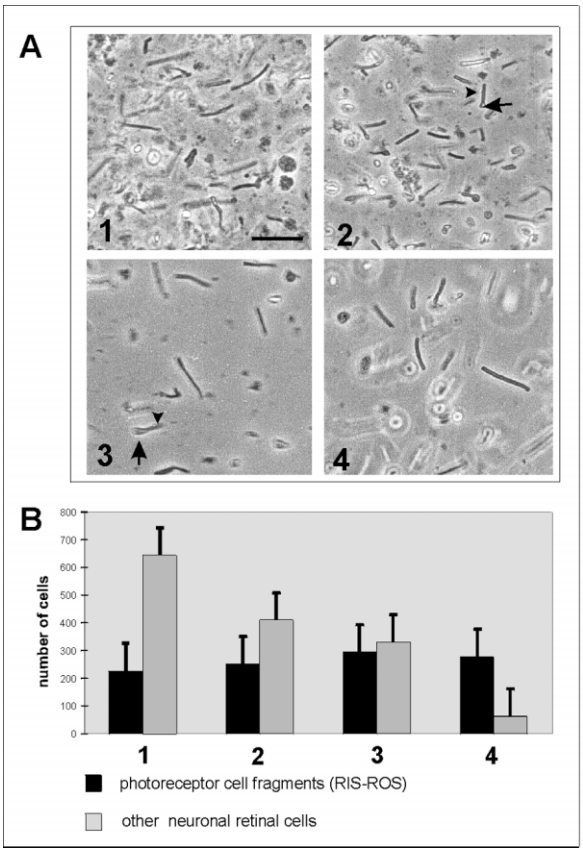

FIG. 3. Purification of photoreceptors RIS-ROS. (A) Retinas were suspended in HERT, filtrated (1), and purified using sucrose density centrifugations (2)–(4). Arrow: photoreceptor cell inner segment; arrowhead: photoreceptor cell outer segment. (B) The purification process was monitored via counting photoreceptor RIS–ROS and other retinal cells of the four purification steps. Histograms indicate the mean number of RIS–ROS and retinal contaminants per 500 μM^2, error bars indicate standard error of the mean. The amount of photoreceptor cell RIS–ROS was nearly stable during the purification process, but the number of retinal cells decreased. But even in the last probe (4), a small portion of retinal contaminant was still present.

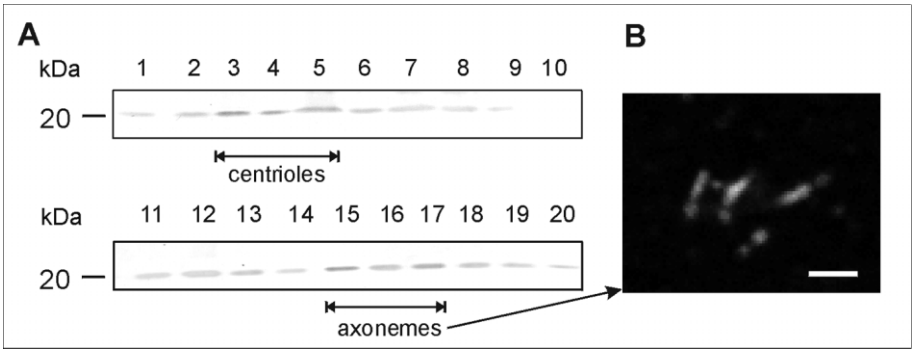

FIG. 4. Western blot analysis and direct immunocytochemistry with anti-centrin antibodies for the identification of gradient fractions containing photoreceptor cell connecting cilia. (A) The 20 fractions obtained from the axoneme fractionation using a discontinuous sucrose gradient were analyzed by Western blots with anti-centrin antibodies (MAb 20H5). Centrin is an unconventional cytoskeletal protein enriched in photoreceptor cell axonemes and commonly used as a molecular marker of photoreceptor cilia (cf. Wolfrum, 1995; Liu et al., 1997; Wolfrum and Salisbury, 1998). Centrin is enriched in the fractions 3–5 based on centriole-sedimentation, and in the fractions 15–17 based on axoneme sedimentation, but also present at lower concentrations in other fractions as a soluble protein. (B) Indirect anti-centrin immunolabeling of isolated bovine photoreceptor cells axonemes present in gradient fraction 16. Bar = 6 μM.

