Cysteine Mutagenesis Reveals Transmembrane Residues Associated with Charge Translocation in Prestin

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The solute carrier transmembrane protein prestin (SLC26A5) drives an active electromechanical transduction process in cochlear outer hair cells that increases hearing sensitivity and frequency discrimination in mammals. A large intramembranous charge movement, the nonlinear capacitance (NLC), is the electrical signature of prestin function. The transmembrane domain (TMD) helices and residues involved in the intramembrane charge displacement remain unknown. We have performed cysteine-scanning mutagenesis with serine or valine replacement to investigate the importance of cysteine residues to prestin structure and function. The distribution of oligomeric states and membrane abundance of prestin was also probed to investigate whether cysteine residues participate in prestin oligomerization and/or NLC. Our results reveal that 1) Cys-196 (TMD 4) and Cys-415 (TMD 10) do not tolerate serine replacement, and thus maintaining hydrophobicity at these locations is important for the mechanism of charge movement; 2) Cys-260 (TMD 6) and Cys-381 (TMD 9) tolerate serine replacement and are probably water-exposed; and 3) if disulfide bonds are present, they do not serve a functional role as measured via NLC. These novel findings are consistent with a recent structural model, which proposes that prestin contains an occluded aqueous pore, and we posit that the orientations of transmembrane domain helices 4 and 10 are essential for proper prestin function.

Prestin is an essential component of the voltage-sensitive molecular motor of outer hair cells that converts changes in transmembrane potential into mechanical forces. This transduction causes outer hair cells to undergo a characteristic length change termed electromotility that enhances sound amplification and frequency selectivity (1, 2). In the absence of prestin, hearing thresholds are effectively elevated 40–60 dB in mice (3). A large displacement current, analogous to the gating current in ion channels, has been established as a reliable electrical signature for electromotility (4, 5). This electrical signature is referred to as the nonlinear capacitance (NLC)4 and requires the presence of intracellular chloride (6).

Very little is known about the structure of prestin and its relationship to prestin function. Several topologies have been proposed based on hydrophobicity and sequence conservation between prestin and the family of solute anion transporters to which it belongs. Proteins of the solute carrier family 26 (SLC26) probably span the membrane 10–14 times (7–9). Because prestin (SLC26A5) has been shown to have intracellular N and C termini (10), topologies have been proposed having an even number of transmembrane domains. Ten-pass and reentrant loop 12-pass models of prestin secondary structure have been proposed (Fig. 1 and supplemental Table S1) and refined based on knowledge of phosphorylation sites (11), evolutionary analysis (12), and conflicting reports of N-linked glycosylation (13, 14), a result possibly due to expression in different model cell systems. Currently, the interactions, orientations, and residues comprising the transmembrane helices and intramembrane structure in prestin are poorly defined. Biochemical data suggest that prestin self-associates and can exist as a higher order oligomer (15). Recently, transmission electron microscopic imaging of partially purified prestin was used to model a three-dimensional oligomeric structure roughly the expected size of a prestin tetramer, with subunits forming a central pore open on the cytoplasmic face (16). This aqueous pore narrows and is occluded at the external face of the membrane, leading to a proposed bullet shape for the prestin structure.

A majority of the cysteine residues in prestin are contained within or near transmembrane domains, and six of the nine are not shared with PAT1 (SLC26A6), the family member having highest homology with prestin (17). Because PAT1 lacks NLC (6, 18), it is possible that the cysteine residues and/or their position within the helix may be important for prestin NLC. Studies using sulfhydryl reagents, which bind cysteine residues and sterically hinder protein conformational change, alter electro-

4 The abbreviations used are: NLC, nonlinear capacitance; TMD, transmembrane domain; SLC26, solute carrier family 26; HEK, human embryonic kidney; APP, amyloid precursor protein; GFP, green fluorescent protein; WT, wild type; fC, femtocoulombs; pF, picofarads; di-8-ANEPPS, di-8-aminonaphthylethenylpyridinium.
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**FIGURE 1. Proposed prestin topologies.** A and B, 10- and 12-pass models of prestin secondary structure have been proposed based on data suggesting phosphorylation sites (11) and conflicting reports of N-linked glycosylation (13, 14). Residue numbers for cysteine locations are shown, and all but 52, 395, and 679 are unique to prestin in comparison with PAT1 (SLC26A6).

motility and NLC of outer hair cells, again implicating a potential role for cysteines in prestin function. Among these reagents the most severe inhibition of electromotility (19) and the greatest alteration to NLC (20) are caused by organomercurials, which are able to penetrate hydrophobic protein regions. This implies that one or more reactive thiol groups may be present in a functionally important and relatively protected region of prestin.

