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Interaction between the motor protein prestin and the transporter protein VAPA

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ABSTRACT

Prestin is the motor protein responsible for cochlear outer hair cell (OHC) somatic electromotility. Eliminating this abundant basolateral membrane protein not only causes loss of frequency selectivity and hearing sensitivity, but also leads to OHC death. A membrane-based yeast two-hybrid approach was used to screen an OHC-enriched cDNA (complementary Deoxyribonucleic Acid) library in order to identify prestin-associated proteins. Several proteins were recognized as potential prestin partners, including vesicle-associated membrane protein associated protein A (VAPA or VAP-33). VAPA is an integral membrane protein that plays an important role in membrane trafficking, endoplasmic reticulum homeostasis, and the stress-signaling system. The connection between VAPA and prestin was confirmed through co-immunoprecipitation experiments. This new finding prompted the investigation of the interaction between VAPA and prestin in outer hair cells. By comparing VAPA expression between wild-type OHCs and OHCs derived from prestin-knockout mice, we found that VAPA is expressed in OHCs and the quantity of VAPA expressed is related to the presence of prestin. In other words, less VAPA protein is found in OHCs lacking prestin. Thus, prestin appears to modify the expression of VAPA protein in OHCs. Intriguingly, more prestin protein appears at the plasma membrane when VAPA is co-expressed with prestin. These data suggest that VAPA could be involved in prestin's transportation inside OHCs and may facilitate the targeting of this abundant OHC protein to the plasma membrane.

1. Introduction

Outer hair cells (OHCs) are sensory receptor cells exclusively found in mammalian cochleae. They play an essential role in increasing sensitivity and frequency selectivity of mammalian hearing (for review, see [1]). Without OHCs, hearing threshold is elevated by ~50 dB [2], frequency resolution disappears [3,4], and the ear's operation is linearized [5]. It is believed that this local mechanical amplification of the cochlear response to sound is associated with somatic electromotility [6], a unique feature of OHCs. When the voltage across the OHC's basolateral membrane (BLM) is altered, OHCs change their length [7] and stiffness [8]. OHC length is also altered if the voltage change is due to the deflection of the stereociliary bundle (the OHC's mechanosensitive organelle), as it would be with sound stimulation *in situ* [9]. The molecular basis for OHC somatic electromotility is the motor protein, prestin [10]. A series of experiments, including molecular biology, cell biology, biophysics, *in vitro* and *in vivo* physiology have demonstrated that prestin is the motor protein of OHCs and that it is required for cochlear amplification (for review, see [11,12]). For example, OHCs derived from prestin-knockout (KO) mice [13–15] or OHCs that lack fully functional prestin [12], lose somatic electromotility, and ~50 dB of hearing sensitivity, as well as frequency selectivity.

The distinct function of the OHC is associated with its several unusual cellular structural features. For instance, the plasma membrane (PM) has an uncommon lipid composition with extremely low cholesterol. Increasing cholesterol levels in the PM leads to OHC death [16]. In addition to the unique lipid composition, a high density of integral membrane proteins is found in the lateral plasma membrane [17]. In fact, the OHC's PM is densely packed with ~10 nm protein particles, with a density estimated from 2500/μm^2 to 6000/μm^2 [17]. How these abundant protein particles, assumed to be prestin tetramers [18], accumulate in the lateral membrane of OHCs and whether the exceptionally high protein content in the PM is related to the uncommon lipid composition or to the targeting process remain unknown. Furthermore, prestin is exclusively targeted to the BLM of polarized cells in both native cells such as OHCs [19], and non-native cells like CL4 [Zheng, L. et al., unpublished data]. Despite the fact that

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prestin mutants were made to study targeting sequences such as tyrosine-containing (YXXI) and di-leucine motifs [20], little is known about prestin trafficking, including the involvement of any transport protein in this process.

VAPA is an important protein involved in protein trafficking. As first discovered in *Aplysia californica* [21], VAPA proteins are highly conserved among different species. There are two *Vap* genes in mammals: *Vapa* and *Vapb* [21]. VAPA and VAPB proteins are ubiquitously expressed integral membrane proteins associated with intracellular vesicles, the endoplasmic reticulum (ER) and microtubules [22,23]. In humans, VAPA and VAPB show 63% amino acid similarity and have common features in their structures such as a major sperm protein (MSP) domain (~120 amino acids) at their N-termini, a coiled-coil structure in the central domain, and a C-terminal transmembrane domain [23]. Mammalian VAPA is known to interact with proteins involved in regulation of sterol, lipid biosynthesis and trafficking. Proteins in this group include the FFAT (two phenylalanine in an acidic tract)-motif-containing oxysterol-binding protein (OSBP), the oxysterol-binding protein-related protein (ORP) [24], and the ceramide transport protein CERT [25]. The MSP domain of VAPA is known to interact with the FFAT-motif in target proteins and is partially responsible for targeting lipid-binding proteins to the ER [26,27]. Mutation of the MSP domain (P56S) of VAPB causes the formation of large ER aggregates and is believed to be linked to late-onset amyotrophic lateral sclerosis type 8 (ALS8) [28]. In addition, VAPA is also known to interact with proteins that do not have the FFAT-motif, including viral proteins whose transportation from ER to Golgi is modulated by VAPA [29], and the ER-localized transcription factor ATF6 (activating transcription factor 6) [30]. Interactions between VAPA and the other proteins not only allow the efficient transport of VAPA-associated proteins to their target locations, but also influence the associated proteins' function. For example, over-expression of VAPA in L6 myoblasts attenuates the insulin-dependent incorporation of GLUT4 into the PM, and this effect can be suppressed by over-expression of VAMP-2 (vesicle-associated membrane protein 2), another VAPA-associated protein [31]. Thus, interaction between VAPA and an associated protein can lead to outcomes on other VAPA-associated proteins, indicating that VAPA can affect many physiological functions beyond its role as a transporting protein.