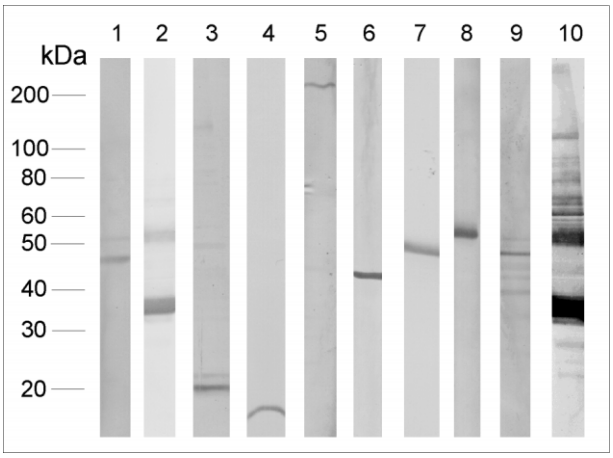

FIG. 5. Western blot analysis of isolated photoreceptor cell axonemes with antibodies against photoreceptor specific proteins and cytoskeletal proteins. For each lane, 20 μg of photoreceptor cell axonemes were separated by SDS–PAGE in a 8–20 % gradient gel and transferred onto PVDF membrane for immunological detection with antibodies specific for arrestin (1), opsin (2), centrin (3), calmodulin (4), myosin VIIa (5), actin (6), α-tubulin (7), acetylated α-tubulin (8), γ-tubulin (9), and the polyclonal AX-4 antiserum (10).

The axoneme fractions were also analyzed with antibodies against several proteins known to be structural components of the cilium, and with probes for photoreceptor specific proteins (Fig. 5). The Western blot analysis revealed that common elements of the ciliary apparatus of vertebrate photoreceptor cells, namely α-tubulin, acetylated α-tubulin, γ-tubulin, actin, myosin VIIa and centrin were present in the fractions of purified photoreceptor cell axonemes. Photoreceptor specific proteins including opsin and arrestin were also identified in these fractions.

Specificity of the Antiserum AX-4 against Photoreceptor Cell Axonemes

The AX-4 antiserum was generated by immunization of a rabbit with purified photoreceptor cell axonemes isolated from 200 bovine eyes (see Fig. 2). Cross-reactivity of the affinity purified AX-4 antiserum was investigated via immunocytochemistry of longitudinal cryosections through a rat retina and Western blot analysis of bovine photoreceptor axonemal proteins. AX-4 immunolabeling was restricted to the photoreceptor cell layer of the rat retina (Fig. 6). Most intense indirect immunofluorescence of AX-4 was observed in the connecting cilium and basal body–centriole complex of the photoreceptor cells. Weak AX-4 immunostaining was also present in photoreceptor outer segments (Fig. 6). As expected, in Western blot analysis of axonemal proteins of bovine photoreceptor cells, AX-4 detected several protein bands from 20 to about 200 kDa (lane 10 in Fig. 5). Immunolabeling was also observed on Western blots of purified photoreceptor RIS–ROS fragments and of the entire neuronal retina. However, in these experiments, the immunoreaction was too intense for the discrimination of individual protein bands, even when probed with the highest antiserum dilutions (antibody dilution 1 : 200 000; data not shown). No reaction to pre-immunoserum was observed in any of the control experiments.

