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Expression of full-length Cochlin p63s is inner ear specific

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Abstract

Objective: The *COCH* gene mutated in DFNA9, murine an autosomal dominant hereditary hearing impairment, encodes Cochlin. Cochlin is also suggested to be the self-antigen of autoimmune sensorineural hearing loss. We previously reported that Cochlin constitutes 70% of the inner ear proteins and is classified into three types of isoform, p63s, p44s, and p40s. To study the specificity of expression of Cochlin isoforms in various organs, here we have investigated expression of the *COCH* gene at both the transcriptional and translational level.
Methods: *COCH* gene expression was studied by RT-PCR and Southern blot analysis. Cochlin isoforms were studied by Western blot analysis using an isoform specific antibody.
Results: At the transcriptional level, *COCH* mRNA was detected only in the inner ear by RT-PCR. Southern blot analysis of RT-PCR products detected a high level of *COCH* mRNA in the inner ear, lower level in spleen, and very low levels in the cerebrum, cerebellum/brain stem, eye, liver and kidney. At the translational level, Western blot analysis showed that a set of isoform, p63s, p44s, and p40s was detected at high levels only in the inner ear. In contrast, multiple proteins were detected at much lower levels in other organs tested. Notably, full-length Cochlin p63s was detected only in the inner ear.
Conclusion: Our findings demonstrate that the *COCH* gene is expressed preferentially in the inner ear and that expression of full-length Cochlin p63s is specific to the inner ear. These results will be central to understanding the function of Cochlin and its role in the pathophysiology of DFNA9.
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Keywords: Hereditary hearing impairment; DFNA9; Cochlin; Inner ear; Isoform; Oragan specificity

1. Introduction

The *COCH* gene, which is mutated in DFNA9 [1–7], an autosomal dominant hereditary sensorineural hearing loss and vestibular disorder, was initially isolated by organ-specific and subtractive approaches [8] and found to be expressed abundantly in the human inner ear [9]. With the aim of elucidating the pathogenesis of DFNA9, we have been focusing on the formation of the different Cochlin isoforms. We have previously shown that Cochlin constitutes 70% of the inner ear proteins and is composed of 16 isoforms that are heterogeneous in charge and size [9]. The Cochlin isoforms can be broadly classified into three types, p63s, p44s, and p40s, according to their molecular weight. Subsequent studies using Cochlin-specific antibodies have identified a novel short isoform, named Cochlin-tomoprotein, in the perilymph [10].

Since Cochlin has been shown to be a self-antigen of T-cell-mediated inner ear specific autoimmune disease [11], studying the patterns of *COCH* gene expression is also important to elucidate the mechanism of immunological inner ear diseases. To better understand the function of Cochlin and its role in the pathophysiology of DFNA9, here we investigated the specificity of the expression of Cochlin isoforms in various organs. The expression of the *COCH* gene was studied at both the transcriptional and translational level in various organs using highly sensitive detection techniques, RT-PCR and Southern blot analysis

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to study the expression at the transcriptional level, and Western blot with Cochlin isoform specific antibody and chemiluminescence detection to study the expression at the translational level.

2. Materials and methods

2.1. Animals

Tissues from Wistar rats (6-week-old females weighing 150 g) were removed under deep anesthesia with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.). The bovine temporal bones were purchased from a slaughterhouse. This study was approved by the Animal Experimentation Ethics Committee of Nippon Medical School.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs from various organs of rats were prepared by using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA from each organ was reverse-transcribed by using Avian Myeloblastosis Virus (AMV) Reverse Transcriptase XL (Takara, Tokyo, Japan) and random primers for 10 min at 30 °C, 30 min at 42 °C, and 5 min at 99 °C (1 cycle). PCR was carried out by using Takara Taq (Takara) and the following protocol: a hot start at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The length of the RT-PCR product was 639 bp and the following p63s specific primers were used (Fig. 1a): sense primer (5′-GTTCCCATTCCTGTCACCTG-3′) corresponding to nucleotides 157–176 in the mouse *COCH* cDNA and amino acid (AA) residues 30 to 35/36 (VPIPVT(C)); antisense primer (5′-TTACCCCCTCGGAAACCTACTT-3′) corresponding to nucleotides 774–795 in the mouse *COCH* cDNA and AA residues 236/237–242/243 ((E)VGFRGG(N)). As a negative control (NC), duplicate cDNA templates were prepared as above without AMV Reverse Transcriptase XL and used in equivalent PCR reactions. PCR products were stained with ethidium bromide (Et–Br).

2.3. Southern blot analysis of RT-PCR products

The RT-PCR products generated from *COCH* cDNA were separated onto 1% agarose gels and transferred to a Hybond N+ membrane (Amersham Biosciences). The blot was hybridized with a non-radio isotope probe according to the manufacturer's instructions and detected with the CDP-star kit (Amersham Biosciences). The probe using this assay was derived from a human expressed sequence tag (EST) clone, IMAGE ID 27789, was obtained from Kurabo Co (Osaka. Japan). The *COCH* cDNA was subcloned from lafmid BA into the pGEM-T easy vector (Promega) by PCR using primers S2 (5′-GCCGCTCCCATTGCTATCACAT-3′), corresponding to AA residues 27–33 (AAPIAIT); and AS2 (5′-TACTCCAGCATCTACCGTGAAG-3′) corresponding to the AA residues 255–262 (FFTVDAGV) (Fig. 1b). The sequence of plasmid pGEM-T-COCH DNA was determined by using the primers S2 and AS2. A digested 708-bp *COCH* DNA was labeled by using an Alkphos direct labeling kit (Amersham Biosciences, Piscataway, NJ, USA). The band

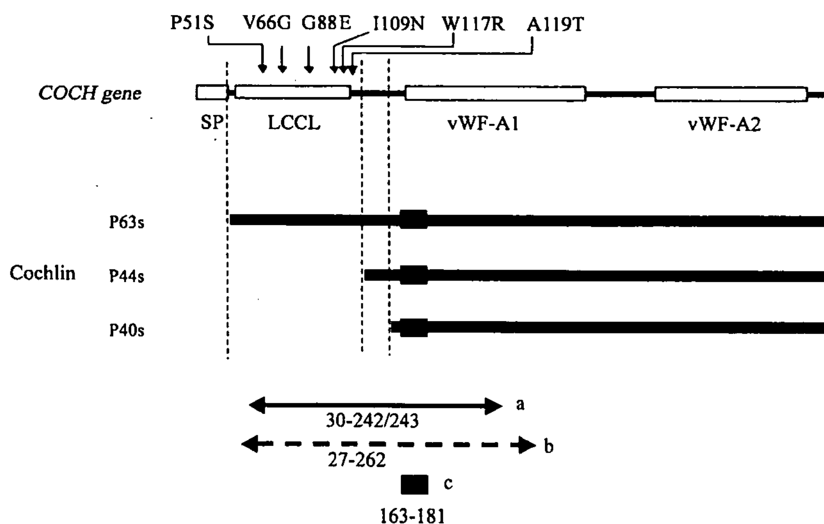

Fig. 1. Antigenic peptide, RT-PCR primer, Southern blot probe and cochlin isoforms. The deduced amino acid sequence of the human *COCH* gene, which encodes the protein Cochlin, shows a predicted signal peptide (SP), followed by a region homologous to a domain in factor C of Limulus, and two vWFA-like domains (vWFA1 and vWFA2). Six missense mutations in the LCCL domain, which cause the DFNA9 deafness and vestibular disorder, are indicated by arrows. (a) The region of the *COCH* cDNA (639 bp) amplified by RT-PCR (AA 30–242/243) contains the LCCL domain. (b) The region of the *COCH* cDNA (a 708 bp) containing human AA 27–262 was used as a probe for Southern hybridization. (c) The peptide sequence corresponding to this region of *COCH* cDNA (AA 163–181) was used as an immunogen to develop an anti-Cochlin antibody.

densities in the three representative blots were quantified by densitometry (CS Analyzer version 2.0; Atto, Tokyo, Japan) and the means and standard deviations of relative ratio with the inner ear signal were calculated.

2.4. *Anti-Cochllin antibody, gel electrophoresis and Western blot analysis*

Generation of an anti-Cochlin antibody, gel electrophoresis and Western blot analysis were performed as previously described [10]. In brief, a 19-mer peptide (KADIA-FLIDGSFNIGQRRF) corresponding to residues 163–181 in the vWF-A1 domain (Fig. 1c) was used to generate an anti-Cochlin antibody (formerly called anti-vWF-A1 antibody). Rabbits were immunized by repeated subcutaneous injections of the keyhole limpet hemocyanin (KLH)-coupled peptide. The serum was purified by a protein column, followed by peptide-affinity chromatography. The specificity of the antibody has been reported previously [10].

We homogenized various rat organs and bovine inner ear in a solubilization mixture containing 0.5% SDS and protease inhibitors. The homogenate was centrifuged and the protein concentration of the supernatant was measured by BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). First, we used 2 ug of the protein from each sample for colorimetric detection. Second, to achieve higher sensitivity, 0.5 ug of inner ear proteins and 50 ug of proteins from other organs were used for chemiluminescence detection. The proteins were resolved by electrophoresis using 15% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated for 2 h with the primary antibody diluted 1:2000 and then incubated for 1 h with a horse radish peroxidase-labeled goat anti-rabbit IgG diluted 1:1000. The blots were developed either with 0.5 mg/ml 3,3′-diaminobenzidine (DAB) in 50 mM Tris Buffer (pH7.6) containing 0.03% hydrogen peroxide or with a chemiluminescence reaction kit (ECL plus, Amersham). The band densities in the three representative blots were quantified by densitometry and the means and standard deviations of relative ratio with inner ear signal were calculated.

3. Results

3.1. *COCH gene expression at the transcriptional level*

We analyzed the expression of *COCH* mRNA in various organs by RT-PCR using primers specific to the isoform p63s. *COCH* mRNA was detected in the inner ear, but not in any of the other organs tested (Fig. 2a). Band intensity values after 25 cycles correlated linearly with the amount of template RNA used in the PCR reaction (data not shown). As a loading control, *GAPDH* mRNA was amplified in all organs in similar amounts.

As a more sensitive assay to detect minimal amounts of *COCH* mRNA, we performed Southern blot analysis.

