Cell Motility and the Cytoskeleton 37:240–252 (1997)

Myosin VIIa, the Product of the Usher 1B Syndrome Gene, Is Concentrated in the Connecting Cilia of Photoreceptor Cells

Xinran Liu,[1] Gordon Vansant,[1] Igor P. Udovichenko,[1]
Uwe Wolfrum,[2] and David S. Williams[1]*

[1]*Departments of Pharmacology and Neurosciences, UCSD School of Medicine, La Jolla, California*
[2]*Zoologisches Institut, Universität Karlsruhe, Karlsruhe, Germany*

Usher syndrome is the most common form of combined deafness and blindness. The gene that is defective in Usher syndrome 1B (USH1B) encodes for an unconventional myosin, myosin VIIa. To understand the cellular function of myosin VIIa and why defects in it lead to USH1B, it is essential to determine the precise cellular and subcellular localization of the protein. We investigated the distribution of myosin VIIa in human and rodent photoreceptor cells and retinal pigment epithelium (RPE), primarily by immunoelectron microscopy, using antibodies generated against two different domains of the protein. In both human and rodent retinae, myosin VIIa was detected in the apical processes of the RPE and in the cilium of rod and cone photoreceptor cells. Immunogold label was most concentrated in the connecting cilium. Here, myosin VIIa appeared to be distributed outside the ring of doublet microtubules near the ciliary plasma membrane. These observations indicate that a major role of myosin VIIa in the retina is in the photoreceptor cilium, perhaps in such a function as trafficking newly synthesized phototransductive membrane or maintaining the diffusion barrier between the inner and outer segments. Our results support the notion that defective ciliary function is the underlying cellular abnormality that leads to cellular degeneration in Usher syndrome. Cell Motil. Cytoskeleton 37:240–252, 1997.

Key words: Usher syndrome; unconventional myosin; cilium; photoreceptor cell; shaker-1 mice

INTRODUCTION

Usher syndrome is an autosomal recessive form of retinitis pigmentosa and hearing impairment [Usher, 1914]. It affects about 1 in 23,000 within the United States [Boughman et al., 1983] and is clinically and genetically heterogeneous. Three clinical types have been described: The most severe is type 1 (USH1), which is characterized by profound hearing loss, vestibular dysfunction, and prepubertal onset of retinitis pigmentosa. Defects in at least four different genes can lead to USH1, but about 75% of USH1 cases (and about 50% of all USH cases) are classified as Usher syndrome 1B (USH1B) and are caused by defects in the gene encoding myosin VIIa [Weil et al., 1995].

The primary sequence of myosin VIIa indicates a 729 amino acid N-terminal domain that has all the main characteristics of a myosin motor domain [Hasson et al., 1995; Weil et al., 1996]. This domain is followed by a 126 amino acid neck domain that contains five IQ motifs and a tail that is the longest among the unconventional

Contract grant sponsor: NIH; Contract grant number: EY 07042; Contract grant sponsor: Faunstiftung, Nürnberg.

*Correspondence to: David S. Williams, Department of Pharmacology, UCSD School of Medicine, Mail Code 0983, 9500 Gilman Drive, La Jolla, CA 92093–0983. E-mail: dswilliams@ucsd.edu

Received 30 December 1996; accepted 21 February 1997.

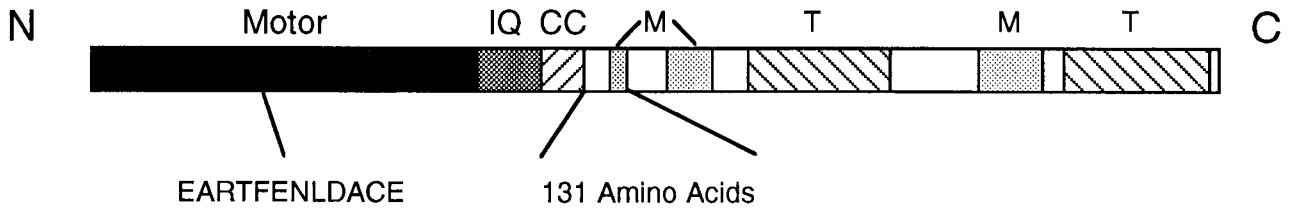


Fig. 1. Diagram illustrating domain organization of myosin VIIa predicted from the full cDNA for human myosin VIIa [Chen et al., 1996; Weil et al., 1996]. The motor domain contains the ATP- and actin-binding regions. The domain indicated by IQ contains five IQ motifs, CC indicates an α-helical segment that appears likely to form a coiled-coil, M indicates myosin tail homology regions, and T indicates regions that are homologous to talin and other members of the protein 4.1 superfamily. The regions against which antibodies were made are indicated. pAb 1 was made against the peptide sequence EARTFENLDACE; pAbs 2.1 and 2.2 were made against a 155 amino acid fusion protein, including 131 amino acids of mouse myosin VIIa that correspond to the region shown.

myosins identified so far. The tail begins with a short (78 amino acids) region that should form a coiled coil; thus, the molecule is likely to dimerize. Farther along the tail, there are myosin tail homology regions and regions that are homologous to the membrane-binding domain of proteins in the protein 4.1 superfamily [Chen et al., 1996; Weil et al., 1996].

To understand the cellular function of myosin VIIa and why defects in it lead to USH1B, it is essential to determine the precise cellular and subcellular distribution of the protein. By immunofluorescence microscopy of rat tissues, Hasson et al. [1995] demonstrated that myosin VIIa is present in the inner and outer hair cells of the cochlea and the pigmented epithelium of the retina (RPE). More recently, El-Amraoui et al. [1996] examined a variety of vertebrate retinae by immunofluorescence microscopy. Interestingly, they detected myosin VIIa in the photoreceptor cells of human, monkey, chicken, and frog but not of rodents. In primate photoreceptor cells, they observed immunofluorescence in the inner and outer segments and synaptic terminals.

In the present study, we investigated the distribution of myosin VIIa in photoreceptor cells and the RPE, primarily by immunoelectron microscopy, using antibodies that we generated against different domains of the protein. Thus, with relatively high resolution, we were able to determine the subcellular distribution of myosin VIIa in human photoreceptor cells and to reassess whether there are differences in the distribution of the protein in human and rodent retinae. Our results indicate that the highest concentration of myosin VIIa is in the region of the photoreceptor cilium that links the inner and outer segments of each cell. They suggest that the basis of the retinitis pigmentosa found in USH1B patients may be a defect in the function of this connecting cilium.

MATERIALS AND METHODS

Preparation of Antibodies

We generated antibodies against a peptide and a fusion protein that correspond to different regions of mouse myosin VIIa (Fig. 1). The peptide sequence ^{337}EARTFENLDACE, which is from part of the motor domain, was chosen from the partial mouse myosin VIIa cDNA sequence that was available at the time we embarked on the present study [Gibson et al., 1995]. Important reasons for selecting this sequence were that we knew from Weil et al. [1995] that it was 100% identical with the corresponding region in human myosin VIIa (so that generated antibodies should cross react with human myosin VIIa) and that it showed no similarity to sequences found in other myosins, as determined by BLAST search of the Genbank database. The peptide was synthesized, purified, and analyzed by mass spectroscopy by Multiple Peptide Systems (La Jolla, CA). It was synthesized with an additional Lys residue at its N-terminal, coupled to bovine serum albumin (BSA) by glutaraldehyde, and injected subcutaneously into rabbits with Freund's adjuvant. Some rabbits were given an additional intravenous injections of BSA peptide that was adsorbed to 70 nm gold particles, according to the method of Pow and Crook [1993]. Antibodies generated in rabbits injected with the peptide were called pAb 1.

The fusion protein was made as follows. Total RNA was purified from mouse testes by using established techniques [Chomezynski and Sacchi, 1987]. mRNA was then isolated from the total RNA by using oligo(dT) cellulose. Reverse transcription to produce cDNA was carried out by using random primers obtained from Invitrogen, San Diego, CA. For polymerase chain reaction (PCR) amplification, degenerate primers were designed based on the human myosin VIIa amino acid sequence [Hasson et al., 1995], because the sequence of the mouse myosin VIIa tail domain was unknown. The primers were designed to amplify a 393 nucleotide portion of the mouse myosin VIIa encoding the N-terminal part of the tail (Fig. 1), which corresponds to amino acids 941–1,071 of human myosin VIIa. The sequences of the PCR primers were 5′-CGGGATCCCGAYATGGTNGAYAARATGTT-3′ and 5′-CCGCTCGAGGGTCATNACNGGDATYTTYTC-3′. They include BamH1 and Xho1

restriction sites, respectively, to facilitate the subcloning of the PCR product. The PCR product was digested and cloned into pET23b (Novagen, Madison, WI), which resulted in an expression vector that encoded a 17-kD protein fragment. This resulting fusion protein consisted of a 14 amino acid leader peptide from the vector; 131 amino acids of myosin VIIa; and the amino acids Thr, Lys, and Glu plus seven histidines added to the C-terminus from the vector. Because the next amino acid of human myosin VIIa is a threonine, we probably have 132 amino acids of mouse myosin VIIa included in the fusion protein. The vector was transformed into BL21 DE3 [lys(s)] cells, which were induced to express the protein with IPTG at a final concentration of 1 mM. The protein was purified using a nickel column (Qiagen, Chatsworth, CA) and was used as an antigen (with Freund's adjuvant) to produce polyclonal antibodies in rabbits by standard methods. Some rabbits were injected (subcutaneously) with soluble protein that was dialyzed against phosphate-buffered saline (PBS) after elution from the nickel column. Others were injected with protein that was denatured and additionally purified by separation in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); in this case, protein in pulverized polyacrylamide was used. Antibodies generated in rabbits injected with fusion protein prior to SDS-PAGE were called pAb 2.1. Antibodies generated in rabbits injected with denatured fusion protein in polyacrylamide were called pAb 2.2.