Immunoscreening and cDNA Sequence Analysis

The obtained AX-4 antiserum with antibodies specifically directed against proteins present in the photoreceptor cell axonemes, was used for immunoscreening an oligo(dT)-primed rat retina cDNA expression library. Out of 240 000 clones of the library screened, antibodies of the AX-4 antiserum reacted with expressed polypeptides of 257 clones. In a first round of analysis, 20 clones were purified by individually rescreening. The 5′- and 3′-terminal nucleotide sequences of each clone was determined by cDNA sequencing. In the following 12 rounds of analysis, the number of redundant clones was reduced by applying a dot blot analysis with digoxigenin (DIG) labeled cDNA probes to identify clones which code for duplicates or for already known proteins of photoreceptor cells (163 AX-4 positive clones). Ninety-four dot blot negative clones were sequenced and further analyzed. Out of these negative clones, 38 do not show any homology to sequences present in the DNA databases. BLAST homology analysis using the NCBI server (Altschul et al., 1997) revealed that 56 of the isolated clones had identities to sequences present in the database, which can be grouped into four categories of clones with homologies to: (i) photoreceptor specific proteins; (ii) ubiquitously expressed proteins; (iii) ESTs (expressed sequence tags); and (iv) cytoskeletal and cytoskeleton-associated proteins (Table I).

The clones encoding cytoskeletal proteins (category iv) were further analyzed by determining open reading frames (ORF), and the partial protein sequences of the nine clones encoding cytoskeletal proteins were used to search the public databases at NCBI (BLASTP) and EMBL (FASTA) for homologous proteins. These analyses revealed that clone AS85 encodes a protein binding single strand RNA (98 % identity to a human homologue), AS97 encodes a homologue of *R. norvegicus* HSP90 protein (94 % identity); the clones AS118 and AS189 code for the microtubule-associated proteins EB2 and MAP4 (86 and 89 % identity); clone AS128 and clone AS42 showed identities to an actin-binding protein (86 %), the human homologue of *Drosophila flightless I*; clone AS129 corresponds to a subunit of the dynactin complex, dynamitin (78 % identity), interacting with cytoplasmic dynein; and clone AS206 was homologous to human cytoplasmic dynein intermediate chain 2C (97 % identity). Furthermore, clone AS11 encodes centrin 3 (96 % identity) (see Table I).

TABLE I

cDNA clones isolated by immunoscreening a rat retina cDNA expression library with the AX4-antiserum