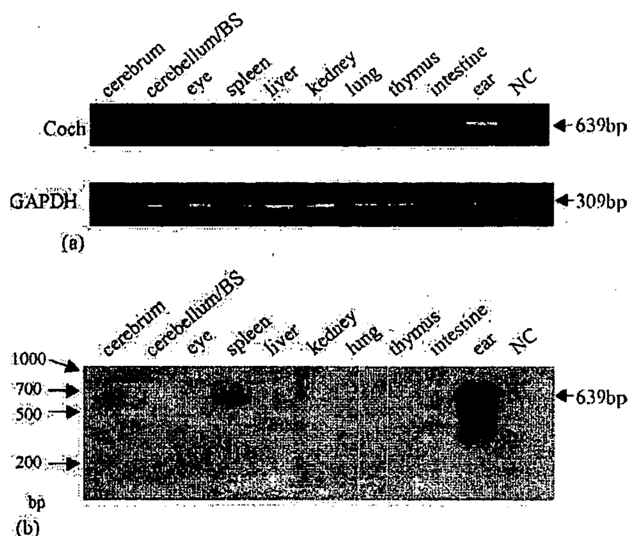

Fig. 2. *COCH* gene expression at the transcriptional level: (a) RT-PCR. PCR products stained with Et-Br. Expression of *COCH* mRNA was detected only in the inner ear, and not in any other organs tested. As a loading control, *GAPDH* was amplified in all organs in similar amounts. (b) Southern blot analysis. *COCH* mRNA was detected in high levels in the inner ear, lower level in spleen (77.9 ± 19.5%), and very low levels in the cerebrum (16.2 ± 12.0%), cerebellum/brain stem (BS) (1.4 ± 0.9%), eye (3.9 ± 0.7%), liver (1.8 ± 0.6%) and kidney (8.9 ± 8.5%). No signals were detected in the lung, thymus or intestine.

A human *COCH* cDNA fragment derived from an EST clone was used as a probe. The band densities in the three representative blots were quantified by densitometry. We detected a high level of *COCH* mRNA in the inner ear, lower level in the spleen (77.9 ± 19.5%), and very low levels in the cerebrum (16.2 ± 12.0%), cerebellum/brain stem (BS) (1.4 ± 0.9%), eye (3.9 ± 0.7%), liver (1.8 ± 0.6%) and kidney (8.9 ± 8.5%) (Fig. 2b). No signals were detected in the lung, thymus or intestine.

3.2. *COCH gene expression at the translational level*

We first analyzed an equal amount of proteins (2 ug) from each organ by Western blot analysis coupled with colorimetric detection (DAB). A set of immunoreactive proteins with sizes of 63, 44 and 40 kDa were observed in the rat and bovine inner ear. In contrast, no immunoreactive proteins were detected in any other organs tested (Fig. 3a).

Next we focused on the cerebrum, cerebellum/BS, eye, spleen, liver, kidney and inner ear, which showed positive signals by Southern blot analysis. We used 100 times more protein (50 ug) from these organs as compared with the inner ear (0.5 ug), and used an enhanced chemiluminescence detection technique (ECL plus) to achieve higher sensitivity. A set of immunoreactive proteins with sizes of 63, 44 and 40 kDa were detected at high levels only in the inner ear. In contrast, multiple proteins were detected at much lower levels in other organs tested. Proteins of 200, 90 and 40 kDa were detected in the cerebrum and cerebellum/BS, proteins

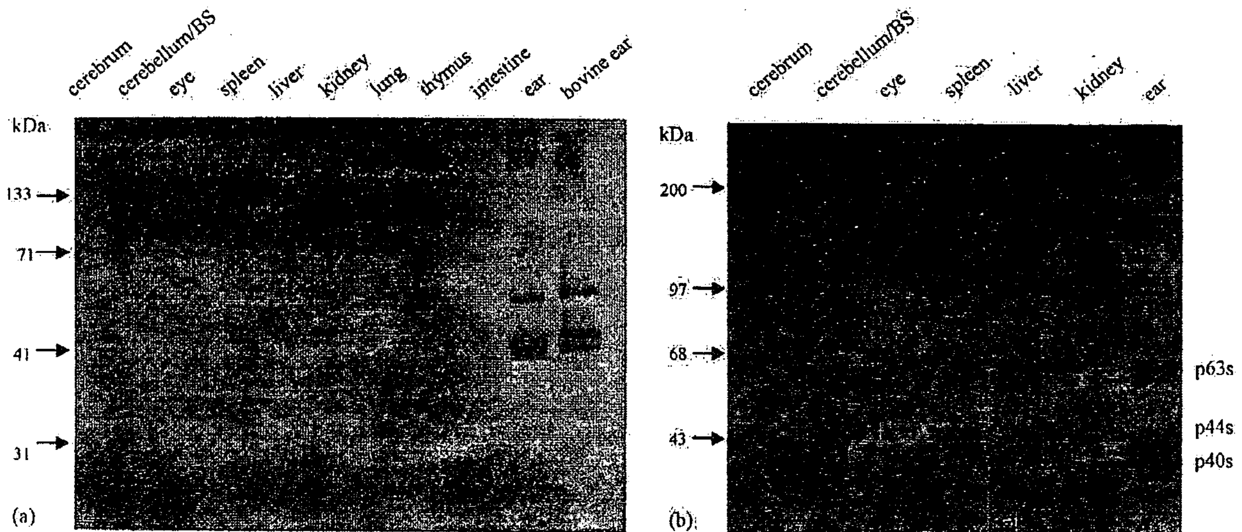

Fig. 3. *COCH* gene expression at the translational level: (a) Equal amounts of proteins (2 ug) were analyzed by immunoblot using colorimetric detection (DAB). A set of immunoreactive proteins of 63, 44 and 40 kDa was observed in the rat and bovine inner ear. In contrast, no immunoreactive proteins were detected in any other organs tested. (b) The organs which showed positive signal in Southern blot analysis, were analyzed at higher sensitivity. We used 100-fold larger amount of proteins (50 ug) of these organs, as compared with the inner ear (0.5 ug), and used enhanced chemiluminescence technique (ECL plus) to achieve higher sensitivity. A set of immunoreactive proteins of 63, 44 and 40 kDa was detected at high levels only in the inner ear. In contrast, multiple proteins were detected at much lower levels in the other organs tested. Proteins of 200, 90 and 40 kDa were detected in the cerebrum and cerebellum/BS, proteins of 80, 55, 40 and 36 kDa were detected in the spleen and liver, and proteins of 80, 55 and 40 kDa were detected in the eye and kidney. Notably, proteins with a molecular mass of 63 kDa corresponding to full-length Cochlin were detected only in the inner ear. We measured intensities of bands in the three representative Western blots. The relative amount of Cochlin in non-inner ear organs were as follows; cerebrum: 65.7 ± 32.3% (mean ± S.D.), cerebellum/BS: 58.7 ± 22.8%, eye: 14.2 ± 0.7%, spleen: 18.0 ± 14.3%, liver: 18.8 ± 12.5%, kidney: 16.3 ± 3.3%.

of 80, 55, 40 and 36 kDa were detected in the spleen and liver, and proteins of 80, 55 and 40 kDa were detected in the eye and kidney. Notably, proteins with a molecular mass of 63 kDa corresponding to full-length Cochlin were detected only in the inner ear (Fig. 3b).

We measured intensities of bands in the three representative Western blots. The relative amount of Cochlin in non-inner ear organs were as follows; cerebrum: 65.7 ± 32.3% (mean ± S.D.), cerebellum/BS: 58.7 ± 22.8%, eye: 14.2 ± 0.7%, spleen: 18.0 ± 14.3%, liver: 18.8 ± 12.5%, kidney: 16.3 ± 3.3%.

4. Discussion

The expression of the genes associated with nonsyndromic deafness is, in general, not restricted to the inner ear. For example, the gene encoding Connexin26, which is mutated in DFNA3 and DFNB1, is known to be expressed in the liver, kidney, intestine, lung, spleen, stomach, testis, and brain, as demonstrated by Northern blot analysis [12].

By contrast, the *COCH* gene was initially isolated by inner ear-specific and subtractive approaches [8]. Expression of the human *COCH* gene was previously studied by Northern blot analysis [8] and by a microarray expression profile analysis of the inner ear [13], proteomic anlysis [9] which showed that *COCH* is highly expressed in the inner ear, in agreement with our present results. Northern blot analysis of mouse organs detected expression of *COCH* mRNA at a high level in the spleen, lower levels in the cerebrum, cerebellum/medulla, and thymus, and faint levels in the eye and lung [14], whereas we did not detect mRNA in the thymus and lung tissues in rat by RT-PCR coupled with Southern blot. This discrepancy may be due to species-specific differences in *COCH* expression.

Cochlin expression at the translational level of mouse ear and spleen has also been reported [15]. Using a rabbit polyclonal antibody against the carboxyl terminus of Cochlin (AA residues 529–544), four proteins of 60, 40, 32 and 18 kDa were detected in the mouse inner ear, and two proteins of 45 and 40 kDa were detected in the spleen. The largest protein of 60 kDa detected in the inner ear, presumably the full-length Cochlin, was not detected in the spleen. These data also support our results to show the full-length Cochlin expression is the inner ear specific. There is a difference between the relative amount of mRNA and protein expression. Especially in the spleen, the *COCH* mRNA was 78%, and Cochlin was 0.18% of that of the inner ear. The discrepancy between the amount of mRNA expression and protein product should be due to the posttranscriptional regulation of *COCH* gene expression and protein metabolism, as often seen in other genes [16].

In the present study, the RT-PCR product was designed to include the LCCL domain [17], which is specific to p63s. Southern blot showed that *COCH* mRNA including this domain is expressed in the cerebrum, cerebellum/BS, eye,

spleen, liver, kidney; however, the full-length Cochlin p63s were detected only in the inner ear by Western blot. This finding suggests that *COCH* is differentially processed in different organs and that the regulation of this processing is organ-specific as previously suggested [15]. Two transcripts approximately 2.0 and 2.5 kb in size were detected with full-length mouse *COCH* cDNA probe [14,15], and the size of the transcripts were the same in the inner ear and in other organs. These two transcripts contain the open reading frame of *COCH* gene. These data indicate that the posttranslational modifications, such as proteolysis at peptide bond or the action of a 'converting enzyme', are mainly responsible for the multiple proteins detected in the present study, especially those smaller than the 63 kDa: full-length Cochlin. There are other possibilities that might explain the formation of those proteins; for example, proteins could be the product of chemical modification of Cochlin such as glycosylation, phosphorylation and/or deamination, or coded from a *COCH* homologue, or products of degradation.

Because the mutations associated with DFNA9 affect only the full-length Cochlin p63s, and not the processed Cochlin isoforms (p44s and p40s) [9], studying the expression patterns of the Cochlin isoforms will help us to investigate the function of Cochlin and the pathogenesis of DFNA9. Cochlin expression specificity is also important to understand the pathophysiology of autoimmune sensorineural hearing loss. Recently, Cochlin has been shown to be a self-antigen of T-cell-mediated autoimmune sensorineural hearing loss in animal model [11]. Our data also support the idea that Cochlin is the possible target antigen of the organ specific autoimmune disorder of the inner ear.

5. Conclusion

At a transcriptional level, *COCH* mRNAs were highly expressed in the inner ear. At a translational level, full-length Cochlin p63s was detected only in the inner ear. Our findings demonstrate that the *COCH* gene is expressed preferentially in the inner ear and that expression of full-length Cochlin p63s is specific to the inner ear. This study provides novel and important information concerning the expression of full-length Cochlin p63s and the differential expression of Cochlin isoforms in the inner ear and non-inner ear organs.