Motivated by these studies and thus the potential for cysteines to participate in prestin function, we performed a cysteine-scanning mutagenesis screen with replacement of each cysteine by either serine or valine and assessed the impact on prestin function. We found varying tolerances for cysteine replacements throughout prestin and discuss the importance of cysteine-scanning mutagenesis screen with replacement of each
tion, and an agar bridge provided electrical continuity with the reference bath containing pipette solution. Ag+/AgCl electrodes were used to record current flow. Only healthy, single cells showing suitable GFP fluorescence were assayed. During the experiment, micropipette placement was controlled using a Burleigh PCS-6200 Micromanipulator (EXFO Life Sciences, Rochester, NY), and pipette pressure was monitored (PM01R digital pressure meter; WPI, Sarasota, FL). Amplifier and electrode offsets were compensated in an open bath, and on-cell seals in excess of 1 gigaohm were obtained. Pipette capacitance was compensated before rupturing the membrane to establish the whole-cell mode. All cells retained for analysis exhibited series resistance less than 10 megaohms and membrane resistance in excess of 1 gigaohm.

Membrane capacitance ($C_m$) and membrane resistance ($R_m$) were determined by applying a sinusoidal command voltage at a superimposed DC offset and then determining the real and imaginary components of the admittance, the complex current response scaled by the command voltage (24). We used a software-based phase-sensitive detector implemented in PatchMaster software (HEKA, Mahone Bay, Canada) to acquire this information. Specifically, we applied an 800-Hz, 10-mV sine wave and measured the current response as DC holding potential was stepped from $-140$ to $+140$ mV (or $-160$ to $+160$ mV for some mutants

**EXPERIMENTAL PROCEDURES**

**Construct Design**—Gerbil prestin (AF230376) was amplified from cDNA (forward/reverse primers: 5′-ggaattccacctggtatcgcacggaag-3′/5′-ccggatccctgccctggtctgttgag-3′), digested (EcoRI and BamHI), and inserted into the multiple cloning site of the pEGFP-N1 vector (Clontech, Palo Alto, CA) to create a C-terminal fusion protein. A point mutation was made to the GFP (A206K) to prevent nonspecific interaction of the fusion proteins (21, 22). The mutation denoted A206K is actually located at residue 207 of the Clontech vector due to an insertion that enhances mammalian translation efficiency. Prestin-GFP plasmid DNA served as a template, and PCR primers were used to produce the mutant constructs required in our study (serine and valine replacements; see supplemental Table S2). Each mutant construct was sequence-verified (3130XL Genetic Analyzer, Applied Bio-systems (Foster City, CA)) to ensure that prestin, the linker region, and GFP did not contain any unintended polymerization errors (Baylor Sequencing Core, Houston, TX).

**Plasmid Expression and Cell Culture**—HEK293 cells were grown in T75 flasks and 6-well plates containing Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (Invitrogen), 1% penicillin-streptomycin, 14.3 mM HEPES, and 16.1 mM NaHCO$_3$. Only cells below passage 20 were used for any of our studies, and this is below conservative limits established for electrophysiological studies (20–30 passages) (23). Cells were transfected with wild type (WT) or mutant prestin-GFP plasmids using 2 μg of DNA and 3 μl of FuGene 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. For electrophysiology and imaging studies, cells were trypsinized and replated onto number 1.5 coverslips or MatTek dishes (MatTek Corp., Ashland, MA) 8–12 h after transfection and then evaluated 24–48 h post-transfection. Mutants with lower than WT charge density, however, were only evaluated at 48 h. In select experiments, cholesterol depletion was accomplished through application of 10 mM methyl-β-cyclodextrin (C4555, Sigma) suspended in Dulbecco’s modified Eagle’s medium to the cells for 15 or 30 min (at 37 °C) prior to patch clamp evaluation. For Western blot experiments, protein was collected 24 or 48 h post-transfection.

**Electrophysiology**—2–4-megaohm micropipettes were formed from Kimax-51 capillary tubes (1.5/0.8 mm outer/inner diameter) on a P-97 puller (Sutter, Novato, CA). The patch pipettes and extracellular bath both contained common blocking solutions (pipette: 130 mM CsCl, 2 mM MgCl$_2$, 10 mM EGTA, 10 mM HEPES; extracellular: 99 mM NaCl, 20 mM TEA-Cl, 2 mM CaCl$_2$, 1.47 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM HEPES) to minimize ionic conductance (11). All solutions were titrated to pH 7.3, and dextrose was added to obtain 300 ± 3 mosM (Osomette A, Precision Systems (Natick, MA)). Coverslips seeded at low cell density formed the base of a patch clamp chamber in which the cells were bathed in extracellular solution, and an agar bridge provided electrical continuity with the reference bath containing pipette solution. Ag$^+$/AgCl electrodes were used to record current flow. Only healthy, single cells showing suitable GFP fluorescence were assayed. During the experiment, micropipette placement was controlled using a Burleigh PCS-6200 Micromanipulator (EXFO Life Sciences, Rochester, NY), and pipette pressure was monitored (PM01R digital pressure meter; WPI, Sarasota, FL). Amplifier and electrode offsets were compensated in an open bath, and on-cell seals in excess of 1 gigaohm were obtained. Pipette capacitance was compensated before rupturing the membrane to establish the whole-cell mode. All cells retained for analysis exhibited series resistance less than 10 megaohms and membrane resistance in excess of 1 gigaohm.

