Photoreceptor expression of the Usher syndrome type 1 protein protocadherin 15 (USH1F) and its interaction with the scaffold protein harmonin (USH1C)

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Purpose: The human Usher syndrome (USH) is the most common form of deaf-blindness. Usher type I (USH1), the most severe form, is characterized by profound congenital deafness, constant vestibular dysfunction and prepubertal onset of retinitis pigmentosa. Five corresponding genes of the seven USH1 genes have been cloned over the years. Recent studies indicated that three USH1 proteins, namely myosin VIIa (USH1B), SANS (USH1G), and cadherin 23 (USH1D) interact with the USH1C gene product harmonin. In these protein-protein complexes harmonin acts as the scaffold protein binding these USH1 molecules via its PDZ domains. The aim of the present study was to analyze whether or not the fifth identified USH1 protein protocadherin 15 (Pcdh15) also binds to harmonin and where these putative protein complexes might be localized in mammalian rod and cone photoreceptor cells.

Methods: In vitro binding assays (GST pull-down, yeast two-hybrid assay) were applied. Antibodies against bacterial expressed USH1 proteins were generated. Affinity purified antibodies were used in immunoblot analyses of brain fractions and isolated retinas, in immunofluorescence studies, and in immunoelectron microscopic studies of rodent retinas.

Results: We showed that Pcdh15 (USH1F) interacted with harmonin PDZ2. Immunocytochemistry revealed that Pcdh15 is expressed in photoreceptor cells of the mammalian retina, where it is colocalized with harmonin, myosin VIIa, and cadherin 23 at the synaptic terminal. Colocalization of Pcdh15 with harmonin was found at the base of the photoreceptor outer segment, where newly synthesized disk membranes are present.

Conclusions: Our data indicate that harmonin-Pcdh15 interactions probably play a role in disk morphogenesis. Furthermore, we provide evidence that a complex composed of all USH1 molecules may assemble at the photoreceptor synapse. This USH protein complex can contribute to the cortical cytoskeletal matrices of the pre- and postsynaptic regions, which are thought to play a fundamental role in the structural and functional organization of the synaptic junction. Defects in any of the USH1-complex partners may result in photoreceptor dysfunction causing retinitis pigmentosa, the clinical phenotype in the retina of USH1 patients.

The Usher syndrome (USH) is the most frequent cause of combined deaf-blindness in developed countries. According to the severity of symptoms USH is distinguished into three clinically distinguishable forms USH1, USH2 and USH3 [1]. Usher type 1 (USH1) is the most severe form, characterized by profound congenital deafness, constant vestibular dysfunction and prepubertal onset of retinitis pigmentosa [2]. USH1 is genetically heterogeneous and out of seven mapped USH1 genes (USH1A-USH1G), five of the corresponding genes have been identified, namely USH1B, USH1C, USH1D, USH1F, and USH1G [2,3]. USH1B encodes the molecular motor protein myosin VIIa [4]. USH1C encodes harmonin, a scaffold protein containing PDZ domains [1,5,6]. PDZ modules are known in other polypeptides as organizers of membrane associated supramolecular protein complexes [7]. In general, binding to the PDZ domains is mediated by specific PDZ binding motifs within those protein complexes. USH1G was shown to encode SANS [2,8]. Mutations in genes encoding two cadherin related cell-cell adhesion proteins, cadherin 23 (USH1D) [9,10] and protocadherin 15 (USH1F) [11,12] underlie two other USH1 syndrome variants.

Recent studies indicated that three of USH1 proteins, namely myosin VIIa (USH1B), SANS (USH1G) and cadherin 23 (USH1D) directly interact with the USH1 gene product harmonin (USH1C) [8,13-15]. In these protein-protein complexes, harmonin acts as the scaffold protein binding the other USH1 molecules via its PDZ domains. Furthermore, harmonin also performs homomorphic interaction by binding to its C-terminus and its PDZ-1 domain [13,14]. These findings prompted us to seek molecular interaction between the harmonin and the USH1F gene product protocadherin 15 (Pcdh15) [15].