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Expression of Cochlin in the Vestibular Organ of Rats

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Key Words
Hereditary hearing impairment · DFNA9 · COCH gene · Cochlin · Inner ear · Vestibular organ of rats · Immunohistochemistry

Abstract
The COCH gene mutated in autosomal dominant sensorineural deafness (DFNA9) encodes cochlin, a major constituent of the inner ear extracellular matrix. Cochlin constitutes 70% of the inner ear protein and cochlin isoforms can be classified into three subgroups, p63s, p44s and p40s. Symptoms of some DFNA9 patients are consistent with those of Ménière's disease. Here, we report the expression of cochlin in the vestibular organ of rats using isoform-specific antibodies that recognize all three isoforms. Cochlin is highly expressed in the stromata of the maculae of otolithic organs and cristae of semicircular canals, and in the channels in the bony labyrinth that transmit the dendritic innervation to the cristae and maculae. Cochlin cannot be detected in the sensory cells, dark cells, nor in the acellular structures, otolithic membrane or in the cupula. These findings support the theory that deposition of acidophilic substance in the inner ear caused by mutation of cochlin can induce a secondary retrograde dendritic degeneration of the vestibular nerves.

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Introduction

The COCH gene mutated in autosomal dominant sensorineural deafness (DFNA9) encodes cochlin, a major constituent of the inner ear extracellular matrix [1, 2]. Sequence analysis of cochlin from DFNA9 patients demonstrated the existence of six missense mutations and one in-frame deletion mutation within a conserved region (the LCCL domain) of cochlin [3]. DFNA9 is characterized by a hereditary progressive sensorineural hearing loss and concomitant vestibular impairment. The onset of symptoms is relatively late and eventually leads to profound deafness and vestibular failure [1, 4–10]. Vertigo attacks may occur and progressive hearing loss may show fluctuations and asymmetry. Clinically, these symptoms of DFNA9 are consistent with the symptoms characteristic of Ménière's disease [9, 10].

Cochlin has several unique characteristics. In order to characterize gene products associated with deafness, we previously performed a proteomic analysis of bovine inner ear proteins [2]. Our results showed that the protein product of the bovine COCH gene constitutes 70% of the inner ear proteins and that cochlin isoforms can be classified into three groups, namely p63s, p44s and p40s, on the basis of their molecular weight. In further studies using isoform-specific antibodies, we demonstrated that human cochlin isoforms are also expressed as p63s, p44s

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and p40s, and that there is an additional isoform, a shortened 16-kDa cochlin tomoprotein in the perilymph [11]. Recently in studies using animal models, cochlin has been shown to be a self-antigen of T-cell-mediated autoimmune sensorineural hearing loss [12]. The pattern of inner ear restricted expression of the full-length cochlin p63 also supports the idea that cochlin is the possible target antigen in organ-specific autoimmune disorders of the inner ear [13].

Histopathologic examinations of temporal bones have revealed the presence of acidophilic deposits in the cochlea, macula, and crista of DFNA9 patients [5, 14]. There was also severe degeneration of the vestibular and cochlear sensory elements and dendrites. In situ hybridization studies to detect cochlin in the inner ear of human embryos and immunohistochemical studies of human adult cochlea have revealed that cochlin is expressed at similar sites as acidophilic deposits in DFNA9 patients [15]. These findings have led to the hypothesis that these deposits may cause strangulation of the nerve endings [1, 5, 14].

Despite the fact that the cochlear and vestibular structures are embryologically, anatomically and functionally closely related, only a very small number of hereditary disorders affect both cochlear as well as vestibular function. Until now, DFNA9 is the only autosomal dominant type of nonsyndromic sensorineural hearing impairment with concomitant vestibular impairment [2]. COCH mRNA expression in the crista ampullaris has been studied histologically by in situ hybridization using COCH antisense riboprobes in chicken [1] and human fetal inner ear [15]. However, the expression of COCH protein, i.e. cochlin, in the semicircular canals and otolithic organs has not yet been studied. To facilitate the understanding of spatial specificity of cochlin expression in the normal vestibular organ and in the pathophysiology of vestibular disorders of DFNA9, we report the expression of cochlin in the vestibular organ of the rat using isoform-specific antibodies that recognize all the three isoforms detected in the inner ear.

Materials and Methods

Animals

Temporal bones of 4 PVG rats (Saitama Experimental Animal Supply, Saitama, Japan) were used in this study. For the experimental procedure, each rat was deeply anesthetized with a combination of sodium pentobarbital (Nembutal, 30 mg/kg, i.p.) and fentanyl-droperidol (Fentanest, 0.3 ml/kg, i.m.) according to the ethical regulations for treatment of animals.

Histological Examination

Rats were sacrificed after intracardiac perfusion with cold saline followed by 4% periodate lysine paraformaldehyde fixative (PLP). The temporal bones were excised and fixed in PLP for 3 h. Temporal bones were then decalcified in phosphate-buffered saline with 5% EDTA at –4°C for 7 days. Temporal bones were embedded in paraffin, and serial sections of 3–4 μm thickness were cut using a microtome (Rotationsmikrotom, Leika, Nussloch, Germany).

Anticochlin Antibody (Anti-vWF-A1 Antibody)

We have reported about the generation of four rabbit anticochlin polyclonal antibodies to recognize distinct domains of cochlin, namely anti-LCCL-N, anti-LCCL-C, anti-ivd1 and the anti-vWF-A1 [12]. In this study, we used the anti-vWF-A1 antibody since this antibody recognizes all the three cochlin isoforms. Hereafter, this anti-vWF-A1 antibody which was used in this study to detect cochlin will be referred to as the anticochlin antibody. The generation of this antibody has previously been described [11]. Using the amino acid sequence of the bovine cochlin isoforms, we designed the antigenic peptide. A 19-mer (KADIAFLIDGSF-NIGQRRF) peptide corresponding to residues 163–181 in the vWF-A1 domain was used to generate antibody. This sequence is present in all three cochlin isoforms and is completely homologous with the sequences in both human and mouse proteins, as deduced from the sequence of the corresponding genes and the sequence of bovine cochlin [11]. Rabbits were immunized and the serum was purified by a protein A column, followed by peptide-affinity chromatography. The specificity of the antibodies for the corresponding antigenic peptides was confirmed by dot blot analysis and a peptide absorption test (data not shown).

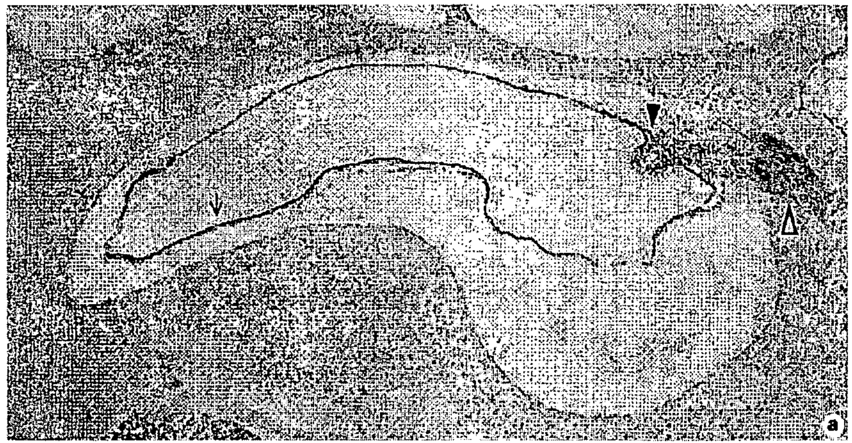
Immunohistochemistry for the Detection of Cochlin

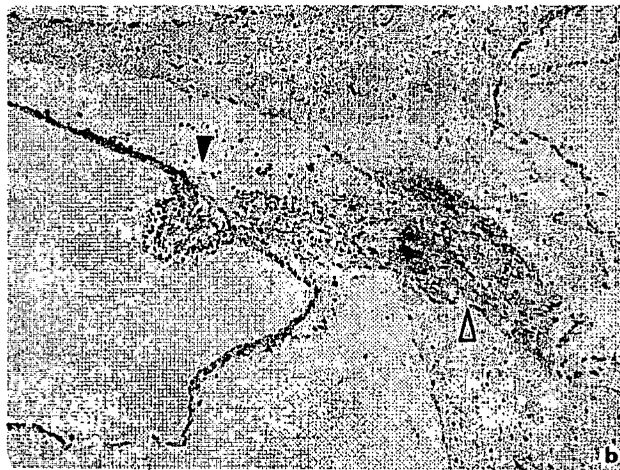
The immunoperoxidase method was used for the detection of cochlin. Paraffin sections were deparaffinized, and the sections were then treated with 0.3% H_2O_2 in methanol in order to block the endogenous peroxidase activity. After treatment with 10% normal goat serum, the sections were incubated with 1:1,000 dilution of the anticochlin antibody overnight at 4°C. The sections were then incubated for 60 min with the biotinylated goat antirabbit IgG antibody and then treated with the streptavidin-biotin-peroxidase complex. Between each incubation the sections were rinsed in phosphate-buffered saline. Finally, the reaction was visualized using the substrate 3,3′-diaminobenzidine in H_2O_2, and after rinsing twice in distilled water, the sections were counterstained with Mayer's hematoxylin. Preimmune serum was used instead of the primary antibody as negative control.

