Hearing Aid for Vertebrates via Multiple Episodic Adaptive Events on Prestin Genes

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Abstract

Auditory detection is essential for survival and reproduction of vertebrates, yet the genetic changes underlying the evolution and diversity of hearing are poorly documented. Recent discoveries concerning prestin, which is responsible for cochlear amplification by electromotility, provide an opportunity to redress this situation. We identify prestin genes from the genomes of 14 vertebrates, including three fishes, one amphibian, one lizard, one bird, and eight mammals. An evolutionary analysis of these sequences and 34 previously known prestin genes reveals for the first time that this hearing gene was under positive selection in the most recent common ancestor (MRCA) of tetrapods. This discovery might document the genetic basis of enhanced high sound sensitivity in tetrapods. An investigation of the adaptive gain and evolution of electromotility, an important evolutionary innovation for the highest hearing ability of mammals, detects evidence for positive selections on the MRCA of mammals, therians, and placentals, respectively. It is suggested that electromotility determined by prestin might initially appear in the MRCA of mammals, and its functional improvements might occur in the MRCA of therian and placental mammals. Our patch clamp experiments further support this hypothesis, revealing the functional divergence of voltage-dependent nonlinear capacitance of prestin from platypus, opossum, and gerbil. Moreover, structure-based docking analyses detect positively selected amino acids in the MRCA of placental mammals that are key residues in sulfate anion transport. This study provides new insights into the adaptation and functional diversity of hearing sensitivity in vertebrates by evolutionary and functional analysis of the hearing gene prestin.

Key words: prestin, adaptation, selection, electromotility, tetrapods, NLC.

Introduction

The vertebrate auditory system is responsible for the detection of sound signals in external environments, which is essential for survival and reproduction. The ability to hear is typically characterized by parameters that quantify frequency selectivity and sensitivity. These parameters generally include the high-frequency hearing limit ($F_{\text{max}}$) (Masterton et al. 1969). Previous behavioral studies reveal that $F_{\text{max}}$ varies enormously across different vertebrate lineages (Fay 1996). For example, most fishes can detect sounds ranging from ~0.44 to ~0.74 kHz (Fay 1988; Popper and Fay 1997), although a few species are able to detect ultrasonic sounds (Popper 2000; Mann et al. 2001). In most species of frogs, $F_{\text{max}}$ values are about three to five times higher than those in most fishes, but ~5-fold lower than those of most of birds (Fay 1988). In general, mammals detect frequencies above 20 kHz, and this is an important characteristic distinguishing them from other tetrapods (Fay 1988; Manley 1990, 2000).

High-frequency hearing detection tends to increase phylogenetically from fishes to amphibians to mammals. Certainly, a few species in each lineage have higher frequency detection than their cohorts (Popper 2000; Mann et al. 2001; Feng et al. 2006). This trend is also documented in anatomical and electrophysiological studies (Heffner and Heffner 1991; Fay 1996; Coffin et al. 2004), yet little is known about the underlying genetic variation and evolutionary trajectories of the gene(s) involved in high-frequency detection.

Prestin, a membrane motor protein driving the elongation of outer hair cells (OHCs), is a major determinant of cochlear amplification for hearing-frequency hearing (Brownell et al. 1985; Belyantseva et al. 2000; Zheng et al. 2000; Brownell et al. 2001). Prestin, also named SLC26A5, is a member of the solute carrier 26 (SLC26) gene family. It encodes anion exchangers capable of transporting a wide variety of monovalent and divalent anions (Sindic et al. 2007; Detro-Dassen et al. 2008). Some ~10–12 transmembrane domains linked by intra- and extracellular loops in prestin are flanked by cytoplasmic N- and C-termini (Navaratnam et al. 2005; Zheng et al. 2005). Prestin, a motor protein essential for electromotility (Zheng et al. 2000; Dallos and Fakler 2002; Liberman et al. 2002), can be measured by its robust voltage-dependent nonlinear capacitance (NLC) (Santos-Sacchi 1991). In mammals, NLC and high-frequency hearing increase to a greater extent than in other vertebrates without electromotility (Fay 1988; Manley 1990, 2000; Tan et al. 2011). In addition, prestin-knockout mice show significantly reduced...
high-frequency sensitivity (Liberman et al. 2002; Wu et al. 2004). Mutations in this gene lead to nonsyndromic auditory loss in humans (Liu et al. 2003). Phylogenetic analysis of prestin sequence data unite echolocating bats that have the ability of high-frequency sound detection (Li et al. 2008), although these bats are not monophyletic in the species tree (Teeling et al. 2005). Surprisingly, echolocating whales and bats also cluster together in phylogenetic analyses of amino acid sequences (Li et al. 2010; Liu, Cotton, et al. 2010). The number of amino acid replacements is positively corrected with an increase in ability to detect high frequencies in whales (Liu, Rossiter, et al. 2010). Taken together, these studies strongly suggest that prestin is an important factor in high-frequency detection.

