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THE LOCALIZATION OF PROTEINS ENCODED BY *CRYM*, *KIAA1199*, *UBA52*, *COL9A3*, AND *COL9A1*, GENES HIGHLY EXPRESSED IN THE COCHLEA

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Abstract—Genes that are highly expressed in the inner ear, as revealed by cDNA microarray analysis, may have a crucial functional role there. Those that are expressed specifically in auditory tissues are likely to be good candidates to screen for genetic alterations in patients with deafness, and several genes have been successfully identified as responsible for hereditary hearing loss. To understand the detailed mechanisms of the hearing loss caused by the mutations in these genes, the present study examined the immunocytochemical localization of the proteins encoded by *Crym*, *KIAA1199 homolog*, *Uba52*, *Col9a3*, and *Col9a1* in the cochlea of rats and mice. Confocal microscopic immunocytochemistry was performed on cryostat sections. Ultrastructurally, postembedding immunogold cytochemistry was applied using Lowicryl sections. Crym protein was predominantly distributed in the fibrocytes in the spiral ligament, as well as the stria vascularis in rats. KIAA1199 protein homolog was localized in various supporting cells, including inner phalangeal, border, inner and outer pillar, and Deiters' cells. Uba52 protein was restrictedly localized within the surface of the marginal cells of the stria vascularis. Collagen type IX was found within the tectorial membrane as well as fibrocytes in the spiral ligament. The present results showed cell-specific localization of the encoded proteins of these highly expressed genes, indicating that the coordinated actions of various molecules distributed in different parts of the cochlea are essential for maintenance of auditory processing in the cochlea.

Key words: *CRYM*, *KIAA1199*, *UBA52*, *COL9A3*, *COL9A1* cochlea.

The coordinated actions of various molecules are essential for the normal development and maintenance of auditory processing in the cochlea. Thorough analysis of gene expression profiles in human inner ear tissues using a cDNA microarray containing 23,040 genes identified 52 highly expressed genes whose signal intensities were more than 10-fold higher in the cochlea and vestibule than in a mixture of 29 other tissues (Abe et al., 2003a). These genes may have a crucial functional role in the inner ear, therefore, genes with unique function and specific expression in the cochlea are excellent candidates as causative genes of human non-syndromic deafness. Among those genes, we have focused on five, *CRYM*, *KIAA1199*, *UBA52*, *COL9A3* and *COL9A1*, because of their high expression ratio in the cochlea compared with other tissues (119.55, 56.54, 37.41, 17.64, 11.88 respectively) and subsequent mutation screening has detected possible disease causing mutations in some of them.

The *CRYM* gene shows the second highest expression in the human cochlea, after *COCH*, and a search for mutations of *CRYM* among non-syndromic deafness patients identified two mutations found at the C-terminus (Abe et al., 2003a). CRYM protein (crystallin, mu) is known to be identical to T3 (triiodothyronine) -binding protein and it binds to T3 in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), and might retain the intracellular free T3 concentration (Suzuki et al., 2007 for review). Our recent study demonstrated that one mutant has no binding capacity to T3, implicating that *CRYM* mutations cause auditory dysfunction through thyroid hormone binding properties (Oshima et al., 2006). Although thyroid hormone is known to be crucial for normal development as well as maintenance of hearing function, the detailed mechanism of thyroid hormone handling in the inner ear has not been well understood.

The gene encoding *KIAA1199* showed a high level of expression in the inner ear by complementary DNA (cDNA) microarray analysis (Abe et al., 2003a). Previous *in situ* hybridization findings suggested that *KIAA1199* is expressed in Deiters' cells and/or the spiral ligament, and subsequent mutation screening identified mutations in non-syndromic hearing loss patients with high-frequency predominant hearing loss (Abe et al., 2003b). Despite the abundant expression in the inner ear, the precise localization as well as detailed function is not yet known.

Uba52 is a 128-amino acid fusion protein consisting of a 52-amino acid tail fused to a 76-amino acid ubiquitin peptide. The 52-amino acid tail is a ubiquitin carboxyl extension protein and is a component of ribosome that is removed from ubiquitin before maturation of the ribosome. Ubiquitin is a highly conserved protein that plays, through the so-called ubiquitin–proteasome pathway, a fundamental role in mediating intracellular selective protein degradation. In this pathway, ubiquitin molecules attach to protein substrates by a multienzymatic pathway and ubiquiti-

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Abbreviations: cDNA, complementary DNA; HAS, human serum albumin; TBST, Tris-buffered saline containing 0.1% Triton X-100.

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nated substrates are degraded by proteasome (Schwartz and Ciechanover, 1999 for review). Although they are highly expressed in the inner ear, the functional significance and relevance to disorders of ubiquitin in the inner ear are still unknown.

Collagen type IX is a heterotrimer of three genetically different alpha chains, alpha I (IX), alpha II (IX), and alpha III (IX). Because cDNA microarray analysis has shown *COL9A1* and *COL9A3* to be highly expressed in the human inner ear (Abe et al., 2003a), type IX collagen, a member of the FACIT (Fibril-Associated Collagen with Interrupted Triplet helices) group of collagens that bind to the surface of fibril-forming type II collagen, may play an important role in the inner ear, type IX collagen, as well as type II and V collagens, also is an important component in the tectorial membrane of the organ of Corti (Slepecky et al., 1992a,b). The non-syndromic hearing loss patients with the *COL9A3* mutations showed a moderate progressive bilateral sensorineural hearing impairment in all frequencies (Asamura et al., 2005a). We also identified a *COL9A1* mutation in a family showing symptoms characteristic of Stickler syndrome, including moderate-to-severe sensorineural hearing loss, moderate-to-high myopia with vitreoretinopathy, and epiphyseal dysplasia (Van Camp et al., 2006).