Results

Semicircular Canals

Representative immunohistochemistry of cochlin expression in the posterior semicircular canals is shown in figure 1. The lining of the membranous labyrinth showed immunoreactivity to the antibody (arrow). The stroma of the crista showed diffuse staining (arrowhead). The nerve beneath the crista was negative, but the channels in the





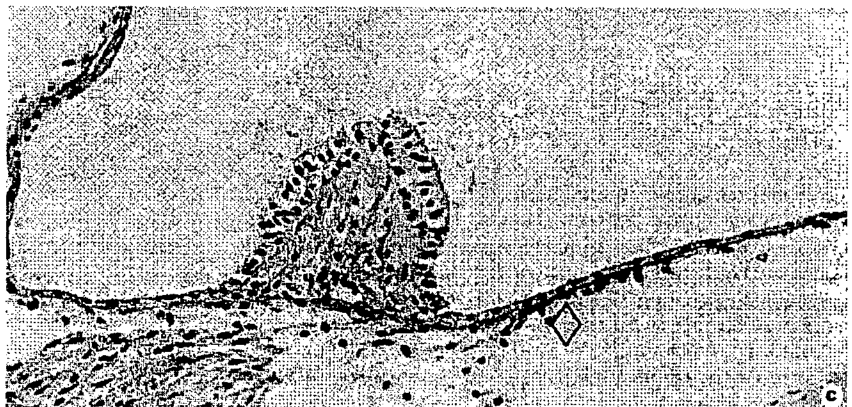
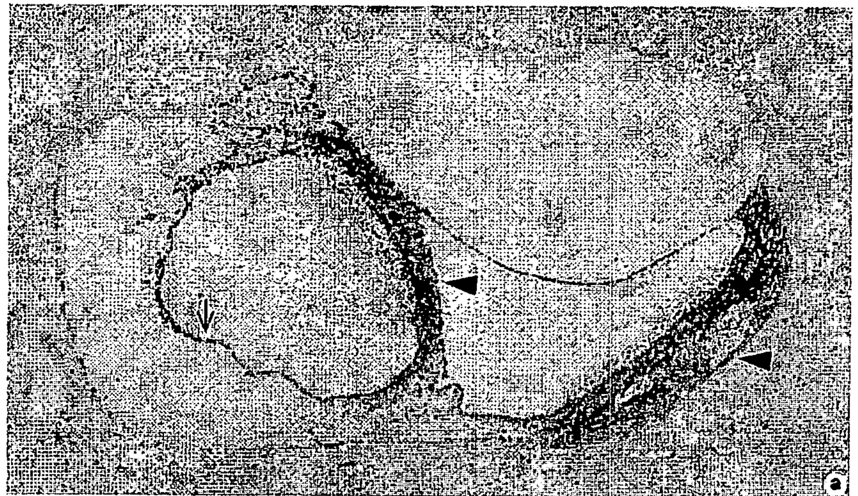
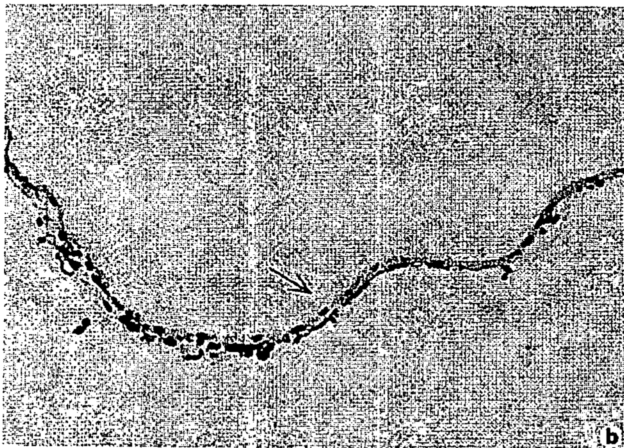
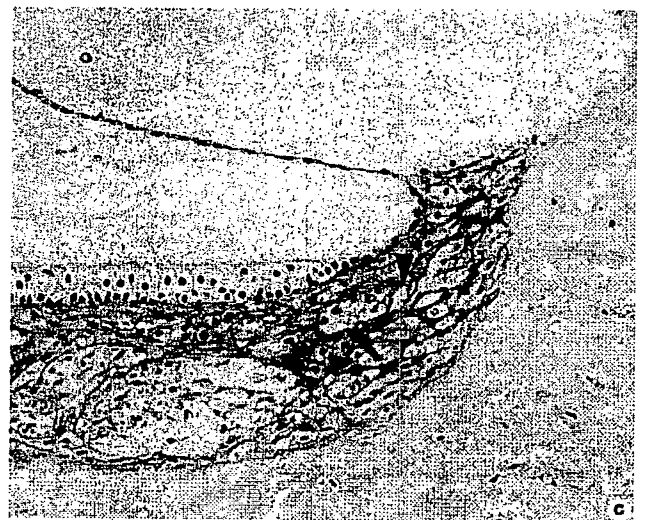


Fig. 1. Representative immunohistochemistry of cochlin expression in the posterior semicircular canals. **a** The lining of the membranous labyrinth showed immunoreactivity to the antibody (arrow). The stroma of the crista showed diffuse staining (arrowhead). The nerve beneath the crista was negative, but the channels in the bony labyrinth that transmit the dendritic innervation to the crista ampullaris were positively stained (open arrowhead). Original magnification ×4. **b** Magnified image of **a**. Original magnification ×20. **c** The stroma of the crista showed diffuse staining. Positive staining was marked in the area beneath the planum semilunatum, dark cells and neurosensory epithelium, especially under the dark cells (open diamond). The apical surface of the sensory epithelia, including the cupula, subcupular space, sensory cells, transitional cells, dark cells, and the supporting cells did not show any immunoreactivity to this antibody. Original magnification ×40.







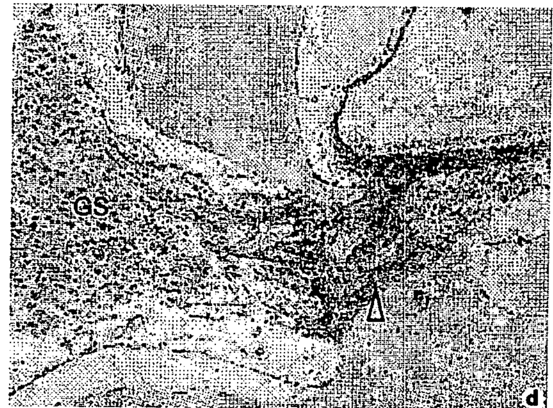


Fig. 2. Representative immunohistochemistry of cochlin expression in the maculae. **a** The stromata of all maculae were positively stained (arrowhead). The lining of the membranous labyrinth was stained with the antibody (arrow). Original magnification ×4. **b** Magnified image of **a**. Original magnification ×20. **c** Magnified image of **a**. Stroma of saccular macula was positively stained (arrowhead). The apical surface of sensory epithelia, including otoconia, gelatin layer, submembranous space, sensory cells, and the supporting cells did not stain positively with the anticochlin antibody. Original magnification ×40. **d** The channels in the bony labyrinth that transmit the dendritic innervation to the macula were stained (open arrowhead). The nerve beneath the maculae, ganglion cells (GS) were negative. Original magnification ×10.

bony labyrinth that transmit the dendritic innervation to the crista ampullaris were positively stained (open arrowhead, fig. 1a, b).

Positive staining in the stroma was marked in the area beneath the planum semilunatum, dark cells and neurosensory epithelium, especially under the dark cells (open diamond). On the other hand, the apical surface of the sensory epithelia, including the cupula, subcupular space, sensory cells, transitional cells, dark cells, and the supporting cells did not show any immunoreactivity to this antibody (fig. 1c).

Negative controls did not show immunoreactivity for cochlin in any of these structures.

Otolithic Organs

Representative immunohistochemistry of cochlin expression in the maculae is shown in figure 2. The stromata of all maculae were positively stained (arrowhead). The lining of the membranous labyrinth was stained with the antibody (arrow, fig. 1a, b).

Stroma of saccular macula was positively stained (arrowhead, fig. 1c). On the other hand, the apical surface of sensory epithelia, including otoconia, gelatin layer, submembranous space, sensory cells, and the supporting cells did not stain positively with the anticochlin antibody. The channels in the bony labyrinth that transmit the dendritic innervation to the macula were stained (open arrowhead). The nerve beneath the maculae, ganglion cells were negative (fig. 1d).

Negative controls did not show immunoreactivity for cochlin in any of these structures.

Discussion

In the present study using a highly specific anticochlin antibody, we have demonstrated that cochlin is highly expressed in the stromata of the maculae of otolithic organs and crista ampullaris, and in the channels in the bony labyrinth that transmit the dendritic innervation to the cristae and maculae. Cochlin was not detected in the sensory cells, dark cells, or in the acellular structures, otolithic membrane and cupula. COCH mRNA expression in the crista ampullaris has been studied histologically by in situ hybridization [1, 15]. COCH mRNA was detected in the stromal cells underlying the sensory epithelium and in the surrounding wall of the semicircular canals. These results are in concert with our present study.

The consistent finding in the DFNA9 subjects is the loss of cellularity and deposition of acidophilic substance in the supporting structures of the auditory and vestibular labyrinths and into the dendritic nerve channels. The infiltrate is seen in the spiral ligament, limbus and stroma of the maculae and cristae where it is associated with loss of cellularity. It is also located in the nerve channels of the osseous spiral lamina and the cribrose areas for the vestibular nerve fibers [5].

In the vestibule, the otolithic membranes show an irregular granular degenerative change with basophilic staining deposits lying on a sensory epithelium with scattered areas of severe hair cell loss. There is thickening and collapse of the ampullary walls and semicircular ducts. The cellularity of the stromata of the maculae and cristae is diminished and replaced by the acidophilic material and is accompanied by a severe loss of dendritic nerve fibers [5, 14, 16].

It seems probable that the severe degeneration of the underlying stroma may have led to these alterations in the sense organs. It is assumed that dendritic fibers atrophied because of the homogenous acidophilic deposits have caused either some or all of the following mechanisms: pressure on the fibers, alterations of their fluid environment, interference with the blood supply. These mechanisms caused severe retrograde dendritic degeneration of the cochlear and vestibular nerves [5].

Our histological findings in the rat vestibular organ also support this theory, since cochlin was detected only in the supporting structures of the maculae and cristae, and not in the sensory cells, dark cells, nerves, ganglion cells.

The sequence of events leading to deafness and dysfunction of balance in DFNA9, the exact composition of the acidophilic material and the relationship to cellular and neuronal atrophy, whether deposition is the cause or the result of structural impairment remain to be elucidated. Immunohistochemical studies of the inner ear of DFNA9 subjects using anticochlin antibodies need to be done.

Immunohistochemical analysis of human and rodent fetal ears and adult rodent ears shows that type II collagen is present extensively in the spiral ligament, spiral limbus, maculae and cristae, areas that correspond to the cochlin expression [17–19]. Mature cochlin is a modular polypeptide consisting of LCCL domain and two vWFA-like domains. vWFA-like domains are found in a large number of proteins, the majority of which are components of the extracellular matrix [20]. The two vWFA modules in cochlin are likely to interact with other connective tissue elements of the cochlea and vestibule, such as type II collagen fibers that are expressed in the same areas of the inner ear as cochlin [1].

Recent electron microscopic examination of DFNA9 sections shows that normal fibrillogenesis is disrupted by an excess in microfibrillar substance, which results in degradation of collagens (such as type II) and extracellular matrix components [21]. The spatial correlation of cochlin expression and type II collagen in normal inner ears, and the abnormal acidophilic deposits in DFNA9 with degraded type II collagen suggest either a direct effect of the mutated protein in these locations, or possible altered binding of cochlin with other proteins such as the fibrillar collagens [15].

Progressive vestibular impairment is a prominent feature of DFNA9. Vestibular symptoms in DFNA9 develop simultaneously with, or slightly later than, hearing deterioration. There is remarkable intersubject variability in signs and symptoms. These signs and symptoms include: recurrent episodes of vertigo accompanied by nausea and vomiting, which resembles Ménière's disease, or special susceptibility for motion sickness and a hyperactive vestibulo-ocular reflex. More advanced stages of vestibular impairment, i.e. vestibular hyporeflexia and complete vestibular areflexia, were eventually found in a number of cases [1, 4–10].