The USH1F protein, Pcdh15, is an atypical member of the large cadherin superfamily of cell surface proteins defined by the presence of a variable number of extracellular cadherin domains termed “EC”. It is commonly accepted that these ECs mediate Ca2+ dependent homophilic binding between cadherin molecules. Cadherins can be grouped into six subfamilies according to size, number and feature of domains, function, and binding partners. Via their cytoplasmic domain, cadherins are linked to the cytoskeleton, but can also be connected to intracellular signal transduction pathways [16].
To understand the cellular function of Pcdh15 and the putative USH1 protein complex, knowledge of the subcellular expression, localization, and interactions of USH1 proteins in the mammalian retina is essential. Our present results show co-expression of Pcdh15 with other disease proteins in photoreceptor cells. We further provide evidence that in ribbon synapse of photoreceptor cells, a supramolecular USH1 protein complex of Cdh23 [3] and myosin VIIa, bridged by harmonin [17] also contains Pcdh15.

METHODS

Animals and tissue preparation: All experiments were conforming to the statement by the Association for Research in Vision and Ophthalmology (ARVO) as to care and use of animals in research. Appropriate tissues of C57BL/6J mice or Wistar rats for total protein extracts and brain crude synaptosomes were prepared as previously described [17].

Antibodies and fluorescent dyes: Pcdh15 antibodies were generated against a part of the cytoplasmic tail of murine Pcdh15 (amino acid positions 1416-1882) bacterially expressed via a pGEX expression vector (Amersham Biosciences, Freiburg, Germany). Expression of the fusion protein and purification of the antibody was performed as described [17]. The antibodies against harmonin (H3), myosin VIIa, synaptophysin, Cdh23 and centrin were previously described [17,18]. The fluorescein labeled lectin peanut agglutinin (PNA) and the anti-His antibody was purchased from Sigma-Aldrich (Deisenhofen, Germany).

Cloning and expression of cDNA: The cDNA for C-terminal of murine Pcdh15 (amino acid positions 1845-1944) was cloned by RT-PCR as previously described [17]. In the Pcdh15C construct, the Pcdh15 C-terminal was truncated using an intrinsic restriction site for Xho I (amino acid positions 1845-1944). Constructs coding for harmonin domains PDZ1 (amino acid positions 82-168), PDZ2 (amino acid positions 207-292), PDZ3 (amino acid positions 419-537), and the full length sequence of murine harmonin a1 (amino acid positions 1-548) were subcloned into a pENTR vector following manufacturer’s instruction (Invitrogen, Karlsruhe, Germany). Using the Gateway™ technology of Invitrogen, inserts were transferred into pDEST15 and pDEST17 vectors and expressed in E. coli BL21 AI following the manufacturer’s instructions.

GST pull-down assay: Equal amounts of glutathione S-transferase (GST) or GST fusion protein were mixed with lysates of His tagged fusion proteins and a protease inhibitor mix (Sigma-Aldrich). Samples were incubated over night at 4 °C followed by incubation with 50 µl/reaction glutathione sepharose beads 4B (Amersham Biosciences) for 45 min with gentle agitation. After centrifugation, beads were washed four times with 50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 0.01% polyoxyethylene-10-lauryl ether, pH 7.5. Bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE and immunoblotting.

Yeast two-hybrid: Proquest™ two-hybrid system (Invitrogen) was used to detect proteins interacting in yeast. Constructs of the Pcdh15 C-terminal (1845-1944 aa) and harmonin a1 (1-548 aa), were subcloned in pDEST22 and pDEST32 vectors using the Gateway™ (Invitrogen) and transformed into yeast strain MaV203 following manufacturer’s instructions. The pEXP-AD502 vector was used as control to exclude self activation. Resulting clones were grown on high stringency medium lacking histidine or uracil.

Western blot analysis: Isolated retinas or protein complexes obtained after pull-downs were separated by SDS-PAGE and transferred to blotting membranes as described [17] Immunoreactivities were detected on blots, with the appropriate primary and secondary antibodies coupled the ECL+ detection system (Amersham Biosciences/GE Healthcare, Freiburg, Germany). For preabsorption, the Pcdh15 antibodies were preincubated for 1 h at room temperature in the presence of 1 mg/ml antigen used for immunization.

