Analysis of membrane structure of the inner ear motor protein prestin by force spectroscopy

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Abstract
The high sensitivity of mammalian hearing is achieved by cochlear amplification. The basis of this amplification is the motility of outer hair cells (OHCs), which are sensory cells in the inner ear. This motility may be due to voltage-dependent conformational changes of the motor protein prestin, which is densely embedded in the lateral membrane of OHCs. However, the membrane structure of prestin has not yet been elucidated. Therefore, the membrane structure of prestin was herein investigated by force spectroscopy using an atomic force microscope (AFM). The gene of prestin fused with an Avi-tag at its C terminus was transfected into Chinese hamster ovary (CHO) cells and the inside-out plasma membrane was isolated. The Avi-tag was enzymatically biotinylated and attached to a streptavidin-coated AFM cantilever via biotin-streptavidin binding. Prestin was then pulled out from the plasma membrane and the relationship between the force applied to the protein and the extension distance, i.e., the force-extension (FE) curve, was assessed. The curves obtained showed saw-toothed patterns. An attempt was then made to analyze these curves using the worm-like chain model. The force caused by stretching of the intracellular C terminus and that due to the extraction of one or several transmembrane domains were identified. The present results imply that the C terminus and the subsequent transmembrane domains of prestin correspond to those of the previously reported model with 12 transmembrane domains.

Keywords: Prestin, Motor protein, Inner ear, Membrane protein, Force spectroscopy, Hearing

1. Introduction
The high sensitivity of mammalian hearing is achieved by cochlear amplification. The basis of this amplification is the motility of mammalian outer hair cells (OHCs), which are sensory cells in the inner ear. OHCs longitudinally elongate and contract in synchronization with changes in the membrane potential. This motility is considered to be based on voltage-dependent conformational changes of the motor protein “prestin,” which is densely embedded in the lateral membrane of OHCs (Ashmore, 1987; Zheng et al., 2000; Murakoshi et al., 2006; Murakoshi et al., 2009; Kumano et al., 2010). Prestin consists of 744 amino acids (aa) with a molecular weight of approximately 81.4 kDa. Based on its amino acid sequence, prestin is classified as a member of anion transporter families. In a series of attempts to clarify its membrane topology, a hydrophobicity analysis in conjunction with the prediction of conserved phosphorylation sites suggested that prestin is a membrane protein associated with 10–14 transmembrane domains with cytoplasmic N and C termini (Oliver et al., 2001; Zheng et al., 2001; Deák et al., 2005; Navaratnam et al., 2005; Rajagopalan et al., 2006; McGuire et al., 2010; Gorbunov et al., 2014; Lovas et al., 2015; Kuwabara et al., 2018). However, the membrane topology of prestin has not yet been elucidated.

Subnanometer resolution is necessary for visualizing the structures of membrane proteins. Methods that achieve this level of resolution are limited to X-ray crystallography and nuclear magnetic resonance (NMR) for membrane proteins with a low molecular weight (~kDa), and negative stain electron microscopy for membrane proteins forming an
oligomeric structure with a markedly larger molecular weight. However, technical difficulties are associated with these methods because they require a relatively large amount of molecular solution and/or the crystallization of molecules. On the other hand, an approach involving the mechanical stretching of membrane proteins using an AFM, so-called single molecule force spectroscopy (SMFS), has been developed. An object molecule is stretched by applying force at a piconewton level and its molecular structure is analyzed based on the measured reaction force and extension length. Although this technique has been utilized in the field of macromolecular materials, it was applied to biological membrane molecules in the 2000s (Rief et al., 1997; Oberhauser et al., 1998; Müller et al., 1999; Müller et al., 2000). However, its application to membrane proteins has been limited to particular proteins that form crystal structures in nature, such as bacteriorhodopsin (the first study to analyze the structure of a membrane protein by SMFS) (Müller et al., 1999) and halorhodopsin (Cisneros et al., 2005) found in archaea, known as halobacteria, and also to relatively small membrane proteins (less than several tens of thousands Da), which had been succeeded to form two-dimensional crystals, such as Na⁺/H⁺ exchanger (Kedrov et al., 2004), human aquaporin (Moller et al., 2003) and 2-adrenergic receptor (Zocher et al., 2012).