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![Diagram](image-url)
with altered NLC) in 2-mV increments (HEKA EPC 10 Plus amplifier with 18-bit digital-to-analog converter). At each DC potential, four complete sinusoidal voltage cycles occurred, and discrete capacitance values were calculated from the last three.

Multiple capacitance versus voltage (DC holding potential) traces were acquired for each cell, and each of the individual recordings was fit to the first derivative of the two-state Boltzmann function using MATLAB (MathWorks, Natick, MA),

\[ C_m = \frac{Q_{max}(ze/kT)}{\exp\left(\frac{ze}{kT} (V - V_{1/2}) \left(1 + \exp\left(-\frac{(V - V_{1/2}) ze}{kT}\right)\right)\right)^2} + C_{lin} \]  

(Eq. 1)

\( Q_{max} \) is the maximum nonlinear charge movement provided by prestin. \( V_{1/2} \) is the voltage at which half-maximal charge transfer occurs or, equivalently, the peak of the capacitance versus holding potential curve. \( z \) is the valence of charge movement by prestin. Boltzmann’s constant \( k \), absolute temperature \( T \), and the charge carried by an electron \( e \), are all constant values. The first term of the fit represents the nonlinear component, caused by prestin-related charge movement, whereas the second term is the linear capacitance \( C_{lin} \), derived from the dielectric properties of the membrane and is proportional to cell size. Since variation in cell size causes differences in the maximal charge transfer \( Q_{max} \), the charge movement is normalized to \( C_{lin} \) and computed on a cell-by-cell basis. This quantity, designated as charge density, has units of fC/pF (25). Mean fit parameters were determined for each cell and pooled for each experimental group. We tested WT prestin and various prestin mutants with amino acid replacements. For comparison of \( V_{1/2} \) or \( z \) between various experimental groups (Figs. 3A, 5A, and 7A), representative NLC curves were reconstructed from averaged \( Q_{max}, V_{1/2} \), and \( z \) values for each group, and each result was normalized to maximal nonlinear capacitance \( Q_{max} ze/4kT \). Normalized curves are not appropriate for comparing charge densities (Figs. 4A, 7B, and 7D), so in this case representative NLC curves were reconstructed from mean \( V_{1/2} \), \( z \), and \( Q_{max}/C_{lin} \), assuming 17.6 pF linear capacitance (mean \( C_{lin} \), of WT prestin-expressing cells).

**Western Blot**—At 24 and 48 h post-transfection, HEK cells were harvested and resuspended in lysis buffer (10 mM Tris, 1 mM EDTA, protease inhibitor mixture (Roche Applied Science), and 0.5 mM phenylmethylsulfon fluoride to collect total protein lysates. Lipid was removed following the procedure of Wessel and Flugge (26). A Bradford assay was used to quantify protein concentrations and to suspend equal amounts in sample buffer (62.5 mM Tris, 10% glycerol (v/v), 2% SDS (w/v), 0.7 M β-mercaptoethanol). After incubation at 95 °C for 5 min, samples were separated by electrophoresis in 4–15% polyacrylamide gradient SDS (0.1%, w/v) gels to collect membrane fractions (Fig. 6, B and C) and membrane protein isolated by surface biotinylation (Fig. 6D). Membrane fractions collected from sucrose gradients were separated on 4–12% gradient SDS-polyacrylamide gels to quantify prestin monomer levels. Total membrane-localized prestin levels were quantified in 7.5% SDS-polyacrylamide resolving gels after chemical biotinylation of surface proteins by sulfo succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate and purification of the biotinylated fraction using NeutrAvidin-agarose beads (Thermo Fisher Scientific, Rockford, IL). Briefly, intact cells were treated with 400 μM membrane-impermeable sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate for 30 min at 4 °C before the reaction was quenched, the cells were lysed, and total protein was collected. Biotinylated product was then captured by NeutrAvidin-agarose beads, and purified proteins were eluted using sample buffer containing 50 mM dithiothreitol. Bradford assays were used to ensure equal gel and avidin bead loading. Both types of blots were labeled with goat anti-prestin (N-20 antibody; Santa Cruz Biotechnology) and rabbit anti-amyloid precursor protein (APP) primary antibodies (detects amyloid precursor protein to track equal loading), and two infrared secondary dyes (LI-COR). Goat anti-rabbit IRDye 680 was excited by 685-nm diode laser light, and the signal was captured at 720 nm (APP channel). Donkey anti-goat IRDye 800CW was excited by 785-nm diode laser light, and the signal was captured at 820 nm (prestin channel). PageRuler molecular weight standards (Fermentas Life Science, Glen Burnie, MD) were also detected fluorescently. Scans were performed at 169 μm resolution and 3.6-order of magnitude intensity values were recorded to effectively prevent signal saturation. Odyssey 3.0 software was used to measure pixel volume in order to quantify the amount of protein in a select region of the gel. Pixel volume (counts/mm²) is the sum of the intensity values for all pixels in a defined region of the gel multiplied by the area of the defined region.