Clone	cDNA size (kb)	Identity to	Genebank accession	Identity (%)	Sequenced (bp)	Overlap (bp)	Organism
Category (i) clones with homology to photoreceptor specific proteins							
AS1, AS4, AS5,	1·4	arrestin/S-antigen	X51781	92	775	380	*R. norvegicus*
AS15, AS32, AS33, AS35, AS39, AS54, AS60, AS36	1·0	rod opsin	RN22180, M55171	95	284	280	*R. norvegicus*
AS56, AS88, AS99	1·5	rod type cGMP-gated cation channel	U93851	97	701	696	*R. norvegicus*
AS115, AS103	3·4	cGMP-gated cation channel	U76220	97	668	534	*R. norvegicus*
AS62	1·0	brain calcium channel	X67655	66	547	112	*O. cuniculus*
AS204	0·9	proton pump polypeptide mRNA	RATPRPU	90	588	545	*R. norvegicus*
AS185	1·0	rod transducin	X63449	70	380	311	*H. sapiens*
Category (ii) ubiquitously expressed proteins							
AS91	1·6	cytochrome C oxidase subunit I	M27315	98	394	387	*R. norvegicus*
AS120, AS180	1·7	cytochrome C oxidase subunit I	S79304	99	237	237	*R. norvegicus*
AS55	1·2	cytosolic aspartat aminotransferase	RNCASPAT	97	599	580	*R. norvegicus*
AS181, AS216	1·4	thioredoxin reductase	RNU73525	95	513	490	*R. norvegicus*
AS93	1·1	serine palmytoyl transferase	X95642	92	342	277	*M. musculus*
AS95	3·2	receptor for hyaluron-mediated transport motility	U87983	92	953	820	*R. norvegicus*
AS89	0·5	calcineurin-B like	C06646	97	460	233	*R. norvegicus*
AS90	1·5	ornithine decarboxylase	D10706	94	764	541	*R. norvegicus*
Category (iii) clones with homologies to ESTs							
AS37	0·8	vh92g03.r1 Knowles solter mouse	MM1311253	57	685	147	*M. musculus*
AS38	1·6	vo69d07.r1 Barstedt mouse	Aa615385	85	817	318	*M. musculus*
AS48	0·3	EST clone	AI030636	98	300	298	*R. norvegicus*
AS58	0·7	zv10d11.r1 soares mouse NhmPuSI	HS1224549	95	650	346	*M. musculus*
AS200	1·0	mRNA for KIAA0266 gene	D87455	68	998	125	*H. sapiens*
AS201	0·7	PPEF-1	AF023456	97	755	273	*H. sapiens*
AS208	1·7	Aa964506 UI-R-CO-gv-e-11-O-UI.Sl UI-R cDNA	AA964506	99	338	273	*R. norvegicus*
AS210	0·9	mx02a05.r1 Soares mouse NM	Aa244510	78	391	390	*M. musculus*
AS213	1·5	vk72h12.sl Knowles Solter mouse EST	Aa547107	76	432	357	
AS87	0·8	yr92a05.s1 cDNA	HS656221	71	594	188	*H. sapiens*
AS94	1·5	EST clone	X92219	43	290	222	*H. sapiens*
AS92	1·2	EST cDNA clone	T84255	52	438	172	*H. sapiens*
AS50	1·2	vi29g10.r1 Barstedt mouse	MM1313496	83	1194	604	*M. musculus*
AS61	1·5	vt52h03.r1 Barstedt mouse	Aa5711009	80	750	423	*M. musculus*
AS62	0·9	brain calcium channel	X67855	89	841	376	*M. musculus*
AS142	3·1	Vf1e03.r1 Soares mouse Nb	Aa420167	85	780	499	*M. musculus*
AS86	2·7	EST cDNA clone	MM1276673	98	706	185	*M. musculus*
AS125	1·8	EST cDNA clone	MM1260536	40	998	245	*M. musculus*
Category (vi) clones with homologies to cytoskeletal and cytoskeleton associated proteins							
AS11	0·9	centrin 3	NM 007684.1	96	335	340	*M. musculus*
AS189	0·8	microtubule-associated protein 4 (MAP4)	M72414	89	376	336	*M. musculus*
AS97	2·6	HSP90	S45392	94	818	814	*R. norvegicus*
AS118	0·4	microtubule-interacting protein EB2	U51204	86	284	269	*M. musculus*
AS42, AS128	1·2	homologue of *Drosophila* flightless I	U01182	86	1200	1188	*H. sapiens*
AS129	0·3	dynamitin	U50733	78	291	156	*H. sapiens*
AS85	0·8	ss-RNA-binding protein	F2541_2	98	781	400	*H. sapiens*
AS206	0·9	cytoplasmic dynein intermediate chain 2C	RNU39046	97	624	70	*H. sapiens*
AS100	1·2	ss-RNA-binding protein	F25451_2	95	980	831	*H. sapiens*