In SMFS, it is important to connect an AFM cantilever with a specific membrane protein because of the presence of many other proteins in the plasma membrane of mammalian cells. We previously examined the membrane topology of prestin molecules, and attempted to unfold a monomeric unit of prestin in the plasma membrane of Chinese hamster ovary (CHO) cells by pulling its C-terminal end tagged with a FLAG peptide using an AFM cantilever coated with an anti-FLAG antibody. However, the majority of prestin molecules were not pulled out from the plasma membrane; binding between the anti-FLAG antibody and the FLAG-tag was broken because the binding force between them (the binding force between an antigen and antibody is approximately 50 pN (Ros et al., 1998)) may have been lower than the interacting force between prestin and the plasma membrane (the interacting force between a membrane protein and the plasma membrane is approximately 220 pN (Müller et al., 2000)). To overcome this issue, the plasma membrane was permeabilized by saponin to reduce the force of the interaction. However, it was not possible to rule out the potential of the saponin treatment to change the membrane topology of prestin in the plasma membrane. Therefore, another method without this treatment is needed.

We herein investigated the membrane topology of prestin molecules without altering the plasma membrane by attaching an Avi-tag (GLNDIFEAQKIEWHE; 15 aa) to the C terminus of prestin. The Avi-tag is a receptor peptide that is enzymatically biotinylated by biotin ligase (Beckett et al., 1999). By biotinylating the Avi-tag, prestin may be connected with a streptavidin-coated AFM cantilever via biotin-streptavidin binding, the force of which is approximately 340 pN (Lee et al., 1994; Zlatanova et al., 2000), which is greater than the interacting force between a membrane protein and the plasma membrane. As a result, prestin may be specifically pulled out from the plasma membrane and FE curves may be obtained. Based on these curves, the membrane topology of prestin was investigated.

2. Materials and methods
2.1 Expression vector of Avi-tagged prestin

To construct Avi-tagged prestin expression vector, a mammalian expression vector pRES-hrGFP-1a (Stratagene, La Jolla, CA) containing gerbil prestin cDNA, which was developed in our previous study (Iida et al., 2003; Iida et al., 2005), was used. The coding sequence for the Avi-tag was modified by adding the XhoI site at its 5’ end and the stop codon and the XhoI site were added at its 3’ end. The DNA coding this sequence was amplified by a polymerase chain reaction (PCR) using a set of primers; 5’–CTCGAGCTTGGTGGCGGTCTGAACGACATCTTCGAGGCTCAGAA-AAT–3’ as the forward primer and 5’–CTCGAGTTATTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGT–3’ as the reverse primer. The gene lengths of PCR products were then confirmed by electrophoresis. PCR products were ligated into T vectors with DNA ligase, and ligation products were transformed into Escherichia coli-competent cells. Transformed E. coli was cultured in a LB medium plate supplemented with ampicillin (50 µg/ml). After the incubation, T vector products were extracted from E. coli using a Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO). The extracted T vector products, which included the Avi-tag gene, were analyzed using an automated DNA sequencer. T vector products were digested with XhoI and phosphates were removed using alkaline phosphatase (Roche, Grenzacherstrasse, Switzerland) to prevent DNA from ligating (the 5’ end attaching to the 3’ end), thereby ensuring that DNA molecules remained linear until ligation. Prestin expression vectors were concurrently digested with XhoI. The Avi-tag gene was then ligated into the XhoI site (located downstream of the prestin gene) of the prestin expression vector,
thereby providing the Avi-Tagged prestin expression vector. The plasmid vector was analyzed using the automated DNA sequencer.

2.2 Stable cell line expressing Avi-tagged prestin

CHO cells were cultured in RPMI-1640 medium (R8758, Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, S1020, Biowest), 100 U penicillin and 100 µg streptomycin per ml (P0781, Sigma-Aldrich), at 37°C with 5% CO₂.

Before transfection, the culture medium was replaced with RPMI-1640 medium only containing 10% FBS. The Avi-tagged prestin expression vector was transfected into CHO cells using Lipofectamine 2000 Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. After transfection, cells were cultured in RPMI-1640 medium at 37°C with 5% CO₂. Transfected cells (1.0 × 10⁶ cells/ml) were harvested from a dish and subjected to a fluorescence-activated cell sorting (FACS) analysis using a flow cytometer equipped with a cell sorting function (S3 Cell Sorter, BIO-RAD Laboratories, Hercules, CA) and Flowjo software (TOMY Digital biology, Tokyo, Japan). GFP fluorescence intensity was measured in all transfected cells, and those exhibiting high GFP fluorescence intensity were selected and collected by FACS. These cells were then cultured for two weeks. Cells with high GFP fluorescence intensity were plated on a 96-well plate. Wells with only one cell were selected and scaled up. The expression of Avi-tagged prestin was confirmed by GFP fluorescence using a confocal laser microscope (FV500, Olympus, Tokyo, Japan).

