Recently the causative gene of autosomal dominant sensorineural nonsyndromic late onset hearing loss (DFNA9) has been identified as the COCH gene, which lies in the DFNA9 region of human chromosome 14 (gene map locus 14q12-q13). Molecular analysis of cases of DFNA9 identified several families with five different mutations in this gene.1,2-3

The cochlin protein encoded by COCH is an extracellular matrix protein that contains an LCCL domain and two von Willebrand type A domains.4 Interestingly, all mutations causing DFNA9 type deafness disorder affect the LCCL domain of cochlin. In view of the central role of this domain in the DFNA9 disorder, we initiated studies on it to explore the molecular basis of the disease.5 We found that most DFNA9 mutations affected conserved structural elements of the LCCL fold and disrupted the proper folding of this domain; recombinant mutant LCCL domains expressed in Escherichia coli failed to adopt the wild type fold and instead formed insoluble aggregates.6 It is noteworthy that insoluble deposits are detected in temporal bone sections of individuals affected by DFNA9.7 These results support the notion that the DFNA9 mutations may act through a gain of function mechanism; it is the presence of the abnormal protein that causes the disease. According to this view, accumulation of deposits in vestibular and cochlear nerve channels leads to stranguulation and progressive degeneration of the dendrites, and loss of cochlear and vestibular neurones.

Our present investigation was aimed at identifying novel mutations affecting the LCCL domain of cochlin in order to gain more insight into the pathomechanism of DFNA9. Hungarian patients selected according to the diagnostic criteria of DFNA9 were screened for the presence of mutations affecting the LCCL domain. These studies identified one person heterozygous for a novel mutation in the LCCL domain of cochlin. The mutation has resulted in the deletion of Val104, a residue conserved in the human, mouse, and chicken cochlin sequences. It affects a critical β strand of the LCCL domain and was found to prevent refolding of a recombinant LCCL domain expressed in E. coli.

**Materials and Methods**
Selection of Patients and Gene Analysis

Patients were selected from the cochlear implantation list of the Department of Otorhinolaryngology of Semmelweis University according to diagnostic criteria of the DFNA9 disorder (OMIM #603196): progressive hearing loss usually begins in the third decade of life as a high frequency loss with concomitant vestibular dysfunction.

**Key Points**

- Autosomal dominant nonsyndromic sensorineural deafness 9 (DFNA9, OMIM #601369) is a rare, late onset hearing loss. In DFNA9 patients, hearing loss usually begins in the third decade of life as a high frequency loss with concomitant vestibular dysfunction.
- DFNA9 is caused by mutations in the COCH gene, which encodes cochlin, an extracellular matrix protein that contains an LCCL domain and two von Willebrand type A domains.
- Molecular analysis of cases of DFNA9 has identified five different mutations in this gene. Interestingly, all mutations causing DFNA9-type deafness disorder affect the LCCL domain of cochlin.
- Here we describe a novel COCH mutation in a Hungarian patient, which results in the deletion of Val104, a residue conserved in the human, mouse, and chicken cochlin sequences. The deletion affects a critical β strand of the LCCL domain and was found to prevent refolding of a recombinant LCCL domain expressed in Escherichia coli.
Expression and characterisation of the recombinant V104del LCCL domain

The DNA fragment encoding the LCCL module of the V104del mutant protein of the proband was produced by a two step PCR strategy. Firstly, we amplified exon 4 and 5 of the mutant COCH gene separately by using 5'-GGGGGATCCGGTGGACACGAGCCCA-3' (sense) and 5'-GAGTTGCTGATTCGCCGTGGACACGAGCCCA-3' (antisense) primers for exon 4, and 5'-TGGGGATCCGGTGGACACGAGCCCA-3' (sense) and 5'-GGGGGATCCGGTGGACACGAGCCCA-3' (antisense) primers for exon 5. For exon 4, the template was the genomic DNA of the proband, while for the mutant exon 5 we used the M13mp19 clone containing the mutant allele of exon 5. The two cDNA fragments were linked in a second reaction with 5'-GGGGGATCCGGTGGACACGAGCCCA-3' (sense), 5'-GGGGGATCCGGTGGACACGAGCCCA-3' (antisense), and 0.1 mol/l EDTA, pH 8.0. The mixtures dissolved in 0.1 mol/l Tris-HCl, 8 mol/l urea, 10 mmol/l EDTA and inclusion bodies containing recombinant protein were the recombinant proteins was induced with 100 μmol/l IPTG and the solubilised proteins were chromatographed on a Sephacryl S-300 column equilibrated with 100 mmol/l Tris-HCl, 8 mol/l urea, 10 mmol/l EDTA, and 0.1% 2-mercaptoethanol. Fractions containing pure fusion proteins were identified by SDS-PAGE, pooled and refolded by dialysis against 0.1 mol/l Tris-HCl and 10 mmol/l EDTA, pH 8.0 at 25°C.