Fluorescence microscopy: Eyes of adult mice were cryofixed in melting isopentane, cryosectioned and treated as described [17,19]. In the appropriate control sections in no case was a reaction observed. Mounted retinal sections were examined with a Leica DMRP microscope. Images were obtained with a Hamamatsu ORCA ER charge coupled device camera (Hamamatsu, Herrsching, Germany) and processed with Adobe Photoshop (Adobe Systems, Version 6.0, San Jose, CA).

Immunoelectron microscopy: Fixation, embedding, and further handling of mouse retinal samples for immunoelectron microscopy were performed as previously described [20]. Nanogold labeling was silver enhanced according to Danscher [21]. Counterstained ultrathin sections were analyzed with an FEI Tecnai 12 electron microscope.

RESULTS & DISCUSSION

We first analyzed the direct binding of protocadherin 15 (Pcdh15) to harmonin in GST pull-down assays [4]. For this purpose recombinantly expressed His tagged harmonin a1 was added to immobilized GST fusion proteins of the cytoplasmic tail domains of Pcdh15 (GST-Pcdh15), and Cdh23, (GST-Cdh23), or GST alone. Subsequently, the pull-downs were analyzed by immunoblotting with anti-His antibodies. In agreement with previous studies [13,14], His tagged harmonin a1 co-precipitated with GST-Cdh23 (Figure 1A, lane 2). Significant recovery of harmonin was also obtained with GST-Pcdh15, but not with GST alone (Figure 1A, lanes 1 and 3). The binding of harmonin to the Pcdh15 tail was also abolished using a truncated Pcdh15 tail construct, lacking the C-terminus, which contains the putative PDZ binding sequence “STSL” (Figure 1A, lane 4). This interaction was confirmed by interaction assays, applying the yeast two-hybrid system. The colonies, which were obtained in two-hybrid experiments using harmonin a1 as bait and the Pcdh15 tail as prey, grew on all stringency media that we employed (data not shown), indicating the in vivo interaction of both proteins. Previous studies showed that Cdh23 binds to harmonin via either PDZ1 or PDZ2 [13,14]. To identify the interacting site in the harmonin molecule to which Pcdh15 binds, different GST harmonin
The well defined layers of the vertebrate retina make it relatively simple to determine the subcellular localization of proteins in a section through the retina even by light microscopy (Figure 3C). To determine the sites of Pcdh15 expression in the retina, cryosections through the eye of mice and rats were analyzed by indirect immunofluorescence microscopy.

Figure 1. Pcdh15 cytoplasmic tail binds to harmonin PDZ2. A: His tagged harmonin a1 was incubated with immobilized GST-Pcdh15 tail, GST-Cdh23 tail, Pcdh15δC, or GST alone. Anti-His immunoblotting revealed recovery of harmonin a1 with the cytoplasmic tails of Cdh23 and Pcdh15, but not with GST alone (arrow). B: His tagged Pcdh15 tail was assayed with each of the immobilized GST PDZ domains of harmonin and GST protein alone and analyzed by anti-His immunoblotting. Binding of the Pcdh15 tail (about 13 kDa, lane 1) is restricted to the PDZ2 domain of harmonin (arrow).
Figure 2. Colocalization of USH1 gene products at photoreceptor synapses. Indirect immunofluorescence of antibodies against myosin VIIa (A), harmonin (B), Cdh23 (C), Pcdh15 (D), and synaptophysin (E) in parallel longitudinal cryosections through a mouse retina. Anti-myosin VIIa (A) labeling was present in retinal pigmented epithelium (RPE) cells, in connecting cilia at junctions between outer segments (OS) and inner segments (IS) and at photoreceptor synapses in the outer plexiform layer (OPL). Harmonin (B) was localized in the OS, IS and OPL. Cdh23 (C) was expressed in the IS and OPL. As shown in previous figures, Pcdh15 (D) was found in the OS, predominantly at its base, and in the OPL. All four USH1 proteins colocalize in the OPL where ribbon synapses of photoreceptor cells are localized. The synaptic region is visualized by the synaptic marker anti-synaptophysin (E). Harmonin, Cdh23, and Pcdh15 are not expressed in the RPE. The outer nuclear layer (ONL) is also labeled. The scale bar represents 5 µm.