2.3 Expression and function of prestin

To confirm the full-length and stable expression of Avi-tagged prestin in the constructed cell line, its expression was confirmed by Western blotting. Cell proteins were separated on a 10% SDS-polyacrylamide gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skimmed milk (190-12865, Wako, Tokyo, Japan) in PBS, the PVDF membrane was incubated with a goat anti-prestin N terminus antibody (sc-22692, Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized using a horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2354, Santa Cruz Biotechnology) and the ECL Select Western Blotting Detection Reagent (RPN2235, Cytiva, Marlborough, MA).

Cells expressing functional prestin exhibit bell-shaped non-linear capacitance (NLC) in response to a change in the membrane potential (Ludwig et al., 2001). Since NLC reflects the voltage-dependent charge transfer of prestin, the anion transport function of prestin may be evaluated based on NLC measured by the whole-cell patch-clamp technique (Dallos and Fakler, 2002). In the present study, the effects of the Avi-tag on the electrophysiological properties of prestin were examined by the whole-cell patch-clamp technique. To assess NLC, electrodes were pulled from a borosilicate glass tube (TW150-4, World Precision Instruments, Inc., Sarasota, FL) by a programmable puller (Model P-97, Sutter Instruments, Novato, CA). Electrodes were filled with an internal solution composed of 140 mM KCl, 3.5 mM MgCl₂, 5 mM EGTA, 5 mM HEPES and 0.1 mM CaCl₂, with pH adjusted to 7.2 using KOH. Cells were plated onto a 35-mm glass-bottomed dish (IWAKI, Chiba, Japan) with an external solution composed of 145 mM NaCl, 5.8 mM KCl, 1.3 mM CaCl₂, 0.9 mM MgCl₂, 10 mM HEPES, 0.7 mM Na₂HPO₄ and 5.6 mM glucose, with pH adjusted to 7.2 using NaOH. Measurements of membrane capacitance were performed using the “membrane test” feature of pCLAMP 8.0 acquisition software of an Axon 200B amplifier (Axon Instruments, Foster city, CA), as previously described (Iida et al., 2005). To assess the voltage dependence of membrane capacitance, the holding potential was swung from −140 to 70 mV. After measurements, membrane capacitance was plotted versus membrane potential. Membrane capacitance recorded from infected cells was fit with the first derivative of the Boltzmann function (Santos-Sacchi, 1991),

\[ C_m(V) = C_{lin} + \frac{Q_{max}}{\alpha \sqrt{1 + \frac{V - V_{1/2}}{\alpha}}} \]

where \( C_{lin} \) is linear capacitance, \( Q_{max} \) is maximum charge transfer, \( V \) is membrane potential, \( V_{1/2} \) is the voltage at which maximum charge was equally distributed across the membrane and \( \alpha \) is the slope factor for the voltage dependence of
charge transfer. Charge density, i.e., the expression level of prestin in the unit cell membrane, may also be obtained by dividing $Q_{\text{max}}$ by $C_{\text{lin}}$, where $Q_{\text{max}}$ reflects the expression level of prestin in the whole cell membrane and $C_{\text{lin}}$ is a linear component of capacitance, which is proportional to the membrane area of the cell.

### 2.4 Inside-out plasma membrane of CHO cells

Cells were detached from a culture flask and deposited on glass-bottomed dishes. After an overnight culture, most of the cells had reattached and become spindle-shaped on the substrate. Cells were washed twice with an external solution (145 mM NaCl, 5.8 mM KCl, 1.3 mM CaCl$_2$, 0.9 mM MgCl$_2$, 10 mM HEPES, 0.7 mM NaHPO$_4$, and 5.6 mM glucose; pH 7.3) warmed to 37°C for the removal of unwanted materials, such as cell fragments and proteins present in the culture medium that attached to the surfaces of the cells and substrate. The dish was immersed for 3 min in hypotonic buffer (10 mM PIPES, 10 mM MgCl$_2$, and 0.5 mM EGTA; pH 7.2) on ice. Cells were then sheared open by gentle exposure to a stream of hypotonic buffer using a 200-µl pipette, resulting in the isolation of inside-out basal plasma membranes. After isolated plasma membranes had been washed with PBS three times, they were incubated in high-salt buffer (2 M NaCl, 2.7 mM KCl, and 1.5 mM KH$_2$PO$_4$; pH 7.2) at room temperature for 30 min to remove cytoskeletal materials and peripheral proteins (Murakoshi et al., 2006; Murakoshi et al., 2009; Murakoshi and Wada, 2009). Isolated plasma membranes were then incubated with 2 mM CM-DiI (Thermo Fisher Scientific) at 37°C for 5 min and then at 4°C for 15 min. Membranes were then washed with PBS.