The vestibular system in DFNA9 ears shows moderate to severe alterations consisting of thickening, atrophy and/or collapse of the ampullae and maculae. In the ears, the pathologic changes in the sense organs are more severe in the vestibular system than in the cochlea [5]. The mild vestibular symptoms in some of the affected subjects are probably related to the slow progression with which the degenerative change in the vestibular system occurs and/or compensatory mechanism of vestibular system.

The mutation site of the COCH gene also affects the vestibular symptoms. Mutation analysis of the COCH gene of one large Belgian and two small Dutch families revealed a missense mutation changing Pro to Ser (P51S) [10]. In all three DFNA9 families, >25% of the affected persons suffered from a number of additional symptoms, including recurrent episodes of vertigo, aural fullness, hearing loss and/or tinnitus. These symptoms are consistent with the criteria for Ménière's disease proposed by the American Academy of Otolaryngology and Head and Neck Surgery [22]. According to the criteria, presently the diagnosis of Ménière's disease is a 'diagnosis of exclusion'. Other than autopsy, there is no specific diagnostic test for the diagnosis of 'certain Ménière's disease'. After a full diagnostic workup to exclude other causes, such as perilymphatic fistula, vestibular schwannoma, autoimmune hearing loss, DFNA9, etc., a diagnosis of 'definite Ménière's disease' can be made. A correct differential diagnosis between Ménière's disease and DFNA9 is only possible by analyzing family relationships and/or genotyping. Patients with familial Ménière's disease might be screened for COCH mutations. In addition to the familial history, the audiogram is useful in the clinical setting. The hearing loss of DFNA9 usually starts in high frequencies as shown in the age-related typical audiogram [23]. The possibility of a COCH mutation should also be considered in sporadic Ménière's disease cases [10]. However, a recent report showed that 30 patients with definite Ménière's disease have no mutations in exons 4 and 5 of the COCH gene [24].

In many types of tissues, the extracellular matrix plays an essential role in building their own structures. The complex network of secreted extracellular macromolecules has many functions, but first and foremost it forms a supporting framework. Due to the more recent introduction of technological advances in molecular biology, several novel noncollagenous extracellular matrix proteins in the inner ear have been identified, such as cochlin, otogelin [25], tectorin alfa and beta [26]. At present, we do not know the precise function of these newly found extracellular matrix proteins. Studying these novel proteins will shed light on this new field of inner ear study.

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Cochlin immunostaining of inner ear pathologic deposits and proteomic analysis in DFNA9 deafness and vestibular dysfunction

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Seven missense mutations and one in-frame deletion mutation have been reported in the *co*agulation factor *C* *h*omology (*COCH*) gene, causing the adult-onset, progressive sensorineural hearing loss and vestibular disorder at the DFNA9 locus. Prevalence of *COCH* mutations worldwide is unknown, as there is no systematic screening effort for late-onset hearing disorders; however, to date, *COCH* mutations have been found on four continents and the possibility of *COCH* playing an important role in presbycusis and disorders of imbalance has been considered. Cochlin (encoded by *COCH*) has also been shown as a major target antigen for autoimmune sensorineural hearing loss. In this report, we present histopathology, immunohistochemistry and proteomic analyses of inner ear tissues from post-mortem DFNA9 temporal bone samples of an individual from a large Dutch kindred segregating the P51S mutation and adult human unaffected controls, and wild-type (+/+) and *Coch* null (−/−) knock-out mice. DFNA9 is an inner ear disorder with a unique histopathology showing loss of cellularity and aggregation of abundant homogeneous acellular eosinophilic deposits in the cochlear and vestibular labyrinths, similar to protein aggregation in well-known neurodegenerative disorders. By immunohistochemistry on the DFNA9 temporal bone sections, we have shown cochlin staining of the characteristic cochlear and vestibular deposits, indicating aggregation of cochlin in the same structures in which it is normally expressed. Proteomic analysis identified cochlin as the most abundant protein in mouse and human cochleae. The high-level expression and stability of cochlin in the inner ear, even in the absence and severe atrophy of the fibrocytes that normally express *COCH*, are shown through these studies and further elucidate the pathobiologic events occurring in DFNA9 leading to hearing loss and vestibular dysfunction.

INTRODUCTION

A large number of loci have been mapped for syndromic and non-syndromic hereditary hearing loss, and the gene mutations responsible for these disorders are being continually discovered and characterized (1). Elucidation of the functions of these genes and their roles in the inner ear and in pathogenesis of hearing and balance disorders are important ongoing endeavors.

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Table 1. *COCH* mutations in DFNA9

Origin	Exon with mutation	Nucleotide change[a]	Amino acid change[b]	Protein domain	References
The Netherlands[c]; Belgium[c]	4	C207T	P51S	FCH/LCCL	(11,12)
United States[c]	4	T253G	V66G	FCH/LCCL	(10)
United States[c]; The Netherlands[c]	5	G319A	G88E	FCH/LCCL	(10,14)
Hungary[d]	5	366_368delGTA	V104del	FCH/LCCL	(16)
Australia[c]	5	T382A	I109N	FCH/LCCL	(13)
United States[c]	5	T405C	W117R	FCH/LCCL	(10)
Japan[d]	5	G411A	A119T	FCH/LCCL	(15)
United States[c]	12	G1681T	C542F	vWFA2	(9)

[a]Numbering of nucleotides is according to the human *COCH* cDNA sequence (GenBank Accession No. AF006740), which starts in the 5′ untranslated region, 56 bp upstream of the start ATG.
[b]Numbering of amino acids begins at the start methionine.
[c]Familial (autosomal-dominant) cases.
[d]Simplex cases.

The autosomal-dominant deafness disorder at the DFNA9 locus has been described and the clinical aspects extensively characterized, showing adult-onset, progressive sensorineural hearing loss and vestibular dysfunction (2–9). Different missense mutations in the *COCH* (*co*agulation factor *C* *h*omology) gene were found initially in three families in the United States, and subsequently in families in the Netherlands, Belgium and Australia (Table 1 and Fig. 1) (10–14). Two simplex cases of another missense mutation and an in-frame deletion in the same domain of *COCH* [*f*actor *C*-*h*omology (FCH)/*l*imulus factor *C*, *c*ochlin, *l*ung gestational protein (LCCL) domain] have been reported in Japan and Hungary, respectively (15,16). A recent report describes the first finding of a *COCH* mutation outside of the FCH/LCCL domain in the von Willebrand factor A-like (vWFA) domain in a large DFNA9 kindred in the United States (9). The prevalence of *COCH* mutations worldwide is not known, as systematic genetic testing of adult-onset hearing loss is not currently performed.

COCH was isolated initially by organ-specific subtractive approaches from a human fetal cochlear cDNA library and found to be expressed at high levels in the inner ear by northern blot, tissue *in situ* hybridization and immunohistochemistry (17–20). The secreted protein, cochlin, was detected by proteomic analysis as the most abundant protein in the bovine inner ear (21). Histopathological analyses of DFNA9-affected temporal bones in the three original US families have revealed very valuable information about the endpoint changes in these inner ears (3,22). A striking and unique finding in these temporal bones, which actually allowed initial identification of several of these families as DFNA9 kindreds, is the presence of homogeneous extracellular eosinophilic deposits in the same areas as fibrocyte atrophy. Other well-characterized neurodegenerative disorders with aberrant protein accumulation include Alzheimer disease (β-amyloid precursor protein) (23), Huntington disease (huntingtin) (24–26) and Parkinson disease (α-synuclein) (27). However, DFNA9 is the only known inner ear disorder showing this type of aggregate as the signature pathological finding, whereas findings in other disorders of the inner ear, such as endolymphatic hydrops, and degeneration of structures, such as the sensory epithelium, ganglion cells, spiral ligament and the stria vascularis, are observed in a variety of different conditions showing hearing loss and vestibular dysfunction.

The cochlear and vestibular fibrocytes, which are severely atrophied in DFNA9, are cells expressing *COCH*, and the homogenous acellular deposits are found in the same areas as cochlin immunostaining in the normal inner ear (19). However, previous to this report, it has not been shown whether this eosinophilic substance in DFNA9 is the abnormal cochlin which has precipitated and aggregated or whether it is another component of the inner ear, a cochlin-interacting protein, or some other downstream effect of the *COCH* mutations.

A recent post-mortem donation of a temporal bone from an individual from the Netherlands with DFNA9 (*COCH* P51S mutation) provided the opportunity to obtain additional histopathological data for DFNA9. By using an antibody to the vWFA domain of cochlin, which detects all known size isoforms of cochlin (Fig. 1), we have undertaken a thorough characterization of cochlin immunostaining in the cochlear and vestibular labyrinths. We present proteomic analysis of DFNA9-affected and unaffected adult human temporal bone sections, as well as of wild-type (+/+) and *Coch* null (−/−) knock-out mouse inner ears. These studies have enabled examination of the content of the abnormal deposits seen in DFNA9 and provided insight into the role of cochlin in the inner ear and the mechanism of pathobiology underlying DFNA9 by *COCH* mutations.

RESULTS AND DISCUSSION

Histopathology of DFNA9 temporal bones with the P51S cochlin mutation

Histopathologic findings in the P51S DFNA9 temporal bone (Fig. 2) are consistent with those previously reported for other DFNA9 families designated 1W (V66G mutation), 1Su (G88E mutation) and 1St (W117R) (3,22,28), suggesting that the P51S mutation causes the same pathologic changes and acts via the same mechanism as the other mutations in the FCH/LCCL domain of *COCH*. A marked reduction in the number of fibrocytes is observed throughout the spiral