**Confocal Imaging**—Localization of the prestin-GFP fusions to the membrane was verified using confocal microscopy. To selectively label the cell membrane, transfected HEK cells were incubated with 25 μM di-8 (Invitrogen) for 10 min at room temperature. Cells were washed with PBS, and imaging was performed on an LSM 510 microscope (Zeiss, Thornwood, NY) using a ×63, 1.4 numerical aperture objective. Both GFP and di-8 were excited with a 488-nm argon laser. Using dichroics and band pass filters, the GFP and di-8 fluorescent signals were isolated in separate channels ranging from 500 to 530 nm and from 650 to 710 nm, respectively. The pinholes for the GFP and di-8 channels were set to 1.38 and 1.00 Airy units (respectively).
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FIGURE 2. Cysteine replacement mutants membrane localize. GFP, di-8 (membrane), and bright field images (columns from left to right) are shown for WT and C192S prestin-expressing HEK cells (first and last row, respectively). In both cases, the fluorescence of the prestin-GFP fusion protein coincides with that of di-8, a lipid-like probe that partitions into the cell membrane. This demonstrates successful trafficking of the protein, which is clearly evident when thresholding is performed to remove low intensity pixels from each fluorescent channel (the lowest −20%), and the remaining colocalized signal is displayed (fourth column). Native HEK cells labeled with di-8 (second row) and unlabeled HEK cells expressing prestin (third row) are shown to demonstrate efficient spectral separation of GFP and di-8 fluorescence into their respective channels. Despite reduced NLC density and the latter concentration (28, 29), we determined if C395S prestin retained the cholesterol sensitivity of WT prestin. The extent of membrane localization is visually indistinguishable from WT prestin. The membrane localization of C192S prestin is representative of the trafficking observed for each of our mutant prestin constructs (see supplemental Fig. S1). Scale bars, 20 μm.

corresponding to optical slices of less than 1.0 μm. Colocalization of fluorescent signals was assessed by thresholding to remove low intensity pixels from each channel and then displaying pixels having both GFP and di-8 fluorescence.

RESULTS

Prestin Cysteine Mutants Are Localized to the Plasma Membrane—To determine if cysteine residues are necessary for prestin function, we created single serine replacement mutants (C52S, C124S, C192S, C196S, C260S, C381S, C395S, C415S, and C679S), single valine replacement mutants (C192V, C196V, C395V, and C415V), and a double replacement mutant, C192S/C196S (see supplemental Table S2). WT prestin-GFP and each prestin-GFP mutant were characterized for expression at the plasma membrane surface using confocal imaging 24–48 h post-transfection in HEK cells. The membrane intercalating dye, di-8, was used to delineate the plasma membrane of the prestin-expressing HEK cells. Fig. 2 shows the results for an experiment performed with C192S prestin. It is clear that both WT and C192S prestin are expressed and localize to the region of the plasma membrane based upon coincidence of di-8 and GFP fluorescence, the former distributed within the external leaflet of the lipid bilayer and the latter fused to prestin and the prestin mutants (Fig. 2). These results are representative of the membrane localization observed for each of our mutant constructs (see supplemental Fig. S1). Misfolded proteins tend to have poor membrane localization in heterologous expression systems (27), but each of our cysteine replacement mutants successfully reached the cell membrane.

Functional Implications of Cysteine Replacement with Serine—We next determined the impact of cysteine replacement on prestin NLC function using whole-cell patch clamping of WT and prestin cysteine mutants in HEK cells. Our results revealed alterations in the NLC that can be segregated into three distinct classes as follows.

