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IDENTIFICATION OF CHICKEN TRANSMEMBRANE CHANNEL-LIKE (TMC) GENES: EXPRESSION ANALYSIS IN THE COCHLEA

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Abstract—Mutations of the human gene encoding transmembrane channel-like protein (TMC)1 cause dominant and recessive nonsyndromic hearing disorders, suggesting that this protein plays an important role in the inner ear. In this study, we cloned chicken Tmc2 (GgTmc2) from a cochlear cDNA library and we annotated four additional TMC family members: GgTmc1, GgTmc3, GgTmc6, and GgTmc7. All chicken TMCs possess the defining TMC signature motif and display high conservation of their genomic structure when compared with other vertebrate TMC genes. GgTmc1 is localized on the chicken sex chromosome Z at a locus that displays conserved synteny with the loci of mammalian orthologues residing on autosomes. In contrast, the locus of GgTmc2 does not exhibit conserved synteny with its mammalian orthologues. Because murine TMC1 and TMC2 are restrictively expressed in cochlear hair cells, we determined the expression of the chicken orthologues in the basilar papilla, the avian equivalent of the organ of Corti. While GgTmc2 was present throughout the basilar papilla and in other tissues, GgTmc1 transcript was detected specifically in the basal portion of the basilar papilla and was not detectable in any other tissue or organ studied. GgTmc3 and GgTmc6 were detectable in all organs analyzed. Antibody labeling revealed that GgTmc2 is predominantly associated with the lateral membranes of hair and supporting cells. The expression of GgTmc2 by both cell types was further confirmed by RT-PCR using isolated cells. This expression and subcellular localization of GgTmc2 is in agreement with the proposed potential role of this novel class of transmembrane proteins in ion transport.

Key words: deafness gene, hearing, hair cell, vestibular, basilar papilla, tonotopic.

The transmembrane channel-like protein (TMC) protein family consists of eight vertebrate members. The name-giving gene of the family, *TMC1*, has been shown to cause the non-syndromic dominant and recessive human deafness disorders DFNA36 and DFNB7/11, when mutated (Kurima et al., 2002). The murine dominant and recessive inner ear defects in the mouse mutants Beethoven (*Bth*; Vreugde et al., 2002) and Deafness (*dn*; Pujol et al., 1983) are also caused by single nucleotide mutation or single exon deletion of the mouse orthologue, *MmTmc1*. Tmc1 is expressed by cochlear hair cells and hearing loss in *Bth* and *dn* mice is accompanied by hair cell degeneration (Vreugde et al., 2002). Analysis of hair cell and auditory function before the onset of degeneration in *dn*/*dn* mice revealed defective electrophysiological responses in *dn*/*dn* mice (Deol, 1968), suggesting a role of TMC1 in hair cell ion homeostasis. The onset of hearing loss in youth in DFNA36 human patients and the normal development of basolateral calcium and potassium currents in hair cells of early postnatal *Bth* mice (Vreugde et al., 2002) suggest that TMCs are essential for proper hair cell function but not for hair cell and cochlear development. The eight vertebrate TMC genes are classified into three subfamilies based on their sequences and their genomic structures (Keresztes et al., 2003). Human TMC subfamily A includes *TMC1*, *TMC2*, and *TMC3*; subfamily B comprises to *TMC4* and *TMC7*; and subfamily C consists of *TMC5*, *TMC6*, and *TMC8*. *TMC6* and *TMC8* (also described as *EVER1* and *EVER2*) are linked to epidermodysplasia verruciformis, an autosomal recessive disease with increased susceptibility to human papilloma virus that causes skin carcinoma (Ramoz et al., 2002).

The cellular function of TMCs is unknown. Nevertheless, it has been speculated that the TMC proteins are either ion channels, transporters, or modulators of such (Keresztes et al., 2003). A proposed function for Tmc1 as a modulator of K^+ currents in murine cochlear hair cells (Steel et al., 2003) led us to investigate expression and tonotopical distribution of avian TMCs in the basilar papilla, the avian equivalent of the mammalian organ of Corti. Here we report the identification and cloning of cDNAs encoding chicken TMCs and their distribution along the longitudinal axis of the cochlea.