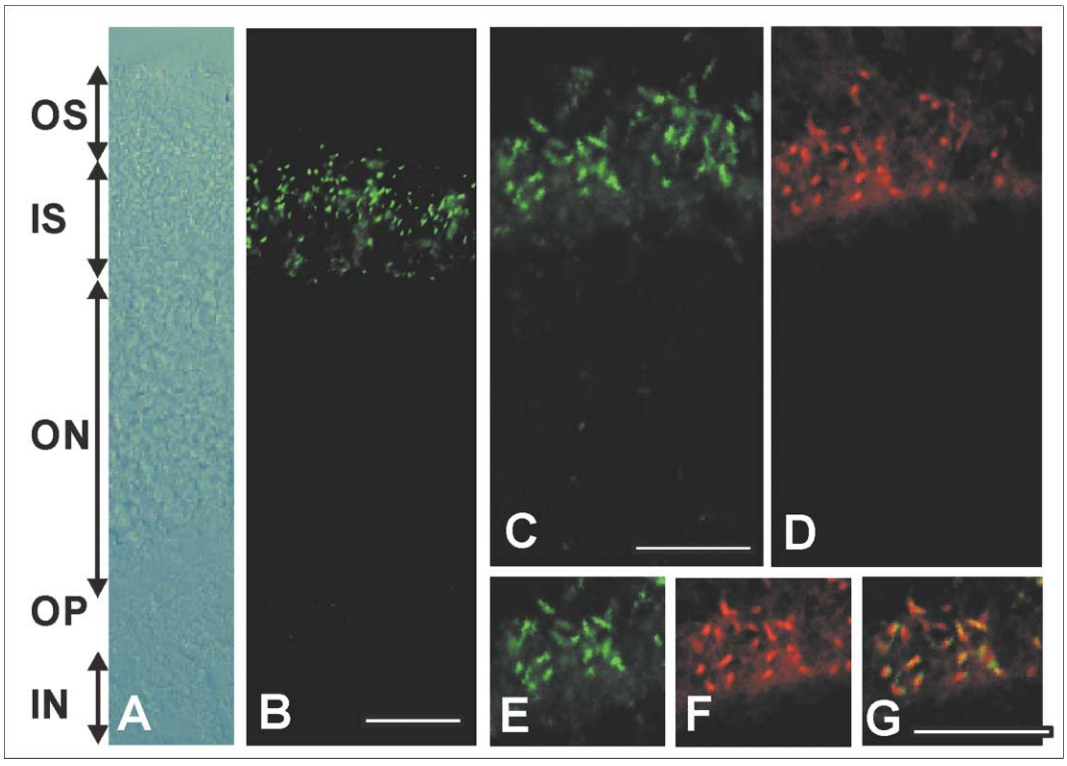

FIG. 6. AX-4 antiserum and centrin double-immunofluorescence labeling of a longitudinal cryosection through the rat retina. (A) Differential interference contrast (DIC). (B) Indirect FITC-immunofluorescence of centrin in a rat retina. Anti-centrin staining is restricted to the connecting cilium and the basal body-centriole complex of photoreceptor cells. (C) and (D) Double-immunofluorescence labeling of centrin (C) and AX-4 (D) in the photoreceptor layer of a rat retina. (C) Indirect FITC-immunofluorescence of centrin. (D) Indirect rhodamin immunofluorescence of AX-4 antiserum. Anti-AX-4 immunofluorescence co-localizes with centrin in the connecting cilium and basal body-centriole complex. (E) and (F) Fluorescence double-labeling of the ciliary apparatus of photoreceptor cells at a higher magnification. (E) Immunolabeling with MAb 20H5 (anti-centrin) used as a specific marker for the ciliary apparatus. (F) Immunolabeling with the AX-4 antiserum. The antibodies in the AX-4 antiserum react with epitopes of proteins in the ciliary apparatus of photoreceptor cells shown by the overlay of (E)–(G) Bar = 6 μm.

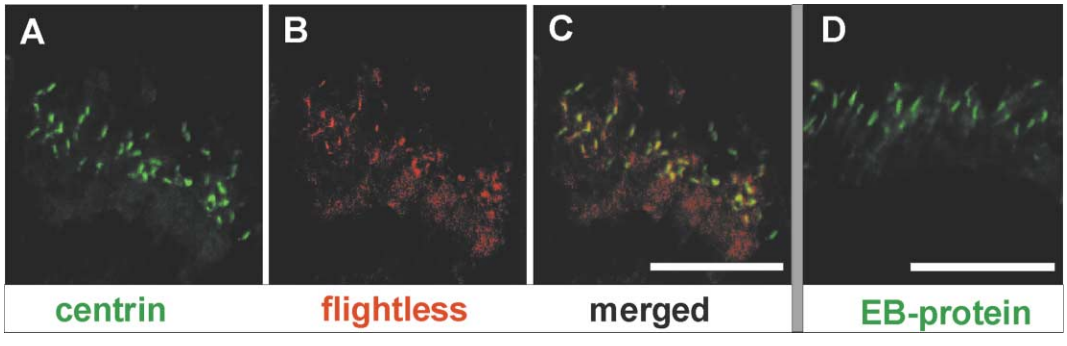

FIG. 7. Immunocytochemical localization of flightless and EB-protein family members in the photoreceptor connecting cilium. (A)–(C) Longitudinal cryosection through the photoreceptor layer of a rat retina double-labeled by indirect immunofluorescence with anti-centrin (20H5) (A), anti-flightless (B). (C) Merged images of (A) and (B) demonstrate co-localization of centrin and flightless in photoreceptor connecting cilia and basal bodies. (D) Indirect immunofluorescence of anti-EB in the photoreceptor layer of a rat retina revealing that EB-proteins were also detected in the ciliary apparatus of photoreceptors. Bar = 14 μm.