Herein, we examine the evolutionary dynamics and selection pressure on prestin genes in vertebrate lineages. We focus on detecting selection pressure in the initial emergence of land vertebrates because these animals tend to detect higher frequencies of sounds than do fishes. This is followed by an investigation of the unique gain of somatic electromotility in mammalian OHCs, the basis for cochlear amplification (Brownell et al. 1985; Ashmore 1987). This amplification system gives mammals the greatest ability to detect the highest frequencies among vertebrates. Our analyses of 48 vertebrate prestin genes detect multiple episodes of adaptive evolution among vertebrates, suggesting that its function might change as organisms evolve. Furthermore, our results from whole-cell patch clamp functional experiments on platypus, opossum, and gerbil prestin support this hypothesis. This study provides insights into the adaptation and functional diversity of high frequency hearing in vertebrates.

Materials and Methods
Identification of prestin Genes and Collection of $F_{\text{max}}$ Data in Mammals
Thirty-four prestin sequences obtained from GenBank included two birds, two fishes, and 30 mammals. In addition, we searched for prestin sequences in Ensembl (http://www.ensembl.org/) and NCBI (http://www.ncbi.nlm.nih.gov/) from 14 vertebrate genomes that have high genome coverage ($\geq 6 \times$). The taxa included three fishes (medaka, Orzias latipes; stickleback, Gasterosteus aculeatus, and fugu, Takifugu rubripes), one amphibian (frog, Xenopus tropicalis), one nonavian reptile (lizard, Anolis carolinensis), one bird (turkey, Meleagris gallopavo), and eight mammals (chimpanzee, Pan troglodytes; gorilla, Gorilla gorilla; orangutan, Pongo pygmaeus; common marmoset, Callithrix jaccus; elephant, Loxodonta africana; guinea pig, Cavia porcellus; panda, Ailuropoda melanoleuca; and horse, Equus caballus) (supplementary table 1, Supplementary Material online). We used our previous pipeline for identifying genes with multiple exons (Yang, Shi, et al. 2005; Liu et al. 2011) as briefly follows: TBlastN was employed to search prestin sequences in the genome databases using previously known prestin protein sequences, and known protein sequences of prestins and the best hit genomic sequences were used to conduct a protein–DNA comparison using Wise2 (http://www.ebi.ac.uk/Tools/Wise2/index.html), which provided the exon/intron structures and the full-length protein sequences and cDNA sequences of the putative genes. We did not search low-coverage mammalian genomes (about $2 \times$ coverage) from each major vertebrate lineage because sequence mining of multiexon genes was not feasible without chromosomal assemblies.

Two analyses were performed in order to exclude false prestin homologies. First, we blasted the putative genes in GenBank to ensure the best hits were known prestin genes. Second, we constructed a tree using the neighbor-joining method (Saitou and Nei 1987) with protein Poisson distances (Nei and Kumar 2000). We employed SLC26A6, the closest related gene to prestin in the SLC26 gene family (Franchini and Elgoyhen 2006) as the outgroup to root the tree (supplementary fig. 1, Supplementary Material online).

We collected $F_{\text{max}}$ values, which measured high-frequency hearing and defined the highest frequency audible at 60 dB sound pressure level (SPL) (Fay 1996), from mammals in order to detect differences among species. Values were collected from two monotremes, the platypus (Gates et al. 1974) and echidna (Mills and Shepherd 2001), three marsupials, the opossum (Reimer 1995), quolls (Dasyurus; Aitkin 1995) and brush-tail possum (Trichosurus; Aitkin 1995), and 23 placentals, including the elephant (Heffner and Heffner 1982), guinea pig (Heffner et al. 1971), horseshoe bat (Long 1977), rat (Gourevitch 1965), bottlenose dolphin (Herman and Arbeil 1973), mouse (Ehret 1976), chinchilla (Clack 1966), macaque (Clack 1966), cat (Nienhuys and Clark 1979), human (Wier et al. 1977), squirrel monkey (Green 1975), yellow baboon (Hienz et al. 1982),lemur (Mitchell et al. 1971), bushbaby (Heffner et al. 1969b), tree shrew (Heffner et al. 1969a), dog (Heffner 1983), ferret (Kelly et al. 1986), kangaroo rat (Heffner and Masterton 1980), gerbil (Ryan 1976), rabbit (Heffner and Masterton 1980), little brown bat (Dalland 1965), common harbor seal (Mohl 1968), and frog-eating bat (Ryan et al. 1983).

Evolutionary Analyses
The prestin sequences were initially aligned using ClustalW (Chenna et al. 2003) followed by manual adjustments. Pairwise comparisons of the numbers of synonymous substitutions per synonymous site ($d_S$) and nonsynonymous substitutions per nonsynonymous site ($d_N$) were estimated by the modified Nei–Gojobori method (Zhang et al. 1998) in MEGAS (Tamura et al. 2011). Tree-based selection tests were calculated by the branch-site likelihood method implemented in PAML4 (Yang, Wong, et al. 2005; Zhang et al. 2005) because this test is conservative and robust against violations of various model assumptions, unlike the previous version of the test (Yang and Nielsen 2002), which has a relatively high rate of false positives (Zhang 2004). This method compared two models. One model defined four classes of sites in terms of $\omega$, the ratio of $d_S$ to $d_N$. Codons conserved throughout the tree were assigned to site class 0 (estimated $0 < \omega_0 < 1$). Site class 1 contained codons that were neutral throughout the tree
Adaptive Evolution of Vertebrate prestin Genes

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Shindyalov and Bourne 1998, and SO1, which value \( C \) corresponded to an estimated 0.001. Root sequences representing all major branches but positive selection on the foreground branch. The corresponding null model fixed \( \omega_2 = 1 \), which differed from the alternative model. A likelihood ratio test was used to compare the two models. When the likelihood of the alternative model was significantly higher than that of the null model, it was assumed to indicate positive selection on the foreground branch. Where tests indicated positive selection, we recorded the sites under selection according to high posterior probabilities (≥0.95) following Bayes empirical Bayes (BEB) prediction (Yang, Wong, et al. 2005).

