Ergic2, a Brain Specific Interacting Partner of Otoferlin

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Abstract
Background: Otoferlin, a postulated calcium sensor of 230 kDa, was proposed to trigger calcium dependent fusion of vesicles with plasma membrane in the ribbon synapses of cochlear IHCs. Otoferlin’s interaction with Rab8b and Myo6, proteins involved in the intracellular membrane trafficking, extended the previous hypothesis assigning Otoferlin an additional role in trans-Golgi trafficking. Here, we present another Otoferlin binding partner, Ergic2, a protein with a still unknown function but presenting sequence homology to other proteins involved in ER/Golgi vesicle trafficking. Methods: Novel binding partners of Otoferlin were searched by yeast two-hybrid screening in a rodent cochlear cDNA library (P3-P15). RT-PCR, western blot, immunohistochemistry staining and co-immunoprecipitation were applied to analyze and confirm an interaction between Ergic2 and Otoferlin. Results: The Y-2-H screening, using baits covering parts of Otoferlin’s C2D domain, identified Ergic2 as an interacting protein for Otoferlin. Both are co-expressed (mRNA and protein level) in rodent cochlea and brain before- and after-onset of hearing. By RT-PCR Ergic2 was detected in cochlear IHCs and OHCs and in brain regions where Otoferlin is known to be present. Co-localization studies revealed an overlap of Ergic2 and Otoferlin signals in IHCs and neurons of cerebral cortical layer I making Ergic2 the promising binding candidate. However, while Ergic2 was co-precipitated by an anti-Otoferlin antibody in protein lysates from murine brain, this specific protein-protein interaction was not detected in cochlea. Conclusion: Our new data on Otoferlin’s interactome suggest that Otoferlin can form different, tissue-specific protein complexes.

Introduction

Hearing impairment is the most frequent neurosensory disorder with many still unknown physiological mechanisms leading to different types of clinical manifestation. Since hearing like many other biological processes requires protein–protein cooperation,
identification of components of protein complexes is the key to understanding the molecular processes associated with deafness.

Following nonsyndromic hearing impairment termed DFNB1, the autosomal recessive nonsyndromic prelingual deafness (DFNB9) is the second most common type of hearing loss [1]. It is caused by mutations in the OTOF gene and more than 50 of them have been described so far [2]. Otoferlin is present in cochlear inner and outer hair cells as well as in the brain and vestibular type I sensory hair cells [3, 4].

Initially Otoferlin was believed to play a key role in calcium-dependent exocytosis of the synaptic vesicle in ribbon synapses of cochlear inner hair cell (IHCs) [5]. However, additional data showing Otoferlin’s co-localization with endosomal (EEA1) or Golgi proteins (GM130) in the IHCs, central neurons and nerve fibers extended Otoferlin’s localization beyond synaptic regions [4]. At the same time, interaction of Otoferlin with Rab8b and Myo6 strengthened the current opinion about a more ubiquitous role in endosomal trans-Golgi dynamics. Rab8b in complex with Otoferlin may be involved in vesicle endocytosis by controlling the delivery of recycling endosomes to vesicles transported to the basolateral plasma membrane [6]. Myo6, a protein playing a key role in the endocytotic trafficking is another Otoferlin binding partner that was also reported as an interactor of Rab8b [7]. These data strongly suggest that Otoferlin, forming a complex with Rab8b and Myo6, controls the targeting of intracellular vesicles to the basolateral cell membrane. Additionally, Brand et al. reported that the hypothyroid (TH) mouse model lacking Otoferlin in hair cells displays exocytosis in cochlea before and after onset of hearing. This observation could reduce Otoferlin’s importance in Ca²⁺-dependent membrane fusion at the ribbon synapse of IHCs [8]. However, under conditions of TH deficiency the P19 hypothyroid IHCs were similar to IHCs of P9 hypothyroid model and their control counterparts. Therefore the mature hair cells exhibited exocytosis with an efficiency typical for immature IHCs [8]. Since the more efficient exocytose in post-hearing hair cells may be still dependent on Otoferlin, the results presented by Brand et al. cannot be used to completely disprove its essential role in exocytosis [8].