Class 1 mutants exhibited shifted $V_{1/2}$ compared with WT prestin. Substitution of either cysteine in transmembrane domain (TMD) 9 altered the operating set point for prestin charge movement by depolarizing $V_{1/2}$ (Fig. 3A). The prestin cysteine mutants C381S ($−57.2 \pm 3.2$ mV) and C395S ($−6.1 \pm 2.0$ mV) had $V_{1/2}$ values that were shifted by $−21$ and $73$ mV (respectively) compared with WT controls ($−78.6 \pm 3.6$ mV) (mean ± S.E.; $p < 0.01$). The altered $V_{1/2}$ was not accompanied by any other functional abnormality, because charge density and $z$ values were consistent with WT prestin. The measured $V_{1/2}$ value for each prestin mutant is reported in Fig. 3B. $V_{1/2}$ is known to be sensitive to membrane cholesterol concentration (28, 29). Because the shift observed in the C395S mutant was of similar magnitude to that observed following cholesterol depletion of HEK cells expressing WT prestin ($−74$ mV upon 30-min incubation with methyl-β-cyclodextrin at $37 \degree C$ (28)), we determined if C395S prestin retained the $V_{1/2}$ cholesterol sensitivity. Despite already being shifted $73$ mV from WT prestin, C395S prestin $V_{1/2}$ was further depolarized $69$ and $111$ mV upon 15- or 30-min depletion of cholesterol, respectively (Fig. 3C). The retained cholesterol sensitivity indicates that the C395S mutant prestin $V_{1/2}$ shift does not share a common mechanism with WT prestin $V_{1/2}$ shift upon cholesterol depletion.

Class 2 mutants have decreased area under the NLC curve, representing reduced charge movement. Compared with WT controls (18.27 ± 1.89 fC/pF), substitution of prestin cysteines
intracellular cholesterol by 10 mM methyl- 
unrecovered in intensity (strong and partial recovery for M 
shown in Fig. 5A). We can only accurately assign 
order states, the dimer, trimer, and tetramer are expected to be 
Serine Replacement Mutants Have Altered Oligomeric 
A) Intramembrane Residues Involved in Prestin Function 
B) Cysteines of TMD 9 affect \( V_{1/2} \) of NLC. A, NLC curves constructed 
- FIGURE 3. Cysteines of TMD 9 affect \( V_{1/2} \) of NLC. A, NLC curves constructed 
- FIGURE 4. Cysteines of TMD 4 and 10 are required for full charge movement. A, NLC curves constructed from mean fit parameters show that C192S/ C196S and C415S prestin have reduced area under the curve, indicating reduced charge movement compared with WT prestin. \( V_{1/2} \) and \( z \) for these mutants are consistent with WT measures (see Figs. 3 and 5). Mutants with normal charge movement capabilities show similar area under the curve compared with WT prestin, and C679S prestin is shown as an example. B, most serine substitutions provided normal charge movement of ~15 fC/pF; however, C192S/C196S and C415S prestin exhibited significantly reduced levels compared with WT controls (\( p < 0.01 \)) as follows: WT, 18.27 ± 1.89 fC/pF (n = 21); C52S, 15.46 ± 2.15 fC/pF (n = 11); C124S, 11.46 ± 2.28 fC/pF (n = 8); C192S/C196S, 7.37 ± 0.81 fC/pF (n = 7); C679S, 16.34 ± 2.75 fC/pF (n = 12); C381S, 15.79 ± 2.14 fC/pF (n = 7); C395S, 15.86 ± 2.19 fC/pF (n = 10); C415S, 8.37 ± 1.33 fC/pF (n = 12); C679S, 16.99 ± 2.69 fC/pF (n = 10). All values are 
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In an attempt to determine if there was a correlation between the expression of prestin cysteine mutants at the membrane and NLC, we quantified the intensities of the glycosylated monomer band using an infrared gel imaging system. The absence of glycosylation, in and of itself, does not impair prestin function. Charge density for this mutant did not differ from WT prestin (see Fig. 4). The origin of NLC and charge density loss upon serine substitution within the Class 2, TMD 4 double replacement mutant (C192S/C196S) was probed with two single replacement mutants (C192S and C196S) and individual valine substitutions (C192V and C196V). Substitution of either cysteine by serine had the same effect as the double mutant, reducing peak NLC (Fig. 7B) and significantly decreasing charge density by about 11.6 fC/pF or 63% (Fig. 7C) from WT. Valine replacement at either the 192 or 196 location resulted in charge density levels indistinguishable from those of WT prestin, suggesting that, if present, disulfide bonding is not essential at either Cys-192 or Cys-196. V₁⁄₂ and z values of C196V were slightly altered from values obtained from WT, but nominal V₁⁄₂ and z values were observed for each of the other mutants (Fig. 7B).