Gene Synthesis, Cell Culture, and Transient Transfection

The entire coding regions of platypus and opossum were synthesized and cloned into the expression vector pcDNA3.1(−) (Invitrogen Inc.). Correct orientation and reading frames were verified by sequencing analysis. In addition, the expression vector of gerbil’s prestin was gifted from Dallos’ lab (Northwestern University).

HEK293 cells were grown in 35-mm dishes containing Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (Invitrogen Inc.). When cell confluence reached roughly 50–60% of the surface area of the dishes, cotransfection of the expression vectors of the prestins and pEGFP-N1 (GFP) were accomplished using lipofectamine 2000 transfection reagent (Invitrogen Inc.). We used a ratio of 1 µg (GFP)/3 µg (prestin), added to 10-µl lipofectamine. The pEGFP-N1 plasmid generated a cytoplasmic EGFP protein as an independent marker for successful transfection of cells. After 24- to 48-h incubation, the successfully transfected cells were used for NLC measurements.

Electrophysiological Experiments for NLC Measurements

NLC was measured using whole-cell patch-clamp recordings that were performed by HEKA EPC 10 USB (HEKA Instruments Inc.) at room temperature (22–26 °C). Electrodes were pulled from borosilicate glass with resistances of 2.5–4 MΩ and filled with the internal solution containing: 140 mM CsCl, 2 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES. The cells were bathed during the recordings in an external solution containing 120 mM NaCl, 20 mM TEA-Cl, 2 mM CoCl, 2 mM MgCl₂, 10 mM HEPES, and 5 mM glucose. Both solutions were adjusted to pH 7.2. Osmolarities of the internal and external solutions were adjusted to 300 and 320 mOsmol⁻¹ with glucose, respectively. Voltage-dependent capacitance was measured using the stair-step voltage protocol to obtain the parameters of charge movement (Huang and Santos-Sacchi 1993). Voltage was stepped from −140 to 100 mV in 10 mV increments of 10 ms each. The capacitive currents were sampled at 100 kHz and low-pass filtered at 5 kHz using PatchMaster software (HEKA Instruments Inc.). For each voltage, the measured membrane capacitance \( (C_m) \) was plotted as a function of membrane voltage \( (V_m) \) and fitted with the derivative of a two-state Boltzmann function:

\[
C_m = \frac{Q_{\text{max}}^2}{\exp[a(V_m - V_{1/2})]/(1 + \exp[-a(V_m - V_{1/2})]^2) + C_{\text{lin}}}
\]

where \( Q_{\text{max}} \) was the maximum charge transfer, \( V_{1/2} \) was the voltage at which the maximum charge was equally distributed across the membrane, \( C_{\text{lin}} \) was the linear capacitance, and \( a = ze/kT \) was the slope factor of the voltage dependence of charge transfer where \( k \) was Boltzmann’s constant, \( T \) was absolute temperature, \( z \) was valence, and \( e \) was electron charge. The \( C_{\text{lin}} \) was proportional to the surface area of the membrane (cell size). To compare the magnitude of NLC obtained from different cells with different levels of prestin expression as a function of cell size, we normalized the NLC by the linear capacitance of the cells. Because differences in \( Q_{\text{max}} \) could have been caused by cell size, the charge movement was normalized to \( C_{\text{lin}} \). This quantity, designated as charge density, had units of fC/pF.

Predicting Prestin Protein Tertiary Structure and Structural Superposition

The tertiary structure of prestin was predicted online by the profile–profile matching algorithms implemented in Phyre (http://www.sbg.bio.ic.ac.uk/−phyre/html/casp8.html). The quality of the predicted proteins was estimated by E value, where an E value < 0.001 corresponded to an estimated precision >95% (Kelley and Sternberg 2009).

Structural superposition was performed using the CE software package (Shindyalov and Bourne 1998). Root mean square deviation (RMSD) values between two protein structures were used to measure the degree of structural similarity for superposition. RMSD values <4 Å were assumed to indicate similar overall structures (Tung and Yang 2007).

Binding Affinity of Prestin Protein and Anions

The binding affinity between prestin and anions (Cl⁻, HCO₃⁻, and SO₄²⁻) was assessed using CDocker (Wu et al. 2003) in Accelrys Discovery Studio 2.1 (Accelrys Software Inc.), a Charmm-based molecular docking tool for analyzing receptor-ligand interactions. The binding affinity was quantified by CDocker interaction energy (CIE), the interaction energy between the proteins and their binding ligands. Generally, CIE < 0 denoted the attraction of proteins and ligands; the lower the value the higher the attraction. In contrast, CIE > 0 indicated repulsion between ligands and proteins, that is, ligands could not bind to acceptor proteins; the higher the CIE value the stronger the repulsive force.