To extend our understanding of Otoferlin’s function, we searched for additional binding partners. Using yeast-two-hybrid screening, co-localization and co-immunoprecipitation studies, we identified Ergic2 as a new candidate. Ergic and golgi 2 (Ergic2) is a ubiquitously expressed protein with still unclear function. Ergic2 was initially identified as a candidate tumor suppressor of prostate cancer [9]. However, Ergic2 was found to be a homolog of yeast Erv41p, a protein that, together with Erv46p, (homologous to mammalian Ergic3) forms a functional complex stabilized by Ergic32 (also known as Ergic1) [10, 11]. Ergic2 shows characteristic structure of the Ergic family with two transmembrane domains, one big luminal loop and specific C and N terminus with putative essential ER export signals (Fig. 1A) [12]. All these proteins are ascribed to proper functioning of endoplasmatic reticulum–golgi intermediate compartment (ERGIC), a complex system of tubulovesicular membranes localized between rough endoplasmic reticulum (ER) and cis-Golgi (Fig. 1B). ERGIC is a first station in protein trafficking, responsible for concentration, folding and quality control of newly synthesized peptides [10, 12]. ERGIC membranes are enriched in Ergic53 and in proteins from two families known for affiliation to Otoferlin; SNARE (syntaxin 5, syntaxin 17, syntaxin 18, rBet1, Sec22) as well as small GTPases (Rab1 and Rab2) [13, 14].

In yeasts Erv41p–Erv46p interaction is required for efficient package into COPII vesicles transporting their cargo from ER to Golgi. Similar localization and transport motifs suggest functioning of the same protein complex in mammals [10]. Thus interaction between Otoferlin and Ergic2 would be a further evidence for involvement of Otoferlin in general, intracellular trafficking.

Materials and Methods

Animals and tissue preparation

NMRI and Otoferlin [15, 16] mutant mice were used for this study. The mutant line was obtained from Jackson Laboratories (Bar Harbor, Maine, USA).

Before decapitation, animals were asphyxiated with ether. Brain and cochleae were then isolated and dissected as previously described [17]. For RNA and protein isolation, tissues were dissected, immediately frozen in liquid nitrogen and stored at –70°C before use.

Care and use of the animals as well as the experimental protocol were reviewed and approved by the animal welfare commissioner and the regional board for scientific animal experiments in Tübingen.

Yeast-two-hybrid

The interacting partners of Otoferlin were identified using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech Laboratories, Mountain View, California, USA) and a mouse cochlear cDNA library isolated from P3-P15 (kind gift of Prof. Richard J.H. Smith, The University of Iowa, Department of Otolaryngology). For more details see [6].

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RNA isolation and RT-PCR

Total RNA from 6-10 mouse cochleas representing different developmental time points and 3 murine brains was isolated using the RNeasy Minikit (Qiagen, Hilden, Germany), following the manufacturer’s protocol. After RNA isolation reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, München, Germany), according to manufacturers’ instruction. For a single cell RT-PCR analysis, 50 of IHCs and 120 of OHCs were collected with micropipettes and frozen in liquid nitrogen [18]. cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). A 219 bp fragment of Ergic2 and 299 bp fragment of a control housekeeping gene Cyclophilin A were amplified byPCR on cDNA. The sequences of used oligonucleotides: Ergic2 forward primer 5’-CTT GAA GGA GGC CTG TCA GGT ACT-3’ and reverse primer 5’-CAC TTT GCT GCT GAT TTG TTG CCG-3’ (annealing temperature: 54°C, 35 cycles); Cyclophilin A forward 5’-CGA GCT CTG AGC ACT GGA GAG AAA-3’ and reverse 5’-CAT GCC TTC TTT CAC CTT CCC AAA GAC-3’ (annealing temperature: 54°C, 35 cycles). The resulting PCR products were analyzed on 10% polyacrylamide gels and stained with ethidium bromide. The PCR product was sequenced for confirming Ergic2 specificity. The analysis was performed in triplicate.