A similar tolerance of valine substitution was discovered in the Class 2, TMD 10 mutant. Serine at residue 415 (C415S) drastically reduced charge density, whereas valine was neutral compared with WT levels (Fig. 7D). C415V NLC was hyperpo-

![FIGURE 5. Cysteine substitution in TMD 6 alters the mechanics of charge movement. A, NLC curves were constructed from mean fit parameters. Because V₁⁄₂ was consistent with WT prestin (see Fig. 3), C260S prestin NLC was translated and aligned with WT to illustrate the effects of increased valence of charge movement. C260S had significantly higher valence than WT prestin (p < 0.01), which corresponds to a narrower voltage range over which the prestin state transition occurs. Charge density for this mutant did not differ from WT prestin (see Fig. 4). B, for all groups is as follows: WT, 0.67 ± 0.01 (n = 21); C52S, 0.76 ± 0.02 (n = 11); C124S, 0.73 ± 0.03 (n = 8); C192S/C196S, 0.70 ± 0.02 (n = 7); C260S, 0.80 ± 0.01 (n = 12); C381S, 0.70 ± 0.03 (n = 7); C395S, 0.67 ± 0.02 (n = 10); C415S, 0.69 ± 0.02 (n = 12); C52S, 0.72 ± 0.02 (n = 10). All values are dimensionless (mean ± S.E.). WT range is highlighted by a gray horizontal bar.
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Serine Substitution of Cysteine Disrupts Prestin Function—To determine if cysteine residues are necessary for prestin function, serine replacement mutations were introduced. Serine was chosen because it is an isosteric replacement, differing from cysteine by a single atom in the side chain. Our analysis revealed several cysteine residues to be important for prestin function. Specifically, we observed three classes of alterations in the NLC: class 1 mutations changed the voltage range over which charge movement occurs; class 2 mutants have reduced charge movement, and class 3 mutants have increased valence of charge movement.

Because uncoupling of NLC and motility has not been reported for prestin, the charge density mutants probably are of the greatest physiological relevance (in comparison with other forms of functional change, such as $V_{1/2}$ shift or $z$ alteration). Cysteine residues in TMDs 4 and 10 were important for charge movement. Substitution by serine at these locations was not well tolerated and greatly impaired function (Fig. 7, B–D). Replace-
Intramembrane Residues Involved in Prestin Function

**FIGURE 7. Effects of valine substitution.** NLC curves for each mutant group were constructed from mean fit parameters, and all values are reported as mean ± S.E. A, valine obviates the shift induced by serine replacement at residue 395. C395V prestin exhibited z and V_{1/2} values that were consistent with WT but had reduced charge density (p < 0.05). For C395V (n = 15), V_{1/2} = −78.6 ± 3.6 mV and z = 0.67 ± 0.01 (n = 21); for C192S, V_{1/2} = −68.4 ± 1.8 mV and z = 0.72 ± 0.01 (n = 16); for C192V, V_{1/2} = −69.8 ± 1.8 mV and z = 0.75 ± 0.02 (n = 10); for C196S, V_{1/2} = −89.5 ± 2.0 mV and z = 0.74 ± 0.03 (n = 15); for C196V, V_{1/2} = −102.2 ± 1.8 mV and z = 0.76 ± 0.02 (n = 14); for C192S/C196S, V_{1/2} = −68.3 ± 2.8 mV and z = 0.70 ± 0.02 (n = 7). C, any substitution of cysteine by serine in TMD 4 reduced charge density (p < 0.05), whereas valine replacement resulted in WT function. Charge densities (fC/pF) for each of the mutants of B are displayed in C. Bars that do not share a common letter are significantly different (p < 0.05). For WT, Q_{max}/C_{lin} = 18.27 ± 1.89 fC/pF (n = 21); for C196V, Q_{max}/C_{lin} = 15.57 ± 1.88 fC/pF (n = 14); for C192V, Q_{max}/C_{lin} = 11.48 ± 2.83 fC/pF (n = 10); for C192S/C196S, Q_{max}/C_{lin} = 7.37 ± 0.81 fC/pF (n = 7); for C196S, Q_{max}/C_{lin} = 7.05 ± 0.95 fC/pF (n = 15); for C192S, Q_{max}/C_{lin} = 5.65 ± 0.40 fC/pF (n = 16). D, similar to the results for TMD 4, valine substitution in TMD 10 provides WT charge density. C415V prestin has normal charge density (Q_{max}/C_{lin}), hyperpolarized V_{1/2} (p < 0.05), and normal z compared with WT. C415V (n = 15); V_{1/2} = 107.2 ± 2.7 mV; z = 0.68 ± 0.01; Q_{max}/C_{lin} = 15.23 ± 2.43 fC/pF.

ment at TMD 4 residues 192 or 196 or at both locations was equally destructive, providing 63% inhibition (Fig. 7, B and C). Comparable inhibition of function resulted from C415S substitution in TMD 10 (Fig. 7D). The decreased charge density exhibited by C192S, C196S, C192S/C196S, and C415S can be reasonably explained through impaired prestin function or decreased surface expression due to impaired trafficking.