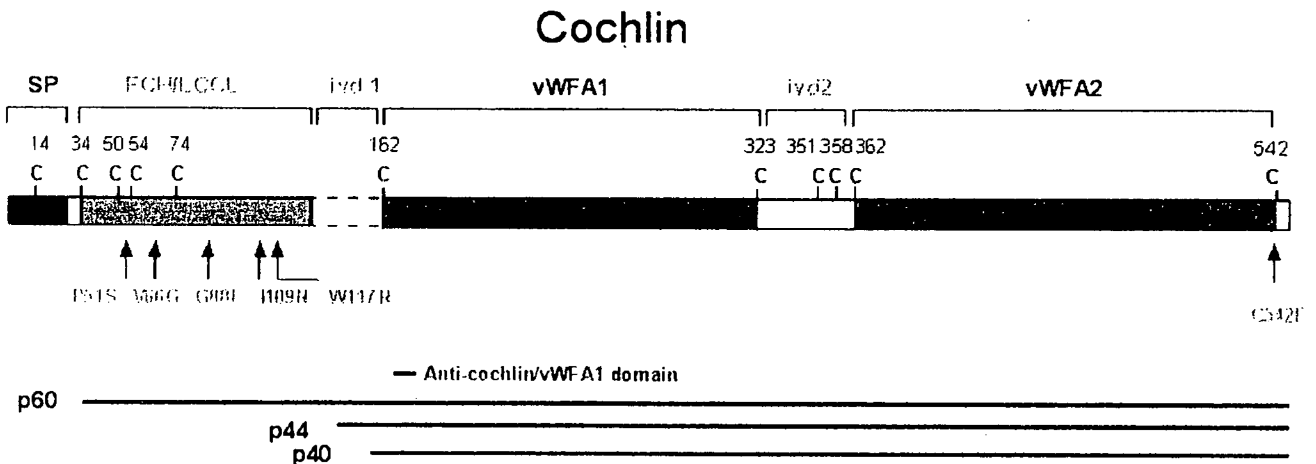

Figure 1. Schematic representation of the deduced amino acid structure of human *COCH*, encoding the protein cochlin, shows a predicted signal peptide (SP), followed by a domain initially designated as FCH, also known as the LCCL domain, followed by an intervening domain (ivd1) and two von Willebrand factor A-like domains (vWFA1 and vWFA2) separated by an intervening domain (ivd2). Six familial missense mutations (five in the FCH/LCCL domain and one in the vWFA2 domain) causing DFNA9 deafness and vestibular disorder are indicated by arrows. The positions of all cysteine residues are shown as 'C'. The three known isoforms of cochlin are represented by horizontal lines corresponding to their sequence and designated as p60 (full-length, excluding the SP) and two shorter isoforms (p44 and p40, both lacking the FCH/LCCL domain). The cochlin antibody used in this study was made against a small peptide in the N-terminus of the vWFA1 domain (amino acid residues 163–181), shown in the figure. This antibody recognizes all three isoforms of cochlin.

ligament and limbus of the cochlear duct and in the vestibular organs. In the same areas of fibrocyte loss, and atrophy is the DFNA9-characteristic eosinophilic-staining extracellular ground substance. The deposits are particularly prominent in the more medial parts of the ligament underlying the stria vascularis and in the area of the insertion of the ligament into the basilar membrane. Eosinophilic material is also present in the osseous spiral lamina, along with loss of dendrites in these channels and in the modiolus.

Cochlin immunostaining in normal inner ear

Prior to performing immunohistochemistry on DFNA9-affected temporal bones, we optimized anti-cochlin antibody staining on normal mouse and human adult tissues, as well as on a *Coch* (−/−) mouse (29,30). In our previous studies, we used a polyclonal antibody to the entire FCH/LCCL and ivd1 domain of cochlin (19). However, given different-sized isoforms of cochlin detected by proteomic analysis and N-terminal sequencing (21) (Fig. 1), four antibodies to small peptides in different regions of cochlin were developed and shown by western blot analysis to be specific for the isoforms that each was expected to recognize (31). The anti-cochlin antibody to the vWFA1 domain reacts with all three known cochlin isoforms: p60 (full-length) and p44 and p40 (both lacking the FCH/LCCL domain) (Fig. 1). We chose this antibody (anti-cochlin/vWFA1 domain) for our studies because it would provide a more complete representation of cochlin localization in unaffected and affected tissues.

In the normal adult mouse cochlea (Fig. 3), cochlin immunostaining is strong in the spiral ligament and spiral limbus. In the ligament, the staining is darkest in the basilar crest, near the basilar membrane and weakest in the spiral prominence. Cells lining Rosenthal's canal and the channels of the osseous spiral lamina are cochlin-positive, whereas the cochlear ganglion cell bodies and the neural processes are cochlin-negative. Distinct cochlin staining of pericytes surrounding blood vessels in the modiolus and throughout the cochlear duct is observed. In contrast, adjacent areas of surrounding bony tissues clearly lack cochlin staining. Cochlin-negative structures in the cochlear duct are the organ of Corti, including the sensory epithelium and tectorial membrane, stria vascularis, Reissner's membrane, cochlear ganglion cells and their neuronal processes. In the vestibular labyrinth, the cristae (Fig. 3G) show intense cochlin staining in the fibrocytes and stroma underlying the sensory epithelium as well as in the ampullary wall. The sensory epithelium, the neuronal processes within the ampullary stroma as well as the surrounding bone and connective tissues are all cochlin-negative.

To confirm antibody specificity, we immunostained sections from a *Coch* (−/−) mouse (29,30) and no staining was detected (Fig. 3E). Negative controls with secondary antibody alone also show no background staining (data not shown). The intense staining for cochlin in the (+/+) mouse inner ear corroborates the finding of cochlin by proteomic analysis as a very abundant and stable protein in cochlea and vestibular organs.

In the unaffected control human adult inner ear (Fig. 4), a similar pattern of cochlin staining is detected. Cochlin immunoreactivity was prominent throughout the spiral ligament, spiral limbus and within the osseous spiral lamina. Immunostaining in the modiolus was also observed around the blood vessels (data not shown). As in the mouse, the organ of Corti, the neuronal cell bodies and central and peripheral axons lack cochlin expression. The surrounding outer bony and mesenchymal tissues are also unstained. In the human adult vestibular labyrinth, the cristae also show immunostaining in the area of the stromal fibrocytes and a lack of staining in the adjacent overlying sensory epithelium. The ampullary wall also contains cochlin, as observed in the mouse sections.

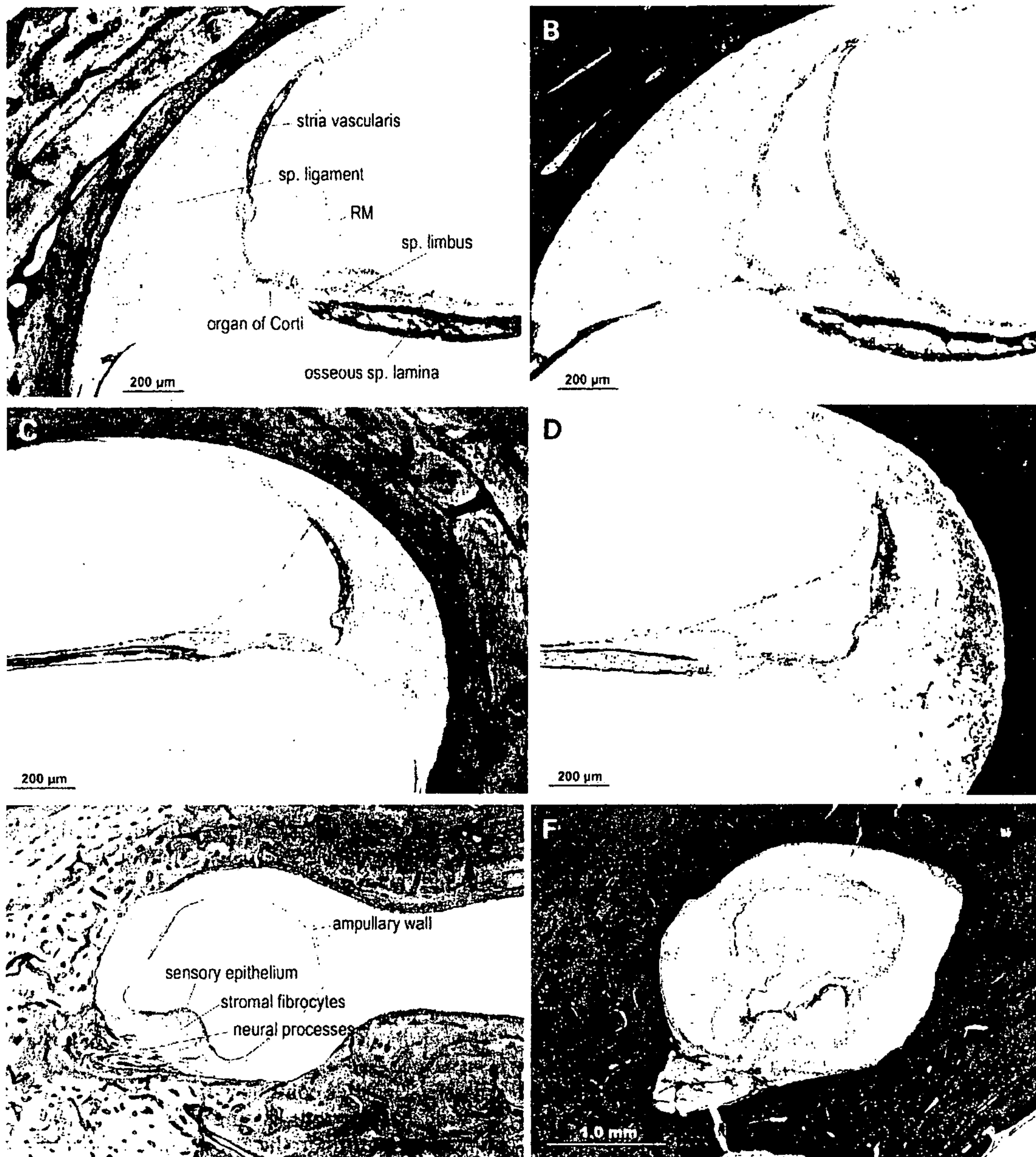

Figure 2. H&E-stained celloidin-embedded temporal bone sections from an individual with DFNA9 from a Dutch kindred segregating the P51S mutation (67-year-old female) (**B**, **D**, **F**) and from an age-matched unaffected control (63-year-old female) (**A**, **C**, **E**). In the cochlear duct of the DFNA9-affected individual (B, D, ×100), as compared with the unaffected control (A, C, ×100), the most striking findings are the presence of abundant extracellular eosinophilic aggregates throughout the spiral ligament, spiral limbus and the osseous spiral lamina, and significant loss and degeneration of fibrocytes in the ligament and limbus. In particular, the more medial parts of the spiral ligament, underlying the stria vascularis and the area of the insertion of the ligament into the basilar membrane are more severely affected with presence of deposits and fibrocyte atrophy. Degeneration of the organ of Corti and of neural processes in the osseous spiral lamina is also observed. In the ampulla of the posterior semi-circular canal in the vestibular labyrinth (E, F, ×40) pathologic changes, similar to those in the cochlear duct, are present in the DFNA9-affected ampulla (F) as compared with the unaffected control (E). Abundant eosinophilic deposition is present within the DFNA9 ampullary stroma, with reduction and atrophy of the stromal fibrocytes, as well as degeneration of the sensory epithelium of the crista, and atrophy of the ampullary nerve. In addition, there is appreciable thickening and partial collapse of the ampullary wall, also showing the presence of eosinophilic aggregates.