Antisera of pAbs 1 and 2 were affinity purified on Affigel 10 (BioRad) columns to which the appropriate peptide or fusion protein was coupled. The procedure followed that described in the BioRad manual, except that peptide or fusion protein were incubated with Affigel 10 for 20 h at 4°C to achieve >95% coupling.

Expression of Partial Myosin VIIa in Insect sf9 Cells

A 3.25-kb length of cDNA, which contained the DNA region encoding amino acids 1–1,075 of human myosin VIIa [Hasson et al., 1995], was given to us by Drs. Tama Hasson and Mark Mooseker of Yale University. By using this cDNA, the DNA for the first 1,075 amino acids of human myosin VIIa was cloned into the baculovirus transfer vector pVL1393 as follows: First, a 320 nucleotide PCR adaptor fragment was produced by using the 3.25-kb cDNA as a template with the primers 5′-CGCGGATCCACCATGGTGATTCTTCAG-CAGGG-3′ and 5′-GGGTTCACAGCCACCAGGATGG-3′. The adaptor includes the initiation site of translation and also has an introduced BamH1 site at the 5′ end and a native BstX1 site at the 3′ end. This fragment was ligated to a 2.9-kb BstX1, EcoR1 cDNA fragment, representing the rest of the clone, and pVL1393 that had been digested with BamH1 and EcoR1. The EcoR1 site, which was at the 3′ end of the encoding sequence, was then digested, filled in with Klenow DNA polymerase, and religated to introduce a stop codon. The transfer vector construct was then used to produce recombinant viruses for protein expression. Briefly, sf9 insect cells were cotransfected with a combination of the transfer vector and linearized wild type baculovirus DNA (Baculogold-Pharmingen) by liposome-mediated transfection. The resultant recombinant virus was harvested and amplified by another propagation to produce high-titer virus. The production of recombinant virus was verified by the expression of a 124-kD protein that was visible on Coomassie blue-stained SDS-polyacrylamide gels (Fig. 2a).

Western Blot Analysis

Retinae were dissected from human, mouse, rat, and bovine eyes. The human eye was from a 98-year-old donor with no history of retinal disease; it was given to us by a local eye bank 7 h after the death of the donor. Rodent eyes were obtained from C57BL/6 mice (postnatal 1 year) and Sprague-Dawley rats (postnatal 21 days) that had been kept on a 12 h light/12 h dark cycle. Bovine eyes were obtained from a local slaughterhouse. Photoreceptor outer segments were purified from bovine retinae by using sucrose density gradients, as described by Azarian et al. [1995]. The purified outer segments were collected by centrifugation; resuspended in 2% Triton X-100, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 24 μM leupeptin, 0.2 mM PMSF, and 20 mM Tris, pH 7.4 (buffer A), at 4°C; and added to the top of a layer of 50% (w/w) sucrose in buffer A, which had been laid on top of 60% (w/w) sucrose in buffer A. Photoreceptor outer segment axonemes were collected from the interface between the two layers of sucrose [cf. Horst et al., 1987]. Retinae, photoreceptor outer segment axonemes, or Sf9 cells were homogenized directly in 62.5 mM Tris buffer, 10% glycerol, and 2% SDS, pH 6.8 (sample buffer), for SDS-PAGE. The separated proteins were electrophoretically transferred to Immobilon-P, blocked, and probed with primary and secondary antibodies. The latter was conjugated to alkaline phosphatase, so that labelling was detected by the formation of the insoluble product of 5-bromo-4-chloroindoyl phosphate hydrolysis.

Immunoelectron Microscopy

Two human eyes with no history of retinal disease were used. One was from a 30-year-old donor; it was given to us by an eye bank 30 min after the death of the donor. The other was from a 25-year-old donor; it was given to us by an eye bank 90 min after the death of the donor. Rodent eyes were obtained from C57BL/6 mice (8–12 months old) and Sprague-Dawley rats (21 days old). Eyes were cut along the ora serrata, and the posterior

eyecups were immersed immediately in primary fixative. The human eyecups were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature. The rodent eyecups were fixed in 0.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature. The tissue was dissected into small pieces during fixation. Fixed pieces of human retina were then dehydrated with an ascending series of ethanol up to 95%, infiltrated in 1:1 mixture of Lowicryl K4M resin and 95% ethanol for 1 h, and then infiltrated overnight in a pure Lowicryl K4M resin mixture at 4°C. Retinal pieces were embedded in the fresh resin, which was cured under UV light for 7 days. Pieces corresponding to the region just peripheral to the parafoveal region were used in the present study. The rodent retinal pieces were embedded in LR White medium and polymerized at 60°C for 24 h.

Ultrathin sections (60–70 nm) were collected on Formvar-coated nickel grids. Sections were first etched with saturated sodium periodate (Sigma, St. Louis, MO), then blocked with 4% BSA in PBS for 30 min. The primary incubation was carried out with myosin VIIa antibodies in PBS containing 1% BSA at 4°C overnight. The grids were rinsed with PBS and then incubated with goat antirabbit IgG conjugated to 10 nm gold (Amersham, Arlington Heights, IL) at a dilution of 1:30, in PBS with 1% BSA for 1 h. The sections were postfixed in 2% glutaraldehyde for 20 min, washed completely in distilled water, and stained with 5% uranyl acetate for 5 min prior to observation. Fusion protein (1 mg/ml) or peptide (50–100 μg/ml) was added to the primary incubation as a negative control. The preimmune sera were also used as a negative control. Sections were observed in a Jeol (model 1200-EX) electron microscope.

Immunogold labelling in different domains was quantified by counting gold particles on negatives. Regions from and around 8–20 randomly selected photoreceptor cells in longitudinal section were counted per block of tissue. Human data were obtained from three different blocks containing tissue from the region just peripheral to the parafoveas of the two human retinae.

