Differential Distribution of Harmonin Isoforms and Their Possible Role in Usher-1 Protein Complexes in Mammalian Photoreceptor Cells

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Purpose. Human Usher syndrome is the most common form of combined deafness and blindness. Usher type I (USH1), the most severe form, is characterized by profound congenital deafness, constant vestibular dysfunction, and prepubertal onset retinitis pigmentosa. Previous studies have shown that the USH1-proteins myosin VIIa, harmonin, and cadherin 23 interact and form a functional network during hair cell differentiation in the inner ear. The purpose of the present study was to analyze the molecular and cellular functions of these USH1 proteins in the mammalian retina.

Methods. Antibodies to USH1 proteins were generated and used in Western blot analysis of subcellular photoreceptor fractions and immunofluorescence and electron microscopy of the retina.

Results. Splice variants of harmonin were differentially expressed in the photoreceptor cell compartments. Whereas harmonin b isoforms were restricted to the light-sensitive outer segment, the harmonin a and c isoforms were more ubiquitously distributed in the photoreceptors. At the synaptic terminal of photoreceptor cells, harmonin a and c colocalized with myosin VIIa and cadherin 23.

Conclusions. USH1 molecules can assemble to a supramolecular complex at photoreceptor synapses. Such a complex may contribute to the cortical cytoskeletal matrices of the pre- and postsynaptic regions, which are thought to play a fundamental role in the organization of synaptic junctions. Dysfunction of any of the USH1 complex partners may lead to synaptic dysfunction causing retinitis pigmentosa, the clinical phenotype in the retina of patients with USH1. Furthermore, in photoreceptor outer segments, harmonin may also contribute to the clustering of outer segment proteins into supramolecular complexes. (*Invest Ophthalmol Vis Sci.* 2003;44:5006–5015) DOI:10.1167/iovs.03-0483

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The human Usher syndrome (USH) is the most frequent cause of combined deafness and blindness. Depending on the degree of clinical syndromes, USH can be divided into three types USH-1, -2, and -3. USH1 is the most severe form, characterized by profound congenital deafness, constant vestibular dysfunction, and prepubertal-onset retinitis pigmentosa. Seven USH1 genes (*USH1A-G*) have been mapped, and five of the corresponding causative genes have been cloned.[1] *USH1B* encodes for the molecular motor myosin VIIa.[2] *USH1C* encodes harmonin, a scaffold protein containing PDZ domains (Fig. 1).[3,4] PDZ modules are thought to act as organizers of membrane-associated supramolecular complexes.[5] Alternatively spliced *USH1C* transcripts predict at least 10 protein isoforms grouped into the three subclasses—harmonin a, b, and c—differing mainly at the C-terminus (Fig. 1A).[3] Mutations in genes encoding two cell–cell adhesion proteins cadherin 23 (*USH1D*; Fig. 1C)[6,7] and protocadherin 15 (*USH1F*)[8–10] cause two other USH1 variants. During completion of this study, *USH1G* was identified to encode SANS, a scaffold protein containing ankyrin repeats and a SAM domain.[11]

In independent parallel studies, interactions of harmonin with USH1 proteins, with cadherin 23 (USH1D)[12] and with cadherin 23 as well as myosin VIIa (USH1B)[13] were identified. Our study on inner ear hair cells revealed that interactions of the USH1 proteins play a fundamental role in differentiation of the actin bundles in stereovilli.[13] A failure of this process leads to stereovilli disorganization, as observed in animal models, and is thought to be responsible for profound congenital deafness in patients with Usher syndrome.[1] However, patients with USH also have visual impairment that occurs much later in life than hearing loss, but is caused by the same genetic defects. Nevertheless, with the exception of myosin VIIa,[14–17] the functions of the proteins underlying USH1 in the retina are still elusive. Although myosin VIIa is expressed in rod and cone photoreceptor cells and in the retinal pigment epithelium, previous studies have indicated expressions of the USH1 proteins harmonin, cadherin 23, and protocadherin 15 in the neuronal retina, but not in the retinal pigment epithelium.[2,3,7,8,10,18]

In this study, we investigated the cellular expression and subcellular localization of the USH1 protein complex—namely, myosin VIIA, harmonin, and cadherin—in the mammalian retina, with a main focus on the harmonin isoforms. Our present results show coexpression of these USH1 proteins in photoreceptor cells and reveal that the harmonin isoforms are localized in spatially different subcellular photoreceptor compartments. We further provide evidence that the supramolecular USH1 protein complex of cadherin 23 and myosin VIIa, bridged by harmonin, may assemble at photoreceptor synapses.

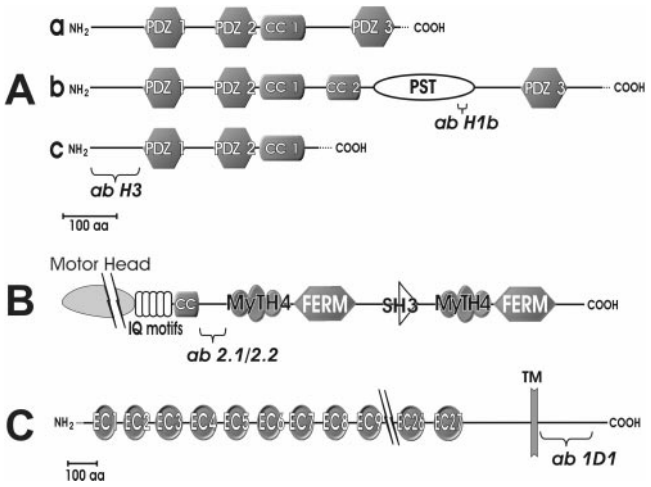

FIGURE 1. The Usher proteins harmonin, myosin VIIa, and cadherin 23. (**A**) Predicted structure of harmonin, grouped into three classes a, b, and c. Common features of all isoforms are the two PDZ domains and one coiled–coil domain (CC1). Harmonin isoforms a and b contain an additional PDZ domain, whereas harmonin isoform b includes a second coiled–coil domain (CC2) and a proline-serine-threonine (PST)–rich region. Each of the classes encloses several isoforms, which predominantly differ in their C-terminal end. (**B**) The unconventional myosin VIIa contains a motor head, a neck region composed of five isoleucine-glutamine (IQ) motifs, and a tail region. This tail region includes a coiled–coil domain (CC), involved in the formation of homodimers, followed by two large repeats separated by a SH3 domain (src homology-3). These repeats consist of a MyTH4 (myosin tail homology 4) and a FERM domain (4.1, ezrin, radixin, moesin). (**C**) Cadherin 23 is composed of 27 extracellular cadherin repeats (EC) and a short intracellular region. We have indicated the domains used to generate specific antibodies against harmonin (H3, H1b), myosin VIIa (2.1/2.2), and cadherin 23 (1D1) (drawings adapted from Ref. 1).

METHODS

Animals and Tissue Preparation

All experiments described herein conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult Wistar albino rats, C57BL/6J mice, and waltzer $v^{alb-/-}$ mice were maintained on a 12-hour light–dark cycle, with food and water ad libitum. After the animals were killed with CO_2, the entire eyeballs were dissected, or the retinas were removed through a slit in the cornea before further analysis. For immunoblot analysis, the appropriate tissues were homogenized in buffer containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were prepared in either radioimmunoprecipitation assay (RIPA) buffer or 500 mM HEPES buffer.