Figure 3. Pcdh15 expression in the retina. A: Immunoblot of total protein lysates of adult murine retina, brain, and brain synaptosomes with anti-Pcdh15. In lanes 1-3 two bands were obtained, one at the estimated molecular mass of holo-Pcdh15 (214 kDa, arrow) and one at about 65 kDa representing the Pcdh15 isoform b. Both bands were abolished by preabsorption of anti-Pcdh15 with recombinant Pcdh15 (lane 4). B: Scheme of a rod photoreceptor cell. Vertebrate photoreceptors are composed of a light sensitive outer segment (OS) linked via a connecting cilium (CC) to an inner segment (IS) which contains the biosynthetic and metabolic machinery. The nucleus (N) is localized in the outer nuclear layer (ONL) and S the synaptic terminal (S) is located in the outer plexiform layer (OPL) of the retina. C-E: Fluorescence microscopy of a longitudinal cryosection through a mouse retina. C: DAPI staining [8] present in the nuclei of the RPE [9] cells, ONL, and the inner nuclear layer (INL) separated by the OPL. D: Indirect immunofluorescence of anti-Pcdh15 was found in photoreceptor synapses within OPL and the photoreceptor outer segments with occasional intense dot-like structures at the outer segment base. Furthermore, the outer limiting membrane just proximal to the ONL was stained by anti-Pcdh15. The scale bar represents 5 µm. E: In the absence of primary antibodies, no fluorescence is detected.
copy using affinity purified antibodies against Pcdh15. Prominent immunofluorescence labeling of Pcdh15 was present in the photoreceptor layers, in the inner and the outer plexiform layer (Figure 3D), and in the ganglion cell layer of the retina (data not shown); these findings support previously obtained results [26]. In contrast, no labeling was observed in the cells of the retinal pigmented epithelium (RPE) of the retina (Figure 3D), where the localization of classical cadherins at cell-cell adhesions are well documented [27,28].

**Pcdh15 localization in photoreceptor cells:** In cone and rod photoreceptor cells, prominent Pcdh15 staining was obtained in the synaptic terminals (outer plexiform layer) and the light sensitive outer segment (Figure 3C,D). Less intense labeling of Pcdh15 was present in the inner segment of photoreceptor cells concentrated to the outer limiting membrane of the retina (Figure 3C,D), a region of cell-cell adhesions between neighboring photoreceptor cells at the junction between the inner segment and the perikaryon. This staining indicated that Pcdh15 is part of these cell-cell adhesion complexes, which also include contacts to the apical part of Müller glia cells. Although the outer segments of cone and rod photoreceptor cells were stained over their entire length the indirect immunofluorescence of anti-Pcdh15 was most prominent at its base, right at the junctions between photoreceptor inner and outer

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**Figure 4. Subcellular localization of Pcdh15 in cone photoreceptor cells.**

A: Schema of a cone photoreceptor cell. In comparison with rods, cone outer segments (OS) are shorter and their OS disks are laterally open to the extracellular space. OS represents outer segment; CC represents connecting cilium; IS represents inner segment; N represents nucleus localized in the outer nuclear layer (ONL), and S represents synaptic terminal located in the outer plexiform layer (OPL) of the retina. B-I: Fluorescence microscopy of a longitudinal cryosection through a mouse retina.