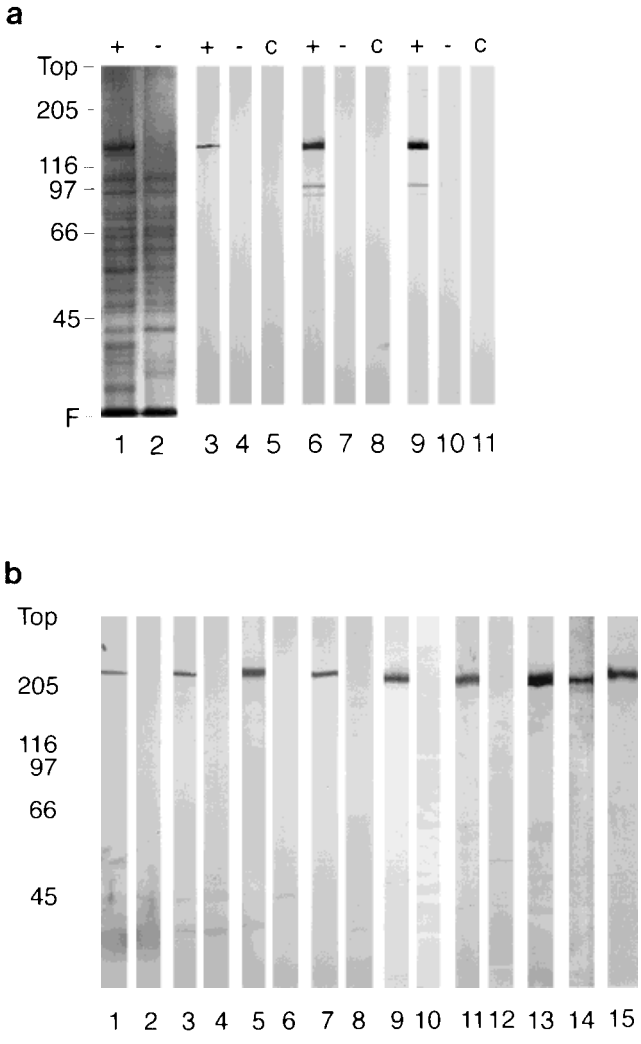


Fig. 2. Western blot analyses of antibodies against myosin VIIa. **a:** Determination that antibodies recognized human myosin VIIa. Western blots [from an 8% sodium dodecyl sulfate (SDS) polyacrylamide gel] were prepared from lysates of sf9 cells either containing or not containing a polypeptide, with an apparent molecular mass of ~124 kD, and corresponding to amino acids 1–1,075 of human myosin VIIa. Each antibody was incubated either with a Western blot strip containing the polypeptide (+), with a strip not containing the polypeptide (−), or with a strip containing the polypeptide but also in the presence of competitive peptide or fusion protein (c). **Lanes 1,2:** Coomassie blue-stained gel of sf9 cells either containing (+) or not containing (−) the 124-kD myosin VIIa prior to transfer to Immobilon. **Lanes 3,4:** Labelled with pAb 1 antiserum. **Lane 5:** Labelled with pAb 1 antiserum + 0.2 mg/ml peptide. **Lanes 6,7:** Labelled with affinity-purified pAb 2.1. **Lane 8:** Labelled with affinity-purified pAb 2.1 + 1 mg/ml fusion protein. **Lanes 9,10:** Labelled with affinity-purified pAb 2.2. **Lane 11:** Labelled with affinity-purified pAb 2.2 + 1 mg/ml fusion protein. **b:** Test for specific cross reactivity of antibodies against myosin VIIa in human, mouse, and rat retinae, and bovine photoreceptor outer segment axonemes. Lanes 1–8 contain Western blotted human retinal proteins, separated by 7% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). **Lane 1:** Labelled with affinity-purified pAb 1. **Lane 2:** Labelled with affinity-purified pAb 1 + 0.1 mg/ml peptide. **Lane 3:** Labelled with affinity-purified pAb 2.1. **Lane 4:** Labelled with affinity-purified pAb 2.1 + 1 mg/ml fusion protein. **Lane 5:** Labelled with pAb 2.2 antiserum. **Lane 6:** Labelled with pAb 2.2 antiserum + 1 mg/ml fusion protein. **Lane 7:** Labelled with affinity-purified pAb 2.2. **Lane 8:** Labelled with affinity-purified pAb 2.2 + 1 mg/ml fusion protein. Lanes 9–12 contain Western blotted mouse retinal proteins, separated by 7% SDS-PAGE. **Lane 9:** Labelled with affinity-purified pAb 2.1. **Lane 10:** Labelled with affinity-purified pAb 2.1 + 1 mg/ml fusion protein. **Lane 11:** Labelled with affinity-purified pAb 2.2. **Lane 12:** Labelled with affinity-purified pAb 2.2 + 1 mg/ml fusion protein. **Lane 13:** Mouse testis proteins labelled with affinity-purified pAb 2.2. **Lane 14:** Rat retinal proteins labelled with affinity-purified pAb 2.2. **Lane 15:** Bovine photoreceptor outer segment axonemal proteins labelled with affinity-purified pAb 2.2.

Mouse data were obtained from three different blocks containing pieces of retina from two different mice.

Immunofluorescence Microscopy of Rat Photoreceptor Cells

Procedures for the isolation of rat photoreceptor inner-outer segments and immunofluorescence microscopy and details of the centrin antibody (monoclonal antibody 20H5) are the same as those described by Wolfrum [1995]. Retinae were obtained from 2-month-old Sprague-Dawley rats that had been maintained on a 12 h light/12 h dark cycle.

RESULTS

Western Blot Analysis

Antibodies were made against a peptide (pAb 1) or a fusion protein (pAbs 2.1 and 2.2) corresponding to different domains of mouse myosin VIIa (Fig. 1). The peptide is 100% identical between human and mouse [mouse: Gibson et al., 1995; human: Weil et al., 1995]. The sequence of mouse myosin VIIa in the region against which pAbs 2.1 and 2.2 were made has yet to be reported. We sequenced 192 of the 393 nucleotides of the cDNA encoding for this region and found that all but one of the encoded 64 amino acids were identical with the reported corresponding amino acids of human myosin VIIa [Hasson et al., 1995; Weil et al., 1996].

The antibodies were tested to confirm that they recognized human myosin VIIa by incubating them with Western blots containing lysate from sf9 cells that had expressed a polypeptide corresponding to amino acids 1–1,075 of the protein. Figure 2a shows that pAb1, pAb 2.1, and pAb 2.2 all specifically recognized the recombinant myosin VIIa. The antibodies did not react with proteins in lysate from sf9 cells that had not been transfected with myosin VIIa DNA (Fig 2a, lanes 4, 7, 10). Moreover, the labelling of the recombinant myosin VIIa was inhibited when pAb 1 was incubated with peptide and when pAbs 2.1 and 2.2 were incubated with fusion protein (Fig. 2a, lanes 5, 8, 11). Two smaller polypeptides were also labelled faintly by pAbs 2.1 and 2.2, but, because they were not labelled in lanes that lacked the expressed myosin VIIa or in lanes that were incubated with antibody plus fusion protein, they most likely represent a small amount of expressed myosin VIIa that had been proteolyzed. They were not recognized by pAb1, indicating removal of the protein's N-terminus.

Next, the antibodies were tested for their specificity for myosin VIIa in Western blots of human retina (Fig. 2b, lanes 1–8). All of them recognized a single polypeptide that migrated above the 205-kD molecular mass marker. In the case of pAb 2.2, even unpurified antisera reacted specifically with this polypeptide at dilutions as high as 1:50,000 (with an alkaline phosphatase-conjugated secondary antibody; for 1:10,000 dilution, see Fig. 2b, lane 5). Labelling of the polypeptide was inhibited when pAb 1 was incubated with peptide and when pAbs 2.1 and 2.2 were incubated with fusion protein (Fig. 2b, lanes 2, 4, 6, 8).

In rodent retinae, the antibodies also recognized a single polypeptide. Western blots of mouse retina (Fig. 2b, lanes 9–12) and rat retina (Fig. 2b, lane 14) labelled with pAbs 2.1 and 2.2 are shown in Figure 2b. Mouse and rat myosin VIIa migrated slightly faster than human myosin VIIa in SDS-PAGE but at a rate similar to that of myosin VIIa in mouse testis, which contains a greater concentration of myosin VIIa than retina (Fig. 2b).

Antibodies also recognized a single polypeptide in an axonemal preparation from bovine photoreceptor outer segments (Fig. 2b, lane 15). This polypeptide migrated slightly slower than the rodent myosin VIIa, more similar to the human myosin VIIa. The detection of myosin VIIa in photoreceptor outer segment axonemes is consistent with the immunocytochemical observations below.

Immunolabelling of Human Photoreceptor Cells and RPE

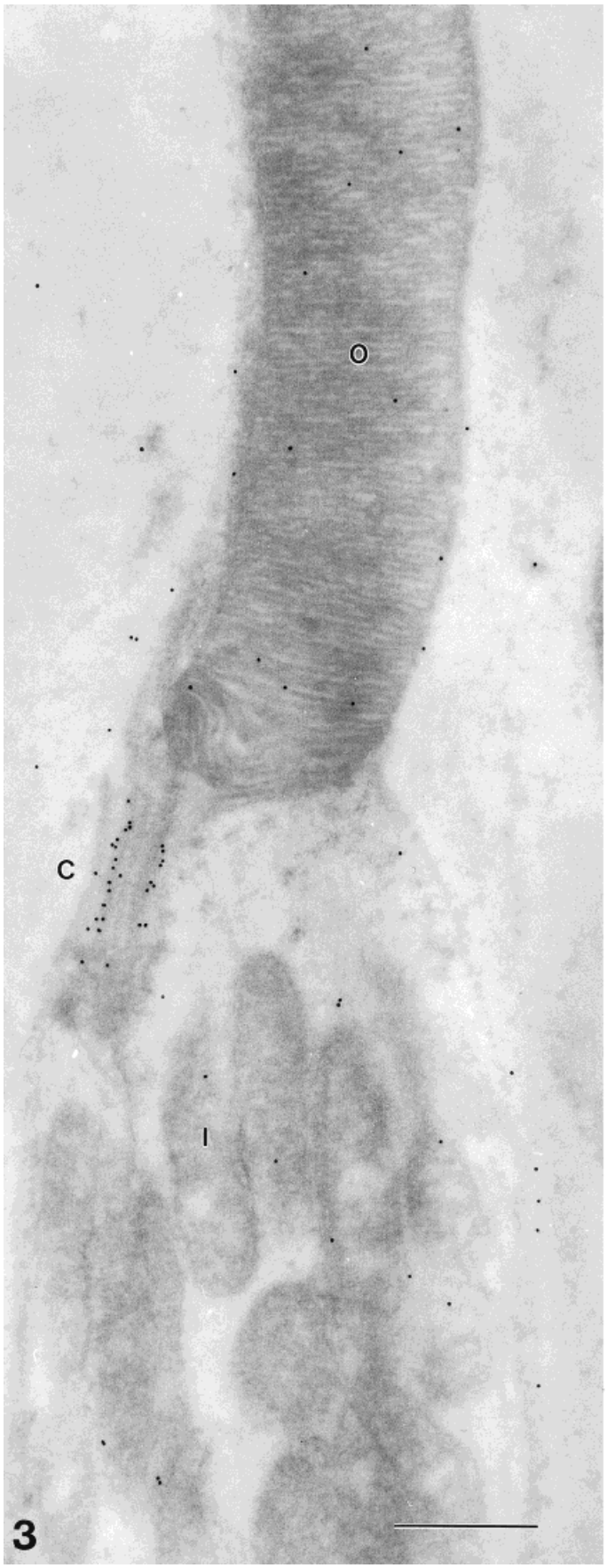
Affinity-purified pAbs 1, 2.1, and 2.2 and pAb 2.2 antiserum were used for immunogold labelling of ultrathin human retinal sections. They all gave the same labelling pattern, although antiserum and affinity-purified fractions of pAb 2.2 (at dilutions of 1:500–1:1,000) gave the strongest signal. No significant labelling was observed with any of the preimmune sera (diluted 1:200).