Immunolocalization of Identified Gene Products

To confirm that the isolated clones encode gene products which indeed co-localize in the photoreceptor axoneme, indirect immunofluorescence was performed with polyclonal antibodies generated against recombinant expressed rat flightless (Schmitt and Wolfrum, in preparation) and a commercially available MAb against the conserved region of EB1- and EB2-proteins (Fig. 7). In cryosections through rat retinas both antibodies reacted with stripe-like structures in the photoreceptor cell layer [Fig. 7(B) and (D)]. Further immunofluorescence double-labeling experiments and antibodies against the ciliary marker

centrin convincingly demonstrated co-localization of flightless, EB-protein and centrin in ciliary apparatus of photoreceptor cells [Fig. 7(A)–(C)].

4. Discussion

In order to analyze the molecular composition of the photoreceptor cell axoneme, an immunoscreening approach was applied. The present approach was based on the assumption that the protein fraction of detergent-extracted axonemes contains molecular components of the ciliary apparatus of photoreceptor cells. A rat retina cDNA expression library was screened with the AX-4 antiserum which was generated against photoreceptor cell axonemes. Identified clones were analyzed by BLAST and FASTA homology searches at NCBI and EMBL and the gene products were further characterized.

With the present strategy, we were able to identify genes which have escaped other molecular genetic methods, namely subtraction cloning (Swaroop et al., 1991; Rodriguez and Chader, 1992), differential hybridization (Bascom et al., 1992), differential display or subtractive hybridization (Bowes et al., 1989; Diatchenko et al., 1996; Swanson et al, 1997; Akhmedov et al., 2000). Using the latter molecular genetic strategies, it is not possible to identify protein components specifically localized in subcellular compartments like the axonemes of photoreceptor cells. For example, differential screening methods are based on the substraction of cDNA libraries generated from different tissue or cell types. However, it is not possible to generate cDNA libraries of clones coding for proteins present in specific cell compartments or composing specific cellular structures.

The limitations of these molecular genetic methods led to the use of biochemical approaches to identify yet unknown ciliary components of photoreceptor cells that were previously applied. In search for structural components of photoreceptor cell subcellular compartments, two different libraries of monoclonal antibodies have been generated, one against photoreceptor cell axoneme components (Horst et al., 1990) and the other against actin-binding proteins of the outer segment of photoreceptor cells (Hallet et al., 1996). However, in both cases only one protein was identified, namely a highly glycosylated protein of the axoneme (Horst et al., 1987, 1990; Besharse and Horst, 1990) and the guanylate cyclase RetGC-1 as an actin binding protein (Hallet et al., 1996). In the present report, a combination of both biochemical and molecular genetic approaches was applied and 257 positive candidate clones potentially coding for protein components of the axonemal subcompartment of photoreceptors were successfully identified. Further analysis revealed that these clones can be grouped in different categories listed in Table I.

Several positive clones code for partial amino acid sequences of photoreceptor specific proteins, e.g. opsin and arrestin, and another category of clones code for ubiquitously expressed proteins. The positive identification of these clones can be interpreted in different ways. On one hand, proteins of both categories were present in the axonemal fraction of photoreceptor cells which was used for antiserum generation as contaminants in the biochemical purification procedure. However, such contaminants cannot be excluded in biochemical fractionation of photoreceptor axonemes (Fleischmann et al., 1980; Pagh-Roehl and Burnside, 1995). On the other hand, at least the photoreceptor specific proteins of the visual transduction cascade are temporarily present in the connecting cilium. It is commonly accepted, that after their de novo synthesis in proximal compartments of the inner segment, they are vectorally transported along cytoskeletal elements in the inner segment and through the connecting cilium to the site of disk formation at the base of the outer segment (Papermaster, Schneider and Besharse, 1985; Deretic et al., 1998; Liu et al., 1999; Tai et al., 1999; Wolfrum and Schmitt, 1999, 2000; Marszalek et al., 2000). Therefore, during their passage through the connecting cilium, transduction cascade proteins could interact with cytoskeletal proteins of the axoneme and they are probably co-purified with these cytoskeletal elements in axonemal preparations. The Western blot analysis presented here as well as in the report by Fleischmann et al. (1980) identified opsin as a prominent protein in axoneme preparations.