EXPERIMENTAL PROCEDURES

cDNA library screening

Chicken Tmc2 (GgTmc2) cDNA clones were isolated from a chicken inner ear cDNA library (Heller et al., 1998) with a chicken TMC cDNA probe generated by PCR from cochlear cDNA with a degenerate primer pair (TMC forward [5′-ATG MTG ATG GCN AAR AAR TGG-3′] and TMC reverse [5′-GCC CAR CAY TGR AAR TAC AT-3′]). The 1392 bp-probe used for library screening corresponds to amino acids 155–619 of chicken Tmc2. GgTmc2 clones were also isolated using a partial MmTmc1 cDNA probe corresponding to amino acids 1–559 (a gift from Dr. A. Griffith, NIDCD). The cDNA sequence of GgTmc2 was confirmed by complete sequencing of both strands. For other Tmc genes, the sequences were reconstructed through alignment of EST sequences, genomic DNA, and ultimately by PCR amplification and

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Abbreviations: EST, Expressed Sequence Tag; gapdh, glyceraldehyde 3-phosphate dehydrogenase; Mb, megabase; MYRIP, *Myosin VIIA*-Rab interacting protein; PBS, phosphate-buffered saline; PI, pre-immune; sca, supporting cell antigen; TM, transmembrane; TMC, transmembrane channel-like protein; TV, tegmentum vasculosum.

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sequencing. The GenBank accession numbers of the cDNA sequences reported here are AY581308 (GgTmc1), AY581309 (GgTmc2), AY581310 (GgTmc3), AY581311 (GgTmc6), and AY581312 (GgTmc7).

Semiquantitative RT-PCR

Tissues were dissected from E19 White Leghorn chicken embryos (Hy-Line North America, Elisabethtown, PA, USA). Experiments complied with the Public Health Service Policy on the use of laboratory animals and were approved by the institutional animal care and use committee. All efforts were made to minimize the number of animals used and their suffering. The apical region 1 mm from the tip of the basilar papilla and the subsequent 1 mm-region were microdissected and designated as BP1 and BP2. The remaining basilar region of the papilla was denoted as BP3. Collection of batches of individual hair and supporting cells was done by digesting basilar papilla sensory epithelium for 2 min with 0.125% trypsin and by pooling of 20 morphologically identifiable hair or supporting cells. Total RNA from whole basilar papillae, or batches of pooled hair or supporting cells was extracted using spin columns (RNeasy micro; Qiagen, Valencia, CA, USA). Total RNA from larger amounts of microdissected tissues was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized by using SuperScriptase II (Invitrogen). PCR fragments were amplified with *Taq*DNA polymerase (Invitrogen) using the following conditions: a denaturation step at 94 °C for 2 min was followed by *x* cycles of 94 °C for 20 s, 53 °C for 20 s, and 72 °C for 40 s. The number of cycles (*x*) was 35 for Tmcs and inward rectifier potassium channel 1 Kir2.1/IRK1, 25 for glyceraldehyde 3-phosphate dehydrogenase (gapdh), and 60 for all the PCR experiments for batches of 20 individual hair or supporting cells. Comparisons were done by performing parallel reactions using equal volumes of the same RT template for controls and specific amplification reactions. To better visualize amplification products from hair and supporting cells, 10 nCi of ^{32}P-α-dCTP was incorporated in each PCR reaction mix. Primer sequences were: GgTmc1: 5′-ATT GGC CAA GCT GAA AGA AGA G-3′ as a forward primer and 5′-TAG CCA ATA GTC CCA ACA CCA AC-3′ as a reverse primer; GgTmc2: 5′-ATC TTC GGC TAT GTG GCA AAT CC-3′ and 5′-GTA TGT ATC GTG GCT GTC CTC TCC-3′; GgTmc3: 5′-GGC GCT CGG GAG ACA CTA T-3′ and 5′-TGG GGC ACT TAT CAG ACA TTT G-3′; GgTmc6: 5′-CTG GCT TGG CGT TCT CTT TG-3′ and 5′-ATC CGG TGC TGC CCT CTC-3′, Kir2.1: 5′-TCT TCA GCC ACA ATG CCG TG-3′ and 5′-AGA CCA GGA ATA TGC GGT C-3′, gapdh: 5′-ATG GGA AGC TTA CTG GAA TGG-3′ and 5′-TGT CAT ACC AGG AAA CAA GC-3′, supporting cell antigen (sca): 5′-ACA CAA TGG AGA GCC ACA CA-3′ and 5′-TCC ATG ACA CAC TGG TTT AG-3′. PCR fragments were fractionated on 1.2% agarose gels and stained with ethidium bromide; amplification products from batches of 20 hair or supporting cells were fractionated on 10% polyacrylamide gels and exposed to X-ray film for visualization.