Immunohistochemistry

For immunohistochemistry, mouse cochlear sections were stained and imaged as described [19]. Briefly, thawed and dried tissue sections were permeabilized with 0.5% Triton X-100 in PBS (Sigma-Aldrich) for 10 min followed by blocking with 4% normal goat serum in PBS and incubated overnight at 4°C with primary antibodies diluted in 2% NaCl, 1% normal goat serum and 0.1% Triton X-100 (all Sigma-Aldrich) in PBS. For the colocalization studies, two different primary antibodies were simultaneously used for identical time periods. The following antibodies were used: mouse monoclonal anti-Otoferlin (Abcam, 53233; 1:50), rabbit polyclonal anti-Ergic2 (Abcam, ab50847; 1:200). The primary antibodies were detected with Cy3- (Jackson Immuno Research Laboratories, West Grove, Pennsylvania, USA; 1:500), and Alexa488- (Molecular Probes, Leiden, The Netherlands; 1:1500), and Alexa532- (Invitrogen, Karlsruhe, Germany). The membranes were blocked using 1x Blocking Buffer (Sigma-Aldrich), and incubated with the secondary antibody for 1 hour at room temperature (Amersham ECL anti-mouse from sheep, anti-rabbit from donkey IgG, Horseradish Peroxidase linked, GE Healthcare, München, Germany, 1:2000). The results were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection Reagent (Amersham, GE Healthcare, München, Germany).

Co-immunoprecipitation, using mouse monoclonal anti-Otoferlin antibody (Abcam, 53233, 1μg) along with brain and cochlea protein lysates (200-300 μg), was performed using Matchmaker co-IP Kit (Clontech Laboratories, Mountain View, California, USA), according to manufacturer’s protocol. Briefly, isolated proteins were incubated overnight with primary antibody at 4°C. On the next day, reaction was mixed with Protein A beads (Clontech Laboratories, Mountain View, California, USA) and incubated for 1.5 h at room temperature with agitation. After incubation beads were washed with buffers 1 and 2 provided with the Matchmaker co-IP Kit (Clontech Laboratories, Mountain View, California, USA). The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto the PVDF membrane and detected by the antibody against Ergic2. To confirm expression of both proteins of interest in cochlear lysates used for the co-immunoprecipitation, about 40 μg protein from the same cochlear lysate was separated on gel. The specificity of the co-immunoprecipitation was controlled using mouse monoclonal anti-Qa1 antibody (Sigma-Aldrich, Q4962, 1μg), as well as Protein A beads (Clontech Laboratories, Mountain View, California, USA) incubated with the protein lysates, and protein samples from Otof ko mouse.

Western blot and co-immunoprecipitation

RNAlater stabilized cochleas and brains isolated from mice were homogenized and lysed in lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100) containing Pefabloc (10 μl/5 ml, Sigma-Aldrich). Nuclei and cell debris were pelleted by centrifugation at 300xg for 5 min at 4°C and the supernatant was used for western blotting and co-immunoprecipitation.

