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ORIGINAL ARTICLE

Functional Analysis of Pendrin (SLC26A4) and its Pathogenic Mutations in cos-7 cells

Hailin Zhanga, Yong Feng, Kun Xia, Lu Jiang, Chufeng He, Lingyun Mei

Department of Otolaryngology, Xiangya Hospital, Central South University, Changsha, Hunan, China (HZ, YF, LJ, CH, LM)
Head and neck surgery, Hunan Provincial Tumor Hospital, Changsha, Hunan, China (HZ)
State Key Laboratory of Medical Genetics, Central South University, Changsha, Hunan, China (YF, KX,
Hunan Key Laboratory of Otolaryngology critical diseases, Changsha, Hunan, China (YF, KX, LJ, CH, LM)

Objective: To investigate the mechanism of the deafness-causing mutation S448X in the pendrin protein through measurement of the expression levels and functional activities of the wild-type (WT) and mutant proteins.

Materials and Methods: pEGFP N1 SLC26A4 S448X (S448X-EGFP) and wild-type pEGFP N1 SLC26A4 (WT-EGFP) were transfected into cos-7 cells and their expression detected by western blotting. Immunostaining and confocal microscopy were used to determine the subcellular localization of WT and S448X proteins. The effects of overexpression of WT and S448X pendrin on ion transport in the cell were measured by whole-cell patch clamp analysis.

Results: S448X was expressed in the cytoplasm, and co-localized with the ER, while WT pendrin was efficiently transported to the cell membrane. The chloride ion current was significantly weaker ($P < 0.05$) in cells expressing the S448X mutant protein than those expressing the wild-type protein.

Conclusion: The S448X mutation in pendrin could not be transported to the cell membrane after translation and remained in the endoplasmic reticulum (ER). This mutation had a reduced ability to transport chloride ions, suggesting that inhibition of chloride ion transport might lead to the pathologies associated with the S448X mutation.

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Introduction

SLC26A4 (Pendred's syndrome gene) is the most common gene after GJB2 to be associated with deafness resulting from ear deformities. The SLC26A4 gene is the fourth member of a family of genes called SLC (solute carriers) 26. The SLC26A4 gene is located on the long arm of chromosome 7 at position 31 (7q31), which consists of 21 exons and encodes the pendrin protein. The pendrin protein is a glycosylated transmembrane protein containing 12 transmembrane domains, and pendrin monomers can associate to form anion channels that mediate the transport of Cl^-, I^- and HCO_3^- across cell membranes. Thus, SLC26A4 belongs to the family of genes that maintain ion balance in the cochlea.

Diseases caused by SLC26A4 mutations are inherited in an autosomal recessive manner. More than 200 SLC26A4 mutations have been identified that are associated with both Pendred syndrome and with DFNB4 (OMIM 600791), which causes non-syndromic hereditary deafness. People with Pendred syndrome are deaf and often have an enlarged thyroid gland (thyroid function is normal or slightly lower). Deafness caused by SLC26A4 mutations are often accompanied by inner ear malformations, the most common of which are an enlarged vestibular aqueduct (EVA) and Mondini deformity. Pendrin in the inner ear takes part in Cl- and HCO_3^- ion transport [1,2] to maintain cochlear homeostasis. However, the pathogenic mechanism by which SLC26A4 mutations leads to inner ear malformations and deafness is still unclear. The deafness-causing mutation S448X in pendrin was first discovered in our laboratory. In this study, we transfected WT-EGFP and S448X-EGFP plasmids into cos-7 cells to detect their expression. We

Corresponding address:
Lingyun Mei
Department of Otolaryngology, Xiangya Hospital, Central South University
87 Xiangya Road, Changsha 410008, Hunan Province, PR China
Phone: (00)86-731-89753745, Fax: (00)86-731-89753545
E-mail: entmly@163.com

immunostained the endoplasmic reticulum (ER), Golgi apparatus and microtubule network in order to determine the distribution of the pendrin protein in these organelles. Furthermore, to explore the effect of S448X on chloride ion transport ability in cells, we recorded the current differences between cells expressing WT and S448X proteins with patch clamp technology.

Materials and Methods

Materials and antibodies

pEGFP N1 SLC26A4 S448X and pEGFP N1 SLC26A4 WT plasmids were constructed by Dr. Lingyun Mei in the early trials of this study and their sequences were verified. Cos-7 cells were provided by the State Key Laboratory of Medical Genetics of Central South University. Primary antibodies used are rabbit polyclonal anti-GFP, mouse monoclonal anti-calnexin (an ER marker), mouse monoclonal anti-Golgi58 (a Golgi marker), and mouse monoclonal anti-β-tubulin (a microtubule marker). Secondary antibodies used were horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG. All antibodies were purchased from Sigma.