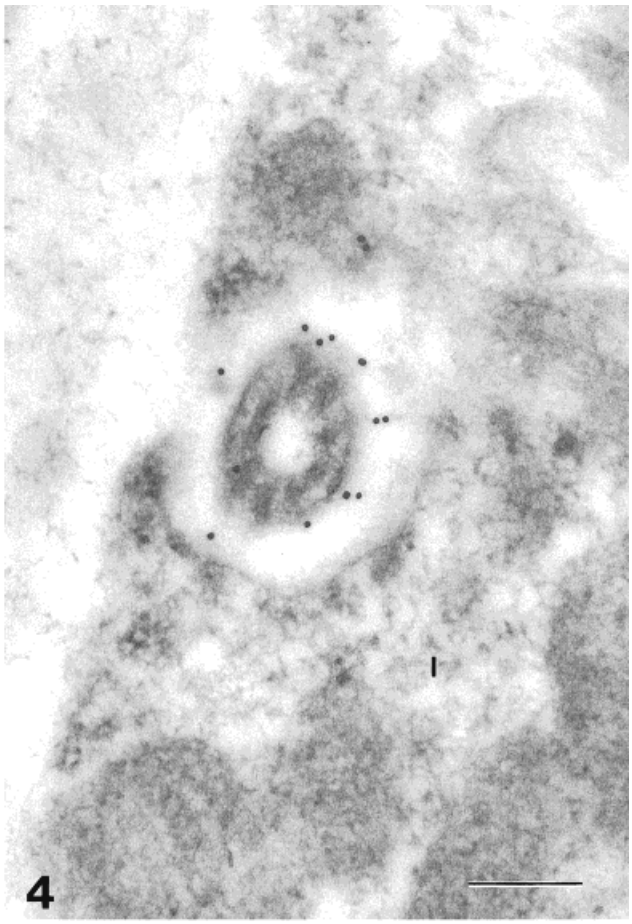
Myosin VIIa was detected predominantly in the proximal cilia (i.e., connecting cilia) of human photoreceptor cells (for an example of an intensely labelled cilium in longitudinal section, see Fig. 3). Within this domain, it was found only near the plasma membrane, outside the ring of microtubules (Figs. 3, 4). In transverse sections of connecting cilia, label was not detected inside the ring of

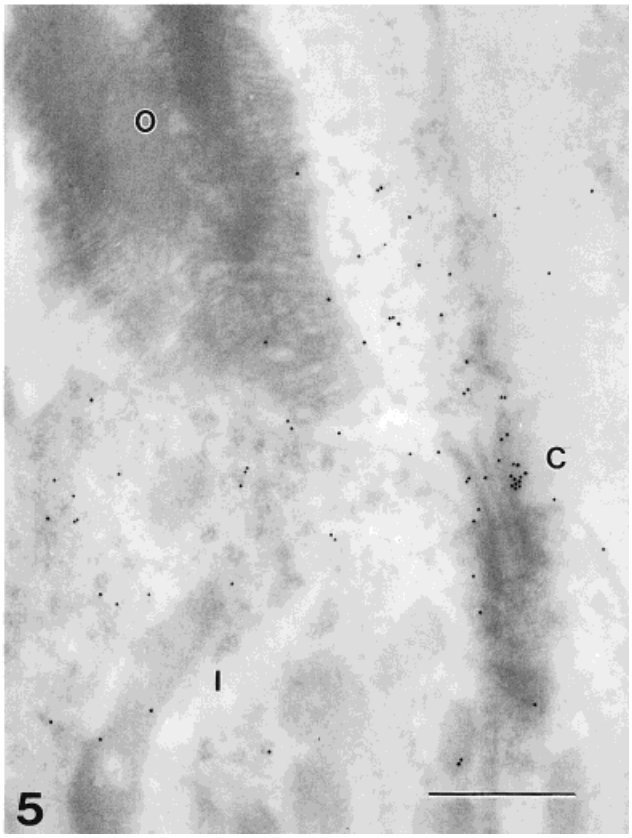
Fig. 3. Longitudinal section of part of a human rod photoreceptor cell immunogold labelled with myosin VIIa antibody pAb 2.2. O, outer segment; C, connecting cilium; I, inner segment. Scale bar = 500 nm.

Fig. 4. Transverse section of connecting cilium of human rod photoreceptor cell immunogold labelled with myosin VIIa antibody pAb 2.2. Note that, in this particular micrograph, most of the gold particles appear to be in the extracellular space between the cilium and the inner segment (I). However, because of the indirect detection of the antigen, the gold particle can be up to two lengths of an immunoglobulin molecule from the antigen; i.e., up to 35–40 nm. Together, this and other micrographs of transverse sections (see also Fig. 14 for mouse) indicate that the antigen is near the cytoplasmic surface of the ciliary plasma membrane. Scale bar = 200 nm.

Fig. 5. Longitudinal section of part of a human cone photoreceptor cell immunogold labelled with myosin VIIa antibody pAb 2.2. The region around the connecting cilium (C) is shown. O, outer segment; I, inner segment. Scale bar = 500 nm.







Figures 3–5.

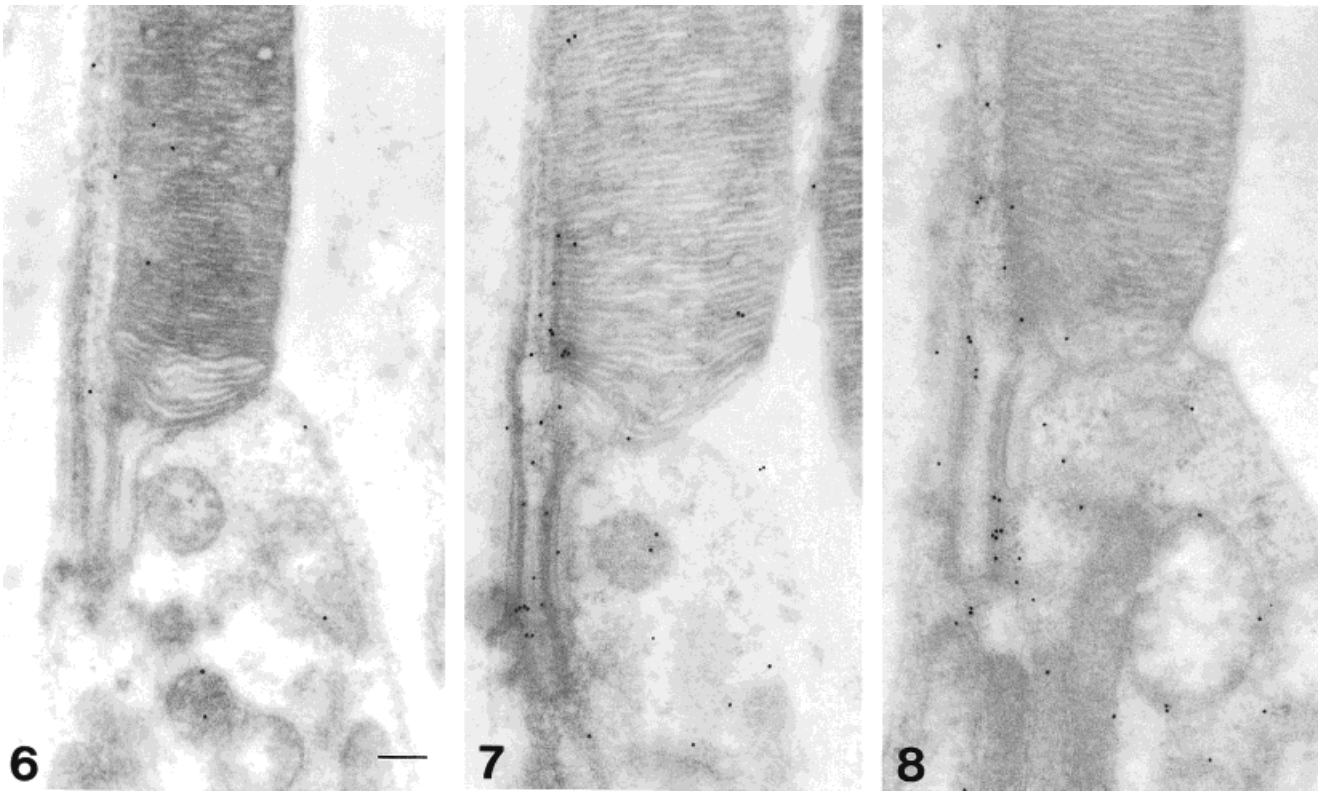


Fig. 6. Longitudinal section of the cilium of a human rod photoreceptor cell immunogold labelled with myosin VIIa antibody pAb 2.2 plus 1 mg/ml fusion protein. In this negative control, the connecting cilium is not labelled. Scale bar = 200 nm.