The C terminus of Avi-tagged prestin was biotinylated using biotin ligase (BirA, Avidity, Aurora, CO). The isolated membranes of Avi-tagged prestin-expressing CHO cells and those of FLAG-tagged prestin-expressing CHO cells, the negative control, were used in this experiment. After the isolation of the plasma membrane, Avi-tagged prestin on the cell surface was enzymatically biotinylated with PBS, 5 mM MgCl$_2$, 0.3 µM BirA, 1 mM biotin and 10 mM adenosine triphosphate (ATP) at room temperature for 60 min.

Isolated plasma membranes were incubated in 10 nM streptavidin-QD655 (Thermo Fisher Scientific) in PBS containing 5 mM MgCl$_2$ and 1% BSA at 4°C for 10 min. After washing with PBS, membranes were incubated with a goat anti-prestin N-terminus antibody (sc-22692, Santa Cruz Biotechnology) at a dilution of 1:100 in PBS at 4°C overnight. Membranes were then washed twice with PBS and incubated with a FITC-conjugated anti-goat IgG antibody (F4891, Sigma-Aldrich) at a dilution of 1:200 in PBS at 37°C for 1 h. Membranes were then washed twice with PBS and observed by fluorescence microscopy in PBS.

### 2.5 Force spectroscopy

A gold-coated AFM cantilever with a spring constant of 0.03 N/m (BL-RC150VB-C1, Bio-lever, Olympus) was incubated in a 500-µl drop of a mixture of two alkane thiols, i.e., a protein-resistant oligoethylene glycol (OEG) thiol (Sigma-Aldrich) and biotinylated alkane thiol (Nanoscience Instruments, Phoenix, AZ, 1 mM in ethanol) at room temperature for 48 hours. The mixture of OEG and biotinylated thiols at a ratio of 8:2 was used to decrease the reactive area on the cantilever (Zhang and Yadavalli, 2009). After the incubation with mixed thiols, surfaces were rinsed with ethanol and incubated in streptavidin (Thermo Fisher Scientific, 20 µg/ml in PBS) at room temperature for 3 hours to conjugate streptavidin with biotin.

After the isolation of the plasma membrane and biotinylation of Avi-tagged prestin, the streptavidin-coated cantilever was brought close to the isolated plasma membrane of CHO cells expressing Avi-tagged prestin using an AFM (Multimode V, Bruker, Billerica, MA). The cantilever was then retracted at a velocity of 300 nm/s, resulting in the unfolding of prestin. The relationship between the force $F$ loaded onto the cantilever and the movement of the cantilever, i.e., the extension length $z$ of the protein, a so-called force-extension (FE) curve, was then measured. As the cantilever is retracted from the sample, the transmembrane domains of a protein may be sequentially extracted from the membrane. When the transmembrane domains are unfolded and/or extracted from the plasma membrane, the cantilever deflects toward the plasma membrane and a “force peak” is observed. In this experiment, the isolated plasma membranes of untransfected CHO cells were used as the negative control.

Each force peak detected in the force curves obtained was fit using the worm-like chain (WLC) model (Rief et al., 1997), which is known to fit to non-linear increases in force curves when the extensional force is higher than 50 pN. The WLC model is represented by the following formula:
This formula describes the relationship between the tensile force $F$ and extension length $z$ of an ideal entropic chain with a contour length $L$ and persistence length of $b$. $k_B$ and $T$ represent the Boltzmann constant and temperature (in Kelvins), respectively. The persistence length reflects polymer stiffness. In the present study, a persistence length of 0.4 nm was employed (Rief et al., 1997) and the length of 1 aa was assumed to be 0.36 nm (Kessler and Gaub, 2006). The contour length $L$ of each force peak, which is calculated by fitting the force curves obtained using this formula, is only one free parameter that describes the length of the stretched portion of the molecule. The WLC fitting of the contour lengths of each force peak was attempted using self-written procedures in MATLAB (Mathworks, Natic, MA).