The denatured protein samples were separated under the reducing conditions by SDS-polyacrylamide gel electrophoresis using the XCell Sure Lock Mini Cell and NuPage Novex 4-12% Bis-Tris Gels (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. As a protein marker All Blue Standard from Bio-Rad was used. After separation, proteins were transferred onto 0.2 μm polyvinylidene difluoride (PVDF) transfer membranes using the XCell I Blot Module (Invitrogen, Karlsruhe, Germany). The membranes were blocked using 1x Blocking Buffer (Sigma-Aldrich), and incubated with the primary antibody at 4°C overnight. We used the following antibodies: rabbit polyclonal anti-Ergic2 (Abcam, ab50847; 1:1000), rabbit polyclonal anti-Otoferlin (1:2000) [4] and polyclonal anti-Actin A (20-33) antibody from rabbit (Sigma-Aldrich, 1:5000). On the next day, membranes were incubated with the secondary antibody for 1 hour at room temperature (Amersham ECL anti-mouse from sheep, anti-rabbit from donkey IgG, Horseradish Peroxidase linked, GE Healthcare, München, Germany, 1:2000). The results were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection Reagent (Amersham, GE Healthcare, München, Germany).

Ergic2 as an Interacting Partner of Otoferlin

Cell Physiol Biochem 2012;29:941-948 943
Results

Identification of Ergic2 as a new interacting partner for Otoferlin

We used the yeast-two-hybrid system to screen a cochlea-specific cDNA library (from mice P3-P15) with several Otoferlin domains as baits (for details see Heidrych et al., 2008). This screening, using bait 3 covering part of the Otoferlin C2D domain, resulted in identification of Ergic2 as an interacting protein (Fig. 2).

Co-expression of Ergic2 and Otoferlin in cochlea and brain

Initially, Ergic2 was identified in prostate cancer. However, its participation in protein trafficking suggested its ubiquitous presence and expression in various tissues. To confirm its validity as a potential binding partner of Otoferlin, Ergic2 expression in cochlea and brain was tested. Expression on the mRNA level was tested by RT-PCR analysis. Using a collection of cochlear cDNA samples representing different time points during cochlear maturation before (postnatal day 10, P10) and after onset of hearing (P23, P33), we demonstrated spatial and temporal co-transcription of Ergic2 and Otoferlin in this organ (Fig. 3A). However, the cochlea is a sophisticated auditory instrument with a complicated structure in which Otoferlin is present only in the hair cells localized in the organ of Corti. To specify Ergic2 expression we used inner (IHC) and outer (OHC) hair cells isolated from mouse P23 (Fig. 3A). Positive results obtained in both,
IHCs and OHCs, verified spatial activity of the analyzed genes. Ergic2 mRNA was also detected in mouse brain, and more precise studies demonstrated presence of Ergic2 in different analyzed regions: inferior colliculus, superior colliculus, hippocampus, cerebellum and in cortex (Fig. 3B). Otoferlin’s expression in the same brain regions was already reported [4].

Using a polyclonal anti-Ergic2 antibody we analyzed brain and cochlea samples representing different developmental stages before (P5) and after onset of hearing (cochlea: P44; brain: P16). Blotting resulted in a signal at an apparent molecular weight of 34 kDa observed in lysates from both developmental time points (Fig. 4). PCR results along with western blot data showing presence at the same time, tissues and specific cells suggest Ergic2 as a promising candidate for Otoferlin binding.

**Fig. 5.** Distribution of Ergic2 protein within cochlear hair cells. Before onset of hearing (P6) Ergic2 (red) is localized in stereocilia of both IHCs and OHCs (white arrowhead), whereas after onset of hearing (P16) protein is distributed in apical and supranuclear part of IHC and apical part of OHCs (white arrowhead). Cell nuclei were counterstained with DAPI (blue). Scale bars indicate 20 μm.

**Fig. 6.** Analysis of co-localization in cochlea and brain. A) After onset of hearing (P17) Ergic2 (red) is observed in the apical and supranuclear part of IHC (arrows), anti-Otoferlin immunostaining (green) was used as a marker for the IHC. Merged images display co-localization (yellow) as indicated in enlarged boxed area (white arrowhead). Cell nuclei were counterstained with DAPI (blue). Scale bars indicate 20 μm. B) The deconvoluted stack image presenting the immunostaining in the brain of adult mice (P120). Overlapping of Ergic2 (red) and Otoferlin (green) signals in neurons of cortical layer I demonstrated, comparing with cochlea, higher percentage of the total area of co-localization (yellow color indicated by white arrowheads). Scale bars indicate 10 μm.