B: Indirect immunofluorescence of anti-Pcdh15 was present in dot-like structures of distinct sizes in the layer of photoreceptor outer segments. C: Double labeling with a fluorescein conjugated lectin, PNA, was used as a marker for the matrix sheath of cones. D: Overlay of B and C, triple labeled with DAPI (blue). Intense Pcdh15 staining was present in cones (yellow), while small dots were localized to rod inner and outer segments. E,F: Double immunofluorescence labeling of sections through photoreceptor cells at the inner-outer segment joint with anti-Pcdh15 and anti-centrin (clone 20H5). E: Centrins are localized in the connecting cilia and basal bodies of rods and cones [43]. F: Merged images revealed Pcdh15 localization in outer segment the bases distally to anti-centrin stained connecting cilia (arrow). G-I: Indirect immunofluorescence double labeling of a cryosection through the outer plexiform layer of a mouse retina with anti-Pcdh15 and fluorescein conjugated PNA. G: Dots of different diameters are stained by anti-Pcdh15. H: Fluorescent conjugated PNA stained cone synapse pedicles. I: Overlay of images G and H shows intense anti-Pcdh15 labeling of large dots, present in the notch of the PNA labeling in cone synapses (white arrow). Beneath the cone pedicles numerous dots of smaller diameter are only stained by anti-Pcdh15 (green), which represent the smaller rod spherule. The scale bars in D and G represent 5 µm; the scale bars in F and the inset of I represent 2 µm. Use the scale bar in D for B-D, the scale bars in G for G-I, and the scale bars in F and the inset of I for E,F and the inset of I.
Double labeling with anti-Pcdh15 and fluorescently conjugated peanut agglutinin (PNA) [6], which specifically labels the extracellular matrix that ensheathes cone photoreceptor cells [29], showed that Pcdh15 was more abundant in the cone than in the rod outer segment bases (Figure 4B-D). Counterstaining of the retinal photoreceptor cells with antibodies to centrin, a molecular marker of the connecting cilium [30,31], revealed that Pcdh15 was not a component of the connecting cilium itself, but was localized in a structure differentiation of the outer segment, distal to the connecting cilium (Figure 4E,F). The staining pattern and the subcellular position of Pcdh15 in photoreceptor cells correspond with the localization of prCAD [7] previously identified in retinal cDNA libraries by subtractive hybridization [32]. This indicates that both cadherins are co-localized at the base of the outer segment of retinal photoreceptor cells.

Mammalian photoreceptor outer segments continually turn over throughout lifetime [33]. The formation of new membranous disks at the proximal compartment of the outer segment is part of this renewal process. The localization of prCAD and Pcdh15 in the proximal compartment of the outer segments and their potential function in membrane-membrane adhesion indicates that both adhesion molecules are involved in the largely unknown molecular processes of the formation, stacking of the maturing outer segment disks, and the organization of the uniform growth of the outer segment. In this context, both transmembrane proteins may connect outer segment membranes to the actin cytoskeleton, which is known to play a role in outer segment disk genesis [34-36]. A prominent function of cadherins is further supported by the fact that dysfunctions in both cadherins lead to retinal degeneration [11,12,32].

In immuno-electron microscopic analysis, Pcdh15 was found to be concentrated at the plasma membrane of the outer segment base, but was also distributed over the disk membranes of the outer segment (Figure 5A), which confirmed our immunofluorescent data (Figure 3 and Figure 4). As far as we know Pcdh15 is the first cadherin, which is localized as a membrane-membrane adhesion molecule at the outer segment disk membrane. Here at disk membranes, we have previously demonstrated harmonin as a prominent protein [17] (Figure 2B). To our knowledge harmonin is to date the only known PDZ protein found in the outer segment of vertebrate photoreceptor cells. Other PDZ domain proteins are described as scaffold proteins organizing protein networks at the synapses of the inner and the outer plexiform retinal layer and at the