We identified VAPA as a potential partner of prestin through a high throughput membrane-based yeast two-hybrid screening [32]. In this report, we confirm interaction between VAPA and prestin in mammalian cells through co-immunoprecipitation (co-IP) experiments. By comparing VAPA expression in wild-type (WT) and prestin-KO OHCs, we discovered that lack of prestin, which is normally abundantly expressed in OHCs, affects VAPA expression. Additionally, we examined the possible role of VAPA in prestin transport inside mammalian cells.

2. Materials and methods

2.1. Antibodies and plasmids

Polyclonal rabbit anti-mPres antibody was raised against the carboxy terminal motif of the mouse protein as an antigen, which has been previously characterized [33]. Monoclonal anti-VAPA antibody was purchased from BD Biosciences (San Jose, CA). Anti-Frizzled3 (FZD3) antibody was purchased from Sigma (St. Louis, MO). Monoclonal anti-V5 and anti-myc antibodies were from Invitrogen (Carlsbad, CA). Monoclonal anti-GFP antibody was from BD Biosciences (San Jose, CA). Anti-Na^+/K^+ATPase antibody was purchased from Upstate Biotechnology, Lucerne. Anti-HIS and anti-HSC70 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-HA antibody 12CA5 was kindly provided by Dr. Robert A. Lamb at Northwestern University. Texas Red-X phalloidin was purchased from Molecular Probes (Eugene, OR). Secondary antibodies, AlexaFluor-488 and AlexaFluor-546 conjugated anti-rabbit IgG, AlexaFluor-488 conjugated anti-mouse IgM, AlexaFluor-546 conjugated anti-mouse IgG, donkey anti-mouse IgM-HRP, and goat anti-mouse IgG-HRP were purchased from Invitrogen, Pierce (Rockford, IL) or Jackson ImmunoResearch (Bar Harbor, ME). Vectashield mounting media with DAPI was purchased from Vector (Burlingame, CA). Plasmids encoding GFP-prestin and V5-prestin were used with GFP- and V5-tags attached to the C-terminus of prestin, respectively [19,20]. pBK-NmycTecta plasmid encoding the myc-α-tectorin was kindly provided by Dr. K. Legan and Dr. G. Richardson of the University of Sussex. HA-HIS-VAPA plasmid was purchased from GeneCopoeia (Germantown, MA).

2.2. Yeast two-hybrid analyses

Details of this protocol have been described before [32]. In summary, full-length mprestin (1–744 a.a.) was inserted into the bait-expression vector pAMBV4 (Dualsystems Biotech, Switzerland) with CUB-LexA-VP16 downstream of and in frame with mprestin. The bait vector carries the *LEU2* gene for auxotrophic selection. The sequence of the prestin-bait vector was confirmed through DNA sequencing. Expression of the mprestin-Cub-LexA-VP16 fusion protein was further verified by Western blot analysis with the anti-mPres antibody. Partial *Vapa* (244–993 a.a.) or partial *Fzd3* (182–356 a.a) was inserted into the prey expressing vector pDL2-Nx (Dualsystems Biotech) with NubG upstream of and in frame with *Vapa* or *Fzd3*, respectively. The prey vector carries the *TRP1* gene for auxotrophic selection. pMBV-Alg5 is a negative control-bait construct, which expresses the Cub-LexA-VP16 fusion protein in the correct orientation in the yeast membrane. The prestin-bait construct and the negative control, pMBV-Alg5, were transformed into yeast strain NMY51 (*MATa his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)$_4$-HIS3 ura3::(lexAop)$_8$-lacZ ade2::(lexAop)$_8$-ADE2 GAL4*) (Dualsystems Biotech) and grown on leucine selective plates (SD-L), respectively. A *Vapa*-prey/*Fzd3* prey construct was transformed into prestin-bait-expressing yeast or pMBV-Alg5 expressing yeast and grown on leucine-tryptophan double selective plates (SD-LT). Positive interactions were identified by the ability of yeast to grow on leucine-tryptophan-histidine-alanine selective plates (SD-LTHA) in the presence of 2 mM 3-aminotriazole, and by β-galactosidase expression, indicated by the blue color observed in the presence of X-gal.

2.3. Immunofluorescence experiments

For cochlear tissue: All surgical and experimental procedures were conducted in accordance with the policies of Northwestern University's Animal Care and Use Committee and the NIH Safety Guidelines. Anesthetized mice were cardiac perfused first with phosphate buffered saline (PBS) containing heparin and then fixed with 4% formaldehyde (EM grade). For cross-section samples, the cochleae were post-fixed in 4% formaldehyde for 1 hour at room temperature and placed in 10% EGTA/PBS at 4 °C overnight. The decalcified cochlear samples were placed in 30% sucrose/PBS and embedded in cold OCT compound. Samples were cut in 10–20 μm sections, placed on glass slides, fixed in 4% formaldehyde for 10 min and blocked at room temperature for 30 min in blocking solution (1% BSA, 0.2% saponin in PBS). Samples were then incubated with anti-VAPA (1:50–1:100) or anti-FZD3 (1:200) followed by incubation with anti-rabbit-IgG/anti-mouse IgM conjugated with AlexaFluor-488 (1:400) and Texas Red-X phalloidin (1:2000). Samples were then mounted on glass slides with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) or Vectashield mounting media with DAPI and were observed using a Leica confocal system with a standard configuration DMRXE7 microscope. For whole-mount samples, EGTA and cryosectioning were omitted from the procedure.

For transfected mammalian cells: Plasmids encoding HA-HIS-VAPA/GFP-prestin + HA-HIS-VAPA were transiently transfected/co-