Antibodies

The harmonin H3 polyclonal antibody was generated by cloning a cDNA coding for an N-terminal epitope (amino acids [aa] 1-89) of mouse harmonin. The cadherin 23 1D1 antibody was generated against an epitope located in the intracellular region of human CDH23 (3287–3465 aa, 178 aa) bacterially expressed via a pGEX expression vector (Amersham Biosciences, Freiburg, Germany). Expression and purification of the glutathione *S*-transferase (GST) fusion protein was performed according to the manufacturer's instructions. After cleavage of the fusion protein with thrombin on the column, the eluted peptides were used to immunize rabbits. Derived antibodies were affinity purified with *N*-hydroxysuccinimide (NHS)–activated columns (HiTRAP; Amersham Biosciences) loaded with the appropriate antigen. The H1b antibody for harmonin isoform b was generated against a peptide of an epitope located in the PST domain of the protein (aa 636-649) and has been described.[13] The antibody D6 was produced against the human harmonin orthologue AIE-75.[19] Also previously described were the anti-myosin VIIa (2.1/2.2)[14] and anti-opsin[17] antibodies. The anti-cytochrome *c* (H-104) and anti-synaptophysin (SVP38) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of ROS

Rod outer segments (ROS) were purified from 14 rat retinas by the discontinuous sucrose gradient method described by Papermaster[20] in a smaller volume. Because immunofluorescence microscopy of retinas showed no differences between dark and light adaptation, the procedure was performed under light. The purity of the ROS was confirmed by immunoblot analysis. Briefly, the retinas were homogenized in 1.4 mL homogenizing medium (1 M sucrose, 65 mM NaCl, 0.2 mM $MgCl_2$, 5 mM Tris-acetate [pH 7.4]) and centrifuged at 2000*g* for 4 minutes at 4°C. The supernatant was diluted with twice its volume in 0.01 M Tris-acetate (pH 7.4) and gently mixed. The ROS were pelleted at 2000*g* for 4 minutes at 4°C and resuspended and homogenized in the first density gradient solution (1.10 g/mL sucrose, 0.1 mM $MgCl_2$, 1 mM Tris-acetate [pH 7.4]). The crude ROS were carefully overlaid on a three-step gradient (1.11 g/mL, 1.13 g/mL, and 1.15 g/mL sucrose with 0.1 mM $MgCl_2$ and 1 mM Tris-acetate [pH 7.4]) and centrifuged at 85,000*g* for 30 minutes. The interface containing the ROS was recovered and diluted with 0.01 mM Tris-acetate (pH 7.4), and the ROS were pelleted again at 50,000*g* for 20 minutes. The pellet with the ROS was resuspended and stored at −80°C before use in immunoblot analyses.

Isolation of Crude Synaptosomes

Crude synaptosomes were prepared as described by Hirao et al.[21] In brief, one adult rat brain, or 11 rat retinas (with the following volumes scaled down by a factor of 10), were homogenized in 8 mL extraction buffer 1 (0.32 M sucrose in 4 mM HEPES, [pH 7.4]) and centrifuged at 800*g* for 10 minutes at 4°C. The supernatant was centrifuged at 9200*g* for 15 minutes at 4°C. The pellet was resuspended in 8 mL of extraction buffer 1 and centrifuged at 10,200*g* for 15 minutes at 4°C. The pellet, representing the crude synaptosomal fraction, was resuspended with 8 mL of a buffer containing 20 mM HEPES/NaOH (pH 8.0), 100 mM NaCl, 5 mM EDTA, and 1% Triton X-100 and centrifuged at 100,000*g* for 30 minutes at 4°C. The supernatant was used in the immunoblot analyses.

Serial Tangential Sectioning

The immunoblot analysis of serial tangential sections was performed as described by Sokolov et al.,[22] with slight modification. Briefly, dissected rat eyecups, after removal of the vitreous, were cut at four opposite sites and flatmounted between two glass slides separated by 0.5-mm spacers. The bottom slide facing the basal membrane of the retina was roughened with sandpaper to improve adhesion. The top slide facing the outer side of the eyecup was covered with Teflon spray to improve later release. The glass slide and eyecup sandwich was held together by two small binder clips and frozen immediately on dry ice. The bottom slide with the eyecup was mounted in a cryomicrotome, and sequential 10-μm tangential sections of the eyecup were collected in 100 μL SDS-PAGE sample buffer. Aliquots of 10 μL per lane were subjected to immunoblot analyses.

Immunoblot Analyses

For denaturing gel electrophoresis, the samples were mixed with SDS-PAGE sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% mercaptoethanol, 1 mM EDTA, and 0.025% bromphenol blue). Proteins (30 μg per lane) were separated on 3.5% to 8% Tris-acetate–buffered (125 mM [pH 7.0]) or 4% to 12% MES/MOPS (2-(*N*-morpholino)ethane sulfonic acid/3-(*N*-morpholino)propanesulfonic acid)–buffered bis-tris polyacrylamide gradient gels (NuPage) according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany) and blotted

onto polyvinylidene difluoride (PVDF) membranes (Amersham Biotech). Immunoreactivities were detected on Western blot analysis, with the appropriate primary antibodies and donkey anti-rabbit secondary antibodies coupled to horseradish peroxidase by a chemiluminescence detection system (ECL+; Amersham Biosciences). For the competition experiment, the antibodies H3 and 1D1 were preincubated for at least 1 hour at room temperature in the presence of 1 mg/mL antigen used to immunize the rabbit. As a molecular marker, a gold label (peqLab Gold; Peqlab, Erlangen, Germany) and a prestained marker (Sigma-Aldrich, Diesenhofen, Germany) were used, ranging in size from 14 to 180 kDa. A mixture of keyhole limpet hemocyanin (KLH) subunits (kindly provided by Wolfgang Gebauer, University of Mainz, Germany) was used to determine the position of 400 kDa on the gel. Band sizes were calculated electronically (TotalLab software; Phoretix, Newcastle-upon-Tyne, UK).

Reverse Transcription–Polymerase Chain Reaction

For reverse transcriptase–PCR (RT-PCR), eyes and brains of mice and rats were isolated and frozen with liquid nitrogen. Total RNA was isolated from frozen tissue using the EZNA Total RNA kit (PeqLab). Subsequently, transcripts of harmonin isoforms were amplified applying the One-Step Long-Template RT-PCR kit (Invitrogen), analyzed on 1% agarose gels and cloned using the Topo TA Cloning kit (Invitrogen). The primers used were 5′-TACATCTGGTGTCTCTCAGG-3′ as reverse primer, located in all harmonin transcripts in 3′-untranslated region (UTR), and 5′-ATGGACCGGAAGGTGGC-3′, 5′-ATGTATCACCAGACCATGGATGTGG-3′, or 5′-AATGTCCTGAAGAGCAGCCGC-3′ as forward primer. The resultant plasmids were sequenced (GENterprise, Mainz, Germany) and results were compared using the BLAST algorithm within the public databases of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/ NCBI, Bethesda, MD). To exclude amplification of genomic DNA, negative controls were performed by processing samples with *Taq* polymerase only and by confirming that no introns were present in the sequences received.

Immunofluorescence Microscopy

Eyes of adult mice and rats were either prefixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 hour at room temperature or cryofixed directly in melting isopentane. Prefixed tissue was washed, infiltrated with 30% sucrose in PBS overnight, cryofixed in melting isopentane, and cryosectioned as described by Wolfrum.[23] Cryosections were placed on poly-L-lysine–precoated coverslips and incubated with 50 mM NH_4Cl and 0.1% Tween 20 in PBS. PBS-washed sections were blocked with blocking solution (0.5% cold-water fish gelatin plus 0.1% ovalbumin in PBS) for 30 minutes, and then incubated with primary antibody in blocking solution overnight at 4°C. Washed sections were subsequently incubated with secondary antibodies conjugated to Alexa 488 or Alexa 546 (Molecular Probes, Leiden, The Netherlands) in blocking solution for 1 hour at room temperature in the dark. After they were washed, sections were mounted in Mowiol 4.88 (Hoechst, Frankfurt, Germany). In the appropriate controls, in no case was a reaction observed. Mounted retinal sections were examined by microscope (DMRP; Leica, Bensheim, Germany). Images were obtained with a charge-coupled device camera (ORCA ER; Hamamatsu, Herrsching, Germany) and processed (Photoshop; Adobe Systems, Mountain View, CA).