RESULTS AND DISCUSSION

Sequence analysis of the region encoding the LCCL domain of COCH of one hearing impaired patient indicated that the patient was heterozygous for a deletion in this region. To determine the precise location and size of the deletion we purchased from Biolabs (Beverly, MA, USA). E. coli strain JM-109 was used to propagate the phage.

Figure 1 shows the strand specific sequence of the LCCL domain of human cochlin. The coordinates of the LCCL domain (1JBI) were taken from the Protein Data Bank. The β strands (yellow) are numbered as in fig 2. The α helix is shown in red. The position of Val104 (deleted in the novel V104del mutant) is shown as a blue segment of the helix in the LCCL domain of human cochlin. The locations of the β strands and the α helix in the LCCL domain of human cochlin ' are shown at the top. The conserved cysteines of the LCCL domains of human (coch_hum, residues 28–124), mouse (coch_mouse, residues 30–126), and chicken cochlin (coch_chick, residues 24–120) are shaded. The top sequence (V104del) represents the sequence of the mutant protein identified in the present study. In the bottom line, asterisks mark the locations of residues where mutations in the human cochlin protein have been found to result in the DFNA9 disorder.
problem and her genetic analysis did not identify a mutation in the LCCL domain of COCH. Although the possibility that the proband’s father carried the mutation cannot be ruled out, it is also possible that the mutation arose de novo.

In order to examine the effect of the mutation on the structure of the LCCL domain we expressed the mutant domain using the same protocol as for the wild type protein. Whereas in the case of the wild type recombinant LCCL domain, this protocol yields a soluble protein that adopts a unique three dimensional structure, the same refolding protocol did not yield any soluble protein for the V104del mutant LCCL domain, and dialysis led to complete precipitation of the recombinant protein. As the deletion of Val104 affects a conserved residue of the β6 strand structure of the LCCL domain (figs 1 and 2), it seems likely that this deletion affects this essential structural element of the LCCL fold, impairing its ability to form the native structure. In this respect, the V104del mutation is similar to most other DFNA9 causing mutations identified so far, which also impair refolding of recombinant LCCL domain (table 1).

In our opinion, this novel mutation provides additional support for the notion that the characteristic ossiconophilic deposits in DFNA9 affected inner ear structures could be the result of accumulation and aggregation of aberrant, mutated cochlin. Recent studies have indeed shown that DFNA9 mutations do not impair secretion and post-translational processing of cochlin produced by mammalian cells.” It seems likely that in vivo the mutations may lead to a slow aggregation of cochlin over a longer time course, consistent with the late onset and progressive nature of this disorder.

**Table 1** Comparison of the V104del mutation with other DFNA9 mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype</th>
<th>Age of onset</th>
<th>Refolding of LCCL domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>V104del</td>
<td>Heterozygous</td>
<td>4th decade</td>
<td>Impaired*</td>
</tr>
<tr>
<td>1109N</td>
<td>Heterozygous</td>
<td>2nd–3rd decade</td>
<td>–</td>
</tr>
<tr>
<td>V66G</td>
<td>Heterozygous</td>
<td>2nd–3rd decade</td>
<td>Impaired*</td>
</tr>
<tr>
<td>G88E</td>
<td>Heterozygous</td>
<td>5th decade</td>
<td>Impaired*</td>
</tr>
<tr>
<td>W117R</td>
<td>Heterozygous</td>
<td>5th decade</td>
<td>Preserved*</td>
</tr>
<tr>
<td>P51S</td>
<td>Heterozygous</td>
<td>4th decade</td>
<td>Impaired*</td>
</tr>
</tbody>
</table>

*Present work.

**ACKNOWLEDGEMENTS**

This work was supported by grant no. 1/044/2001 of the National Research and Development Fund of Hungary.

**Authors’ affiliations**

I Nagy, M Trexler, L Patthy, Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary

M Horváth, G Répássy, Department of Otorhinolaryngology, Head and Neck Surgery, Faculty of Medicine, Semmelweis University, Budapest, Hungary

Correspondence to: Dr L Patthy, Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Karolina u 29, Budapest, H-1113 Hungary; patthy@enzim.hu

Received 10 July 2003
Accepted 18 August 2003

**REFERENCES**