Results and Discussion

Vertebrate prestin Genes

Herein, a total of 48 prestin sequences representing all major vertebrate lineages except for turtles and crocodilians were subjected to analyses (fig. 1). Alignment of the amino acid sequences (supplementary fig. 2, Supplementary Material
revealed that the N- and C-termini contained the highest levels of variability. We computed Poisson-corrected evolutionary distances for each region in each major lineage to further quantify regional sequence diversity. Gene regions were assigned according to the prestin membrane topology proposed by several lines of functional evidence (Ludwig et al. 2001; Matsuda et al. 2004; Deak et al. 2005; Rajagopalan et al. 2006). The mean sequence distance in the N-terminal region (NR) (fig. 2) was significantly higher than that of the full-length sequence in both teleosts ($P = 0.0047$, Fisher’s Exact Test) and tetrapods ($P = 0.0027$). The same was true for the C-terminus (teleosts: $P = 0.0005$; tetrapods: $P = 0.0001$). In contrast, the sequences of the transmembrane region (TR) in both lineages were more conserved than those of other regions. These comparisons revealed that NRs and C-terminal regions (CRs) evolved faster than TRs at the protein sequence level in both major vertebrate lineages. This result taken together with a previous result in mammals (Okoruwa et al. 2008) suggested high levels of variations in NRs and CRs might comprise a general pattern in vertebrate prestin genes.

Adaptive Evolution of prestin Genes during the Emergence of Tetrapods

To further understand the evolutionary dynamics and selective pressure on prestin genes between fishes and tetrapods, we compared the mean nonsynonymous nucleotide substitution distances between teleosts and tetrapods in NR, CR, TRs, extracellular regions (ERs), and intracellular regions (IRs) using the modified Nei–Gojobori method (Zhang et al. 1998). As presented in figure 3, $d_N$ values were higher in NRs and CRs than in ER, IR, and TR. This suggested either that positive selection had acted to favor amino acid replacements in the terminal regions or that some of the amino acids in these regions enjoyed fewer functional constraints.

To distinguish between positive selection and the absence of functional constraints, we compared $d_N$ and $d_S$, because the former was expected to exceed the latter in cases of positive selection. We estimated the mean synonymous distance for the five regions between teleosts and tetrapods and found that the mean $d_N$ values were much lower than $d_S$ values in ER, IR, and TR. In contrast, the $d_N/d_S$ ratio was larger than 1.0 in NR, although the mean $d_N$ value was not significantly higher than $d_S$ (fig. 3). The absence of significance between $d_N$ and $d_S$ might have owed to the saturation of synonymous and nonsynonymous substitutions (Tanaka and Nei 1989).

Positive selection between teleosts and tetrapods was difficult to detect by pairwise comparisons at this high level of sequence divergence ($0.5 < d_S < 1$) because subsequent substitutions may have hidden the signal of positive selection, especially if the emergence of tetrapods occurred in a relatively short evolutionary time span (Zhang et al. 1998; Shi et al. 2003). To rectify this methodological limitation,
We used an improved branch-site likelihood method (Zhang et al. 2005) to look for selection signal while restricting our signal test to the ancestral branch of tetrapods. This method more reliably and powerfully detected positive selection at this level of sequence divergence and saturation by computational simulation (Anisimova et al. 2001, 2002).

We assigned the ancestral branch of tetrapods as the foreground branch and all others as background branches (fig. 1). This approach obtained a significantly higher likelihood for the alternative model than that of the null model ($P < 0.001, \chi^2$ test), suggesting positive selection on prestin in the ancestor of tetrapods. Conservative BEB (Yang, Wong, et al. 2005; Zhang et al. 2005) identified 20 sites under positive selection and with posterior probabilities > 0.95 (table 1). In addition, almost half of these sites were located in NRs and CRs. This could have explained the higher $d_H$ values found in these two regions.

Functional and morphological audition innovations in tetrapods are believed to be adaptations for processing airborne sound (Fritzsch 1991). For example, when the ancestral tetrapods moved onto land, the spiracular pouch in fishes transformed into a tympanic middle ear, the hyomandibular bone transformed into the stapes (Gaupp 1898, 1913; Werner 1960; Thomson 1966; Lombard and Bolt 1988), and the basilar papilla formed as a unique sensory adaptation for airborne sound detection (Retzius 1881, 1884). Consistent with these morphological innovations, the rate of amino acid replacements in prestin genes, which has been shown to be highly related to high-frequency sensitivity, dramatically changed at the same time. The positive selection tests indicated an accelerated rate of amino acid changes in the most recent common ancestor (MRCA) of tetrapods, suggesting that prestin genes might have been involved in the functional shift from low-frequency hearing in fishes to higher frequency audition in tetrapods. To our knowledge, this analysis provided the first evidence that positive selection on prestin...
genes might have enhanced the high-frequency hearing of tetrapods.