In both mouse and human cochlea and vestibular organs, cochlin immunostaining is restricted to tissues that are mesodermal in origin; neuroectodermally derived structures clearly lack cochlin expression. Within the mesodermal structures, there is widespread and high-level expression of cochlin in areas such as the spiral ligament, which comprises a large percentage of the total mass of the membranous cochlea, in agreement with findings of high levels of cochlin mRNA by EST, northern blot and tissue *in situ* hybridization analyses (17,19,32), and with abundance and stability of cochlin

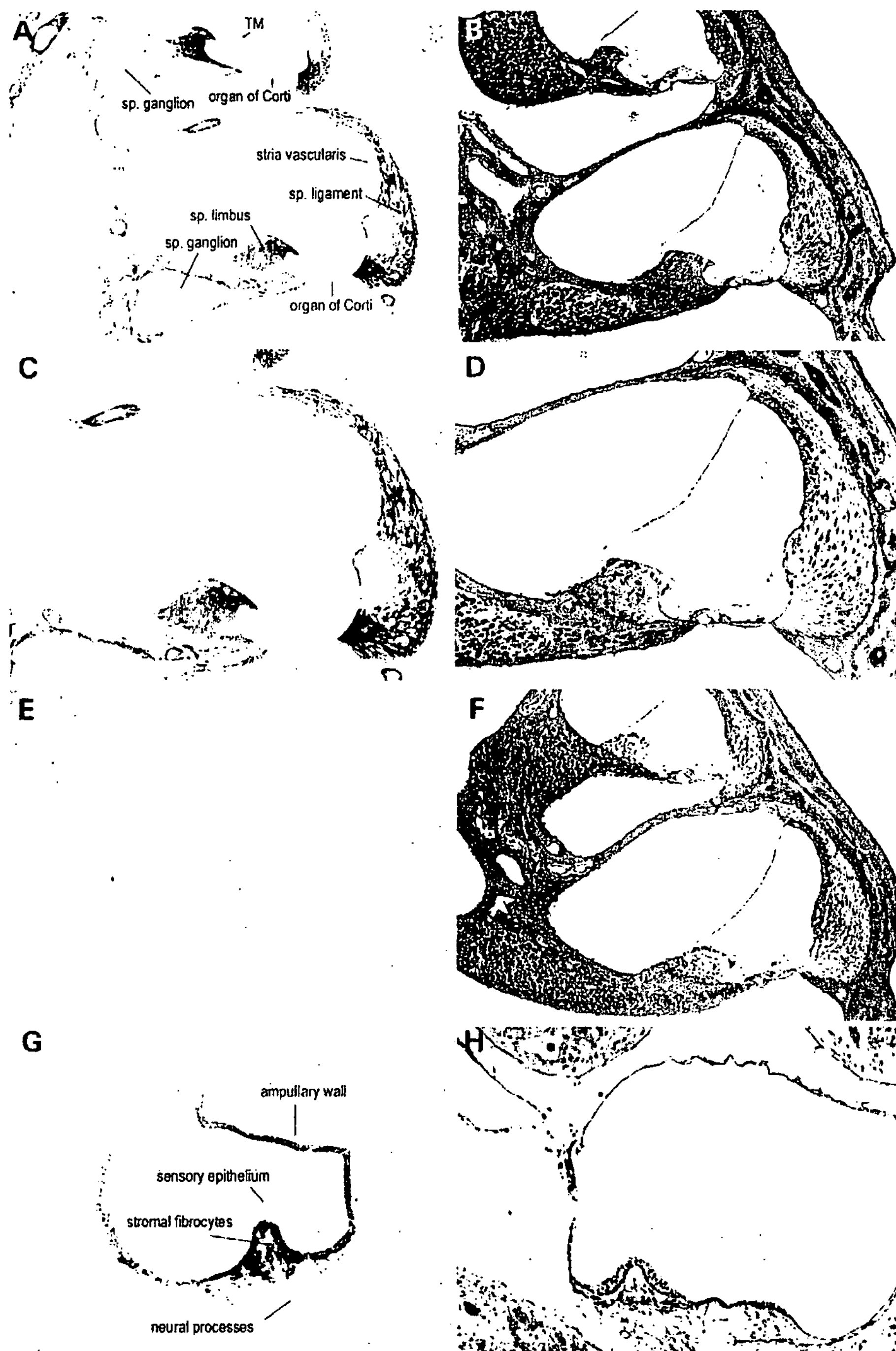

Figure 3. Immunohistochemistry on postnatal (5-month-old) (+/+) (**A**, **C**, **G**) and *Coch* (−/−) (**E**) mouse inner ear sections with anti-cochlin. Immunostaining (on the left panels) appears as a reddish brown DAB reaction product; no counterstain was applied on these sections. Serial sections stained with H&E are shown in parallel on the right panels (**B**, **D**, **F**, **H**). In the (+/+) cochlear duct (A, × 100; C, × 150), prominent cochlin immunostaining is present in fibrocytes and in the ECM throughout the spiral ligament and spiral limbus. Cells lining Rosenthal's canal (surrounding the spiral ganglion) and the channels of the osseous spiral lamina also contain cochlin, whereas the cochlear ganglion cell bodies and the neural processes are negative for cochlin immunostaining. Distinct perivascular rings around blood vessels in the modiolus and throughout the cochlear duct are stained. In contrast, adjacent areas of surrounding bony tissues clearly lack cochlin staining. The structures of the cochlea that show the absence of cochlin expression are the organ of Corti, including the sensory epithelium and tectorial membrane (TM), stria vascularis, Reissner's membrane and spiral ganglion cells. The cochlear duct in the *Coch* (−/−) mouse (E, × 100) was used as a negative control and lacks any cochlin immunostaining, confirming the specificity of this antibody. In the (+/+) crista of the posterior ampulla in the vestibular labyrinth (G, × 200), intense cochlin staining is observed in the fibrocytes and stroma underlying the sensory epithelium, as well as in the ampullary wall. Neuronal processes within the ampullary stroma as well as the surrounding bone and connective tissues lack any immunostaining. The sensory epithelium is also completely devoid of any cochlin staining, as observed in the cochlear duct.

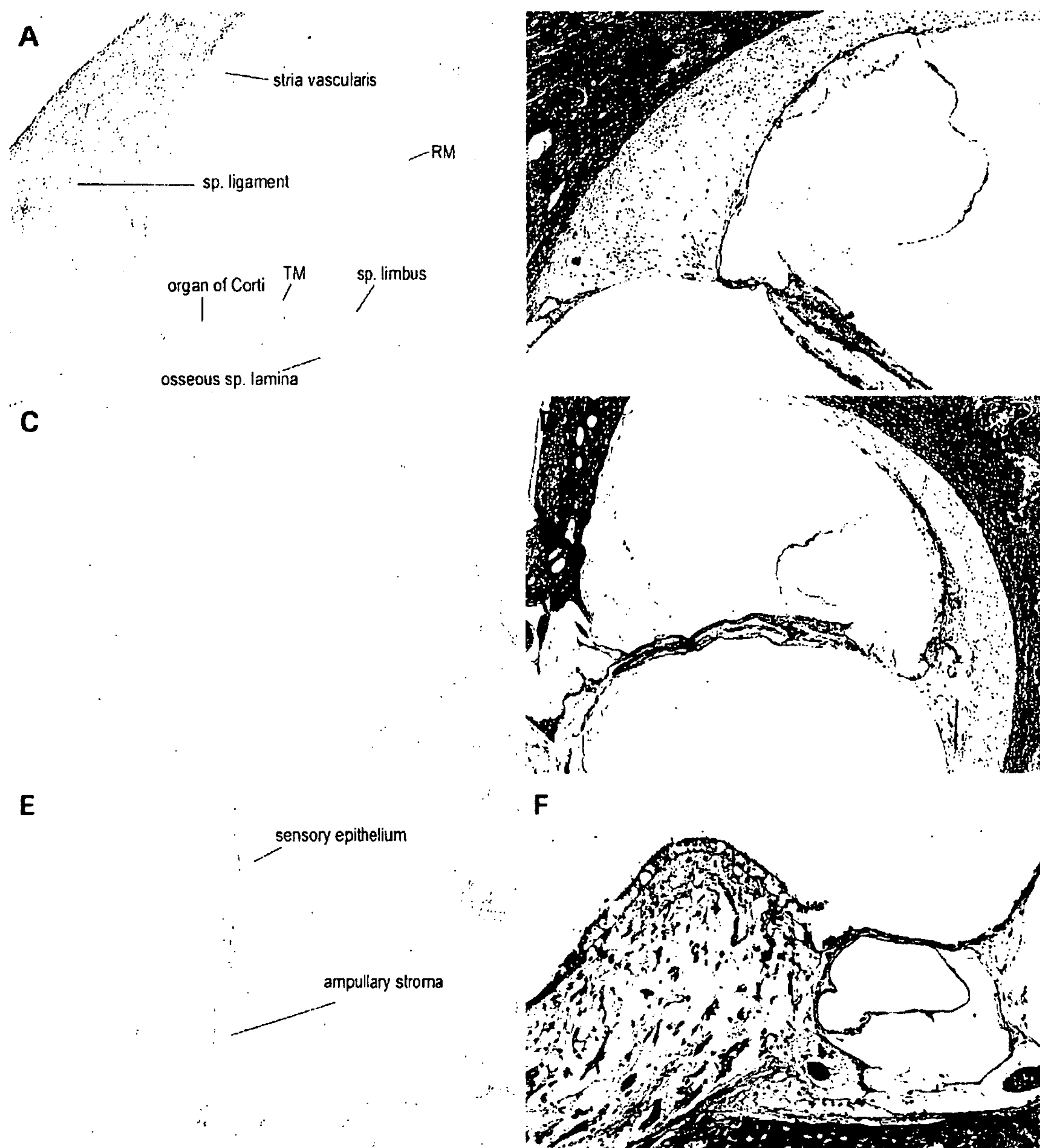

Figure 4. Immunohistochemistry on unaffected human adult (75-year-old male) temporal bone sections with anti-cochlin (**A**, **C**, **E**). No counterstain was used on these sections; serial H&E sections are shown (**B**, **D**, **F**). In the cochlear duct (A, C, × 100), cochlin immunostaining is prominent throughout the spiral ligament, spiral limbus and the channels of the osseous spiral lamina. Adjacent areas of surrounding bony tissues are not stained with the anti-cochlin antibody. Structures of the cochlea shown in this figure, which lack cochlin expression, are the organ of Corti, including the sensory epithelium and tectorial membrane (TM), stria vascularis and Reissner's membrane (RM). Some of these structures show artifactual disruption as a result of paraffin embedding of adult temporal bones. In the posterior crista ampullaris of the vestibular labyrinth (E, × 200), intense cochlin staining is observed in the fibrocytes and stroma underlying the sensory epithelium. The sensory epithelium is completely devoid of any cochlin expression, as was observed in the cochlear duct.

protein as observed by western blot and proteomic analyses (19,21).