**Co-localization analysis**

In the next step, co-localization of Ergic2 and Otoferlin was studied in the IHC of mouse cochlea (P17) and in the brain (P120). As previously reported [4] Otoferlin is ubiquitously distributed in the cell bodies of IHC and OHC in first postnatal days and, with time, its expression is down-regulated. Antibody staining showed differences in the localization of Ergic2 during cochlear maturation. Before onset of hearing Ergic2 is present only in the stereocilia of IHC and OHCs (Fig. 5), excluding possible co-localization with Otoferlin. After onset of hearing Ergic2 was expressed in the apical part of OHCs (Fig. 5), and mostly in the apical and supranuclear regions of IHC (Fig. 5). Co-immunostaining of anti-Ergic2 and anti-Otoferlin antibody revealed a weak overlap (Fig. 6A). However, co-localization studies in mouse brain

Ergic2 as an Interacting Partner of Otoferlin

**Cell Physiol Biochem 2012;29:941-948**
Fig. 7. Co-immunoprecipitation of interacting partners. A) Otoferlin and Ergic2 do not co-immunoprecipitate in cochlear lysates. B) Ergic2 was co-precipitated by the Otoferlin-specific antibody only in brain sample. A, A2, B, B2) Otoferlin knock-out (ko) mouse model, anti-Qa1 antibody and beads were used as controls of precipitation specificity. A3, B3) Immunoblotting confirms Otoferlin expression in lysates from wild type (wt) mouse and lack of protein in samples derived from Otoferlin ko mice.

demonstrate overlapping expression to a great extent as shown here in neurons of cerebral cortical layer I (Fig. 6B). This positive result in mouse brain is in a marked contrast to a smaller percentage of the total area of co-localization in cochlea.

Co-immunoprecipitation of Ergic2 and Otoferlin

The physical interaction between Ergic2 and Otoferlin was studied by co-immunoprecipitation using mouse derived brain and cochlear protein lysates (Fig. 7). The antibody against Ergic2 recorded protein bands in both, brain and cochlea samples (input lane in the Fig. 7A, A2, B, B2). Presence of Otoferlin in above mentioned lysates was tested by the Otoferlin specific antibody, resulting in one signal of the expected size of 230 kDa in brain and two signals corresponding to two isoforms (230 kDa and 240 kDa) in mouse cochlea (Fig. 7A3, B3). The same samples were used for the co-immunoprecipitation analyses. When anti-Otoferlin antibody was used for precipitation of specific protein in cochlear lysate, we did not detect any co-precipitated Ergic2 (Fig. 7A). However, the Otoferlin precipitation using the brain lysates, resulted in detection of co-immunoprecipitated Ergic2 (Fig 7B). Despite being spatially and temporally co-expressed in these tissues both proteins interact only in the brain and not in the cochlea suggesting that Ergic2 is a conditional binding partner of Otoferlin.

Discussion

For several years Otoferlin was believed to be a protein triggering the calcium-dependent fusion of the synaptic vesicle in the auditory system. This notion was supported by the observed interaction of Otoferlin with syntaxin1 and SNAP25 [5]. However, identification of further interacting partners, Rab8b and Myo6, expanded this idea, extending Otoferlin’s function and connecting it to intracellular trafficking of the vesicles to basolateral cell membrane in the cochlear IHC [6, 21]. This finding, shedding light on new mechanisms, encouraged our search for additional binding proteins that can help to correctly identify the role of Otoferlin in the intracellular trafficking.