Antibody to GgTmc2, Western blotting and immunohistochemistry

GgTmc2 cDNA encoding the carboxyl-terminal region (amino acid residues 713–864) was subcloned into the probaculovirus vector pFASTBAc-Hta (Invitrogen), which was used to produce recombinant baculovirus for expression of GgTmc2 carboxyl-terminus, tagged with an amino-terminal hexahistidine tail, in Sf9 insect cells. The recombinant protein was purified under denaturing conditions with Co^{2+}-conjugated agarose (BD-Talon affinity resin; Clontech, Palo Alto, CA, USA). Immunizing two rabbits each with 300 μg antigen followed by five boosts with 150 μg antigen at 2- to 3-week intervals generated the antisera. Immunoglobulins were purified from preimmune (PI) and from antisera using proteinA-conjugated agarose (AffiGel; BioRad, Hercules, CA, USA). To confirm the specific immunoreactivity, 5 ng of the antigen or 150 μg of whole protein extract from lung was diluted in 1× sample buffer (Mutai et al., 2000) and fractionated in 4–20% gradient or 7.5% SDS–polyacrylamide gels. Gels were blotted onto nitrocellulose membrane, and each lane was incubated separately with PI, antiserum to GgTmc2 at 1:2000 dilutions, or antiserum preincubated with 5 μg of the antigen for 1 h at room temperature. Signals were detected with horseradish peroxidase-conjugated secondary antibodies followed by incubation in chemiluminescence substrate (ECL Plus; Amersham, Piscataway, NJ, USA). For immunohistochemical studies, cochleae were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.2, embedded in TissueTek OCT compound (Sakura Finetek, Torrance, CA, USA) and cut in 10 μm sections. The specimens were incubated overnight at 4 °C in antibody diluted in PBS including 0.05% Tween, 1% bovine serum albumin, and 5% goat serum. Signals were visualized with secondary antibodies conjugated with fluorescein-isothiocyanate. Counterstaining of the specimen was committed with anti-SNAP25 (Covance, Princeton, NJ, USA) followed by secondary antibody conjugated with tetramethylrhodamine-isothiocyanate, and DAPI to visualize nuclei. Image acquisition was done with a digital camera (AxioCam; Zeiss, Thornwood, NJ, USA) connected to an epifluorescence microscope (Axioskop2; Zeiss).

RESULTS

Cloning of chicken Tmc2 and annotation of chicken Tmcs 1, 3, 6, and 7

Since all eight TMC genes are expressed in mouse inner ear (Keresztes et al., 2003; Kurima et al., 2003), we used the chicken cochlea as a source to identify chicken Tmc cDNA. We first employed RT-PCR on cochlear RNA prepared from late embryonic and post-hatch chicken with degenerate oligonucleotide primers corresponding to highly conserved amino acids among the eight TMC protein family members of human, mouse, and puffer fish (Keresztes et al., 2003). PCRs with four possible primer pair combinations exclusively resulted in the amplification of cDNA fragments encoding a single chicken TMC isoform that displayed the highest sequence similarity with vertebrate TMC2 and Tmc2 amino acid sequences. We used the longest PCR-generated chicken Tmc2 probe for low stringency screening of 2×10^6 plaque-forming units of an embryonic chicken cochlea cDNA library. All eight positive clones encoded the same cDNA, albeit only two cDNAs appeared to contain complete open reading frames. The longest cDNA of 3066 base pairs encoded a protein with 864 amino acid residues that displayed high identity with mouse Tmc2 (MmTmc2, 73.4%) and substantially lower identity to any other proteins including MmTmc1 (59.5%). P-SORT II software (Nakai and Horton, 1999) predicts eight transmembrane domains (TMs) at positions 208–224 (TM1), 285–301 (TM2), 379–395 (TM3), 418–434 (TM4), 453–469 (TM5), 532–548 (TM6), 642–658 (TM7), and 703–719 (TM8), which is in close agreement with the predicted positions of TMs in human TMC2 and mouse Tmc2. Based on this high similarity, we conclude that the cloned cDNA encodes chicken Tmc2 protein (GgTmc2) and corresponds to the *Gallus gallus Tmc2* (*GgTmc2*) gene. As is the case among mouse and human TMCs, GgTmc2 is highly conserved (approximately 90% identity)