Apart from these clones, nine clones that code for rat homologues of cytoskeletal proteins or cytoskeleton-associated proteins were identified. Two of these cDNA clones (AS128 and AS42 of 1·2 kb) show identities to the 3′ terminus of *human flightless II* gene (86·2 %), *C. elegans flightless* (82·4 %) and *Drosophila melanogaster flightless I* gene (68·2 %). The *flightless* genes code for actin-binding proteins consisting of two functional domains: a C-terminal domain with significant homology to members of the gelsolin/villin superfamily, and an N-terminal LRR-domain (LRR: *l*eucine *r*ich *r*epeat) with identities to LRR proteins of the Cyr1p-subfamily (Claudianos and Campbell, 1995; Buchanan and Gay, 1996). In *Drosophila* embryos, flightless I protein localizes to actin rich membranes and membrane invaginations (Straub, Stella and Leptin, 1996; Davy et al., 2000). The gelsolin-like domain of the fli-I related proteins of human and *C. elegans* show Ca^{2+} independent G-actin binding activity, and F-actin binding and severing activities (Orloff et al., 1995; Liu and Yin, 1998; Goshima et al., 1999; Campbell, Young and Matthaei, 2000).

The amino acid sequence of the rat flightless fragment deduced from the partial cDNA sequence is similar to the C-terminus of human flightless II (88 % identity), *C. elegans* flightless (54·1 % identity), and *Drosophila* flightless I (52 % identity). It has previously been shown that in addition to the core microtubule

cytoskeleton, a cluster of actin filaments are localized in the distal portion of the connecting cilium. These actin filaments are suggested to be involved in disk morphogenesis (Chaitin et al. 1984; Chaitin and Bok, 1986; Arikawa and Williams, 1989). This actin-based process is probably supported by α-actinin (Arikawa and Williams, 1989) and myosin II (Williams et al., 1992). More recently, axonemal actin has been identified at the entire ciliary membrane (Wolfrum and Schmitt, 1999, 2000). This actin cytoskeleton may contribute to myosin VIIa mediated ciliary transport (Liu et al., 1997; Wolfrum and Schmitt, 1999, 2000). The present preliminary light microscopy analysis reveals that flightless is localized in the connecting cilium of photoreceptor cells in mammalian retinas (see Fig. 7). More detailed investigations into the subcellular localization will demonstrate that flightless and actin indeed co-localize in a subdomain of the photoreceptor cilium (Schmitt and Wolfrum, in preparation). These findings indicate that flightless may act as a gelsolin-like actin-modulator protein of the ciliary actin cytoskeleton in photoreceptors.

Two clones (AS118 and AI189) were isolated coding for microtubule-associated proteins. The 284 bp cDNA insert of clone AS118 shows sequence identity of 83 % to the 3′ terminus of *M. musculus* adenomatous polyposis coli-(APC) binding protein EB2 gene. The deduced partial amino acid sequence is 95 % identical to the mouse EB2 protein. The present indirect immunofluorescence labeling verifies the localization of EB-family proteins in the connecting cilium of photoreceptor cells. Although nothing is yet known about EB2 function, high sequence identities to EB1 and the yeast homologues (Bim1p, Mal3p) indicate that EB2 may also interact with microtubules (Beinhauer et al., 1997; Schwartz, Richards and Botstein, 1997; Berrueta et al., 1998).