Before applying the WLC model, the careful pre-processing of experimental data was performed. Some FE curves do not contain unfolding signals or some curves show peaks due to non-specific interactions. In this experiment, the AFM cantilever is designed to bind with the C-terminal end of a molecule via biotin-streptavidin binding. Therefore, the molecule attached to the cantilever may result in an FE curve with its total molecular length or with a smaller length when some domains in the molecule are simultaneously pulled out from the membrane. In consideration of this point, the maximum length of the unfolded prestin molecule is expected to be 744 aa (~268 nm) when the whole molecule is unfolded. If the STAS domain (189 aa) located at the C-terminal side of the molecule retains its shape, the maximum length obtained will be smaller at 555 aa (~200 nm). The classification of FE curves exhibiting a length less than 50 nm was complicated due to the partial unfolding of unspecified molecules attached to the tip via the C-terminal end of prestin. Therefore, only FE curves showing a length greater than 50 nm were selected and analyzed. In addition, since the binding force between biotin and streptavidin was previously reported to be approximately 340 pN (Lee et al., 1994; Zlatanova et al., 2000), the maximum force of each peak is expected to be smaller than this force. Therefore, FE curves including peaks with a force less than 340 pN were selected. The standard deviation of noise was calculated based on the linear fit of the final part of the FE curves, the non-contact part with a free vibration of the cantilever, and the zero-force baseline was evaluated. All FE curves were aligned based on this baseline.

3. Results
3.1 FACS and limiting dilution cloning

To obtain cell lines that highly express prestin, cells with high GFP fluorescence intensity were collected using the flow cytometer because the expression level of prestin correlated with GFP expression in our cell line (Iida et al., 2004). As shown in Fig. 1, GFP fluorescence intensity was higher in transfected CHO cells (red line) than in untransfected CHO cells (blue line). Cells with GFP intensity larger than 40 arbitrary units (a.u.) were collected by FACS, called 1st step cells, and cultured for 2 weeks. Cells with a GFP intensity larger than 40 a.u. were collected from 1st step cells by FACS, called 2nd step cells, and cultured for 2 more weeks (green line). Using this procedure, GFP intensity increased by approximately 10-fold.
line). Using this procedure, GFP intensity increased by approximately 10-fold. To generate a monoclonal cell line by limiting dilution method, 2nd step cells with GFP fluorescence intensity higher than $3 \times 10^2$ a.u. were plated on two 96-well plates at a concentration of one cell/well. Wells containing a single colony were observed for 2 weeks and incubated at 37°C with 5% CO$_2$. As a result, single cells were confirmed from 7 wells and were scaled up. GFP fluorescence was confirmed in all constructed cell lines as shown in Fig. 2(a). The average intensities of each constructed cell line are shown in Fig. 2(b). Since the H-D2, H-F2 and H-F8 cell lines showed high GFP intensities, they were used in subsequent analysis.

### 3.2 Expression and function of Avi-tagged prestin

Cells expressing Avi-tagged prestin harvested every 24 h during cultivation were subjected to Western blotting analysis (Fig. 3). Bands were detected at approximately 90 and 65 kDa. Similarly, FLAG-tagged prestin-expressing cells, as a positive control, showed two dominant bands at approximately 90 and 65 kDa. These bands were likely to be glycosylated and unglycosylated prestin, respectively (Iida et al., 2005). On the other hand, untransfected CHO cells, as a negative control, did not show bands representing prestin. These results suggested that Avi-tagged prestin was expressed in the generated cell line. The luminescence intensity of the band increased and was saturated by 2 days of culture, and then decreased by 4 days of culture. Luminescence intensity remained stable until 10 days.

![Fig. 3](image_url)

**Fig. 3** The expression of Avi-tagged prestin in the generated CHO cell line. Positive control, FLAG-tagged prestin expressing CHO cells; Negative control, untransfected CHO cells. The luminescence intensity of the band increased and was saturated by 2 days of culture, and then decreased by 4 days and became stable until 10 days.