Cochlin immunostaining in DFNA9-affected inner ear

The cochlin staining pattern in the DFNA9 temporal bone sections (Fig. 5) is similar to that in the unaffected control sections. Immunostaining is strong throughout the spiral ligament, spiral limbus, stroma of the crista ampullaris and the ampullary wall. As a negative control, no staining was detected using the secondary antibody alone (data not shown). There is no detectable background staining in the tissues immediately adjacent to the spiral ligament lateral wall and tissues surrounding the ampulla. The regions of the osseous spiral lamina normally occupied by cochlear peripheral axons are immunopositive for cochlin, as are perivascular areas in the modiolus. Other

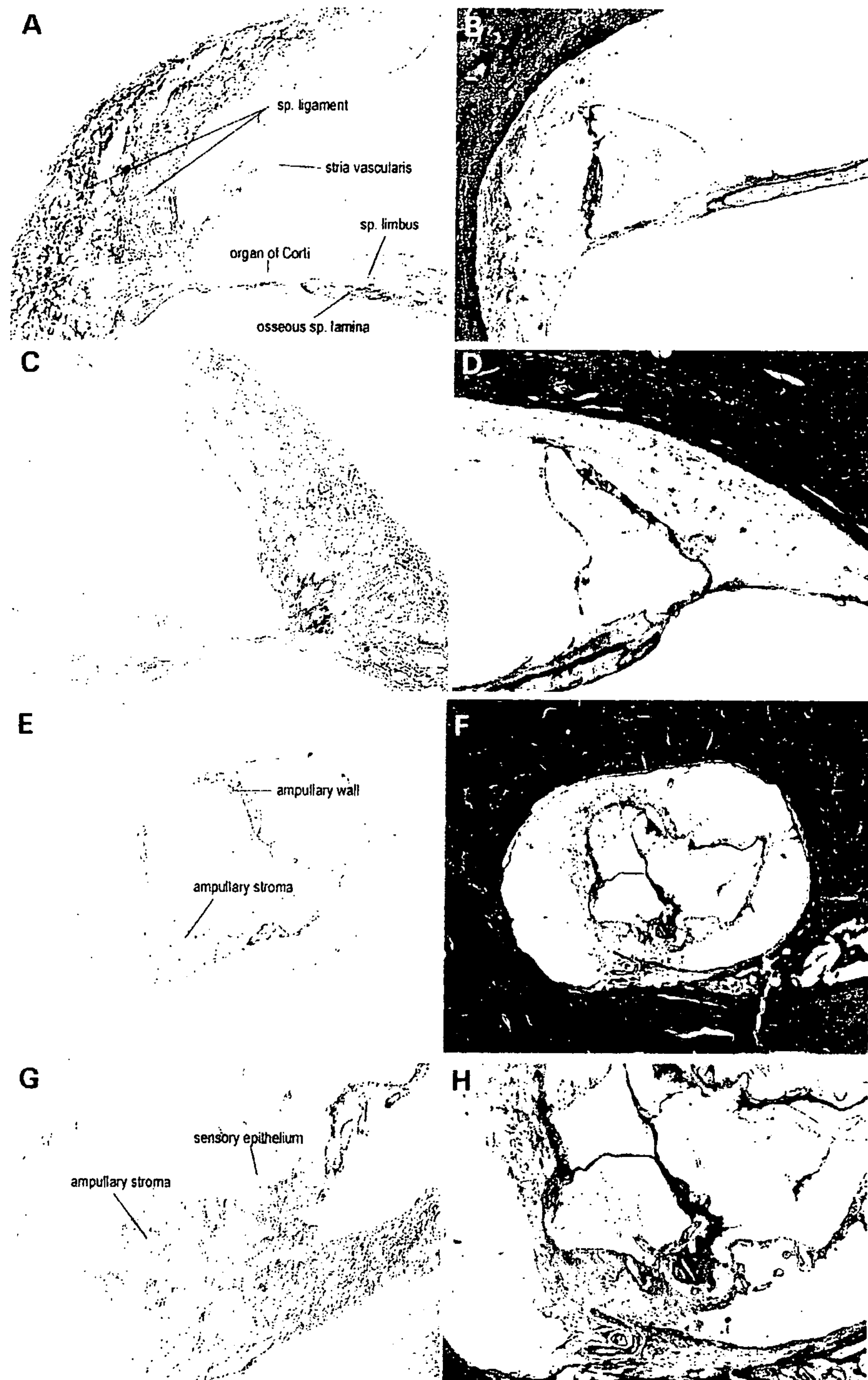

Figure 5. Immunohistochemistry on DFNA9-affected human adult (67-year-old female) temporal bone sections with the anti-cochlin antibody (**A**, **C**, **E**, **G**). No counterstain was used on these sections; serial H&E sections are shown (**B**, **D**, **F**, **H**). In the cochlear duct (A, C, ×100), cochlin immunostaining is observed throughout the spiral ligament, spiral limbus and the channels of the osseous spiral lamina. The homogeneous eosinophilic deposits seen on the H&E sections are stained darkly and evenly. The cochlin immunostaining of this acellular material is prominent in the spiral ligament, particularly in the area of insertion into the basilar membrane, the spiral limbus and within the channels of the osseous spiral lamina. The organ of Corti and the stria vascularis are negative for cochlin staining. Some stained tissue underlying the stria vascularis appears to be a part of the spiral ligament that was detached along with the stria. Adjacent areas of surrounding bony tissues do not show any immunostaining. In the posterior ampulla of the vestibular labyrinth (E, ×4; G, ×100), cochlin staining of the ampullary stroma containing the eosinophilic deposits is observed. The collapsed ampullary wall showing prominent thickening and acellular deposition (F) also contains cochlin (E). The sensory epithelium does not show cochlin expression, as seen in the cochlear duct in both DFNA9 and unaffected control inner ears.

structures such as the organ of Corti, vestibular sensory epithelium and stria vascularis, which are cochlin-negative in normal tissues, also lack staining.

The large amounts of eosinophilic acellular deposits contained throughout the spiral ligament, limbus and osseous spiral lamina are darkly and evenly immunostained with anti-cochlin, but completely lack non-specific staining with secondary antibody alone. The ampullary stroma and wall, which show distortion, collapse and thickening, and contain the acellular material, also show prominent cochlin staining. These results are consistent with the view that cochlin is intimately associated with the eosinophilic deposits characteristic of the temporal bone histopathology in DFNA9.

Proteomic analysis in mouse inner ear

Proteomic analysis of the cochlear and vestibular labyrinths of (+/+) and *Coch* (−/−) mice were performed and an abridged list of the representative peptide matches is presented (Table 2). The number of tryptic peptides identified from mass spectrometry analysis reflects the relative abundance of proteins detected within each tissue by this method. A striking finding is the presence of cochlin peptides as the most abundantly detected protein in the cochlea of (+/+) mice. In the vestibular organs, cochlin is the second most frequently detected protein, with albumin being primary. In both tissues, cochlin is more abundant than β-hemoglobin. Findings for two other proteins, α-tectorin and keratin 9, are representative of structural proteins expressed in these tissues. Our proteomic analysis combines results of four gel fractions where digestion and mass spectrometry were performed separately. Identification of cochlin as the most abundant protein in mouse inner ear lysates corroborates previous proteomic analysis by the alternative method of 2D gel electrophoresis, also revealing cochlin as the most prevalent protein in bovine inner ear (21). As a negative control, we studied the *Coch* (−/−) mouse. No cochlin peptides were detected in either cochlear or vestibular tissues, confirming the lack of cochlin protein as shown also by our immunohistochemistry and by previous western blot analysis (30).

In (+/+) mouse cochlea, a total of 123 cochlin tryptic peptides representing 38 unique peptides are detected, ranging from 7 to 35 amino acid residues each. Extensive peptide coverage for cochlin was observed (Fig. 6) throughout all domains of the full-length protein (excluding the signal peptide). In conjunction with detection of cochlin as a highly abundant and stable protein in the cochlear and vestibular organs, and its fairly restricted expression at a high level in the inner ear, it is interesting to note that several studies have implicated cochlin as a target antigen for autoimmune sensorineural hearing loss via both immunoglobulin and T-cell-mediated mechanisms. Elevated serum levels of anti-cochlin immunoglobulins have been detected in a number of patients with autoimmune hearing loss (33,34). In addition, cochlin has been shown to co-immunoprecipitate with choline transporter-like protein 2 as targets of antibody-induced hearing loss (35,36). Studies have also demonstrated experimentally induced CD4+ T-cell-mediated autoimmune hearing loss with cochlin as the target antigen (37,38). Furthermore, recent investigations have revealed significantly higher

Table 2. Relative abundance of inner ear peptides in wild-type +/+ and *Coch* −/− mice

	Cochlea Wild-type +/+	*Coch* −/−	Vestibular Wild-type +/+	*Coch* −/−
Cochlin	123	0	50	0
Albumin	68	109	55	38
β-Hemoglobin	76	81	47	28
α-Tectorin	12	19	1	1
Keratin 9	18	20	31	36

frequencies of cochlin-specific circulating T-cells as well as elevated cochlin-specific serum antibody titers in individuals with autoimmune sensorineural hearing loss, as compared with unaffected age-matched controls (39). These reports implicate cochlin as a prominent inner ear target antigen in both antibody and T-cell-mediated autoimmune hearing loss.

Proteomic analysis in human adult unaffected and DFNA9-affected and temporal bones

Because the only material available from the DFNA9-affected and unaffected human samples is formalin-fixed, paraffin-embedded temporal bones, we had to employ a different approach than that used in the mouse. Thus, proteomic analysis was performed on proteins extracted from 8-μm paraffin-embedded sections, whereas fresh whole tissue lysates or frozen tissues are typically used.

In the human adult unaffected temporal bone sample, cochlin is also the most abundantly detected protein by mass spectrometry, as was the case in the mouse using fresh cochlear lysates. A total of 66 cochlin peptides, representing 17 unique peptides, are detected (Table 3), ranging from 10 to 35 amino acid residues each. Cochlin peptides identified in the human sample are also representative throughout the protein in all domains of cochlin, although peptide coverage is not as complete as that found in the mouse sample (Fig. 6). This is not surprising given that peptide extraction from archival formalin-fixed, paraffin-embedded sections (40) is much more difficult than from fresh total tissue lysates. Nonetheless, these results indicate that cochlin is also a prevalent and stable component of the human adult inner ear.

A complete alphabetical listing of all proteins detected from the proteomic analysis of the human adult temporal bone sections is presented in Table 3. The major classes of proteins found are extracellular and structural components of the cochlea, cochlin being the primary. Many of the proteins are known to be expressed in the spiral ligament, which comprises a major amount of the total cochlear mass. Collagen types I, II, IX and XI, which are also abundantly detected in our analysis, are very representative of the known and stable components of cochlear tissue and involved in both non-syndromic and syndromic types of hearing loss (1). Other proteins frequently represented in our analysis are those comprising cytoskeletal elements such as keratins, β-actin and tubulin, which are also known to be expressed in the cochlea. A total of 13 unique peptides are identified, representing five distinct