Fig. 7. Longitudinal section of the cilium of a human rod photoreceptor cell immunogold labelled with myosin VIIa antibody pAb 2.1 (same magnification as in Fig. 6).

Fig. 8. Longitudinal section of the cilium of a human rod photoreceptor cell immunogold labelled with myosin VIIa antibody pAb 1 (same magnification as in Fig. 6).

microtubules (see, e.g., Fig. 4). The connecting cilia of both rod (see, e.g., Fig. 3) and cone (Fig. 5) photoreceptor cells were labelled. Figures 3–5 were obtained by using pAb 2.2. Incubation of either antiserum or affinity-pure pAb 2.2 with 1 mg/ml fusion protein prevented labelling of the cilium (Fig. 6). Rod photoreceptor cells labelled with pAb 2.1 and pAb 1 are shown in Figures 7 and 8; most label is in the connecting cilia. Label was also evident in the distal cilium (the region of the cilium that is adjacent to the outer segment disk membranes), although it was clearly less than in the connecting cilium. Outside the photoreceptor cells, the apical surface of the RPE was labelled above background levels. Myosin VIIa was detected in the apical processes that extend around the outer segment (Fig. 9).

Immunolabelling of Rodent Photoreceptor Cells and RPE

Freshly isolated, unfixed rat rod photoreceptor outer and inner segments were double-labelled with a mixture of centrin mouse monoclonal antibody and myosin VIIa pAb 2.1 or 2.2. The centrin antibody, as described by Wolfrum [1995], labeled the entire photoreceptor cilium plus the basal bodies (Fig. 10c). In contrast, myosin VIIa was detected only in the proximal part of the cilium (Fig. 10b).

In ultrathin sections of mouse and rat retinae, the connecting cilia of photoreceptor cells (Figs. 11–13) and the apical processes of the RPE (not shown) were the most obviously immunolabelled structures. The cilia were labelled in the same manner as that observed with human photoreceptor cells; gold particles were concentrated around the periphery of the connecting cilium (see Fig. 14, which shows mouse connecting cilia in transverse section). In rat (Fig. 12) and mouse (Fig. 13) retinae, we were able to observe regions of the retina in which connecting cilia of adjacent photoreceptor cells were profiled in longitudinal section. Occasionally, a connecting cilium appeared unlabelled over a significant part of its length (see, e.g., the middle cilium in Fig. 12). This observation could be explained if the myosin VIIa molecules were spaced at intervals around the cilium rather than in a continuous ring. Thus, it would be possible for the surface of a section (LR White-embedded

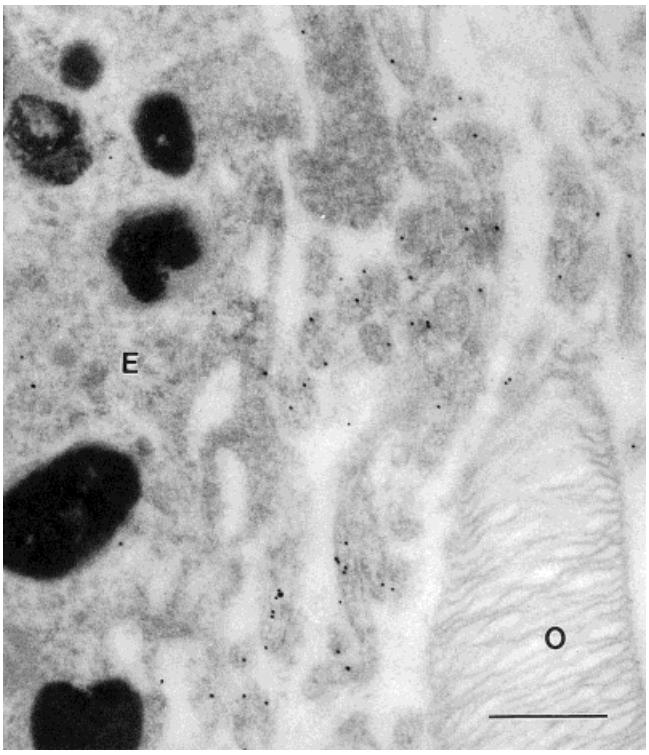


Fig. 9. The apical region of a retinal pigment epithelium cell (E) and the distal tip of a rod outer segment (O) in a human retinal section that was immunogold labelled with myosin VIIa antibody pAb 2.2. Scale bar = 500 nm.

sections are only labelled at the etched surface) to coincide with where myosin VIIa is absent in some cilia.

Quantification of Immunolabelling

The number of gold particles per μm^2 was determined for different regions of photoreceptor cells and the apical processes of the RPE in human retina for pAbs 1 and 2.2 and in mouse retina for pAb 2.2. The sections that were used contained the photoreceptor cells in longitudinal section. Figure 15 shows that the connecting cilium contained the most label in both human and mouse photoreceptor cells, irrespective of the antibody used. The only other region in the photoreceptor cells that was labelled above background levels was the distal cilium; this domain contained a concentration of label that was almost as high as the apical processes of the RPE.

DISCUSSION

Our results indicate that, in human and rodent outer retinae, myosin VIIa is located primarily in the photoreceptor cell connecting cilium and the RPE (Fig. 16). Localization of myosin VIIa in the RPE has been reported previously [Hasson et al., 1995; El-Amraoui et al., 1996]; however, this is the first report of localization in the connecting cilium. Immunolabelling of the connecting cilium may have been missed by the earlier studies, because they used immunofluorescence of thick sections, in which identification of the connecting cilium (only ~200 nm in diameter) would be difficult. Although they did not observe labelling of the connecting cilium, El-Amraoui et al. [1996] did report immunolabelling in the outer and inner segments and synaptic terminals of human (but not rodent; see below) photoreceptor cells. In contrast, our quantitative analysis of immunogold labelling showed that these regions contained only background levels of labelling (Fig. 15). A possible explanation for the differences between our results and previous reports is that there may be a number of different isoforms of myosin VIIa in the retina that arise from alternative splicing, as proposed by Weil et al. [1996], and, perhaps, different isoforms have different distributions. This explanation seems unlikely, however, because the antibodies used by Hasson et al. [1995] and El-Amraoui et al. [1996] were made against an N-terminal part of the tail of myosin VIIa, which, in both cases, included the region against which our pAbs 2.1 and 2.2 were made. Our antibodies appeared to react with only a single polypeptide on Western blots, indicating that they do not recognize more than one isoform of myosin VIIa (or other forms of myosin VII) in retinal tissue. Unfortunately, tissue from USH1B patients or mutant mice that could be used to test the specificity of our antibodies was not available. Nevertheless, positive evidence that our antibodies do react with myosin VIIa was obtained from their specific reactivity on Western blots of lysates from sf9 cells, which expressed recombinant partial myosin VIIa (Fig. 2a).

An additional disagreement between our results and those of El-Amraoui et al. [1996] is that El-Amraoui et al. found differences in myosin VIIa distribution between human and rodent retinae. These researchers proposed that such differences may explain why the photoreceptor cells of shaker-1 mice do not appear to degenerate in contrast to human USH1B photoreceptor cells. Shaker-1 mice, like USH1B patients, have defects in their myosin VIIa gene [Gibson et al., 1995]. They suffer from deafness and lack of balance [Steel and Bock, 1983], but their retinae have not been reported to undergo degeneration [Gibson et al., 1995]. In the present study, we have found that myosin VIIa has the same distribution in human, mouse, and rat retinae, so our results indicate that any differences in susceptibility to photoreceptor degeneration cannot be explained by species differences in myosin VIIa distribution.