Immunoelectron Microscopy

Freshly isolated rat and mouse retinas were fixed and embedded in resin (LR White; Electron Microscopy Sciences, Fort Washington, PA) as previously described by Wolfrum and Schmitt.[17] Ultrathin sections collected on polyvinyl formal (Formvar; SPE, West Chester, PA)–coated nickel grids were etched with saturated sodium periodate (Sigma-Aldrich) before immunogold labeling as described.[17] Immunogold label (Nanogold; Nanoprobes, Yaphank, NY) was silver-enhanced according to Danscher.[24] Counterstained ultrathin sections were analyzed by electron microscope (model EM 900 or EM 906; Carl Zeiss Meditec, Oberkochen, Germany; or model Tecnai 12; Eindhoven, The Netherlands). The quantification of silver-enhanced gold particles was accomplished by counting particles in the inner and outer segments of 30 randomly selected photoreceptor cells of three independent samples (image processing: Photoshop; Adobe Systems, and ImageJ; available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). To confirm the significance of the distribution, a paired Student's *t*-test ($\alpha = 0.001$) was applied.

Results

Harmonin Isoform Expression in the Retina

Previous RT-PCR investigations have indicated that harmonin expression in the mouse retina is restricted to the isoforms of the subclasses a and c.[3] In the current study, we reexamined the expression of harmonin isoforms in the retina by additional RT-PCR approaches and by Western blot analyses with isoform-specific harmonin antibodies.

To address the expression of the harmonin isoform b in the retina, RT-PCR was performed with the RNA of rat and mouse retinas. Several forward primers were chosen in the beginning of the coding sequence and a reverse primer in the 3′-UTR common to all isoforms, resulting in amplification products from 1 to 3 kb that overlapped multiple exons (data not shown). Different PCR products were subcloned and sequenced. In agreement with Verpy et al.,[3] isoforms a and c were found to be expressed in the retina in the current study. In addition to the a and c isoforms, harmonin b isoforms were transcribed in the retina. Sequence analysis of the expressed harmonin b isoforms revealed, besides harmonin b2 and b3 sequences, a sequence nearly identical with harmonin b3, which we named b4. The b4 sequence includes an insertion of 8 bp at the border between the exon G′ and exon 21 at position 2563. It has been deposited under GenBank accession number AY246556 (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

For our immunoblot analyses, we applied a polyclonal rabbit antiserum (H3) against the recombinantly expressed N-terminus of harmonin containing both potential start codons and a previously characterized peptide antibody (H1b) against a domain specific for harmonin b (Fig. 1A).[13] In analyses of retina and brain homogenates, affinity-purified pan-harmonin H3 antibodies detected two major bands at 73 and 113 kDa, as well as several minor bands (Fig. 2A, lanes 1 [retina] and 2 [brain]). The band at 73 kDa matches the molecular weight of the human orthologues of harmonin a, previously reported as AIE-75 (75 kDa) and PDZ-73 (73 kDa).[19,25] As previously shown by Kobayashi et al.[19] this 73-kDa polypeptide was also expressed in the kidney, whereas a band at 113 kDa was missing (Fig. 2A, lane 3 [kidney]). To determine whether the larger 113-kDa band represents the harmonin isoform b with the calculated mass of 100 kDa, we dissected transblotted protein lanes and incubated them with the pan-harmonin H3 antibodies or the harmonin b–specific H1b antibody (Fig. 2B). The data obtained by Western blot analysis show that the detected 113-kDa band represents the harmonin isoform b. Below the two main bands are some weaker bands that comigrate with the molecular sizes of harmonin isoforms c with 45, 54, and 59 kDa, which were previously reported by Scanlan et al.[25] However, because the intensity of these bands increases rapidly over time in tissue lysates, they may also represent

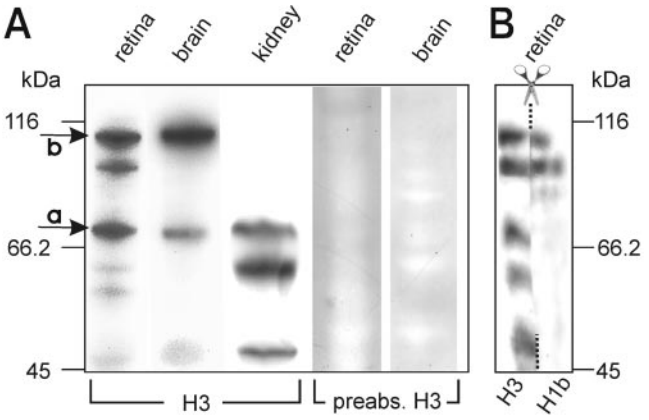

FIGURE 2. Immunoblot analysis of harmonin isoform expression in rat tissues. (**A**) Total protein extracts of rat retina, brain, and kidney were immunoblotted with the pan-harmonin H3 antibodies (*lanes 1* to *3*). The molecular weights of the two major bands (indicated with a and b) detected in brain and retina lysates correspond with the sizes of the harmonin a and b isoforms (73 and 113 kDa, respectively). Lower molecular weight bands may represent harmonin c isoforms or degradation products of harmonin a and b. Note that in immunoblotted kidney extracts, bands higher than 73 kDa were absent (*lane 3*). In *lanes 4* and *5*, total protein extract of rat retina and brain were immunoblotted with the H3 antibody preabsorbed with recombinantly expressed antigen. All bands present in *lane 1* and *2* were abolished. (**B**) Immunoblot analysis of the 113-kDa band with anti-harmonin H3 and H1b antibodies. For isoform determination, a Western blot wide lane of rat retina extract was cut in half and probed either with H3 antibodies or with the harmonin b isoform–specific H1b antibodies. Both labeled the identical band at 113 kDa.

proteolytic degradation products of harmonin a and b isoforms.

To validate the specificity of the pan-harmonin H3 antiserum to the epitope, affinity-purified antibodies were preincubated with 1 mg/mL of the recombinantly expressed antigen used for immunization before use in Western blot analysis of retina and brain homogenates. Immunoreactive bands were completely abolished (Fig. 2A, lanes 4 [retina] and 5 [brain]), whereas H3 antibodies pretreated with recombinantly expressed unrelated antigen did not show any difference from the samples that were not preabsorbed with any peptide (data not shown).

Expression of Harmonin Isoforms in Subcellular Compartments of Photoreceptor Cells

The vertebrate retina is composed of well-defined layers consisting of specific sets of cell types or subcellular compartments of retinal cells (see differential interference contrast [DIC] image in Fig. 3A). This spatial distribution makes it relatively simple to determine the subcellular localization of proteins in a section through the retina, even by light microscopy. To determine the localization of harmonin in the retina, cryosections through the eyes of mice and rats were analyzed by immunofluorescence microscopy with the pan-harmonin H3 antibodies. Harmonin isoforms were located in the photoreceptor layer, in the inner and the outer plexiform layers, and in the ganglion layer of the retina (Fig. 3B). In contrast, no labeling was observed in the cells of the pigmented epithelium (Fig. 3B) or when the antibodies were preincubated with the N-terminal peptide of harmonin before they were added to the cryosections (data not shown).

We next wanted to explore the subcellular distribution of harmonin isoforms in photoreceptor cells. Vertebrate photoreceptor cells are highly polarized sensory cells composed of their light-sensitive outer segments, metabolically active inner segments, perikarya, and synapses. These subcellular compartments can be easily assigned to specific horizontal layers in retinal cryosections. Indirect immunofluorescence analysis with affinity-purified H3 pan-harmonin antibodies of the photoreceptor layers of retinas revealed harmonin localization in the outer segment, to a lesser extent in the inner segment, and in the outer plexiform layer containing the photoreceptor synapses (Fig. 4B). These findings were confirmed by immunocytochemical experiments with an additional pan-harmonin D6 antibody that has been used for the characterization of the harmonin orthologue AIE-75 in human (Fig. 4C).[13,19] Immunoelectron microscopy analysis of ultrathin retina sections further supported harmonin localization in the photoreceptor cell compartments. In both types of photoreceptor cells, silver-enhanced immunogold labeling was predominantly localized at the membranous disks of the outer segment, whereas the inner segment was stained to a significantly lesser extent (Fig. 4E). For quantification of the immunolabeling, silver-enhanced gold particles were counted per square micrometer of both photo-