Multiple Episodic Adaptive Events on Mammalian prestin Genes

Sound amplification via electromotility gives mammals a superior ability to hear high-frequency sound (Fay 1988; Manley 1990, 2000). This represents a major evolutionary advancement. Therefore, it is important to evaluate the evolutionary tempo and mode of prestin in mammals because the protein is responsible for somatic electromotility (Brownell et al. 1985; Ashmore 1987; Zheng et al. 2000).

Using placental and marsupial prestin sequences, Franchini and Elgoyhen (2006) report positive selection signals in the MRCA of mammals. However, because mammals consist of three major clades of monotremes, marsupials, and placentals, the absence of monotre prestin sequences in their study precludes understanding of the evolution of this gene and its function in mammals.

The available platypus genome (Warren et al. 2008) and a clone of platypus prestin (Okoruwa et al. 2008) provide an opportunity to reexamine selective pressures on prestin genes for the MRCA of all mammals. The test assigns the ancestral branch, that leading to all mammals, to the foreground and all other amniotes to background branches (fig. 1). The results (table 1) show a significantly higher likelihood of the alternative model than that of the null model (after multiple testing correction $P < 0.05$, $\chi^2$ test), indicating that the MRCA of mammals experienced positive selection. This result supports the conclusion that the origin of electromotility in mammals likely happened in the MRCA of all mammals (Franchini and Elgoyhen 2006).

Furthermore, analyses of all available audiogram data from monotremes, marsupials, and placentals reveals that the average upper hearing limit in monotremes (12 kHz) is significantly lower than that of therians (38 kHz, $P < 0.01$, t-test) and placentals (61.9 kHz, $P < 0.01$). These observations lead to the hypothesis that prestin experienced additional adaptive selection for detecting high-frequency sound shortly after the origin of electromotility during the evolution of mammals.

To test this hypothesis, we examined selection pressures on prestin genes on the ancestral branches that lead to therians and placentals respectively. The two branches were separately assigned as foreground branches and all other amniotes as background branches (fig. 1). Significant signals of positive selection were detected on the ancestral branches of therians and placentals, respectively (table 1).

Table 1. Detection of Positive Selection in the Different Lineages of Prestin Orthologous Genes.

<table>
<thead>
<tr>
<th>Foreground Branches</th>
<th>$2\Delta L^a$</th>
<th>$P$ Value$^b$</th>
<th>Estimates of the Parameters in the Modified Model A$^c$</th>
<th>Positively Selected Sites$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancestral branch of tetraps</td>
<td>34.56</td>
<td>$P = 1.65 \times 10^{-8}$</td>
<td>$p_0 = 0.80027$, $p_1 = 0.09229$, $p_{2a} = 0.09633$, $p_{2b} = 0.01111$, $\omega_0 = 0.08133$, $\omega_2 = 306.77$</td>
<td>30E, 49A, 73A, 155D, 169E, 184L, 192C, 273L, 292L, 305A, 312S, 368Q</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>449K, 460F, 584N, 594K, 602E, 608K, 608E, 627E</td>
</tr>
<tr>
<td>Ancestral branch of mammals</td>
<td>5.27</td>
<td>$P = 0.022$</td>
<td>$p_0 = 0.06826$, $p_1 = 0.07127$, $p_{2a} = 0.22263$, $p_{2b} = 0.02324$, $\omega_0 = 0.06303$, $\omega_2 = 2.02367$</td>
<td>44D, 47K, 50F, 59N, 68T, 73A, 75N, 76F, 151L, 247T, 257L, 338L, 415C, 493L, 540I, 588A, 598E, 599V, 618P, 631R, 634P, 662G, 690N</td>
</tr>
<tr>
<td>Ancestral branch of therian mammals</td>
<td>26.68</td>
<td>$P = 7.20 \times 10^{-6}$</td>
<td>$p_0 = 0.87403$, $p_1 = 0.09010$, $p_{2a} = 0.03252$, $p_{2b} = 0.00335$, $\omega_0 = 0.06793$, $\omega_2 = 32.66711$</td>
<td>124C, 225M, 260C, 330N, 460F, 521L, 583G, 617P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68T, 76F, 196C, 240I, 268V</td>
</tr>
</tbody>
</table>

$^a$ Twice the difference between the log likelihood of the alternative model and that of the null model. The modified model A with $\omega_2$ fixed at 1 is the null model. The modified model A is used as the alternative model.

$^b$ Multiple testing corrections are performed.

$^c$ $\omega$ values are the nonsynonymous/synonymous rate ratios. $p_0$ is the proportion of codons that have $\omega_0$ in all branches, $p_1$ is the proportion of codons that have $\omega_1 = 1$, in all branches, $p_{2a}$ is the proportion of codons that have $\omega_2$ in the background branches but $\omega_0$ in the foreground branches, and $p_{2b}$ is the proportion of codons that have $\omega_2$ in the background branches but $\omega_0$ in the foreground branches. Note that as long as $\omega_1$ significantly exceeds 1 (as indicated by the likelihood ratio test), its exact value has little biological meaning due to the large estimation error.

