Molecular biology of the DFNA9 hereditary hearing loss

Ph.D theses

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Introduction

Hearing loss is the most common sensory disorder. Genetic causes of hearing loss can be found in the majority of cases. More than 70% of hereditary hearing loss is monogenic and nonsyndromic. Over the past decade a large number of loci have been mapped for hereditary hearing loss and many genes have been identified as related to deafness. Identification of new genes and elucidation of their functions and roles in the inner ear will provide further knowledge about the pathways involved in the hearing process and will allow for better genetic diagnostics and therapies for hearing loss.

In 2000, using in silico methods, the Functional Genomics Group of the Institute of Enzymology, BRC, defined the LCCL module as an autonomous folding domain that has been used for the construction of various modular proteins. Because the best characterized proteins that were found to contain this module were *Limulus polyphemus* factor C, cochlear protein Coch-5b2 (cochlin), and late gestation lung protein Lgl1 the name LCCL was proposed for this domain-family.

The secreted protein, cochlin is the most abundant protein in the extracellular matrix of the inner ear. It contains one LCCL domain and two von Willebrand factor type A domains. It has been shown that mutations in the LCCL domain of cochlin cause autosomal dominant nonsyndromic hearing loss, DFNA9, therefore our group initiated structure-function studies on this domain. In collaboration with a swedish NMR group, the solution structure of the LCCL domain was determined and it was shown, that mutations causing DFNA9 disrupt conserved elements of the LCCL fold and cause misfolding and aggregation of the recombinant proteins.

Aims

1. Identification of novel disease causing mutations in the Hungarian population in order to shed more light on the pathomechanism of the disease.

    1.1. Selection of hearing impaired persons according to the diagnostic criteria of DFNA9.

    1.2. Mutation screening of the LCCL domain region of the *COCH* gene.

    1.3. Expression of the mutant LCCL domains to explore the effect of these mutations.

2. Exploring the normal function of human cochlin protein in the extracellular matrix of the inner ear to clarify its role in the hearing process. I aimed to examine the interaction of the different domains of cochlin with other components of the extracellular matrix of the inner ear. These results can contribute to understand the normal function of cochlin.

2.1. Expression and characterization of the LCCL and von Willebrand factor type A domains of cochlin.

2.2. Studies on the interactions of the LCCL domain and the two von Willebrand factor type A domains of cochlin with various extracellular matrix proteins. Determination of the kinetic parameters of interactions.

Methods

Genetic 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. DNA from 17 hearing impaired person from 14 different families was used for genetic analysis and compared with DNA from 50 unrelated controls. Mutation screening was performed for exons 4 and 5 of *COCH* gene, which encode the LCCL domain of cochlin. To verify the sequence of the genomic DNA of the patient carrying a mutation, the PCR product was cloned and sequenced.

Cloning: The pMed23 expression vector containing the DNA fragment of the COCH/LCCL_V104del and COCH/LCCL_I109N mutant domains; the pPICZαA expression vector containing the DNA fragment of the COCH/LCCL, COCH/LCCL_I109N, COCH/vWFA1 and COCH/vWFA2 were constructed by standard recombinant technology protocols.

Expression and purification of proteins: The *Escherichia coli* strain JM-109 and the *Pichia pastoris* strain GS115 were used for recombinant protein expression. Proteins were purified with Ni-affinity chromatograpy and gel filtration methods.

SDS polyacrylamide gel electrophoresis: The composition of protein samples was analysed by SDS/PAGE using 11-22% linear polyacrylamide gradient slab gels under both reducing and non-reducing conditions.

Protein analyses: N-terminal sequencing was performed on an Applied Biosystems 471A protein sequencer.

The concentrations of the recombinant proteins were determined using the following extinction coefficients: coch_LCCL, 10930 M-1cm-1; coch_vWFA2, 13075 M-1cm-1.

Circular dichroism (CD) spectroscopy: CD spectra of coch_vWFA2 domain were measured over the range of 195-250 nm with a JASCO J-720 spectropolarimeter. The measurements were carried out using protein solutions of 0,2 mg/ml, in 25 mM Tris/HCl, pH:8,0, at 25 ^{o}C. The thermal unfolding of the protein was monitored at 222 nm, from 25 ^{o}C to 85 ^{o}C. Melting temperature was determined by derivative processing of changes in CD using the spectra analysis program for JASCO.

Sequence analysis: Multiple alignments for the LCCL domains of different cochlins were constructed using CLUSTAL W program.

Interaction measurements: Interaction of the coch_vWFA2 domain and coch-LCCL domain with human type I., II., and IV: collagens were measured by surface plasmon resonance (SPR) on a BIAcore X instrument. The kinetic parameters for each interaction were determined by BIAevaluation software 4.0.

To study the effect of different cations on the binding properties of coch_vWFA2 to collagens, additional SPR measurements were carried out.

Results

1. Identification of new mutations in the LCCL domain and examination of the effect of mutations

1.1. Sequence analysis of the region encoding the LCCL domain of cochlin of hearing impaired persons indicated that one patient was heterozygous for a deletion in this region and this new type of mutation caused DFNA9 hearing loss. This is the firstly identified mutation in the Hungarian population which causes DFNA9.