A series of these studies suggested that these five molecules might have crucial functions with regard to hearing. As the first step toward complete understanding of the detailed function of these molecules in the cochlea, the present study reviewed the current knowledge of these genes and examined the cellular and sub-cellular localization of the proteins encoded by *Crym*, *KIAA1199 homolog*, *Uba52*, *Col9a3*, and *Col9a1* in the cochlea of rats and mice.

EXPERIMENTAL PROCEDURES

Tissue preparation

Wistar rats and C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) were used in the present study. The animals were deeply anesthetized with sodium pentobarbital (50 mg/kg). For the confocal microscopic analysis, 1 ml of fixative (4% formaldehyde in 0.1 M phosphate buffer, pH 7.25) was injected through the tympanic membrane and for the electron microscopical analysis perilymphatic perfusion was performed by injection of 1 ml of fixative (4% formaldehyde and 0.5% glutaraldehyde) into the scala tympani. Subsequently, the animals were perfused through the heart by the same fixative used for tympanic injection or perilymphatic perfusion. Then the temporal bones were removed and postfixed in the same fixative (4–8 h, 4 °C). For confocal microscopy analysis, the specimens were immersed in 10% sucrose and 30% sucrose (each overnight) and then serial cryostat sections (15 μm thick) were cut and placed on silane-coated slides. For electron microscopy, the specimens were cryo-protected, quick frozen, freeze-substituted, and low temperature embedded in a methacrylate resin (Lowicryl HM 20; Chemische Werke Lowi, Waldkraiburg, Germany).

Polyclonal antibodies

Preparation of polyclonal antibodies to Crym protein, KIAA1199 protein homolog, and Uba52 protein were as follows. Sequences were chosen from mouse Crym protein (AFLSAEEVQDHLRSC and EGHSNTAVPSHQASC), mouse Uba52 protein (VKAKIQDKEGIPPDC and RKKKCGHTNNLRPKK), and mouse KIAA1199 protein homolog (YRSKKESERLVQYLC and NDFAYIEVDGRRYPC), to synthesize peptides. The peptides, coupled to keyhole limpet hemocyanin (KLH), were mixed with complete adjuvant and injected into rabbits (Crym protein, KIAA1199 protein homolog) or guinea pigs (Uba52 protein). The antiserum was affinity purified on a column carrying the peptide used for immunization. Antibodies against collagen type IX (COSMO BIO Co., Ltd., Tokyo, Japan or Calbiochem, San Diego, CA, USA), von Willebrand factor (to detect alpha-tectorin) (Chemicon, Temecula, CA, USA), and GST7-7(P) (kindly provided by Dr. Shigeki Tsuchida, Hirosaki University) were also used.

Immunocytochemistry

The tissue specimens were incubated as follows: (1) normal goat serum at room temperature for 30 min, (2) rabbit polyclonal antibodies Crym protein, KIAA1199 protein homolog, collagen type IX, GST7-7(P) (1:10,000, 1:250, 1:200 dilution respectively), or guinea-pig polyclonal anti-Uba52 protein antibody (1:200 dilution) diluted with 0.3% Triton X-100, overnight at 4 °C, (3) Rhodamine-conjugated anti-rabbit antibodies (Chemicon; 1:150 dilution for Crym protein, KIAA1199 protein homolog, collagen type IX, GST7-7(P)), or FITC conjugated goat anti-guinea-pig antibodies (MP Biomedicals, Irvine, CA, USA; 1:150 dilution for Uba52 protein), 4 h at room temperature. The specimens were examined with a confocal laser scanning microscope (Leica TCS SP2 ABOS). For control, adsorption experiments were carried out by blocking with the synthetic peptides. Specificity of the antisera was also examined by immunoblotting analysis (Oshima et al., 2006; Kitoh et al., 2007).

Postembedding immunogold staining of ultrathin sections

Ultrathin sections were briefly (2–3 s) immersed in a saturated solution of NaOH in absolute ethanol, rinsed well with double-distilled water, and incubated in the following solutions (at room temperature): (1) 0.1% sodium borohydride and 50 mM glycine in Tris-buffered saline containing 0.1% Triton X-100 (TBST) (10 min), (2) 2% human serum albumin (HSA) in TBST (10 min), (3) primary antibodies (1:10,000 for Crym protein, 1:200 for Uba52 protein, 1:250 for KIAA1199 protein homolog, 1:200 for collagen type IX, 1:100 for von Willebrand factor) in TBST containing 2% HSA (2 h), (4) 2% HSA in TBST (10 min), and (5) secondary 15 nm gold-coupled goat anti-rabbit IgG (AuroProbe EM GAR15, GE Healthcare, Buckinghamshire, UK), or 18 nm gold-coupled anti-guinea-pig IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1:20 in TBST containing 2% HSA and polyethylene glycol (5 mg/ml, 2 h). The sections were rinsed well between steps (3)-(5), counterstained by uranyl acetate and lead citrate, and examined in a JEOL 1200CX electron microscope.

All studies were carried out in accordance with Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Every effort was made to minimize the number of animals used and their suffering.

RESULTS

Crym protein was localized in type II fibrocytes of the spiral ligament in the cochlea in mice and rats (Fig. 1A). In rats, it was also distributed in the stria vascularis (Fig. 1B). Ultrastructurally, Crym protein was localized in the nucleus