Cell culture and transient transfection

Cos-7 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) in a 37 °C incubator containing 5% CO_2. One day before transfection, 0.5–2 × 105 cos-7 cells in 500 μL of DMEM medium were added to each well of a 24-well plate. The next day, 0.8 μg of the plasmid DNA and 2 μL Lipofectamine™ 2000 were separately diluted in 50 μL Opti-MEM. After a 5-min incubation at room temperature, the diluted DNA and diluted Lipofectamine™ 2000 were combined and incubated for another 20 min at room temperature. Finally, we added this mixture to the cells and kept them at 37°C in a CO_2 incubator for 36-48 h prior to testing for transgene expression.

Western blotting

Cells were harvested in 2× SDS sample buffer and heated at 100°C for 5 min. Protein concentrations of the cell lysates were determined using Bradford reagent (Pierce). Protein (30 mg) was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes, followed by blocking for 1 h with 5% milk and overnight incubation with primary antibody (mouse anti-GFP) at 4°C. The membranes were washed 3 times with 0.1% Triton X-100/PBS, and the secondary antibody (HRP-labeled goat anti-rabbit IgG) was added for 1 h at room temperature. The membrane was washed again and the antibody-protein complexes were detected using the ECL PLUS detection kit (Amersham Biosciences) followed by autoradiography.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. After washing 3 times with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, followed by blocking in 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Cells were incubated with primary antibodies (anti-calnexin, anti-Golgi58 and anti-β-tubulin) in BSA for 1 h at room temperature. Following 5 washes with PBS and blocking for 30 min with 5% BSA, the fluorophore-conjugated secondary antibody (goat anti-mouse IgG conjugated to Cy3) in 5% BSA was incubated with the cells for 1 h at room temperature. Cells were then washed 5 times with PBST, and incubated with 1 μg/mL DAPI in PBS for 5 min. After washing twice with PBS, cells were mounted on slides and observed using a confocal microscope.

Whole-cell patch clamp recording

The main instruments used were a patch-clamp amplifier and digital to analog converter EPC-9 (HEKA, Germany), PULSE/PULSEFIT software (HEKA, Germany), and Pipette Puller (Sutter, USA).

The reagents used were 5-nitro-2-3 phenol propylamine benzoate (NPPB) (Sigma) and tetrodotoxin (TTX) (Sigma). The pipette solution contained 125 mM CsCl, 10 mM HEPES, 10 mM EGTA, 5 mM Mg_2 + ATP, and 5 mM $MgCl_2$ that had been adjusted to pH 7.2 with CsOH. The bath solution contained 130 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose and 0.002 mM TTX adjusted to pH 7.4 with NaOH.

Whole-cell patch clamp recording

After transfection for 36 h, cos-7 cells that grew well, had a clear outline, and expressed green fluorescence

under the fluorescence microscope were selected for whole-cell patch-clamp recording. Glass microelectrodes with an open tip diameter of 0.5–1.0 μm were drawn using Pipette Puller. The resistance of the glass microelectrodes was 2–3 MΩ when filled with the pipette solution. Access resistance (Ra), and fast and slow capacitance were compensated and monitored throughout the recordings. The current signal was amplified through the negative feedback amplifier of the patch-clamp amplifier, and the recorded data were filtered at 2 kHz, sampled at 5 kHz, and analyzed with the PULSE/PULSEFIT software package. Statistical analyses of the data via the t-test was carried out with the SPSS15.0 software. Numerical data were expressed as the mean ± the standard error. P values <0.05 were considered significant.

Results

WT-EGFP and S448X-EGFP fusion proteins are expressed in cos-7 cells.

The WT-EGFP and S448X-EGFP fusion proteins were expressed in cos-7 cells, and the resulting bands on the SDS-PAGE gel were of the expected molecular weights (Fig. 1A). The lower molecular weight observed for the S448X mutant is due to the fact that the codon for a serine at position 448 has been replaced with a stop codon and the resulting protein is truncated and missing the 333 amino acids encoded by exons 12 to 21.