The present study demonstrates Ergic2 as a new candidate identified by the yeast-two-hybrid screening
of a cochlear library. Our knowledge about Ergic2 is still limited, most of the data are based on comparative studies with the homologous proteins: yeast Erv41p, Ervp46p and Ergic32 [9, 22]. Localization to ERGIC compartment and the fact that homologs of Ergic2 are involved in vesicle transportation, make this protein a promising binding partner for Otoferlin. The co-localization, co-immunoprecipitation and expressional analyses presented in this study demonstrate an interaction between both proteins.

It is worth noting that in most of the Otoferlin’s projects all the attention focuses on the organ of Corti within cochlea which is the most critical element in the auditory pathway involved in mechano-electrical transduction of sound. In previous studies on Otoferlin interactome both, Roux et al. [5] and Heidrych et al. [6, 21] also restricted their analysis of binding partners to cochlear hair cells. Their results indicated two possible activities into which Otoferlin can be involved; calcium-dependent exocytosis at the hair-cell synapse and intracellular trafficking/vesicle recycling [5, 6, 21]. It remains important to stress that assignment to either one of these processes does not exclude Otoferlin’s activity in the other one. Otoferlin can play roles in both pathways depending on whether action at ribbon synapses, endocytic or secretory trafficking is required.

However, Otoferlin demonstrates a wider expression pattern not only restricted to the auditory organ. Next to cochlea, presence of Otoferlin at both, mRNA and protein levels, was detected in murine vestibular organ and brain but the function of Otoferlin in these organs has not been studied yet [4].

For the first time we demonstrate that Otoferlin can recruit to the complex different proteins depending on the tissue: Otoferlin interacts directly with Ergic2 in brain but not in cochlea. This finding showing distinct properties of a protein in native organs stays in line with observation that in Otoferlin deficient mice exocytosis in IHC is almost completely abolished, while neurotransmitter release at hippocampal synapses remains not affected [23].

Since an impairing mutation in OTOF gene results in elimination of a functional protein which in turn leads to deafness, the hypothetical model of Otoferlin interactome with Otoferlin as a core-forming protein seems to be a logical assumption. However, exocytosis impaired only in IHCs of Otoferlin mouse model may indicate a different importance of Otoferlin in complexes formed in cochlea and brain. Moreover, the tissue-dependent interaction with Ergic2 may suggest that Otoferlin can act as a main protein recruiting binding partners into the cochlear complex, whereas in brain another key-protein establishes functional interactome acting properly even without Otoferlin as a structural element.

Cochlear hair cells and brain neurons are both highly specialized with a set of proteins expressed exclusively within these cells, e.g. Synaptotagamins in brain neurons and Prestin in OHCs [24, 25]. Therefore, a third element characterized by cell-specific synthesis and acting as a linker could also explain formation of tissue-depending complexes. In other words, a protein undergoing translation in neurons but not in IHCs can serve as a connector coupling Ergic2 with Otoferlin in brain. Furthermore, functional differences between neurons and hair cells may result in assignment of both proteins into trafficking at distinct stages or in opposite directions in cochlear IHC.

In our deliberations we cannot exclude the possibility that the well studied roles of Otoferlin in exocytosis and intracellular trafficking may be true only for IHC, whereas in brain Otoferlin interacts with Ergic2 in order to serve another yet unknown function. However, Ergic2 can also be a multifunctional protein presenting distinct activities in cochlea and brain. Ergic2 was already reported to be a candidate tumor suppressor of prostate cancer. Homology to proteins involved in vesicle trafficking suggested Ergic2 contribution to transportation [26]. Furthermore, interaction with β-amyloid, the main constituent of amyloid plaques and apoptotic inducer in neurons responsible for cognitive dysfunction in Alzheimer’s disease, adds another potential affiliation to neurodegenerative impairment and programmed cell death [27].

Taking into consideration the novel brain specific binding partner of Otoferlin it is intriguing to speculate whether previously described cochlear interactors also form complexes in brain, or their collaboration with Otoferlin is restricted only to cochlear hair cells. Finding an answer to this question along with detailed analysis of Otoferlin’s function in the brain are challenging tasks for further studies.

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