Possible Function of Myosin VIIa in Photoreceptor Cells

In a vertebrate photoreceptor cell, phototransduction occurs in the outer segment, which consists primarily

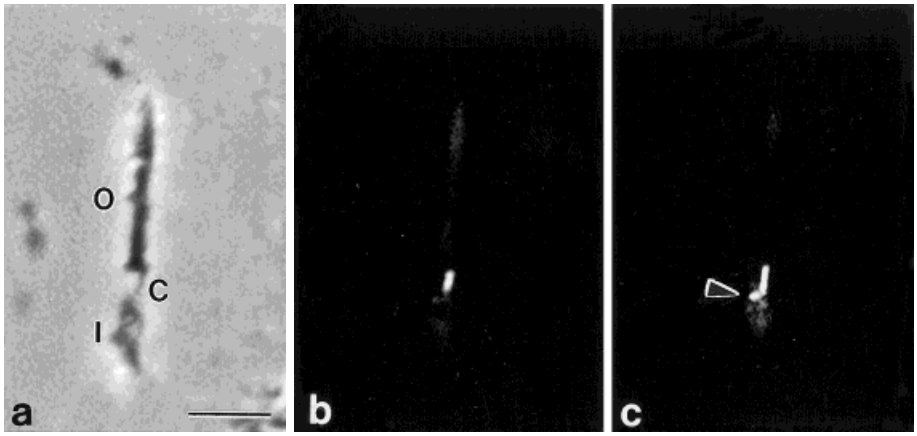


Fig. 10. Double immunofluorescence of an isolated rat photoreceptor cell outer-inner segment. **a:** Phase micrograph. O, outer segment; I, inner segment; C, connecting cilium. **b:** Immunolabelling by pAb 2.2, which was detected by a secondary antibody conjugated to rhodamine. Only the proximal cilium (i.e., connecting cilium) is labelled. **c:** Immunolabelling by antibody against centrin, which was detected by a secondary antibody conjugated to fluorescein isothiocyanate (FITC). Anticentrin labels the basal bodies and both the proximal and distal domains of the cilium. Arrowhead indicates basal bodies. Scale bar = 10 μm.

of a densely packed stack of phototransductive disk membranes (Fig. 16). A major undertaking for photoreceptor cells is the turnover of its disk membrane proteins, mainly opsin [Young, 1967]. These proteins are synthesized de novo in the inner segment of the cell and are transported in membrane vesicles to the base of the connecting cilium [Papermaster et al., 1985]. New disk membranes containing these newly synthesized proteins are formed by outgrowths of the plasma membrane of the distal cilium [Steinberg et al., 1980; Arikawa et al., 1992]. Balancing the addition of new disk membranes, disks at the distal end of the photoreceptor outer segment are phagocytosed by the RPE [Young and Bok, 1969; Williams and Fisher, 1987], where they are subsequently degraded. Defects in the turnover of the phototransductive disk membranes appear to underlie a number of different inherited forms of photoreceptor cell degeneration. For example, some mutations in rhodopsin [Li et al., 1996; Liu et al., 1997] and peripherin/rds [Jansen and Sanyal, 1984; Usukura and Bok, 1987] appear to result in defective disk membrane morphogenesis in transgenic mice and the rds mouse, respectively. In the RCS rat, an unknown genetic defect affects the ability of the RPE to phagocytose outer segment disks [Mullen and LaVail, 1976].

When myosin VIIa was detected by thick-section immunofluorescence in the apical RPE (and nowhere else in the retina), it was suggested that retinitis pigmentosa in USH1B patients might result from a defect in the ability of the RPE to carry out its normal phagocytotic role in disk membrane turnover [Hasson et al., 1995]. Although this suggestion might still be correct, our finding that the connecting cilium has the highest concentration of myosin VIIa in the retina suggests that photoreceptor degeneration in USH1B patients might result from a defective cilium.

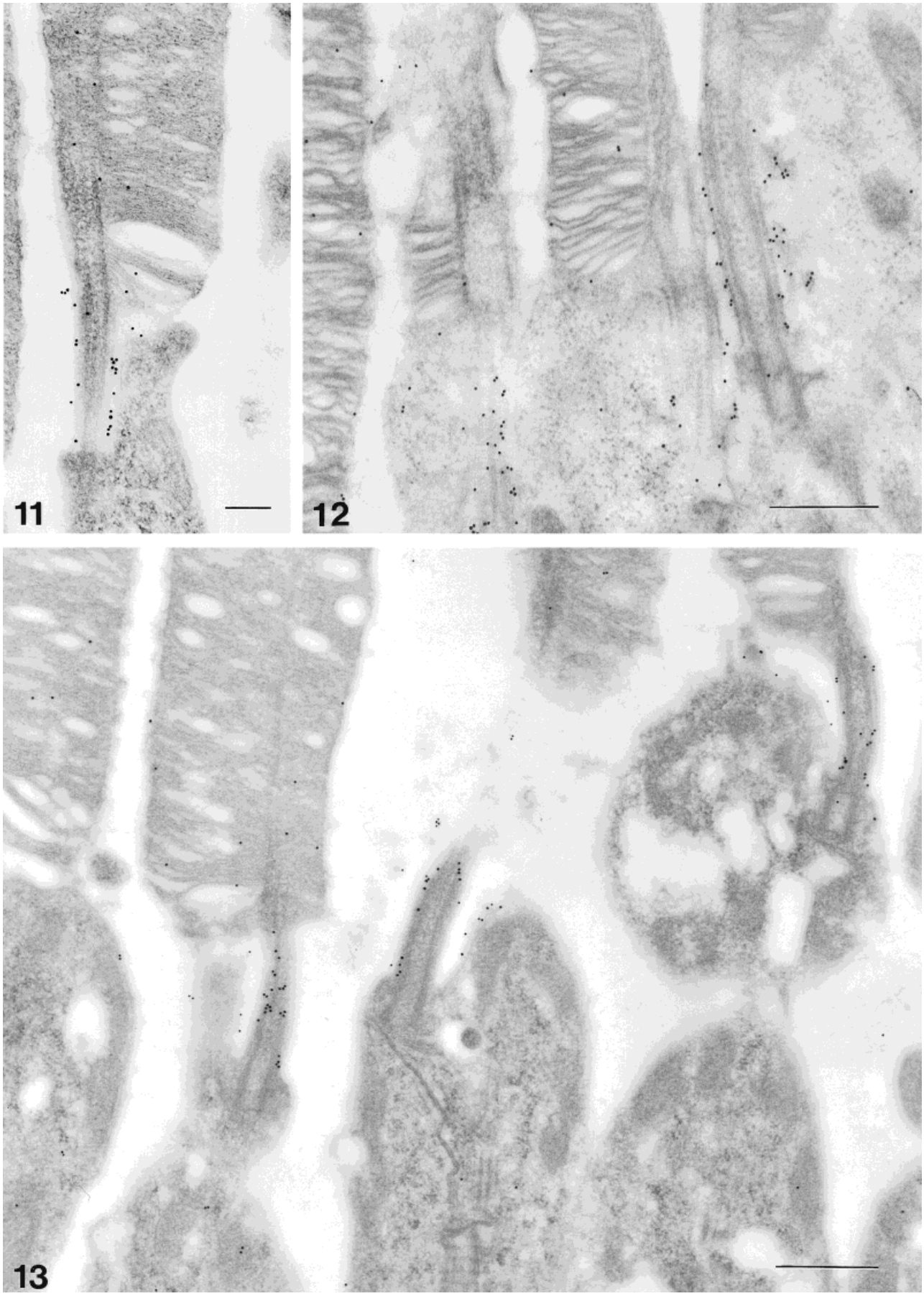
To our knowledge, the present paper is the first to report localization of an unconventional myosin within a cilium. Conventional myosin (myosin II) has been detected in the photoreceptor cilium, but it appeared to be in the center of the cilium and only in the distal cilium, adjacent to the base of the outer segment disks [Williams et al., 1992]. A role for myosin VIIa in the connecting cilium of photoreceptor cells might be the transport of newly synthesized outer segment proteins to the site of disk membrane assembly (i.e., along the length of the connecting cilium). Countering this suggestion, however, is the observation that destruction of the actin filaments in the cilium by cytochalasin D does not prevent newly synthesized phototransductive membrane from reaching the distal cilium, at least in the short term [Williams et al., 1988].

Although newly synthesized outer segment proteins must be transported from the inner segment to the outer segment, an important function of the photoreceptor connecting cilium is to provide a barrier against diffusion between the inner and outer segments [Spencer et al.,

Fig. 11. Longitudinal section of the cilium of a mouse rod photoreceptor cell immunogold labelled with myosin VIIa antibody pAb 2.2. Scale bar = 200 nm.

Fig. 12. Part of a rat retina that was immunogold labelled with myosin VIIa antibody pAb 2.2 showing the connecting cilia of several rod photoreceptor cells in longitudinal section. Scale bar = 500 nm.

Fig. 13. Part of a mouse retina that was immunogold labelled with myosin VIIa antibody pAb 2.2 showing the connecting cilia of several rod photoreceptor cells in longitudinal section. Scale bar = 500 nm.



Figures 11–13.