S448X is distributed mainly in the cytoplasm and co-localizes with the ER

To determine the cellular location of S448X, we transfected WT-EGFP and S448X-EGFP into cos-7 cells and analyzed the distribution of the proteins with a confocal microscope. We simultaneously immunostained the ER, Golgi apparatus and microtubule network in order to determine the colocalization between the S448X protein and cell organelles. We found that the S448X protein was expressed in the cytoplasm and colocalized with the ER, but not with the Golgi apparatus or the microtubule network. Wild-type pendrin was found mainly in the cell membrane, and the small amount in the cytoplasm was likely due to the high expression level of the protein in the cell.

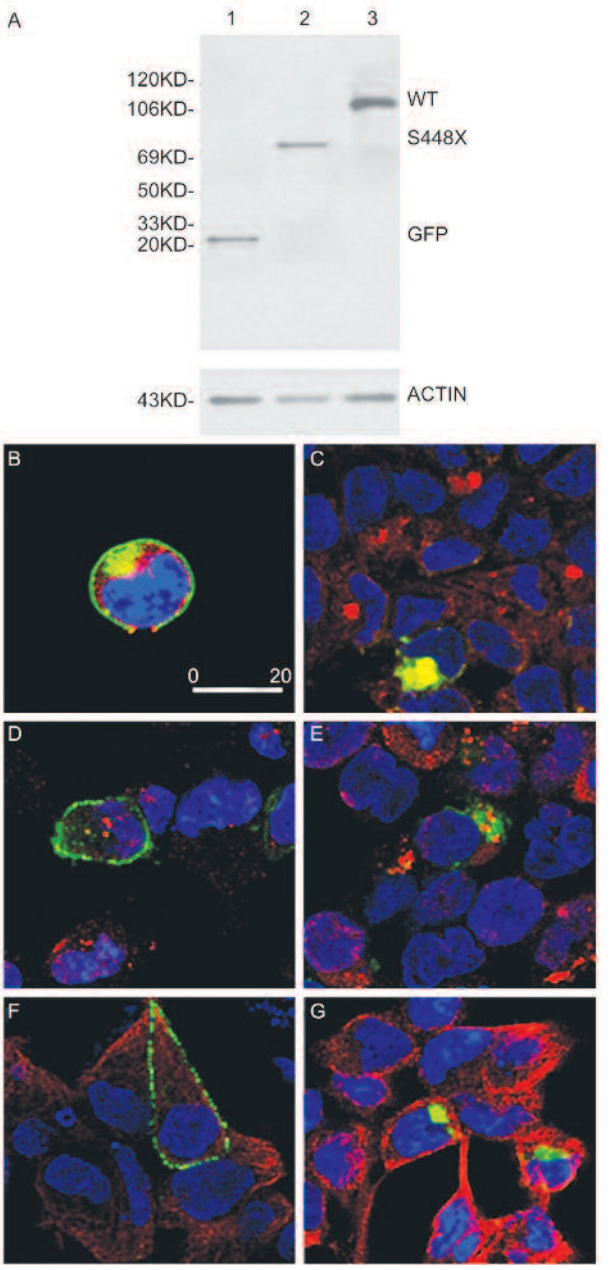

Figure 1. SLC26A4 WT-EGFP and S448X-EGFP fusion protein expression in cos-7 cells. (A) Cos-7 cells were transfected with pEGFP N1 (lane1), SLC26A4 WT-EGFP (lane2) and S448X-EGFP (lane3). Protein expression was detected by western blotting with mouse anti-GFP antibody. β-actin was used as a loading control. (B,C) The endoplasmic reticulum (ER) is labeled in red. (D,E) The Golgi apparatus is labeled in red. (F,G) The microtubule network is labeled in red. The wild-type protein (green) is found in the cell membrane (B,D,F) while S448X (green) accumulates in the cytoplasm (C,E,G) and only colocalizes with the ER (as seen in panel C). The scale bar is 20 ∝m.

Transmembrane chloride currents are reduced by expression of the S488X mutant

The concentration of chloride ions was 130 mM both intracellularly and extracellularly, and a stable current was recorded in cells expressing both the WT and S448X proteins (Fig. 2). Step pulses of 1-s duration were applied from -100 mV to 100 mV in 20-mV steps, and the current amplitude changed with the clamping voltage. Statistical analysis showed that there was a significant difference ($p < 0.05$) in current amplitude between cells expressing WT and S448X at each clamping voltage, and control cells (untransfected cos-7 cells) had a similar curve to the cells expressing the S448X mutant protein ($p > 0.05$) (Fig. 3A). To exclude the effect of sodium ion current, we added TTX in the bath solution. The current amplitudes of cells expressing WT and S448X and the control cells were significantly reduced ($p < 0.05$) 20 min after addition of 100 μM NPPB, a chloride ion inhibitor (Fig. 3B-D).