To evaluate the function of Avi-tagged prestin, the voltage dependence of membrane capacitance was measured by the whole-cell patch clamp method. To estimate the NLC per unit cell surface area, the normalized non-linear capacitance $C_{\text{nonlin/lin}}$ was defined as

$$C_{\text{nonlin/lin}} (V) = \frac{C_{\text{nonlin}}}{C_{\text{lin}}} = \frac{(C_{\text{in}}(V) - C_{\text{lin}})}{C_{\text{lin}}},$$

where $C_{\text{nonlin}}$ is the non-linear component of the measured membrane capacitance. $C_{\text{nonlin/lin}} (V)$ was then normalized by that of wild-type (WT) prestin, termed relative $C_{\text{nonlin/lin}} (V)$. As shown in Fig. 4, Avi-tagged prestin expressed in the cell line exhibited NLC (red line), indicating that newly developed Avi-tagged prestin was functional. Fitting

![Fig. 4](image_url)

**Fig. 4** The expression of Avi-tagged prestin in the generated CHO cell line. Positive control, FLAG-tagged prestin expressing CHO cells; Negative control, untransfected CHO cells. The luminescence intensity of the band increased and was saturated by 2 days of culture, and then decreased by 4 days and became stable until 10 days.
parameters for Avi-tagged prestin were $C_{\text{lin}} = 13.4 \text{ pF}$, $Q_{\text{max}} = 101.8 \text{ fC}$, $\alpha = 41.0 \text{ mV}$ and $V_{1/2} = -61.2 \text{ mV}$, while those for WT prestin were $C_{\text{lin}} = 19.7 \text{ pF}$, $Q_{\text{max}} = 75.5 \text{ fC}$, $\alpha = 38.1 \text{ mV}$ and $V_{1/2} = -74.8 \text{ mV}$. Charge density, which is related to the expression level of prestin in a unit cell surface area, was calculated by dividing $Q_{\text{max}}$ by $C_{\text{lin}}$, where $Q_{\text{max}}$ reflects the expression level of prestin in the whole cell membrane and $C_{\text{lin}}$ is the membrane capacitance proportional to the membrane area of the cell. The charge densities of Avi-tagged prestin and WT prestin were $7.6 \pm 5.3$ and $3.83 \pm 0.9 \text{ fC/pF}$, respectively. No significant differences were observed in electrophysiological properties between cells expressing Avi-tagged prestin and WT prestin, except for a difference in charge density.

**Figure 5** shows fluorescence images of the isolated plasma membranes of CHO cells expressing Avi-tagged prestin and FLAG-tagged prestin. Prestin labeling (red) was observed in both cells (left panels). Biotin labeling (green) was only detected in the CHO cell expressing Avi-tagged prestin (middle panels). Prestin labeling colocalized with biotin labeling (orange; upper right panel).

3.3 Cantilever preparation

Figure 6(a) shows fluorescence images of biotin labeling on cantilevers. The cantilever coated with the biotinylated alkane thiol only showed high intensity fluorescence (top right panel). On the other hand, the cantilever coated with mixed alkane thiols showed lower intensity fluorescence. The cantilever without coating showed no fluorescence. To compare the fluorescence intensity of each cantilever, the average intensity in a microregion ($15 \times 15 \mu\text{m}$) of each cantilever was calculated and normalized by that of the cantilever coated with the biotinylated alkane thiol. As shown in Fig. 6(b), the cantilever coated with the mixed alkane thiol showed 70% lower intensity than that coated with the biotinylated alkane thiol. This result indicated that reactive area on the cantilever was reduced by the incubation with the mixture of alkane thiols.
Fig. 6 The fluorescence of biotin labeling on cantilevers. (a) Fluorescence images of the cantilevers. Left panels, differential interference contrast (DIC) images; right panels, fluorescence images of biotin labeling. The scale bar is 50 μm. Cantilevers coated with the biotinylated alkane thiol and mixed alkane thiols showed fluorescence. (b) Fluorescence intensity ratio of the cantilevers. The cantilever coated with mixed alkane thiols showed 70% lower intensity than that coated with the biotinylated alkane thiol.

3.4 Force spectroscopy of prestin

In the present study, 862 and 99 FE curves were obtained from CHO cells expressing Avi-tagged prestin and untransfected CHO cells, respectively. Figure 7 shows representative FE curves measured from CHO cells expressing Avi-tagged prestin and untransfected CHO cells. Stretching experiments revealed sawtooth patterns in FE curves with approximately one to five force peaks. The FE curves obtained were subjected to the WLC model and curves with a coefficient of determination greater than 0.7 were included in subsequent data analysis, namely, 88 and 48 FE curves were selected from CHO cells expressing Avi-tagged prestin and untransfected CHO cells, respectively. Figure 8 shows a multitude of force-extension traces. In this figure, force peaks were detected from both cells at an extension length less than 50 nm.