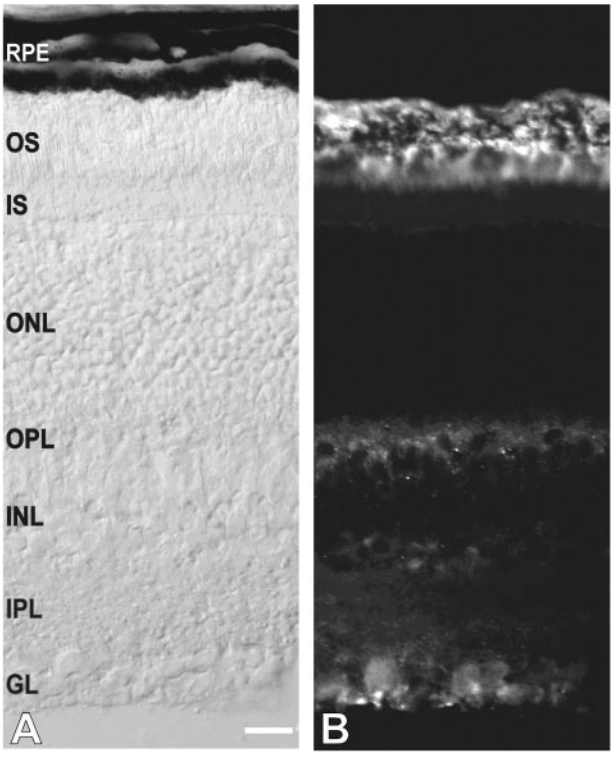

FIGURE 3. Localization of harmonin in the mouse retina. (**A**) The composition of the vertebrate retina is visualized in this DIC image of a cryosection through a light-adapted mouse retina. The retina can be divided into distinct layers of a well-defined content. The layers of photoreceptor cells contain the light-sensitive outer segments (OS); the inner segments (IS); the outer nuclear layer (ONL), where the perikarya or cell bodies of photoreceptor cells are localized; and the outer plexiform layer (OPL), which contains the photoreceptor synaptic terminals. The cell bodies of the secondary retinal neurons are present in the inner nuclear layer (INL) and their synaptic connections are found in the inner plexiform layer (IPL). The ganglion layer (GL) contains the cell bodies of the ganglion cells that extend axons into the optic nerve to the brain. (**B**) Corresponding indirect immunofluorescence image of the cryosection stained with anti-harmonin H3. Anti-pan-harmonin labeling was predominantly found in the OS of photoreceptor cells, the OPL, and the ganglion cell layer. Faint anti-harmonin staining was present in the photoreceptor cell IS and IPL. Bar, 10 μm.

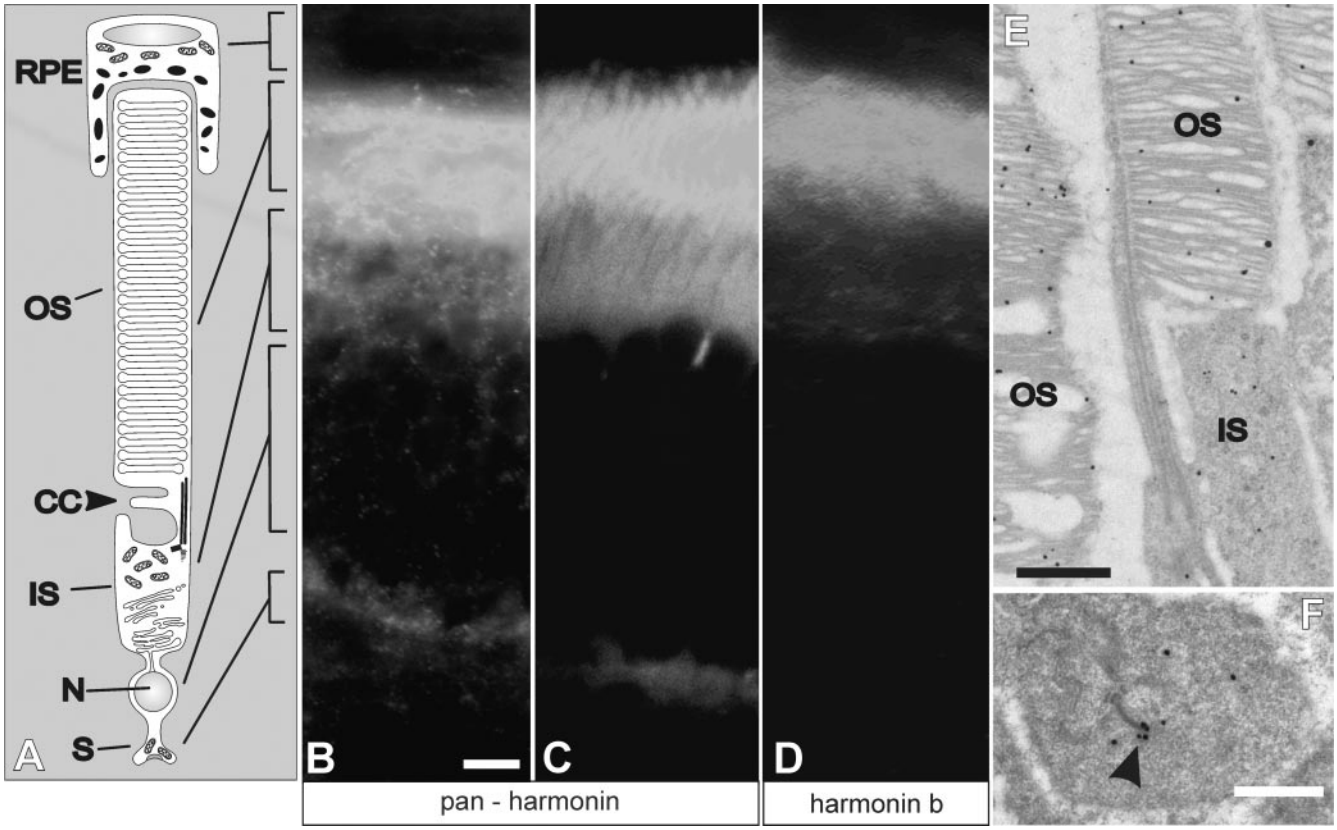

FIGURE 4. Localization of harmonin isoforms in subcellular compartments of photoreceptor cells. (**A**) Schematic representation of a rod photoreceptor cell. Vertebrate photoreceptors are composed of distinct morphologically and functionally compartments. The photosensitive outer segment (OS), which is connected through the connecting cilium (CC) to the inner segment (IS), which contains the biosynthetic machinery of the cell, the cell body, which is localized in the ONL of the retina and contains the nucleus (N) and the synaptic terminal (S) in the OPL of the retina. (**B–D**) Indirect immunofluorescence with harmonin antibodies in cryosections of an unfixed rat retina. Labeling with pan-harmonin antibodies H3 (**B**) D6 (**C**). Immunolabeling was predominantly found in outer segments and inner segments of photoreceptor cells, as well as in their synapses. (**D**) Immunolabeling with harmonin b isoform–specific antibody H1b. Labeling was present in outer segments, but not at synapses, indicating that harmonin b was restricted to photoreceptor outer segments. (**E**, **F**) Electron micrographs of sections through mouse photoreceptor cell compartments, illustrating silver-enhanced immunogold labeling of harmonin (harmonin antibody H3). (**E**) Longitudinal section through a portion of the OS and the apical IS of a rod photoreceptor cell. Labeling was associated with OS disks and in the cytoplasm of the IS. (**F**) Ultrathin section through synaptic terminal of a cone photoreceptor cell. Labeling was localized at the ribbon synapse. Bars: (**B**) 5 μm; (**E**) 0.5 μm; (**F**) 0.4 μm.

receptor compartments. This analysis revealed 44% ± 12% fewer particles in the inner segment than in the outer segment (IS: 6.19 ± 1.4 particles per μm^2; OS: 10.97 ± 0.6 particles per μm^2; $P < 0.001$; $n = 30$). Labeling of harmonin at the membrane of the connecting cilium, where myosin VIIa has been localized,[14] was absent. At the photoreceptor synaptic terminal, labeling was observed close to the membrane of the synaptic ribbon structure of this specialized synapse (Fig. 4F). None of the experiments conducted to determine the distribution of harmonin isoforms in the retina revealed differences between light- and dark-adapted retinas (Fig. 5).