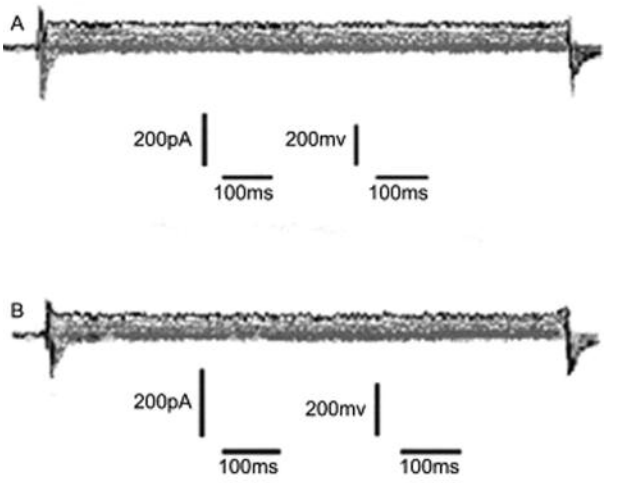

Figure 2. Chloride current in cos-7 cells expressing SLC26A4 WT (A) and S448X (B).

Discussion

Mutations in the SLC26A4 gene result in the autosomal recessive conditions DFNB4 and Pendred

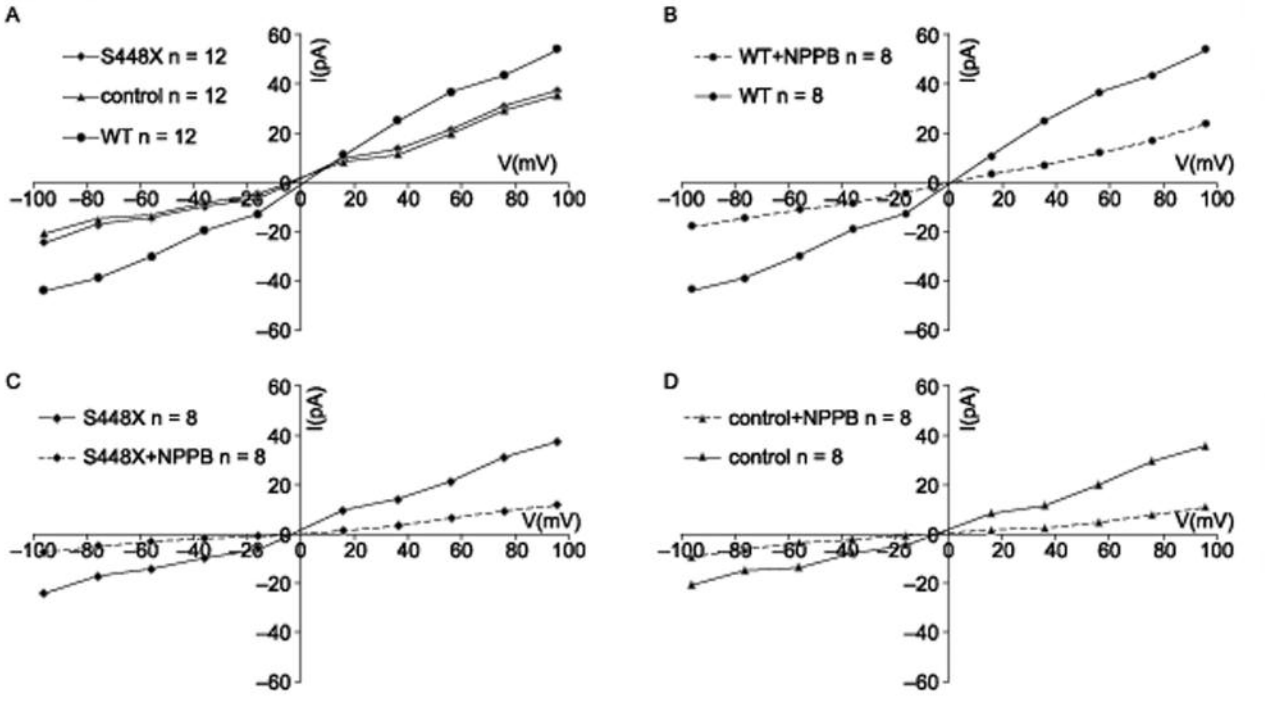

Figure 3. Chloride current measurements in cos-7 cells. The clamping voltage (mV) is on the X-axis and the corresponding chloride current (pA) is on the Y-axis. (A) Wild-type (WT), S448X and control. The current amplitudes with WT were not significantly different ($P > 0.05$) when the clamping voltage was 16 mV but were significantly different ($P < 0.05$) at all other clamping voltages. No significant differences were observed ($P > 0.05$) between S448X and the control cells at any clamping voltage. (B) WT + 100 ∝M NPPB. (C) S448X + 100 ∝M NPPB. (D) Control + 100 ∝M NPPB. Significant differences ($P < 0.05$) were observed for all cells at all clamping voltages after a 20-min incubation with 100 ∝M NPPB.