Fig. 7 Representative FE curves. (a) Avi-tagged prestin-expressing CHO cells. (b) Untransfected CHO cells.

4. Discussion
4.1 CHO cell line expressing Avi-tagged prestin

In the present study, CHO cell lines expressing Avi-tagged prestin were initially obtained by a combination of FACS and limiting dilution cloning. As shown in Fig. 2, CHO cells transfected with Avi-tagged prestin expressed GFP. GFP was stably expressed for more than 4 months in these cell lines. Since the prestin gene and GFP gene were transcribed into sequential mRNA when the constructed expression vector was used, the stable expression of prestin may be achieved in these cell lines.
A previous study reported that deglycosylation alters the function of prestin (Matsuda et al., 2004). Therefore, the glycosylation pattern of Avi-tagged prestin in the constructed cell line needs to be considered. A Western blotting analysis indicated that the full-length Avi-tagged prestin was expressed and its glycosylation pattern was stable after construction of the cell line (Fig. 3). This result indicates that the Avi-tag does not interfere with the glycosylation of prestin at any time point after construction of the cell line. Furthermore, the total amount of Avi-tagged prestin became stable after 4 days of culture. Therefore, Avi-tagged prestin expressing CHO cells after 4 days of culture were used in subsequent experiments.

As shown in Fig. 4, the cell line that stably expressed Avi-tagged prestin (red line) exhibited NLC versus the membrane potential. A previous study reported that N- or C-terminally truncated prestin did not show NLC (Navaratnam et al., 2005). Since NLC was observed in the present study, the functional domains of Avi-tagged prestin were expressed in the membrane of CHO cells. The charge density of the established cell line expressing Avi-tagged prestin was 7.6 ± 5.3 fC/pF, which was larger than that of WT prestin of 3.83 ± 0.9 fC/pF. The value of charge density represents the expression level of prestin; therefore, the constructed cell line was considered to have a higher expression level of prestin than that of WT prestin. In the present study, the procedure used to construct a cell line was different from that in our previous study (Iida et al., 2005), which did not include cell sorting by GFP fluorescence. Therefore, cells with larger amounts of prestin may have been selected by cell sorting in the present study, resulting in the construction of a cell line with a larger amount of prestin. Apart from charge density, no significant differences were observed in electrophysiological properties between the cell line that stably expressed Avi-tagged prestin and that expressing WT prestin. Based on these results, the Avi-tag did not interfere with the function of prestin.

Figure 5 shows fluorescence images of the isolated plasma membrane of CHO cells. In this experiment, prestin was labeled with the goat anti-prestin N-terminus antibody (red) and with streptavidin, which detects the biotinylated Avi-tag at the end of the C terminus of prestin (green). The merged image revealed that prestin labeling colocalized with biotin labeling in CHO cells expressing Avi-tagged prestin (orange). These results confirmed that Avi-tagged prestin was biotinylated in the plasma membrane.

4.2 Force spectroscopy of Avi-tagged prestin

Force spectroscopy using an AFM is useful for measuring forces between molecules and/or between a molecule and the plasma membrane. In a previous study, unfolded proteins behaved in a first approximation similar to random coils, the elasticity of which was well described by the WLC model with an apparent persistence length of 4 Å (Rief et al., 1997). The non-linear force increase along with the extension trace fit well with the WLC model with only one free parameter, the contour length of the stretched portion of the molecule. After unfolding traces had been fit with the WLC model, they were analyzed and sorted into five groups.

The first group of traces only exhibited a peak at 244 aa (Fig. 9(a)). In consideration of the secondary structure of prestin (Fig. 10), which is based on a re-entrant 12-pass model (Zheng et al., 2001; Matsuda et al., 2004; Deák et al., 2005; McGuire et al., 2010), the C-terminal chain was fully stretched, including the STAS domain (Fig. 9(g–h)).

The second group of traces exhibited peaks at 265 and 282 aa (Fig. 9(b)). Separation between 244 and 265 aa was 21 aa, corresponding to the length of transmembrane domain N (Fig. 9(i)). Separation between 265 and 282 aa was 17 aa, possibly exhibiting the extracellular loop between domains N and M (Fig. 9(j)).