Western blot analyses (Fig. 2) indicated that different splice variants of harmonin are expressed in the mammalian retina. To determine whether these harmonin isoforms are similarly distributed in the same subcellular compartments of the photoreceptor cells, we immunostained the harmonin isoform b in unfixed, native retinal cryosections, using harmonin b–specific H1b antibodies. We found that harmonin b was present in the outer segments of photoreceptor cells, but absent at the photoreceptor synapses (Fig. 4D). To occlude the antigen masking occasionally observed in tissue sections, we determined the subcellular distribution of harmonin isoforms in retinal photoreceptor cells by Western blot analyses of subcellular fractions of photoreceptor cells and tangential cryosections through the retina, a method recently introduced.[22] Both biochemical approaches confirmed our immunocytochemical results (Fig. 6). The immunoblots in Figure 6A show harmonin a to be the ubiquitous harmonin isoform present in all retinal fractions. In contrast, the harmonin b isoform was localized in the photoreceptor outer segment fraction, but was absent in crude synaptosome preparations of the retina (Fig. 6A) and the brain (data not shown). The immunoblot analysis of tangential sections through flatmounted rat retinas further strengthened the differential distribution of harmonin isoforms in the photoreceptor outer segments (Fig. 6B). Although harmonin a was present in all tangential sections, harmonin b was restricted to those sections that also contained rhodopsin, the molecular marker for the outer segment of photoreceptor cells.

Colocalization of USH1 Proteins

Previous studies have shown that the USH1 proteins myosin VIIa, harmonin, and cadherin 23 interact through harmonin PDZ domains.[12,13] In the mammalian retina the possible codistribution of these molecules has not yet been investigated. Especially the subcellular localization of harmonin and cadherin 23 in the retina were unknown. We therefore generated the antiserum 1D1 against a recombinantly expressed part of

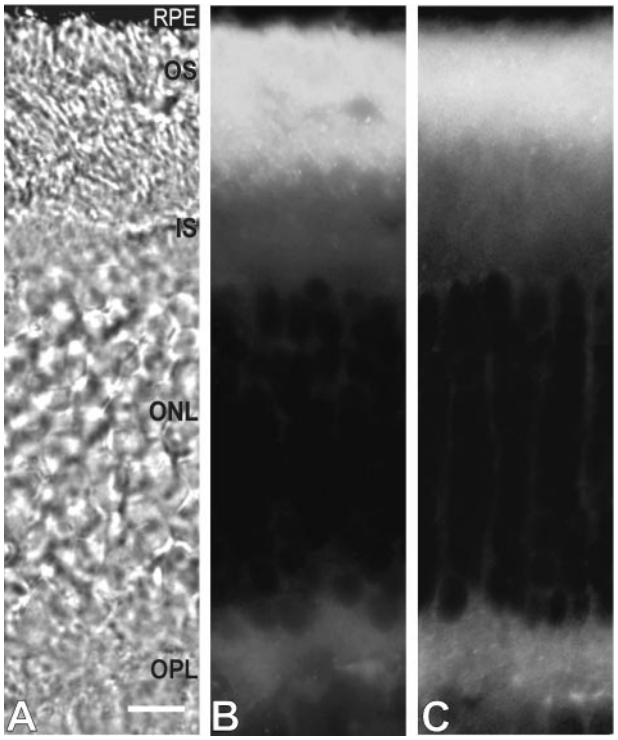

FIGURE 5. Localization of harmonin in light- and dark-adapted mouse retina. (**A**) DIC image of a cryosection through the photoreceptor cell layer of fixed, light-adapted mouse retina. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer. (**B**, **C**) Indirect immunofluorescence micrograph of anti-pan-harmonin of a light-adapted (**B**) and a dark-adapted (**C**) mouse retina. The staining pattern of harmonin did not differ between the two illumination states. Bar, 10 μm.

the cytoplasmic domain of human cadherin 23 for further immunoanalyses (Fig. 1C). The specificity of the antiserum 1D1 was determined in Western blot analysis (Fig. 7). Affinity-purified 1D1 antibodies specifically recognized a single band of approximately 400 kDa in protein extracts of mammalian tissues, including human and rodent retinas (Fig. 7A). This high molecular band was abolished in Western blot analysis after preabsorbance of the 1D1 antibodies with the cadherin 23 antigen and was absent in blots of tissue extracts prepared from cadherin 23 deficient waltzer $v^{alb-/-}$ mice (Fig. 7B).

To study the distribution of the USH1 proteins myosin VIIa, harmonin, and cadherin 23, cryosections through mouse retinas were stained with affinity-purified antibodies against the three USH1 proteins (Fig. 8). Indirect immunofluorescence of anti-myosin VIIa confirmed results that have been obtained in several studies (Fig. 8A). These studies have indicated that myosin VIIa is expressed in the cells of the retinal pigment epithelium and the photoreceptor cells.[2,18] In photoreceptor cells, myosin VIIa has been shown to be localized in the connecting cilium and the synaptic terminal.[14,17,26] The present immunocytochemical experiments showed that harmonin and cadherin 23 were not expressed in the retinal pigment epithelium (Figs. 3B, 4B, 8B). As described earlier in detail, harmonin isoforms were localized in photoreceptor cells in outer and inner segments and at the synaptic terminal (Figs. 3B, 4B, 8B). Immunofluorescence of anti-cadherin 23 1D1 antibodies indicated that cadherin 23 is found in the inner segment and at the ribbon synapses of photoreceptor cells (Fig. 8C). The staining pattern of anti-cadherin 23 antibodies was similar to the immunofluorescence of proteins known as components of the presynaptic cytomatrix at the ribbon synapses in photoreceptors (e.g., bassoon and piccolo shown by Dick et al.[27]). From these analyses and further immunoelectron microscopy results (data not shown) we conclude that all three USH1 proteins myosin VIIa, harmonin, and cadherin 23 are