The cDNA insert of clone AS189 is 89 % identical to human microtubule-associated protein 4 (MAP4). Members of the family of microtubule-associated proteins (MAPs) bind to microtubules without requirement of nucleotides. MAP4 is ubiquitously expressed and can induce microtubule polymerization and participates in microtubule dynamics (Hirokawa, 1994). In addition, clones were identified coding for proteins composing the molecular motor complex of cytoplasmic dynein: clone AS206 is homologous to human cytoplasmic dynein intermediate chain 2C (DIC2C) and clone AS129 shows identity to dynamitin, the basic subunit of the dynactin complex which resembles a subcomplex of cytoplasmic dynein. The multiprotein complex of cytoplasmic dynein is composed of two heavy chains and several light chains, four light intermediate chains, and two intermediate chains which can link the dynein motor via the dynactin complex to cargo vesicles (Valee and Sheetz, 1996; Hirokawa, 1998). As a rule, cytoplasmic dynein mediates minus-end directed microtubule-based intracellular transport. The identification of cytoplasmic dynein components by the screening strategy is consistent with the prominent role of dynein in photoreceptor function. In the photoreceptor cell inner segment, cytoplasmic dynein mediates the translocation of opsin-laden post-Golgi vesicles along cytoplasmic microtubules from the trans-Golgi network to the apical membrane at the base of the connecting cilium (Tai et al., 1999). More recently Besharse, Janson and Liang (1999, 2000) gathered evidence which indicate that cytoplasmic dynein, bearing the dynein heavy chain 1B may also contribute to retrograde ciliary transport of so called raft transport complexes from the outer segment to the base of the connecting cilium. It is worth considering that the gene products of clone AS206 (cytoplasmic dynein intermediate chain 2C) and clone AS129 (dynamitin) are part of these transport rafts.

In the present immunoscreen, a clone (AS11) which is 96 % identical to human centrin 3, a member of the parvalbumin superfamily of the Ca^{2+}-binding proteins has also been isolated. It is known that centrin polymers are involved in Ca^{2+} modulated motility and/or Ca^{2+} dependent signal transduction (Salisbury, 1995; Schiebel and Bornens, 1995). In previous studies, it has been demonstrated that centrin isoforms are differentially localized in the basal body-centriole complex (centrin 1 and 2) and the connecting cilium (centrin 1) of mammalian photoreceptor cells (Wolfrum, 1995; Wolfrum and Salisbury, 1998). Unpublished data demonstrate that the isoform centrin 3 is also a structural component of ciliary apparatus of mammalian photoreceptor cells (U. Wolfrum et al., unpubl. res.). Uzawa et al. (1995) have shown that centrins assemble complexes with large heat shock proteins (HSP). The AS97 clone is homologous to one of these molecular chaperones, the heat shock protein 90 (HSP90). The parallel finding of centrin 3 and HSP90 indicates that both proteins may also assemble complexes in mammalian photoreceptor cells.

Although 240 000 clones were screened, the analysis of the isolated clones revealed that none of them code for conventional cytoskeletal elements, e.g. tubulins and actins. One reason may be the low antigenicity of these conventional cytoskeletal proteins because of their high conservation among vertebrate species. Another reason could be the low expression level of genes encoding cytoskeletal proteins, and as a consequence, the low frequency of the corresponding cDNA clones in the expression library screened. It is well accepted that structural proteins turn over less frequently than other proteins and it has previously been discussed that in photoreceptors, the gene expression of cytoskeletal components is at a much lower level than genes encoding proteins of the visual transduction cascade (Eckmiller, 1997). This may also explain the high number of isolated clones encoding proteins of the visual transduction cascade in the present work. Furthermore, some genes of the

retinal cDNA library may not be expressed in *Escherichia coli* cells, or assemble to aggregates which are not detected by the antibodies in the antiserum raised against native proteins (Huber et al., 1996).

The applied biochemical and molecular genetic strategy described here enables the isolation of yet unknown protein components of photoreceptor cell axonemes, which were not identified by the biochemical or molecular genetic approaches previously applied. Further cell biological and biochemical characterization of the cytoskeletal proteins encoded by the isolated clones will provide additional insight into the molecular structure and function of the connecting cilium and its role in molecular transport, disk morphogenesis, and as a diffusion barrier. The growing knowledge about the molecular composition of the ciliary compartment of photoreceptors will improve the understanding of vertebrate photoreceptor cell function.

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