$^d$ Sites with the Bayes empirical Bayes posterior probabilities higher than 95% are shown. The sites are indexed by the amino acids at the site in the gerbil prestin. The sites in different regions are shown as followings: italic in NR and CR, single underlined in TR, double underlined in ER, and boxed in IR.
As shown in figure 4, functional variations occur in three mammalian prestin genes. For platypus prestin, the parameters of NLC from 12 cells after fitting two-state Boltzmann function are as follows: $Q_{\max}/C_{\text{lin}} = 15 \pm 2.3 \text{fC/pF}$, $V_{1/2} = -38.4 \pm 5.3 \text{mV}$, $1/\alpha = 40.57 \pm 2.4 \text{mV}$ (mean $\pm$ S.E.). These values are consistent with previous reports (Tan et al. 2011). Compared with the platypus’ prestin, $1/\alpha$ value of opossum normalized from 25 cells is significantly lower ($P = 0.01$, Student’s t-test, fig. 4A), suggesting changes in the reactivity of prestin to the membrane charge transfer. Other parameters are also well fitted a two-state Boltzmann function curve, with follow values of $Q_{\max}/C_{\text{lin}} = 9.7 \pm 1.6 \text{fC/pF}$, $V_{1/2} = -30.1 \pm 3.7 \text{mV}$, and $\alpha = 58.98 \pm 4.3 \text{mV}$.

In comparison with platypus and opossum prestin, the peak voltage of NLC ($V_{1/2}$) of gerbil prestin is significantly shifted toward the hyperpolarizing direction ($P < 0.01$, Student’s t-test, fig. 4B), with a value of $-67.9 \text{mV}$ ($n = 20$). Furthermore, the curve-fitting parameters of charge density ($Q_{\max}/C_{\text{lin}} = 19.7 \pm 2.7 \text{fC/pF}$) and $1/\alpha$ value ($35.82 \pm 2.7 \text{mV}$) also diverges significantly from that of opossum prestin ($P < 0.01$, Student’s t-test, fig. 4C).

In addition to functional variation among the three mammalian lineages, functional changes occur between nonmammalian vertebrates and mammals and between fish and tetrapods. For example, whereas the prestin gene of all three mammals exhibits a robust bell-shaped voltage-dependent NLC, those of the zebrafish and chicken do not. Furthermore, the magnitude of NLC in chicken prestin is considerably larger than that of zebrafish (Tan et al. 2011).

The generation of robust bell-shaped NLC in mammalian prestins from platypus and opossum to gerbil, as well as the functional improvement of NLC in nonmammalian vertebrates (e.g. zebrafish and chicken), might owe to multiple positive selection events on the ancestral branches of tetrapods, all mammals, therians, and placentals. This possibility requires that positively selected sites involve functional changes and site-directed mutagenesis studies provide strong support for this. For example, the NLC experiment of chimera gerbil prestin, constructed by exchanging 225M, a positively selected site on the ancestral branch of therians, into the corresponding site of gerbil prestin reveals functional changes of NLC and the motility of prestin-expressing cells (Kumano et al. 2009). Another site 260C on the same branch and site 196C on the ancestral branch of placental also play an important role in functional changes of prestin (Rajagopalan et al. 2006; Kumano et al. 2009; McGuire et al. 2010). The mutagenesis of 415C, a positively selected site in the MRCA of mammals, can significantly decrease the magnitudes of NLC, suggesting 415C is required for the increase of NLC in mammals (McGuire et al. 2010). Amino acid 192C is inferred to be positively selected on the ancestral branch of tetrapods, and it plays an important role in charge movement of prestin (McGuire et al. 2010). All of these sites are in the list of positively selected sites (table 1).

Thus, our sequence analysis is consistent with experimental results, and it may help discover more key functional sites.

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**Functional Variations of prestin in Platypus, Opossum, and Gerbil**

If the ancestral branches of mammals, therians, and placentals are indeed under positive selection as described above, we would expect that functional changes of prestin might be observed in monotreme, marsupial, and placental mammals. Voltage-dependent NLC is one of the unique characteristics of prestin, and it is often used to measure prestin function (Santos-Sacchi 1991). NLC exhibits a bell-shaped dependence on membrane potential, and it can be fitted with the first derivative of a two-state Boltzmann function (Santos-Sacchi 1991, Oliver et al. 2001). Consequently, we measure the NLC of prestin genes from the platypus, opossum, and gerbil, the representative monotreme, marsupial, and placental mammals, respectively.

We also identified 23, 8, and 6 positively selected sites in the MRCA of mammals, therians, and placentals, respectively (table 1). The inferred positively selected amino acid substitutions on these three branches might have provided evidence for functional divergence.

