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Construction of an expression system for the motor protein prestin in Chinese hamster ovary cells

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Abstract

The electromotility of outer hair cells (OHCs) is believed to be a major factor in cochlear amplification that enables the high sensitivity of hearing in mammals. This motility is thought to be based on voltage-dependent conformational changes of a motor protein embedded in the lateral wall of the OHC. In 2000, this motor protein was identified and termed prestin. To obtain knowledge on the function of prestin, research at the molecular level is necessary. For this purpose, a method of obtaining a large amount of prestin is required. In this study, an attempt was therefore made to construct an expression system for prestin. Prestin cDNA was introduced into *Escherichia coli* (*E. coli*), insect cells and Chinese hamster ovary (CHO) cells, and the expression of prestin was examined by Western blotting. As CHO cells expressed prestin well, we generated prestin-expressing cell lines using CHO cells by limiting dilution cloning. The stable expression and the activity of prestin in generated cell lines were then confirmed. Finally, to obtain prestin from these cell lines efficiently, culture conditions of the cells were examined, and it was clarified that cells should be cultured in serum-free medium and harvested around 48 h after passage.
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Keywords: Outer hair cell; Prestin; Chinese hamster ovary cell; Cloning; Stable expression

1. Introduction

Mammalian hearing sensitivity relies on a mechanical amplification mechanism based on the electromotility of outer hair cells (OHCs) (Brownell et al., 1985; Kachar et al., 1986; Zenner, 1986; Ashmore, 1987; Santos-Sacchi and Dilger, 1988). This mechanism enables the high sensitivity, wide dynamic range and sharp frequency selectivity of hearing in mammals (Dallos, 1992). The molecular basis of this mechanism is thought to be voltage-dependent conformational changes of motor proteins, which are embedded in the plasma membrane of the OHC lateral wall (Forge, 1991; Huang and Santos-Sacchi, 1993).

In 2000, Zheng et al. identified the motor protein in the gerbil cochlea and termed it prestin. Since its identification, prestin has been intensively researched to elucidate the characteristic behavior of the OHCs. As a result, it has been confirmed that prestin-transfected mammalian cells show characteristic features of the

Abbreviations: OHC, outer hair cell; *E. coli*, *Escherichia coli*; CHO, Chinese hamster ovary; MBP, maltose-binding protein; GFP, green fluorescent protein

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OHC based on its motor protein, i.e., they exhibit voltage-dependent nonlinear capacitance (Zheng et al., 2000; Ludwig et al., 2001; Santos-Sacchi et al., 2001), electromotility (Zheng et al., 2000) and force generation (Ludwig et al., 2001). It has also been clarified that both the amino and carboxyl termini are located on the intracellular side (Zheng et al., 2001) and that intracellular anions act as voltage sensors of prestin (Oliver et al., 2001). In addition, Liberman et al. (2002) generated prestin knockout mice and showed that targeted deletion of prestin in mice resulted in a loss of OHC electromotility in vitro. Such deletion has also been found to result in a 40–60 dB loss of cochlear sensitivity in vivo (Wu et al., 2004).

However, the motor function of prestin still needs to be understood at the molecular level. For this purpose, a method of obtaining a large amount of prestin as material for such research is required. In this study, an attempt was therefore made to construct an expression system for prestin. Prestin cDNA was introduced into *Escherichia coli* (*E. coli*), insect cells and Chinese hamster ovary (CHO) cells, and the expression of prestin was examined by Western blotting. As CHO cells expressed prestin, stable prestin-expressing cell lines were generated using transfected CHO cells by limiting dilution cloning. The expression of prestin and the activity of prestin in the generated cell lines were then examined by immunofluorescence experiments and patch–clamp measurements, respectively. Finally, to obtain prestin from these cell lines efficiently, the optimal culture condition of the cells was considered.

2. Materials and methods

2.1. Verification of expression in cells

Escherichia coli expression vectors pET28b (Novagen, Madison, WI), pET20b (Novagen) and pMAL-c2 (New England Biolabs, Beverly, MA) were used for the *E. coli* expression system. Gerbil prestin cDNA was inserted into these vectors. The open reading frame of the prestin cDNA was fused in a frame with the His_6-tag coding sequence of the expression vector pET28b or pET20b. For this purpose, the stop codon was removed. To express prestin as a maltose-binding protein (MBP)-fusion protein, prestin cDNA was inserted into the pMAL-c2 expression vector. The *E. coli* strains BL21 and JM109 were transformed by heat shock with pET28b or pET20b and pMAL-c2 *E. coli* expression vectors containing prestin cDNA, respectively. *E. coli* transformed with pET28b or pET20b was cultured in 200 ml 2× YT medium at 28 °C, and *E. coli* transformed with pMAL-c2 was cultured in 200 ml LB medium at 37 °C. When the bacteria had grown to an optical density at 600 nm of 0.6, IPTG was added to a final concentration of 1 mM. After 2–5 h, cells were harvested and the expression of prestin in bacteria was examined by Western blotting. When pET28b or pET20b was used, the expression was examined with anti-His_6 antibody (Invitrogen, Rockville, MD), and when pMAL-c2 was used, the expression was examined with anti-MBP antibody (New England Biolabs).

To use the baculovirus expression system, the pVL1392 transfer vector (PharMingen, San Diego, CA) was employed. Prestin cDNA fused at its 3′ end to the His_6-tag coding sequence was introduced into the pVL1392 transfer vector. Linearized baculovirus DNA (BaculoGold, Pharmingen) and the constructed pVL1392 transfer vector containing the prestin cDNA were co-transfected into Sf9 insect cells using Lipofectin Reagent (Invitrogen). The recombinant baculovirus was then amplified. Sf9 cells plated onto a 35-mm plate with 2 ml of fresh medium were infected with amplified baculovirus. After incubation for 3 days, cells were harvested and the expression of prestin in Sf9 cells was examined by Western blotting with anti-His_6 antibody.

For the mammalian expression system, the pIRES-hrGFP-1a (Stratagene, La Jolla, CA) mammalian expression vector was used. The open reading frame of the prestin cDNA was fused in the frame with the FLAG-tag of the expression vector. CHO-K1 cells (provided by the Cell Resource Center for Biomedical Research, Tohoku University) were transfected with the constructed expression vector using LipofectAMINE 2000 Reagent (Invitrogen). Transfected CHO cells were cultured in RPMI-1640 medium with 10% fetal bovine serum, 100 U penicillin/ml and 100 μg streptomycin/ml at 37 °C with 5% CO_2 for 2 days, and the expression of prestin in CHO cells was then examined by Western blotting with anti-FLAG antibody (Sigma–Aldrich, St. Louis, MO).

When Western blotting was performed, cell proteins were separated on 10% SDS–polyacrylamide gel and electroblotted onto nitrocellulose membrane. After blocking with skimmed milk, membranes were incubated with the primary antibody described above. Bands were visualized using horseradish peroxidase-conjugated secondary antibody and the ECL Western blotting detection system (Amersham–Pharmacia Biotech, Buckinghamshire, UK).

2.2. Cloning of prestin-expressing CHO cells

As prestin was expressed in CHO cells, an attempt was made to generate stable prestin-expressing cell lines using CHO cells. Wild-type prestin cDNA or C-terminal FLAG-tagged prestin cDNA contained in the pIRES-hrGFP-1a mammalian expression vectors was transfected into CHO cells using LipofectAMIN 2000 Reagent. After transfection, cells were plated out at a density of one cell/well in 96-well tissue culture plates. Plates were

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