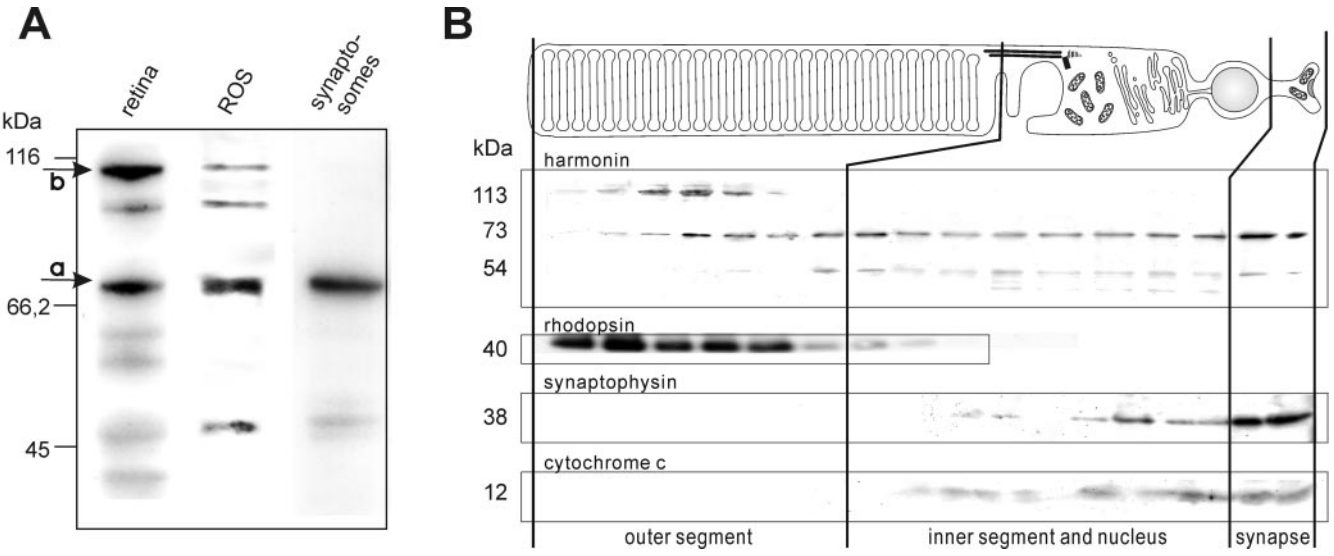

FIGURE 6. Immunoblot of the H3 antibody with different subcellular compartments. (**A**) Immunoblot analysis of subcellular fractions of rat photoreceptors. Total rat retina extract (*lane 1*); fraction of rat rod outer segments (ROS; *lane 2*); crude synaptosome preparation of rat retina. The 73-kDa band corresponding to harmonin isoform a and the low molecular weight variant c were visible in all fractions, whereas harmonin isoform b (113 kDa) was not present in the synaptosome preparation. (**B**) Immunoblot analyses of tangential cryosections through a rat retina. Each *lane* corresponds to a 10-μm-thick slice of the photoreceptor layer, cut from left to right. Blots with antibodies to rhodopsin, synaptophysin, and cytochrome *c* were used to determine the photoreceptor compartments the outer segment, the inner segment, and the synaptic terminal. Cytochrome *c* is known to be expressed in the mitochondria, which are localized in the inner segment and the synapse of photoreceptor cells. Synaptophysin is an integral membrane protein of synaptic vesicles and is commonly used as a synaptic marker. The harmonin isoform b corresponding band of 113 kDa was seen only in the outer segment fractions. In contrast, harmonin isoform a (~73 kDa) was present in all fractions, and the low molecular weight variant c was mostly concentrated in the non–outer segment fractions.

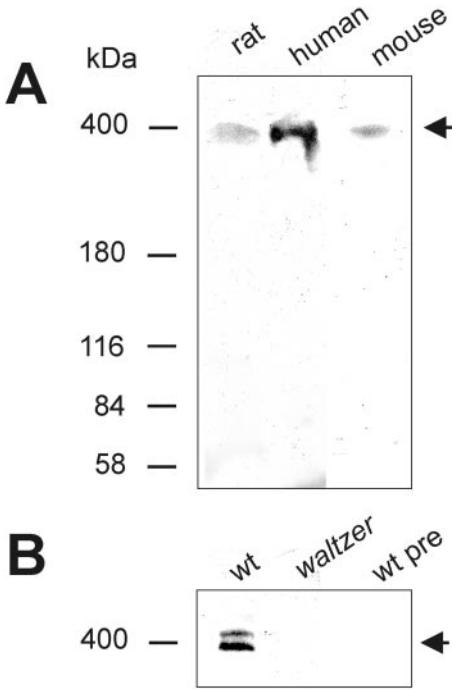

FIGURE 7. Immunoblot analysis of cadherin 23 expression in mammalian retinas. (**A**) Immunoblot analysis of mouse, rat, and human retina with the cadherin 23 antibody 1D1. (**B**) Immunoblot analysis of wild-type mouse (wt) and waltzer $v^{alb-/-}$ mouse testis with affinity-purified cadherin 23 antibodies 1D1 and with the preabsorbed 1D1 antibody (*lane 3*). Cadherin 23 1D1 antibodies detected a band of approximately 400 kDa in retina extracts of mouse, rat, and human (**A**, *arrow*). A double band of the same molecular weight was stained in the analysis of wild-type (wt) mouse testis, but not in the testis of the cadherin 23–deficient waltzer $v^{alb-/-}$ mouse nor in the blots probed with the preabsorbed cadherin 23 1D1 antibodies (**B**, *arrow*).

colocalized in the synaptic terminal of photoreceptor cells. Therefore, a functional network composed of the three USH1 proteins, previously demonstrated in inner ear hair cells may be formed in photoreceptor cells at the synaptic terminal.

Characteristics of Harmonin and Cadherin 23

Phylogenetic analysis of complex components indicates that the USH1-complex may be restricted to the vertebrate phylum. Whereas myosin VIIa genes already exist in genomes of rather low eukaryotes, the orthologue genes to harmonin and cadherin 23 are found throughout the vertebrates but not in lower phyla of eukaryotes. Comparisons of harmonin and cadherin 23 amino acid sequences between vertebrate species indicate that both proteins are highly conserved throughout several hundred million years. The human and murine amino acid sequence of harmonin and cadherin 23 were compared with the orthologue gene products of the Japanese puffer fish *Fugu rupripes* (sequences available from the *Fugu* genome project in the Ensembl database; http://www.ensembl.org/; provided in the public domain by Sanger Centre and European Bioinfomatic Institute, Cambridge, UK) which divided from humans into a different line approximately 450 million years ago.[28,29] The comparison of the amino acid sequence of harmonin shows an identity of 69%, which increases up to 80% by leaving out the coiled–coil region and low-complexity areas. Comparing the intracellular part of cadherin 23 results in 65% identity, including the conserved binding consensus sequence for the harmonin PDZ2 domain at the C-terminus.

DISCUSSION

Independent studies have shown that the USH1C protein harmonin is the key organizer of USH1 protein complexes.[11–13] Harmonin acts as a potent scaffold protein in these complexes by binding myosin VIIa (*USH1B*), cadherin 23 (*USH1D*), and SANS (*USH1G*) through its PDZ domains. The high conservation of these proteins in the vertebrate phylum, reflect the importance of these proteins and their protein–protein com-

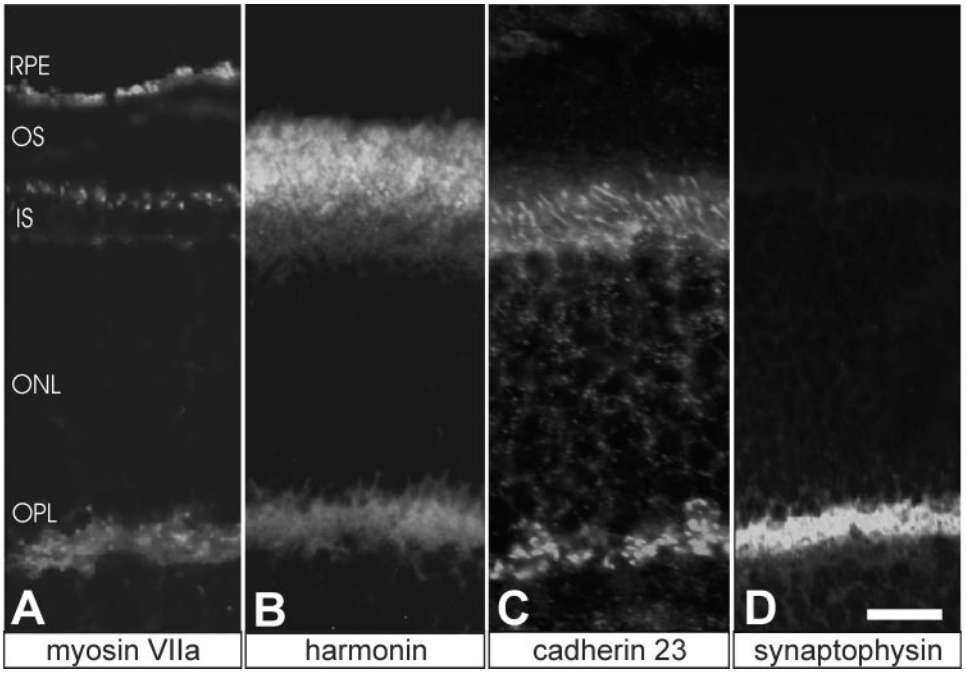

FIGURE 8. Colocalization of USH1 gene products myosin VIIa (USH1B), harmonin (USH1C), and cadherin 23 (USH1D) at the photoreceptor synapses. Indirect immunofluorescence of antibodies to myosin VIIa (**A**), harmonin (H3) (**B**), cadherin 23 (1D1) (**C**), and synaptophysin (**D**) in longitudinal cryosections through a mouse retina. The unconventional myosin VIIa was found in the cells of the retinal pigment epithelium (RPE), in the connecting cilium at the junction between the outer segment (OS) and the inner segment (IS) of photoreceptor cells, and at photoreceptor synapses in the outer plexiform layer (OPL). Harmonin was localized in the OS and at the synapse of photoreceptor cells. Cadherin 23 was expressed in the IS and the synaptic terminal of photoreceptor cells. All three USH1 proteins colocalized in the OPL where the ribbon synapses of photoreceptor cells were localized, stained by anti-synaptophysin. Note that harmonin and cadherin 23 were not expressed in the RPE. Bar, 5 μm.

plexes for the cellular physiological integrity in vertebrate systems.