1.2. To determine the precise location and size of the deletion we cloned the affected region. The mutant allele carried a deletion of nucleotides 367-369 in exon 5. The deletion of three nucleotides resulted int he deletion of a Val104 residue without causing a frameshift. No mutation in the LCCL domain was detected in the other 16 DFNA9 patients or in the 50 control individuals.

1.3. The V104del mutant LCCL domain and the formerly identified I109N mutant LCCL domain were expressed in bacterial expression system.

1.4. Similarly to most other DFNA9 causing LCCL domain mutations examined so far, the same refolding protocol that yielded soluble, native wild type protein did not yield any soluble protein for the V104del and I109N mutants and the mutant proteins precipitated completely.

1.5. As the V104del and I109N mutations affect conserved residues of the β-strand structures of the LCCL domain, it seems likely that these mutations affect the essential structural elements of the LCCL fold, impairing its ability to form the native structure.

2. Structural characterization of the second von Willebrand factor type A domain (vWFA2) of human cochlin

2.1. The vWFA2 domain of human cochlin was expressed in *Pichia pastoris* expression system.

2.2. The circular dichroism spectrum of the coch/vWFA2 domain is very similar to those of other members of the vWFA-domain family. Thermal denaturation of the recombinant protein revealed that the native fold collapses in a highly cooperative fashion with a T_m value of 52 ºC.

3. Protein interaction measurements

3.1. Recombinant coch/vWFA2 domain was found to bind to collagens type I, II, and IV measured by surface plasmon resonance (SPR), whereas the coch/LCCL domain displayed no detectable affinity for these proteins. Evaluation of the sensorgrams revealed that the equilibrium dissociation constants for the interaction of covh/vWFA2 with type I, type II, and type IV collagens were $7{,}97 \times 10^{-8}$ M, $9{,}45 \times 10^{-8}$ M, and $1{,}10 \times 10^{-7}$ M.

3.2. SPR measurements were also performed in the presence of 1 mM Ca^{2+}, 1 mM Mg^{2+}, or 100 μM Zn^{2+}. Addition of these metal ions had no detectable effect on the affinity of coch/vWFA2 to these collagens.

Conclusions

1. Examination of the effect of mutations provided additional support to the notion that the DFNA9 mutations act through a gain-of function mechanism: it is the presence of the

abnormal protein that causes the disease. The characteristic acidophilic non-cellular deposits in DFNA9 affected inner ear structures could be the result of slow aggregation of aberrant, mutated cochlins over a longer time course, causing progressive degeneration of neurons. This is consistent with the late onset and progressive nature of this disorder.

2. Interaction measurements revealed that cochlin has high affinity for type II collagen, thus confirming earlier suggestions that interaction of cochlin and type II collagen plays a crucial role in the structural homeostasis of the cochlea and vestibule. The coch/vWFA2 domain was also found to have high affinity for type I and type IV collagens but the physiological importance of the latter interactions is unclear.

My observation that the affinity of cochlin's vWFA2 domain for type I, II, and IV collagens is not influenced by the addition of metal ions suggests that collagen recognition by this vWFA domain occurs by a mechanism different from that of integrins.

Furthermore, mutations eliminating cochlin's interaction with other constituents of the extracellular matrix of the inner ear may interfere with the normal cochlear function resulting hearing loss.

Publications

Publications discussed in the Ph.D work:

I. Nagy, M. Horvath, M. Trexler, G. Repassy, and L. Patthy. (2004) A novel *COCH* mutation, V104del, impairs folding of the LCCL domain of cochlin and causes progressive hearing loss.

J. Med. Genet. **41**:e9. IF: 4.112

I. Nagy, M. Trexler, and L. Patthy. (2008) The second von Willebrand type A domain of cochlin has high affinity for type I, type II, and type IV collagens.

FEBS Letters. **582**, 4003-7. IF:3.263

Oral presentations

Ildikó Nagy, Mária Trexler, Edvards Liepinsh, Andrei Kaikkonen, Johan Weigelt, László Bányai, Miklós Horváth, Gábor Répássy, Gottfried Otting, László Patthy
„*COCH* gene mutations impair folding of the LCCL domain of cochlin and cause progressive hearing loss."
30th FEBS Congress and 9th IUBMB Conference, Budapest, Hungary, 2005.

Ildikó Nagy, Mária Trexler, Edvards Liepinsh, Andrei Kaikkonen, Johan Weigelt, László Bányai, Miklós Horváth, Gábor Répássy, Gottfried Otting, László Patthy.
„The LCCL module. Presentation of the NMR structure and examination of LCCL domain mutations causing DFNA9 hearing loss."
9th Conference of the Molecular Biology Section of Hungarian Biochemical Society, Sopron, Hungary, 2004.

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I. Nagy, M. Trexler and L. Patthy. (2003) Expression and characterization of the olfactomedin domain of human myocilin.
Biochem. Biophys. Res. Commun. **302**, 554-61. IF: 2.836

Poster presentation

Ildikó Nagy, Mária Trexler, László Patthy
„Structure of the olfactomedin domain of human myocilin and role of its mutations in the development of glaucoma."
8th Conference of the Molecular Biology Section of Hungarian Biochemical Society, Tihany, Hungary, 2003.

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