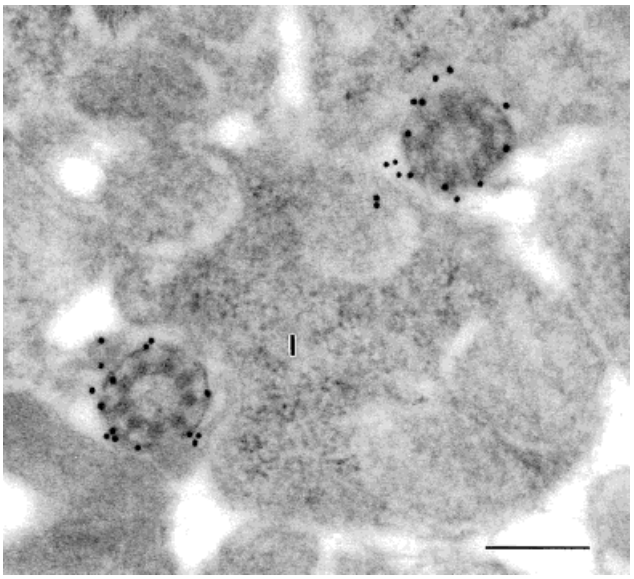


Fig. 14. Transverse section of two connecting cilia from photoreceptor cells in a mouse retina that was immunogold labelled with myosin VIIa antibody pAb 2.2. Each dark-staining structure in the cilia is a microtubule doublet. I, photoreceptor inner segment. Scale bar = 200 nm.

1988; Besharse and Horst, 1990]. Maintenance of this barrier may be an active process and may require a molecular motor, such as myosin VIIa, suggesting an alternative ciliary function for this protein.

Usher Syndrome

In addition to visual and hearing abnormalities, some USH patients have also been found to have deficient olfaction [Zrada et al., 1996], decreased sperm motility [Hunter et al., 1986], abnormal nasal cilia [Arden and Fox, 1979], bronchitis [Bonneau et al., 1993], and asthma [Baris et al., 1994]. Because all of these abnormalities might result from a ciliary defect, it has been suggested that such a defect might be the basis of some forms of Usher syndrome [see, e.g., Hunter et al., 1986; Zrada et al., 1996]. It is unclear from these reports, however, whether any USH1B patients were included in these studies and, thus, whether they have any of these other tissue abnormalities. Consequently, two questions arise.

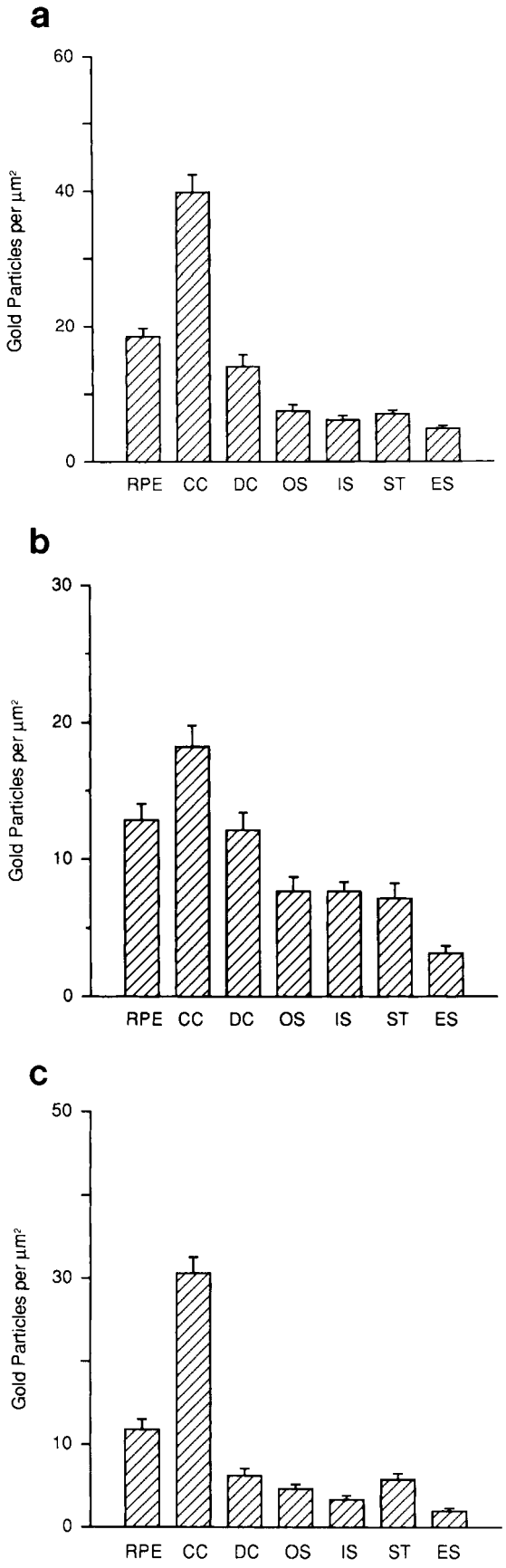


Fig. 15. Histograms of gold particle counts in different domains of the retina. **a:** Human retinae immunogold labelled with myosin VIIa antibody pAb 2.2. **b:** Human retinae immunogold labelled with myosin VIIa antibody pAb 1. **c:** Mouse retinae immunogold labelled with myosin VIIa antibody pAb 2.2. RPE, retinal pigment epithelium; CC, connecting cilium; DC distal cilium; OS, outer segment; IS, inner segment; ST, synaptic terminal; ES, extracellular space surrounding the inner and outer segments (nontissue background control). Histograms indicate mean number of gold particles per μm^2; error bars indicate standard error of the mean.

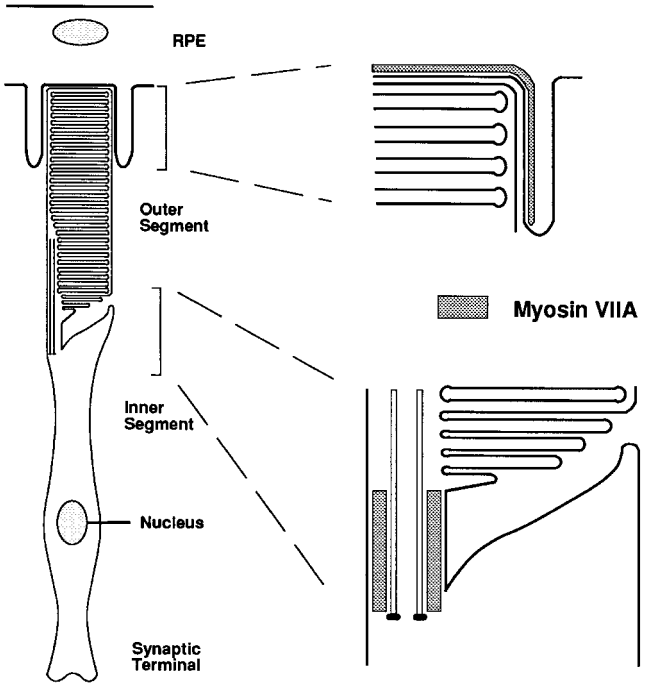


Fig. 16. Diagram of a rod photoreceptor cell and retinal pigment epithelium (RPE). Enlargements on the right summarize the distribution of myosin VIIa, as indicated by immunogold labelling; most labelling was found in the periphery of the photoreceptor cell connecting cilium and the apical surface of the RPE.

First, does myosin VIIa function in cilia in general? The photoreceptor connecting cilium corresponds to the transition zone of a motile cilium [Röhlich, 1975; Besharse and Horst, 1990], and Northern and Western blot analyses and in situ hybridization studies have indicated expression of myosin VIIa in the ear, lung, kidney, testis, and olfactory epithelia in addition to the retina [Gibson et al., 1995; Hasson et al., 1995; Weil et al., 1996]. To date, immunocytochemistry has only been performed on the retina and cochlea. In the latter, it has been found in mature hair cells [Hasson et al., 1995], but the possibility of localization in the kinocilium, which is present only during development of the cochlea [Kikuchi and Hilding, 1965], has not yet been explored.

Second, despite the genetic heterogeneity of Usher syndrome, might defective cilia be a common underlying cause? Abnormal photoreceptor connecting cilia that contain additional microtubules have been described in photoreceptor cells of two USH2 patients [Hunter et al., 1986; Barrong et al., 1992]. Combining these observations with our results leads to the suggestion that photoreceptor degeneration in different types of Usher syndrome might be caused by a defect in the photoreceptor connecting cilium. It is noteworthy that abnormalities in olfaction [Zrada et al., 1996], sperm motility [Hunter et al., 1986], and the structure of nasal cilia [Arden and Fox, 1979] were described from groups containing both USH1 and USH2 patients, and, in the study by Zrada et al. [1996], it was specifically noted that both USH1 and USH2 patients had defective olfaction.

In conclusion, we have described the specific localization of myosin VIIa in the photoreceptor connecting cilium and suggest that retinitis pigmentosa in USH1B patients might result from a defect in this structure. It will be interesting to determine whether myosin VIIa is a component of all cilia and whether the genes that are defective in other types of Usher syndrome encode for other components of the photoreceptor connecting cilium.