**Trafficking and Membrane Localization of Serine Mutants—** Confocal imaging provides evidence that WT prestin and prestin cysteine mutants reach the plasma membrane (Fig. 2). This information is of limited value, however, because it does not indicate the concentration of prestin in the membrane, only its presence. Quantitative fluorescent microscopy is challenging due to differences in cell-to-cell expression levels, and high throughput screens, such as flow cytometry, require surface reactive antibodies to identify the membrane fraction. Due to these challenges, we interpret charge density as a quantitative measure of the amount of functional prestin in the membrane which indicates that the C415S and C196S mutations impaired prestin function without reducing membrane expression levels.

**Valine Substitution of Cysteine Is Tolerated in Hydrophobic Prestin Domains—** Cysteines at amino acid 196 and 415 of prestin appear to be crucial to prestin activity. Western blot analysis with varying reducing treatments has previously demonstrated that prestin dimers might consist of disulfide bond-linked monomers (15), and if this assembly is functionally important, our data suggest that cysteine 196 and/or 415 might be involved. Proof of functionally relevant disulfide bonding, however, requires equivalent charge density reduction with either serine or valine replacement. Instead, we saw nominal function with C196V and C415V (Fig. 7, B–D). This does not preclude disulfide bonds from occurring in prestin but indicates that their loss is not detectable and hence not essential for NLC.

It is most likely that the reduced function of C196S and C415S prestin is the result of disruption to the charge translocation mechanism, because reduced charge density could not
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In prestin, it is known that intracellular Cl\textsuperscript{−} dipoles and side chain groups provide an energy well for anion conduction (30). In prestin, NLC (nonspecific ligand conduction) is thought to occur as a result of changes in intracellular Cl\textsuperscript{−} concentration (31). This evidence implies that Cl\textsuperscript{−} ion(s) bind prestin and are either directly involved in charge translocation (32) or cause an allosteric change that allows outward movement of a positive gating charge in response to depolarized transmembrane potential (32). Our serine replacement at residues 196 and 415 may have altered tertiary structure and thereby altered energy barriers to prevent Cl\textsuperscript{−} binding and/or translocation.

**Tolerated Substitutions Support a Prestin Topology with an Occluded Pore**—Our cysteine mutations can be rationalized in terms of the existing topological models and used to make predictions regarding local residue environments. Because physical size is closely matched between cysteine and serine, hydrophobic character seems to be the crucial property affecting whether cysteine replacement is tolerated. Based on hydrophobicity (35), valine is expected to substitute well for buried residues (36) within the hydrophobic helices, and serine would be suitable near interfaces and other water-exposed regions. Cysteine at locations 192 and 196 are predicted to reside within \(\alpha\)-helices, contained in the hydrophobic core of prestin, by each of the suggested topological models (Fig. 1 and supplemental Table S1). Therefore, it is not surprising that serine replacement inhibited function, evident by decreased charge movement, whereas valine replacement resulted in WT charge movement. Similar results are observed for cysteine substitution at 415, where two of the three current topological models predict the site to be buried within an \(\alpha\)-helix. This trend was reversed at the 395 site, with serine instead being tolerated and valine impairing charge movement. This suggests that the 395 residue is interfacial, as suggested by the 12-pass reentrant loop model, or if contained within an \(\alpha\)-helix, as suggested by the other two models (12-pass evolutionary trace and 10-pass), the residue is probably exposed to an aqueous local environment. Based on this idea, the C260S and C381S prestin mutants may provide information about the putative low density core of tetrameric prestin (16). Their ability to tolerate serine replacement might imply that the residues line a water-filled, occluded pore, an idea that is consistent with TEM data (16).

Serine replacement at cysteine residues 196 and 415 inhibited NLC but maintained normal surface expression, demonstrating that changes in prestin tertiary structure affect prestin charge translocation. Tertiary structure did not appear to be affected when valine was instead used as a replacement, because NLC was not perturbed from WT measures. Our mutations indicated that NLC was maintained when cysteine substitutions did not interfere with native hydrophobicity. Native hydrophobicity could readily be inferred from the proposed topologies with two key exceptions. We were intrigued