![Figure 5. Immunoelectron analysis of Pcdh15 in photoreceptor cells. Silver enhanced immunogold labeling of Pcdh15 in ultrathin sections through parts of mouse photoreceptor cells. A: Longitudinal section through a portion of outer segments (OS) and apical inner segments of rod photoreceptor cells. Silver enhanced immunogold particles were associated with OS disks and accumulated at the OS plasma membrane (arrowhead). B: Section through the apical part of a RPE cell and the tip of an OS. Silver enhanced immunogold labeling was restricted to the OS. A RPE cells the labeling was not above the background. C, D: A section through synaptic terminals of photoreceptor cells. Silver enhanced immunogold labeling of Pcdh15 (C) and harmonin (D) was present in the ribbons of photoreceptor synapses (arrowheads). The scale bars represent 0.5 µm.]
cell adhesion complexes of the outer limiting membrane of the retina [37,38]. Although co-localization of both proteins does not prove a physical interaction, the results of our present in vitro and in vitro binding assays make it plausible that Pcdh15-cytotail may bind to harmonin in the small cytoplasmic cleft between the disk stacks of the outer segment. Since the splice variant harmonin b, which is restricted to the outer segment in photoreceptors, also binds to actin filaments [17], a putative Pcdh15-harmonin b complex may also be ridged to associated the previously described actin cytoskeleton of the outer segment.

Identification of Pcdh15 as an adhesion component of photoreceptor synapses: Our present immunocytochemical experiments further showed abundant localization of Pcdh15 in the outer plexiform layer of the retina where the photoreceptors cells are wired via their synaptic terminals to horizontal and bipolar cells (Figure 3). Double labeling of photoreceptor ribbon synapses in mouse retinal sections with affinity purified anti-Pcdh15 and fluorescent conjugated PNA revealed localization of Pcdh15 in the highly specialized synapses of cones (Figure 4G-I). The merged images in Figure 4F and the inset within Figure 4 showed additional staining beside cone pedicles, which represented labeling of Pcdh15 at rod photoreceptor synapses (Figure 4I). Silver enhanced immunogold labeling revealed a co-localization of Pcdh15 and harmonin at the arciform density, a substructure at the active zone ribbon synapses in photoreceptor cells (Figure 5C,D). Our immunoblot analysis of brain synaptosome fractions indicated that Pcdh15 also is a common component in synapses of nonretinal neurons (Figure 3A, lane 3).

As previously stressed, Pcdh15 is likely a part of cell-cell adhesion complexes. In synaptic terminals of neuronal cells, cadherins are present in the pre- and postsynaptic membranes, interact via their EC domains, and keep in turn the synaptic cleft in close register [39]. Furthermore, cell-cell adhesion molecules regulate the organization of the pre- and postsynaptic cytomatrixes of a synaptic junction and play an important role in synaptogenesis [40-42].

USH1 proteins that physically interact co-localize at photoreceptor cell synapses: In our previous study [17], we have shown that the USH1 proteins myosin VIIa, harmonin, and Cdh23 are co-localized in photoreceptor synapses. Here, we demonstrated a fourth USH1 protein, Pcdh15, as a further molecular component in the specialized ribbon synapse of rod and cone photoreceptor cells. Present indirect immunofluorescent labeling of parallel cryosections through the mouse retina confirmed partial co-localization of all four USH1 proteins (Figure 2). This co-localization together with the physical interaction of the USH1 proteins with the scaffold USH1C protein harmonin strongly supports our hypothesis of the assembly of USH1 protein complexes at the ribbon synapses of cone and rod photoreceptor cells [17]. Defects in one of the USH1 complex components should lead to dysfunction of the entire complex affecting the function of the ribbon synapse. The pathophysiology in the retina of human USH1-patients may originate from defects in photoreceptor synapses.

Nevertheless, it is important to note that the immunofluorescent labeling of harmonin and Pcdh15 overlap not only in the synaptic region, but also in the outer segment (Figure 2B-D). Thus, both proteins, which are potential binding partners, may also interact in the outer segment where harmonin was previously suggested as a potent scaffold protein of supramolecular complexes [17].

In conclusion, in vertebrate photoreceptor cells the cell-cell adhesion molecule and USH1F protein, Pcdh15, interacts via its PDZ binding sequence with the scaffold protein harmonin, which organizes a supramolecular USH1 protein complex at the specialized ribbon synapse containing harmonin (USH1C), myosin VIIa (USH1B) and Cdh23 (USH1D). Mutations in the USH1-complex components may cause dysfunction of photoreceptor cell synapses, which might be responsible for the pathophysiology underlying retinitis pigmentosa in human USH1 patients.

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