ACKNOWLEDGMENTS

We thank Joan Delaat for assistance with Western blots, Dr. Larry Goldstein for helpful discussions, Drs. Marilyn Farquhar and Michael McCaffery for use of their electron microscope facilities, and Drs. Tama Hasson and Mark Mooseker for the gift of the human myosin VIIa (1–1,075) cDNA. This study was supported by NIH grant EY 07042 to D.S.W., by a postdoctoral fellowship from the Fight For Sight Research Division of Prevent Blindness America to X.L., and by the Faunstiftung, Nürnberg (U.W.).

REFERENCES

Arden, G.B., and Fox, B. (1979): Increased incidence of abnormal nasal cilia in patients with retinitis pigmentosa. Nature 279:534–536.

Arikawa, K., Molday, L.L., Molday, R.S., and Williams, D.S. (1992): Localization of peripherin/rds in the disk membranes of cone and rod photoreceptors: Relationship to disk membrane morphogenesis and retinal degeneration. J. Cell Biol. 116:659–667.

Azarian, S.M., King, A.J., Hallett, M.A., and Williams, D.S. (1995): Selective proteolysis of arrestin by calpain: Molecular characterization and its effect on rhodopsin dephosphorylation. J. Biol. Chem. 270:24375–24384.

Baris, B., Ataman, M., Sener, C., and Kalyoncu, F. (1994): Bronchial asthma in a patient with Usher syndrome: Case report. J. Asthma 31:487–490.

Barrong, S.D., Chaitin, M., Fliesler, S., Possin, D., Jacobson, S., and Milam, A. (1992): Ultrastructure of connecting cilia in different forms of retinitis pigmentosa. Arch. Ophthalmol. 110:706–710.

Besharse, J.C., and Horst, C.J. (1990): The photoreceptor connecting cilium: A model for the transition zone. In Bloodgood, R.A. (ed.): "The Photoreceptor Connecting Cilium: A Model for the Transition Zone." New York: Plenum Press, pp. 409–431.

Bonneau, D., Raymond, F., Kremer, C., Klossek, J.-M., Kaplan, J., and Patte, F. (1993): Usher syndrome type I associated with bronchiectasis and immotile nasal cilia in two brothers. J. Med. Genet. 30:253–254.

Boughman, J., Vernon, M., and Shaver, K. (1983): Usher syndrome: Definition and estimate of prevalence from two high-risk populations. J. Chronic Dis. 36:595–603.

Chen, Z.Y., Hasson, T., Kelley, P.M., Schwender, B.J., Schwartz, M.F., Ramakrishnan, M., Kimberling, W.J., Mooseker, M.S., and Corey, D.P. (1996): Molecular cloning and domain structure of

human myosin-VIIa, the gene product defective in Usher syndrome 1B. Genomics 36:440–448.

Chomezynski, P., and Sacchi, N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.

El-Amraoui, A., Sahly, I., Picaud, S., Sahel, J., Abitbol, M., and Petit, C. (1996): Human Usher 1B/mouse *shaker-1*: the retinal phenotype discrepancy explained by the presence/absence of myosin VIIA in the photoreceptor cells. Human Mol. Genet. 5:1171–1178.

Gibson, F., Walsh, J., Mburu, P., Varela, A., Brown, K.A., Antonio, M., Beisel, K.W., Steel, K.P., and Brown, S.D.M. (1995): A type VII myosin encoded by mouse deafness gene *shaker-1*. Nature 374:62–64.

Hasson, T., Heintzelman, M.B., Santos-Sacchi, J., Corey, D.P., and Mooseker, M.S. (1995): Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. Proc. Natl. Acad. Sci. USA 92:9815–9819.

Horst, C.J., Forestner, D.M., and Besharse, J.C. (1987): Cytoskeletal-membrane interactions: A stable interaction between cell surface glycoconjugates and doublet microtubules of the photoreceptor connecting cilium. J. Cell Biol. 105:2973–2987.

Hunter, D.G., Fishman, G.A., Mehta, R.S., and Kretzer, F.L. (1986): Abnormal sperm and photoreceptor axonemes in Usher's Syndrome. Arch. Ophthalmol. 104:385–389.

Jansen, H.G., and Sanyal, S. (1984): Development and degeneration of retina in rds mutant mice: Electron microscopy. J. Comp. Neurol. 224:71–84.

Kikuchi, K., and Hilding, D. (1965): The development of the organ of Corti in the mouse. Acta Otolaryngol. 60:207–222.

Li, T.S., Snyder, W.K., Olsson, J.E., and Dryja, T.P. (1996): Transgenic mice carrying the dominant rhodopsin mutation P347S—Evidence for defective vectorial transport of rhodopsin to the outer segments. Proc. Natl. Acad. Sci. USA 93:14176–14181.

Liu, X., Wu, T.-H., Stowe, S., Matsushita, A., Arikawa, K., Naash, M.I., and Williams, D.S. (1997): Defective phototransductive disk membrane morphogenesis in transgenic mice expressing rhodopsin with a mutated N-terminal domain. Submitted.

Mullen, R.J., and LaVail, M. (1976): Inherited retinal dystrophy: Primary defect in pigment epithelium determined with experimental rat chimeras. Science 192:799–801.

Papermaster, D.S., Schneider, B.G., and Besharse, J.C. (1985): Vesicular transport of newly synthesized opsin from the Golgi apparatus toward the rod outer segment. Invest. Ophthalmol. Vis. Sci. 26:1386–1404.

Pow, D.V., and Crook, D.K. (1993): Extremely high titre polyclonal antisera against small neurotransmitter molecules: Rapid production, characterisation and use in light- and electron-microscopic immunocytochemistry. J. Neurosci. Methods 48:51–63.

Röhlich, P. (1975): The sensory cilium of retinal rods is analogous to the transitional zone of motile cilia. Cell Tissue Res. 161:421–430.

Spencer, M., Detwiler, P.B., and Bunt-Milam, A.H. (1988): Distribution of membrane proteins in mechanically dissociated retinal rods. Invest. Ophthalmol. Vis. Sci. 29:1012–1020.

Steel, K.P., and Bock, G.R. (1983): Inherited hearing defects in mice. Annu. Rev. Genet. 29:675–701.

Steinberg, R.H., Fisher, S.K., and Anderson, D.H. (1980): Disc morphogenesis in vertebrate photoreceptors. J. Comp. Neurol. 190:501–508.

Usher, C. (1914): On the inheritance of retinitis pigmentosa with notes of cases. R. London Ophthalmol. Hosp. Rep. 19:130–236.

Usukura, J., and Bok, D. (1987): Changes in the localization and content of opsin during retinal development in the rds mutant mouse: Immunocytochemistry and immunoassay. Exp. Eye Res. 45:501–515.

Weil, D., Blanchard, S., Kaplan, J., Guilford, P., Gibson, F., Walsh, J., Mburu, P., Varela, A., Levilliers, J., Weston, M.D., Kelley, P.M., Kimberling, W.J., Wagenaar, M., Levi-Acobas, F., Larget-Piet, D., Munnich, A., Steel, K.P., Brown, S.D.M., and Petit, C. (1995): Defective myosin VIIA gene responsible for Usher syndrome type 1B. Nature 374:59–61.

Weil, D., Levy, G., Sahly, I., Levi-Acobas, F., Blanchard, S., El-Amraoui, A., Crozet, F., Philippe, H., Abitbol, M., and Petit, C. (1996): Human myosin VIIA responsible for the Usher 1B syndrome: A predicted membrane-associated motor protein expressed in developing sensory epithelia. Proc. Natl. Acad. Sci. USA 93:3232–3237.

Williams, D.S., and Fisher, S.K. (1987): Prevention of the shedding of rod outer segment disks by detachment from the retinal pigment epithelium. Invest. Ophthalmol. Vis. Sci. 28:184–187.

Williams, D.S., Linberg, K.A., Vaughan, D.K., Fariss, R.N., and Fisher, S.K. (1988): Disruption of microfilament organization and deregulation of disk membrane morphogenesis by cytochalasin D in rod and cone photoreceptors. J. Comp. Neurol. 272:161–176.

Williams, D.S., Hallett, M.A., and Arikawa, K. (1992): Association of myosin with the connecting cilium of rod photoreceptors. J. Cell Sci. 103:183–190.

Wolfrum, U. (1995): Centrin in the photoreceptor cells of mammalian retinae. Cell Motil. Cytoskeleton 32:55–64.

Young, R.W. (1967): The renewal of photoreceptor cell outer segments. J. Cell Biol. 33:61–71.

Young, R.W., and Bok, D. (1969): Participation of the retinal pigment epithelium in the rod outer segment renewal process. J. Cell Biol. 42:392–403.

Zrada, S.E., Braat, K., Doty, R.L., and Laties, A.M. (1996): Olfactory loss in Usher syndrome—Another sensory deficit. Am. J. Med Genet. 64:602–603.