Differential Expression of Harmonin Isoforms

In mammals, the harmonin *USH1C* gene is transcribed in all tissues tested so far, where a variety of alternatively spliced transcripts are differentially expressed.[3] The diversity of harmonin isoforms in combination with their differential tissue expression predicted their involvement in distinct molecular complexes in diverse tissues. The transcripts encoding the longest harmonin b isoforms so far have been detected only in the inner ear.[3] Nevertheless, our results show that subtypes of all three harmonin subclasses including the novel harmonin b4 isoform are expressed in rodent retinas. In the retina, harmonin has been shown to be expressed in photoreceptor cells.[4] Herein we demonstrate the cellular localization of harmonin in diverse subcellular compartments of photoreceptors, the outer segment, the inner segment, and the synaptic terminal. Furthermore, a combination of complementary experiments revealed diverse spatial distribution of the harmonin isoforms in photoreceptors. Whereas the abundant harmonin isoforms a and c were present in all compartments, the localization of harmonin b variants was restricted to the light-sensitive outer segment.

Organization of a Protein Complex in Photoreceptor Outer Segments

The observed diverse spatial distribution among harmonin isoforms in subcellular compartments of photoreceptor cells may reflect differences between harmonin isoforms in their cellular function and their involvement in supramolecular protein complexes. In these compartments, harmonin is associated with membrane specializations, the outer segment disks, or the membrane ribbon synapses, as has been shown for other scaffold proteins containing PDZ modules.[5] Because the known binding partners of harmonin to its PDZ domains, myosin VIIa and cadherin 23, are not present in the outer segment, harmonin may be targeted to this region of the photoreceptor by other binding partners. In signal transduction compartments of other sensory systems, PDZ domain–containing scaffold proteins coordinate the organization of signaling molecules into macromolecular complexes (transducisomes) providing specificity, sensitivity, and speed in intracellular signaling pathways.[30,31] In the rhabdomeric photoreceptors of the invertebrate animal model *Drosophila melanogaster*, the PDZ protein inactivation no afterpotential D (INAD) clusters the components of the visual signal transduction cascade into a signal complex.[32] There is a growing body of evidence that suggests transduction cascades in vertebrates may also be arranged in signaling complexes.[33,34] We recently confirmed this hypothesis by demonstrating that harmonin organizes protein complexes in stereovilli,[13] the apical specializations for signal transduction of vertebrate hair cells.[35] The identification of proteins of the photoreceptor outer segment interacting with the PDZ domains of harmonin should further reveal the role of harmonin in the organization of signal complexes in vertebrates. As in the transducisome of the microvilli of *Drosophila* rhabdomeres, a putative protein complex of vertebrate photoreceptor outer segments is probably associated with the actin cytoskeleton. Our present study revealed that harmonin b isoforms are also expressed in photoreceptor outer segments. We have shown previously that the PST domain found in b isoforms, but not in a or c, can bind directly to actin filaments.[13] Previous studies have indicated the presence of an actin cytoskeleton in photoreceptor outer segments.[36,37] Thus, we propose that harmonin b may interact with this outer segment actin cytoskeleton, and, in consequence, harmonin may bridge a putative protein complex composed of the outer segment proteins binding to its PDZ domains to the actin cytoskeleton.

Harmonin: the Scaffold of a USH1 Complex at the Ribbon Synapse in Photoreceptor Cells

Our analyses demonstrate expression of harmonin a and c isoforms in inner segments and at the specialized ribbon synapses of photoreceptor cells. The observed colocalization of harmonin a and c with cadherin 23 within the inner segment compartment indicates that previously described binding of cadherin 23 to harmonin may occur here.[12,13] As are other members of the cadherin protein family, cadherin 23 is probably part of a cell–cell adhesion complex.[38] Classic cadherins typically mediate adhesion between neighboring cells through their numerous extracellular domains and form linkages to the actin cytoskeleton through their cytoplasmic binding partners catenins and vinculin.[39,40] Cadherin 23 and harmonin may also serve as molecules in cell–cell adhesions between the inner segments of neighboring photoreceptor cells.

The colocalization of harmonin with both of the harmonin interacting partners myosin VIIa and cadherin 23 in photoreceptor cell synapses provides a prerequisite for the assembly of a supramolecular USH1 protein complex at the ribbon synapse of photoreceptor cells. In comparison to normal chemical synapses, the high rate of tonic neurotransmitter release from ribbon synapses demands much faster vesicle release.[41] Clustering of specific synaptic proteins in a supramolecular complex should coordinate their function, which may enhance the speed of vesicle release at ribbon synapses. It is commonly accepted that interactions of the extracellular domains of cell–cell adhesion molecules of the two synaptic sides keep the synaptic cleft in close register and participate in the organization of the cytomatrices of both, the pre- and postsynaptic side.[42] Furthermore, adhesion molecules regulate the organization of the presynaptic zone of a synaptic junction and play an important role in synaptogenesis.[43–45] Cadherin 23–based cell–cell adhesions may contribute to the specific function of the specialized ribbon synapse. From previous studies it is obvious that, in the cytomatrix associated with synaptic membranes of photoreceptor cells, cadherin 23 can also bind to the first two PDZ domains of harmonin through its PDZ-binding interface.[12,13] Nevertheless, the PDZ1 domain of harmonin is also suitable to bind myosin VIIa.[13] This molecular motor is localized at the cell–cell adhesion complexes in several tissues, to which it is recruited by its tail-binding protein vezatin.[46] Vezatin is a transmembrane protein ubiquitously localized at adhesion junctions where it is integrated into the classic cadherin–catenin complex. Preliminary data indicate that vezatin is also a component of the supramolecular USH1 protein complex at photoreceptor synapses (Wolfrum U, unpublished data, 2000). In the synaptic terminal, myosin VIIa may create a tension force between the adhesion complex and the actin cytoskeleton previously discussed for the junction complex in mechanosensitive hair cells.[46] However, as has been suggested for other unconventional myosins, myosin VIIa may also participate in exocytosis and/or endocytosis—for example, the recycling pathway of synaptic vesicle or internalization of membrane proteins at synaptic membranes.

In addition to the molecules that were shown to participate at the USH1 protein complex, other USH proteins may interact with harmonin and contribute to the protein complex. The

USH1F protein protocadherin 15 and the USH3A protein clarin-1 have been suggested to play a role at photoreceptor synapses in the outer plexiform layer of the retina.[10,47] Nevertheless, defects in one of the USH complex components should lead to dysfunction of the entire complex, effecting the function of the ribbon synapse. Although, none of the existing mouse models for USH1 exhibit retinal degeneration seen in patients with USH1,[1] electrophysiological studies of myosin VIIa–deficient *shaker-1* mouse lines have revealed reduction of electroretinograms that is consistent with the dysfunction of the photoreceptor synapse.[48] Thus, the pathophysiology in the retina of patients with USH1 underlying retinitis pigmentosa may originate from defects in photoreceptor synapses.

In conclusion, the scaffold protein harmonin organizes supramolecular protein complexes in a variety of cellular systems in the vertebrate phylum. In mammalian photoreceptors, harmonin clusters proteins in the light-sensitive outer segment and contributes to synaptic organization.