At lengths longer than 60 nm, the third group of traces showed peaks at 312, 343 and 359 aa (Fig. 9(c)). Separation of the peak at 312 aa from that at 282 aa was 30 aa. This may reflect the total length of transmembrane domain M (21
Fig. 9 Unfolding of Avi-tagged prestin. (a) Stretched C terminus. (b) Unfolding of domain N and extension of the extracellular loop. (c) Unfolding of domain M and the intracellular loop, unfolding of domain L, and extension of the extracellular loop. (d) Unfolding of domain K and the intracellular loop, and unfolding of domain J and extension of the extracellular loop. (e) None of the WLC fittings satisfied the criterion in this group. (f) Cysteine in domain L (C415) and cysteine in domain G (C260) may possibly be simultaneously pulled out from the membrane. (g–q) Schema of the possible unfolding pathways of Avi-tagged prestin.
aa) and the intracellular loop between domains M and L (9 aa), i.e., 31 aa (Fig. 9(k)); this result suggests the simultaneous unfolding of domain M and the intracellular loop because there was no force barrier when this intracellular loop was stretched. The peak at 343 aa was 31 aa form that at 312 aa (Fig. 9(l)). These results may reflect the length of domain L although it was 10 aa longer than that shown in the secondary structure model of prestin (Fig. 10). The last peak at 359 aa was separated from the peak at 343 aa by 16 aa, corresponding to the extracellular loop between domains L and K (Fig. 9(m)).

The fourth group had two peaks at 399 and 447 aa (Fig. 9(d)). The difference between the peak at 359 aa and that at 399 aa was 40 aa, possibly indicating the simultaneous unfolding of domain K and the intracellular loop between domains K and J (Fig. 9(n)), which is a similar phenomenon to that found in the peak at 312 aa. Similar to the latter peak at 447 aa, separation from the peak at 399 aa was 48 aa, which corresponded to the total length of transmembrane domain J (21 aa) and the extracellular loop between domain J and I (27 aa) (Fig. 9(o)). This reflects the condition in which domain J and the extracellular loop were unfolded at the same time, suggesting that the interaction between this extracellular loop and the plasma membrane was weaker than those of the other extracellular loops.

The fifth group, with an extension length between 160 and 200 nm, did not show any WLC fitting that satisfied the criterion described above (Fig. 9(e)), suggesting that it was no longer possible to unfold the prestin molecule in this extension range. This whole part remaining in the plasma membrane may be somehow pulled out from the membrane at the same time.

In some cases, a force peak at 571 aa was detected, which was far from those observed in the fourth group (Fig. 9(f)). As shown in Fig. 10, since the gerbil prestin molecule has 8 cysteines (C52, C124, C192, C196, C260, C381, C395 and C415), a disulfide bond (i.e., Cys-Cys bond) may exist between these cysteines, the binding force of which is approximately 3.5 nN (Iozzi et al., 2011; Popa et al., 2013). Due to the position of the force peak at 571 aa, C415 in domain L may possibly be associated with C260 located at domain G (Fig. 9(p)), and this portion was pulled out from the membrane as a lump and most of the molecule was unfolded, as shown in Fig. 9(q).

Based on the present results, a common unfolding principle of prestin may be described by a transmembrane \( \alpha \)-helix and extracellular/intracellular polypeptide loop. In most cases, the \( \alpha \)-helix was unfolded within a single step. However, the extracellular loop following the \( \alpha \)-helix did not extend with the helix at the same time in this experimental design. This may be due to the hydrophobicity of the loop, i.e., a hydrophilic extracellular loop is the resistant part for unfolding.

Fig. 10 A possible secondary structure of Avi-tagged prestin. Since the inside-out plasma membrane was used in the present study, N and C termini were facing up in this figure. In the C-terminal tail, there is a STAS domain, which has 189 aa.
the hydrophobic plasma membrane. After this extracellular loop was extended, the next transmembrane α-helix started to be unfolded and this unfolding process was consecutively followed by the extension of the intracellular loop. This is reasonable because there was no interaction between the intracellular loop and plasma membrane in this experimental design (Figs. 9 and 10). Since unfolding data were limited from the C-terminal end to domain L in the present study, further investigations on subsequent domains are warranted.

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