**Table 1.**

<table>
<thead>
<tr>
<th>Site</th>
<th>Protein</th>
<th>Branch</th>
<th>Selection</th>
<th>Functional Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>225M</td>
<td>NLC</td>
<td>Therian</td>
<td>Positive</td>
<td>Increase</td>
</tr>
<tr>
<td>260C</td>
<td>NLC</td>
<td>Placental</td>
<td>Positive</td>
<td>Increase</td>
</tr>
<tr>
<td>415C</td>
<td>NLC</td>
<td>MRCA</td>
<td>Positive</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

**Fig. 4.** Voltage-dependent membrane capacitance (NLC) of HEK cells transiently transfected with prestin orthologs of platypus, opossum, and gerbil, respectively. The capacitance–voltage plots are fitted with two-state Boltzmann function. (A) $1/\alpha$ values of prestin in three species are analyzed: platypus, $40.57 \pm 2.4 \text{mV}$ ($n = 12$); opossum, $58.98 \pm 4.3 \text{mV}$ ($n = 25$); and gerbil, $35.82 \pm 2.7 \text{mV}$ ($n = 20$). (B) Comparison of $V_{1/2}$ for three mammalian species: platypus, $-38.4 \pm 5.3 \text{mV}$ ($n = 12$); opossum, $-30.1 \pm 3.7 \text{mV}$ ($n = 25$); and gerbil, $-67.9 \pm 4.2 \text{mV}$ ($n = 20$). (C) Charge density for all three species is as follows: platypus, $15 \pm 2.3 \text{fC/pF}$ ($n = 12$); opossum, $9.7 \pm 1.6 \text{fC/pF}$ ($n = 25$); and gerbil, $19.7 \pm 2.7 \text{fC/pF}$ ($n = 20$). All values are mean $\pm$ S.E. *$P < 0.05$, **$P < 0.01$.}
of prestin, which is associated with the gain and subsequent evolution of NLC and electromotility. More importantly, our evolutionary analysis of prestin genes, taken together with behavioral and functional analyses, suggests that the gene experienced at least three adaptive selection events in mammals alone. The functional improvement of prestin might be a very complex stepwise process in mammals.

Positive Selection in the MRCA of Placental Mammals for Transporting Sulfate
In addition to changing NLC of electromotility, the function of prestin differs substantially among vertebrates with respect to its ability to transport sulfate. For example, in the zebrafish and chicken, prestin acts as an electrogenic antiporter exchanging \( \text{SO}_4^{2-} \) for \( \text{Cl}^- \) with a 1:1 stoichiometry (Schaechinger and Oliver 2007). This plesiomorphic function is not known to occur in mammals, including gerbils (Oliver et al. 2001). The vertebrate lineage that experienced this functional change remains to be identified. Regardless, functional data suggest that this transformation might be associated with structural conformation changes of prestin (Schaechinger and Oliver 2007).

The 3D structure of prestin facilitates an evaluation of whether or not structural conformation changes are involved in functional sulfate transport. Whereas the 3D structure of the C-terminus is known (Pasqualetto et al. 2010), it remains unknown for TRs. Because these are important functional domains for anion transport (Bai et al. 2009; McGuire et al. 2010), our understanding of the changes in sulfate transport mechanisms are limited. Upon using Phyre to predict the 3D structure of TRs of prestin, the best hit of the gerbil’s prestin is a chloride channel (PDB ID = 1ots) (Dutzler et al. 2003; \( E \) value = \( 3.1 \times 10^{-5} \)). All other vertebrate prestins hit the same model (1ots) with a predicted high accuracy (\( \geq 95\% \)). Significant \( E \) values are found to range from \( 10^{-3} \) to \( 10^{-5} \), although the similarity between the template and query sequences is not so high (alignments in supplementary fig. 3, Supplementary Material online).

Technological limitations of 3D modeling and the relatively few available crystallographic structures of membrane proteins require us to evaluate the reliability of the predicted 3D structure of prestin. The predicted structure agrees with the key properties of prestin. First, the predicted structure has 12-transmembrane helixes, and this is consistent with the secondary topology demonstrated by most functional and modeling assays (Oliver et al. 2001; Deak et al. 2005; Rajagopalan et al. 2006). Second, both template and prestin are anion channels that function to conduct \( \text{Cl}^- \) across cell membranes in all vertebrates (Oliver et al. 2001; Dutzler et al. 2003; Schaechinger and Oliver 2007). Third, the accuracy of our 3D model can be validated by comparisons of the predicted functions by molecular docking and functional assays. Whereas a positive CIE value implies little or no affinity between prestin and anions, a negative value suggests that prestin can drive anions freely through the membrane. If our predicted prestin structure holds true, then all of the prestins in non-mammals and mammals should have a high affinity for \( \text{Cl}^- \) and \( \text{HCO}_3^- \) and negative CIE values. Here, \( \text{Cl}^- \) and \( \text{HCO}_3^- \) should be freely transported by prestins in all vertebrates, as evidenced by functional experiments on the zebrafish, chicken, and gerbil (Oliver et al. 2001; Schaechinger and Oliver 2007). As expected, the CIEs between prestins and \( \text{Cl}^-/\text{HCO}_3^- \) in the zebrafish, chicken, and gerbil are all negative (data not shown).

For \( \text{SO}_4^{2-} \), functional assays (Oliver et al. 2001; Schaechinger and Oliver 2007) predict that the zebrafish and chicken prestins will have high-binding affinities and
the gerbil’s prestin should have a lower affinity. Consistent with the functional assays, CIE values of prestin-SO$_4^{2-}$ in the zebrafish and chicken are $-10.968$ and $-10.068$, respectively, suggesting that their prestins can easily bind and transport SO$_4^{2-}$. In contrast, the CIE value of prestin-SO$_4^{2-}$ in the gerbil is 5.19, indicating that this mammal cannot as easily transport SO$_4^{2-}$ (fig. 5A). Therefore, our modeled structure for prestin is congruent with the key aspects of transporting SO$_4^{2-}$, and Cl$^-$/HCO$_3^-$. The structure appears to provide a reliable means for determining the functional changes for permeability to SO$_4^{2-}$ and for inferring positively selected amino acid sites associated with changes in permeability in mammals.