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**FIGURE 8.** Comparison of monomer band intensities between serine and valine replacement mutants. A, the glycosylated monomer bands were evaluated from membrane fractions isolated on sucrose gradients as described in the legend to Fig. 6b. Lane 1, WT; lane 2, C192S; lane 3, C192V; lane 4, C196S; lane 5, C196V; lane 6, C192S/C196S; lane 7, C395S; lane 8, C395V; lane 9, C415S; lane 10, C415V; lane 11, untransfected HEK. B, normalized glycosylated monomer band intensity (gray bars) is plotted in order of decreasing charge density (dotted line with S.E. bars and n values), with the corresponding lane numbers of A shown in parentheses. C192S/C196S, C192V, C196S, C196V, and C192S prestin all show significantly reduced charge density compared with WT \((p < 0.05)\). C, charge density varies linearly with prestin membrane concentration. The glycosylated monomer band intensities from Figs. 6 (B and C) and 8 (A and B) (normalized for equal protein loading) were plotted as a function of mean measured charge density. All values were normalized to the band intensity of WT prestin, and in cases of replicate measures \((C395S, C192S/C196S, C415S, C196S, and C192S)\), the mean band intensity was used. Loss-of-function charge density mutants \((C415S, C395V, and C196S)\) and mutants with abnormally high band intensity \((C395S)\) were omitted \((open squares)\). Linear regression of the remaining functional prestin mutants \((closed squares)\) provided \(y = 0.0611x + 0.091\) \((R^2 = 0.68)\).
that serine was tolerated at cysteine residues 260 and 381, because these residues are located near the midpoint of purported TMDs. This finding supports exposure of these sites to a water-filled internal pore in prestin. Prestin NLC has been suggested to arise from incomplete Cl– (6) or charged residue (32, 37) translocation, and both mechanisms rely on interaction of Cl– with prestin. Therefore, we speculate that the orientation of helices 4 and 10 is critical for prestin function, and when it is disrupted, by hydrophobic mismatch, both Cl– interaction and prestin function are inhibited.

Charge Density Varies with Prestin Membrane Concentration—We observed a linear relationship between the prestin concentration in the membrane and prestin activity after eliminating mutants in which residue replacement caused loss of function (C415S, C196S, and C395V) (Fig. 8C). This is important because it proves that point mutagenesis is not intrinsically disruptive to prestin function. The slight variation in charge density among mutants that are not statistically different from WT prestin is disrupted, by hydrophobic mismatch, both Cl– interaction and prestin function are inhibited.

Effects of Cysteine Substitution on V1/2 and Valence—Since the V1/2 shift measured in the C395S mutant was of similar magnitude and polarity to the shift induced in cholesterol-depleted HEK cells expressing WT prestin (28), we investigated if the two phenomena shared a common mechanism. C395S did not lose sensitivity to membrane cholesterol levels, and cholesterol depletion depolarized V1/2 in a time-dependent manner (Fig. 3C). This highlights the ability of membrane material properties to fine tune prestin function (28, 38), even in cases in which mutation has already modified NLC. Protein-membrane interaction is an ongoing area of investigation in understanding prestin and auditory function (28, 29, 38–40). Interestingly, valine replacement at 395 had normal V1/2 (Fig. 7A) but caused loss of function because the mutation resulted in significantly decreased charge density despite normal membrane expression of glycosylated monomer (Fig. 8B). Whereas loss-of-function mutants indicated that hydrophobicity was necessary at residues 196 and 415, this result shows that hydrophilicity is needed at residue 395 for normal charge movement.

The physiological significance of charge movement valence is unknown. The valence of the Boltzmann function characterizes the voltage range over which prestin undergoes a hypothesized two-state transition. The transition is commonly thought of as a prestin conformational change, so conceptually z may be thought of as a descriptor of the rate, or abruptness (tightness of voltage range), of conformational change. C260S significantly altered the mechanics of the prestin two-state transition, elevating valence and reducing the voltage range over which charge transfer occurs (Fig. 5), while maintaining normal V1/2 and charge density. Since C260S moved the same total charge as WT prestin, the increase in valence can also be interpreted as a more complete translocation of charge across the membrane. For example, one common interpretation of valence in WT prestin (z = 0.67 ± 0.01) is that the protein moves the charge carried by an electron 67% across the membrane. This is increased to nearly complete translocation of elementary charge in the case of the prestin C260S mutant (80%; see Fig. 5). Thus, we have shown that altering residue hydrophobicity can impair prestin function (C196S and C415S), and in this case (C260S) we conclude that although residue hydrophobicity was compatible, the substitution affected the mechanics of charge translocation. This effect may be due to increased tendency for hydrogen bonding.

In conclusion, cysteine-scanning mutagenesis has provided new insights regarding the residues and helices that participate in the mechanism of charge translocation by prestin. Maintenance of hydrophobicity at residues 196 and 415 appears to be critical for prestin function. Additionally, because valine substitutions were tolerated at locations where replacement of cysteine by serine impaired charge movement (192, 196, and 415), we show that disulfide bonding, if involved in prestin at all, does not serve a functional role that can be detected via NLC. Our analysis motivates further study of the orientation of transmembrane domains 4 and 10 and their contribution to intramembrane charge movement. These results advance our knowledge of the molecular basis of outer hair cell electromotility and mammalian audition and also provide additional topological information to guide further studies on structure-function relationships in prestin and other members of the SLC26 family.

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