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References

1. Petit C. Usher syndrome: from genetics to pathogenesis. *Annu Rev Genomics Hum Genet*. 2001;2:271–297.
2. Weil D, Blanchard S, Kaplan J, et al. Defective myosin VIIA gene responsible for Usher syndrome type 1B. *Nature*. 1995;374:60–61.
3. Verpy E, Leibovici M, Zwaenepoel I, et al. A defect in harmonin, a PDZ domain-containing protein expressed in the inner ear sensory hair cells, underlies Usher syndrome type 1C. *Nat Genet*. 2000;26:51–55.
4. Bitner-Glindzicz M, Lindley KJ, Rutland P, et al. A recessive contiguous gene deletion causing infantile hyperinsulinism, enteropathy and deafness identifies the Usher type 1C gene. *Nat Genet*. 2000;26:56–60.
5. Sheng M, Sala C. PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci*. 2001;24:1–29.
6. Bolz H, von Brederlow B, Ramirez A, et al. Mutation of *CDH23*, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. *Nat Genet*. 2001;27:108–112.
7. Bork JM, Peters LM, Riazuddin S, et al. Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene *CDH23. Am J Hum Genet*. 2001;68:26–37.
8. Ahmed ZM, Riazuddin S, Bernstein SL, et al. Mutations of the protocadherin gene *PCDH15* cause usher syndrome type 1F. *Am J Hum Genet*. 2001;69:25–34.
9. Alagramam KN, Murcia CL, Kwon HY, Pawlowski KS, Wright CG, Woychik RP. The mouse Ames waltzer hearing-loss mutant is caused by mutation of *Pcdh15*, a novel protocadherin gene. *Nat Genet*. 2001;27:99–102.
10. Alagramam KN, Yuan HJ, Kuehn MH, et al. Mutations in the novel protocadherin *PCDH15* cause Usher syndrome type 1F. *Hum Mol Genet*. 2001;10:1709–1718.
11. Weil D, El-Amraoui A, Masmoudi S, et al. Usher syndrome type I G (USH1G) is caused by mutations in the gene encoding SANS, a protein that associates with the USH1C protein, harmonin. *Hum Mol Genet*. 2003;12:463–471.
12. Siemens J, Kazmierczak P, Reynolds A, et al. The Usher syndrome proteins cadherin 23 and harmonin form a complex by means of PDZ-domain interactions. *Proc Natl Acad Sci USA*. 2002;99:14946–14951.
13. Boëda B, El-Amraoui A, Bahloul A, et al. Myosin VIIa, harmonin and cadherin 23, three Usher 1 gene products that cooperate to shape the sensory hair cell bundle. *EMBO J*. 2002;21:6689–6699.
14. Liu XR, Vansant G, Udovichenko IP, Wolfrum U, Williams DS. Myosin VIIa, the product of the Usher 1B syndrome gene, is concentrated in the connecting cilia of photoreceptor cells. *Cell Motil Cytoskeleton*. 1997;37:240–252.
15. Liu XR, Ondek B, Williams DS. Mutant myosin VIIa causes defective melanosome distribution in the RPE of shaker-1 mice. *Nat Genet*. 1998;19:117–118.
16. Liu X, Udovichenko IP, Brown SD, Steel KP, Williams DS. Myosin VIIa participates in opsin transport through the photoreceptor cilium. *J Neurosci*. 1999;19:6267–6274.
17. Wolfrum U, Schmitt A. Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells. *Cell Motil Cytoskeleton*. 2000;46:95–107.
18. Hasson T, Heintzelman MB, Santos-Sacchi J, Corey DP, Mooseker MS. Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. *Proc Natl Acad Sci USA*. 1995;92:9815–9819.
19. Kobayashi I, Imamura K, Kubota M, et al. Identification of an autoimmune enteropathy-related 75-kilodalton antigen. *Gastroenterology*. 1999;117:823–830.
20. Papermaster DS. Preparation of retinal rod outer segments. *Methods Enzymol*. 1982;81:48–52.
21. Hirao K, Hata Y, Ide N, et al. A novel multiple PDZ-domain-containing molecule interacting with *N*-Methyl-D-aspartate receptors and neuronal cell adhesion proteins. *J Biol Chem*. 1998;273:21105–21110.
22. Sokolov M, Lyubarsky AL, Strissel KJ, et al. Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. *Neuron*. 2002;33:95–106.
23. Wolfrum U. Centrin- and α-actinin-like immunoreactivity in the ciliary rootlets of insect sensilla. *Cell Tissue Res*. 1991;266:231–238.
24. Danscher G. Histochemical demonstration of heavy metals: a revised version of the sulphide silver method suitable for both light and electron microscopy. *Histochemistry*. 1981;71:1–16.
25. Scanlan MJ, Williamson B, Jungbluth A, et al. Isoforms of the human PDZ-73 protein exhibit differential tissue expression. *Biochem Biophys Acta*. 1999;1445:39–52.
26. El-Amraoui A, Sahly I, Picaud S, Sahel J, Abitbol M, Petit C. Human Usher 1B mouse shaker-1: the retinal phenotype discrepancy explained by the presence/absence of myosin VIIA in the photoreceptor cells. *Hum Mol Genet*. 1996;5:1171–1178.
27. Dick O, Hack I, Altrock WD, Garner CC, Gundelfinger ED, Brandstätter JH. Localization of the presynaptic cytomatrix protein Piccolo at ribbon and conventional synapses in the rat retina: comparison with Bassoon. *J Comp Neurol*. 2001;439:224–234.
28. Venkatesh B, Gilligan P, Brenner S. Fugu: a compact vertebrate reference genome. *FEBS Lett*. 2000;476:3–7.
29. Aparicio S, Chapman J, Stupka E, et al. Whole-genome shotgun assembly and analysis of the genome of Fugu rubripes. *Science*. 2002;297:1283–1285.
30. Tsunoda S, Sierralta J, Sun Y, et al. A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature*. 1997;388:243–249.
31. Zuker CS, Ranganathan R. The path to specificity. *Science*. 1999;283:650–651.
32. Montell C. Visual transduction in Drosophila. *Annu Rev Cell Dev Biol*. 1999;15:231–268.
33. Neer EJ. Heterotrimeric G proteins: organizers of transmembrane signals. *Cell*. 1995;80:249–257.
34. Hamm HE. The many faces of G protein signaling. *J Biol Chem*. 1998;273:669–672.
35. Gillespie PG, Walker RG. Molecular basis of mechanosensory transduction. *Nature*. 2001;413:194–202.
36. Körschen HG, Beyermann M, Müller F, et al. Interaction of glutamic acid-rich proteins with components of the cGMP-signaling pathway in rod photoreceptors. *Nature*. 1999;400:761–766.

37. Kajimura N, Harada Y, Usukura J. High-resolution freeze-etching replica images of the disk and the plasma membrane surfaces in purified bovine rod outer segments. *J Electron Microsc*. 2000;49: 691–697.
38. Di Palma F, Pellegrino R, Noben-Trauth K. Genomic structure, alternative splice forms and normal and mutant alleles of cadherin (Cdh23). *Gene*. 2001;281:31–41.
39. Bolz H, Reiners J, Wolfrum U, Gal A. The role of cadherins in Ca^{2+}-mediated cell adhesion and inherited photoreceptor degeneration. *Adv Ex Med Biol*. 2002;514:399–410.
40. Perez-Moreno M, Jamora C, Fuchs E. Sticky business: orchestrating cellular signals at adherens junctions. *Cell*. 2003;112:535–548.
41. Schmitz F, Königstorfer A, Südhof TC. RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. *Neuron*. 2000;28:857–872.
42. Garner CC, Nash J, Huganir RL. PDZ domains in synapse assembly and signalling. *Trends Cell Biol*. 2000;10:274–280.
43. Bruses JL. Cadherin-mediated adhesion at the interneuronal synapse. *Curr Opin Cell Biol*. 2000;12:593–597.
44. Scheiffele P, Fan J, Choih J, Fetter R, Serafini T. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell*. 2000;101:657–669.
45. Togashi H, Abe K, Mizoguchi A, Takaoka K, Chisaka O, Takeichi M. Cadherin regulates dendritic spine morphogenesis. *Neuron*. 2002; 35:77–89.
46. Küssel-Andermann P, El-Amraoui A, Safieddine S, et al. Vezatin, a novel transmembrane protein, bridges myosin VIIA to the cadherin-catenins complex. *EMBO J*. 2000;19:6020–6029.
48. Libby RT, Steel KP. Electroretinographic anomalies in mice with mutations in Myo7a, the gene involved in human Usher syndrome type 1B. *Invest Ophthalmol Visual Sci*. 2001;42:770–778.