syndrome. The expression, cellular localization, and ion transport ability of the pendrin protein in eukaryotic cells are widely used to study functional deficiencies or functional changes caused by SLC26A4 mutations. It has been reported that mutations in the pendrin protein can be classified into 3 distinct groups. The first group of mutations cannot correctly translocate to the cell membrane and ion transport function is lost. For example, G497S [3], H723R [4], and E737D [5] accumulate in the cytoplasm and G102R [6], T717A [7], and I487YFSX39 (526X) [8] [9] are retained in the ER. The second group of mutations such as Y556C [6] and R409H [10] can partially translocate to the cell membrane, but ion transport function is lost or diminished. The third group of mutations, such as L579S [11], F335L [12], R776C [13] [14], are silent mutations and the resulting proteins are efficiently transported to the cell membrane with little or no loss of ion transport function.

The S448X mutation was first discovered by our group in a family afflicted with hereditary EVA deafness [15] [16]. The S448X mutation arises from a C to A transversion at position 1343 in the SLC26A4 gene, resulting in a change from a serine to a stop codon. The encoded protein is only 448 residues in length and does not contain the 333 amino acids encoded by exons 12 to 21. In this mutant protein, the 11th and 12th transmembrane regions, 6th extracellular domain, and the C-terminal are lost. In this study, S448X-EGFP and SLC26A4 WT-EGFP were transfected into cos-7 cells and their expression levels were analyzed by western blotting. To determine the cellular localization of S448X and WT protein, we stained the ER, Golgi apparatus and microtubule organization with specific antibodies. These immunoflourescent experiments showed that the truncated protein could not be transferred to the Golgi apparatus for processing after it was synthesized in the ER. The S448X mutant protein accumulated around the nuclei and colocalized with the ER but not with the Golgi apparatus or the microtubule network, while the WT protein was found in the cell membrane. We had previously presumed that the truncated protein was misfolded and could therefore not be transported to the cell membrane, thereby leading to loss of ion transport function. Our current results, however, show that the S448X protein could be properly folded and processed in the ER, but could not be transferred to the Golgi apparatus for further processing and subsequent transport to the cell membrane.

The pendrin protein is mainly expressed in the endolymphatic aqueduct, saccule, utricle, and lateral sulcus of the cochlea. It plays an important role in adjusting the pH value of the endolymph and keeping ion levels balanced. Point mutations in the SLC26A4 gene can cause malfunction of the pendrin protein that results in failure to transport chloride ions across the cell membrane . In this study, we recorded the chloride ion current of cells expressing WT and the S448X mutant of pendrin using whole-cell patch-clamp techniques. The results showed that chloride ion current, as verified by inhibition with NPPB, could be recorded in cells expressing WT or S448X. Statistical analysis suggested that the chloride ion current of cells expressing S448X is significantly weaker than that of cells expressing the wild-type protein, while there was no significant difference in current amplitude between control cells and cells expressing S448X. The chloride ion current recorded in cells expressing S448X was the same as that in the control cells, and probably represents the endogenous chloride channel currents in cos-7 cells.

Till date, the mechanism by which pendrin transports I-, Cl-, HCO_3^- anions has been unclear. The chloride ion current could not be detected by the Dossena group in HEK293 cells expressing pendrin, which suggests that anion transport mediated by pendrin in human cells is electrically neutral [17]. SLC26A4 WT was transfected into cos-7 cells by Yoshida [18], who showed that chloride and iodide ion currents could be recorded using whole-cell patch-clamp methods, which is in accordance with our results in the current study. The functional mechanism of the pendrin protein varies in different expression systems, which explains the different experimental results obtained.

In summary, the S448X mutation in pendrin was first discovered by our group in a core family afflicted with heritable EVA deafness. Our study on the expression and function of S448X revealed that S448X mutant proteins were retained in the ER and could not reach the cell membrane after translation. The decreased

chloride ion transport ability of S448X-expressing cells suggests that the S448X mutation may lead to ear deformities and deafness by acting as a chloride ion transport inhibitor.

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