CIE values of prestin-SO$_4^{2-}$ can be used to evaluate when functional change might have occurred in the vertebrates. The values for the zebrafish, frog, lizard, chicken, platypus, and opossum are negative, ranging from $-11.26$ to $-10.939$. In contrast, placental mammals have positive CIE values, ranging from $4.36$ to $5.951$ (fig. 5A). Thus, the functional change in sulfate transport most likely has its origin in the MRCA of placental mammals and the positively selected amino acids in the MRCA of these animals might be associated with changes in the ability to transport sulfate.

We tested whether the functional change depended on the structural conformation caused by positive selection or not by performing structural analyses after artificially exchanging positively selected sites identified on the branch of placental MRCA. First, we created two chimera prestins: chimera opossum prestin (CP1) and chimera gerbil prestin (CP2). CP1 was constructed by inserting the positively selected sites of placental mammals into the corresponding sites of opossum prestin. CP2 was established by exchanging the positively selected sites of gerbil prestin with the corresponding sites of opossum prestin. Second, the 3D structures of CP1 and CP2 were modeled and reliability of the modeling was validated. Both chimera prestins also hit the same model (1ots) with highly predicted accuracy and significant $E$ values (supplementary table 2 and alignments in supplementary fig. 3, Supplementary Material online). When inferred positively selected sites in the prestin sequence of opossum were replaced by those from the gerbil prestin, the CIE value changed from $-9.85$ to $43.89$ (fig. 5B), suggesting these positively selected amino acids affected the ability to transport sulfate. This might have owed to positively selected amino acids changing the local structural conformation of the pore region of the channel.

Superposition of the opossum prestin and CP1 structures detected a mismatch between one region in opossum prestin (256–260) and the corresponding part in the CP1 (256–260). The side chain of LYS256 in the CP1 projected into the pore, and this might have blocked the entryway of the anion channel for the sulfate (fig. 6A). The ability of the gerbil prestin to transport sulfate was fully rescued by replacing positively selected sites with the amino acids of opossum prestin (fig. 5B). Superposition of the structures of the gerbil prestin and CP2 showed that the helix turn region (431–436) in the CP2 was similar to that of the opossum prestin (fig. 6B). It might have swung out of the channel’s pore to allow sulfate penetration and, hence, obtained a negative CIE value (fig. 5B).

Results from the docking analysis and artificial mutagenesis of positively selected sites on the ancestral branch of placental mammals support the above findings to some extent. However, exactly how the positively selected residues influence the changes of SO$_4^{2-}$ transport ability remains to be detailed. One possible scenario is that the positively selected residues are located on the pore of the channel, and they directly bind to SO$_4^{2-}$ and Cl$^-$. Another scenario is that the positively selected sites surround the pore and indirectly bind the anions; this would change the conformation and, thus, result in defective sulfate transport. The absence of functional data precludes the
unambiguous selection of one scenario. Regardless, our docking results more strongly support the latter possibility.

The loss of SO$_4^{2-}$ transport ability seems to have happened in the MRCA of placental mammals. The positively selected amino acids in this MRCA appear to be related to changes in sulfate transport ability. The prestin of the MRCA of placental mammals, as well as that in the majority of, if not all, placental mammals, has a novel anion transport function that might further enhance the ability to detect high frequencies. Interestingly, our audiogram analysis supports this conjecture. Placental mammals generally possess a superior ability to detect higher frequencies (average upper hearing limit 61.9 kHz) than do marsupials (average upper hearing limit 38 kHz).

**Conclusion**

Our evolutionary analysis of prestin genes from 48 vertebrates provides evidence for multiple instances of positive selection and functional divergence events during vertebrate evolution. Prestin appears to have undergone positive selection during the emergence of tetrapods and, for the first time, adapted hearing for a terrestrial lifestyle. Moreover, our analyses indicate three independent adaptive events in the evolution of mammalian prestin genes. The first is predicted to have occurred in the MRCA of mammals and this possibly resulted from the gain of NLC and electromotility. The second adaptive event seems to have occurred in the MRCA of therian mammals and this might be related with the functional improvement of electromotility. The third appeared in the MRCA of placental mammals, which is associated with the ability of prestin to transport solutes, further enhances high-frequency detection. Our functional experiments support sequentially functional enhancements of prestin in monotrems, marsupials, and placents, respectively. Combined with other results on bats and whales (Li et al. 2008, 2010; Liu, Cotton, et al. 2010; Liu, Rossiter, et al. 2010), these findings suggest that prestin genes underwent at least six positive selection events during the evolution of vertebrates. This discovery represents an unusually detailed understanding of how adaptation leads to functional diversity for the perception of high-frequency sound.

**Supplementary Material**

Supplementary figures 1–3 and tables 1–2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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