doi:10.1093/brain/awq273 Brain 2011: 134; 210–219 | 210

BRAIN
A JOURNAL OF NEUROLOGY

Riboflavin-responsive oxidative phosphorylation complex I deficiency caused by defective *ACAD9*: new function for an old gene

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Mitochondrial complex I deficiency is the most common oxidative phosphorylation defect. Mutations have been detected in mitochondrial and nuclear genes, but the genetics of many patients remain unresolved and new genes are probably involved. In a consanguineous family, patients presented easy fatigability, exercise intolerance and lactic acidosis in blood from early childhood. In muscle, subsarcolemmal mitochondrial proliferation and a severe complex I deficiency were observed. Exercise intolerance and complex I activity was improved by a supplement of riboflavin at high dosage. Homozygosity mapping revealed a candidate region on chromosome three containing six mitochondria-related genes. Four genes were screened for mutations and a homozygous substitution was identified in *ACAD9* (c.1594C>T), changing the highly conserved arginine-532 into tryptophan. This mutation was absent in 188 ethnically matched controls. Protein modelling suggested a functional effect due to the loss of a stabilizing hydrogen bond in an α-helix and a local flexibility change. To test whether the *ACAD9* mutation caused the complex I deficiency, we transduced fibroblasts of patients with wild-type and mutant *ACAD9*. Wild-type, but not mutant, *ACAD9* restored complex I activity. An unrelated patient with the same phenotype was compound heterozygous for c.380G>A

Received May 26, 2010. Revised August 6, 2010. Accepted August 11, 2010. Advance Access publication October 7, 2010

and c.1405C>T, changing arginine-127 into glutamine and arginine-469 into tryptophan, respectively. These amino acids were highly conserved and the substitutions were not present in controls, making them very probably pathogenic. Our data support a new function for *ACAD9* in complex I function, making this gene an important new candidate for patients with complex I deficiency, which could be improved by riboflavin treatment.

Keywords: *ACAD9*; oxidative phosphorylation; riboflavin

Abbreviations: ACADVL = very long chain acyl-CoA dehydrogenase; FAD = flavine adenine dinucleotide; PCR = polymerase chain reaction

Introduction

Mitochondrial oxidative phosphorylation disorders are among the most common groups of metabolic disorders with a prevalence of ~1 in 5000–10 000 live births in man (Skladal *et al.*, 2003). Of all enzymatic oxidative phosphorylation disorders, complex I deficiency is the most prevalent and is found either to be isolated or in combination with other complexes (McFarland *et al.*, 2004). Complex I is the largest complex of the respiratory chain and carries electrons from NADH to ubiquinone. Simultaneously, protons are transported across the inner membrane, creating a membrane potential that is needed for ATP synthesis and the transport of metabolites. Mammalian complex I structurally consists of 45 subunits of which seven are encoded by mitochondrial DNA and the other 38 subunits are encoded by nuclear DNA (Carroll *et al.*, 2006). Additionally, nuclear DNA encodes assembly chaperones that are required for proper assembly and stability of this complex (Vogel *et al.*, 2007).

Mitochondrial complex I disorders are clinically heterogeneous, leading to a broad variety of clinical manifestations ranging from neonatal death to late-onset neurodegenerative disorders. Clinical phenotypes include encephalopathy, Leigh syndrome, leucodystrophy, Leber's hereditary optic neuropathy, myopathy, cardiomyopathy, liver failure and fatal infantile lactic acidosis. Apart from the clinical heterogeneity, complex I deficiencies also show extensive genetic heterogeneity. Disease-causing mutations have been described both in mitochondrial DNA-encoded subunits *ND1* (Huoponen *et al.*, 1991), *ND2* (Schwartz and Vissing, 2002), *ND3* (Taylor *et al.*, 2001), *ND4* (Wallace *et al.*, 1988), *ND5* (Taylor *et al.*, 2002) and *ND6* (Johns *et al.*, 1992), as well as in the nuclear DNA-encoded subunits *NDUFV1* (Schuelke *et al.*, 1999), *NDUFV2* (Benit *et al.*, 2003), *NDUFS1* (Benit *et al.*, 2001), *NDUFS2* (Loeffen *et al.*, 2001), *NDUFS3* (Benit *et al.*, 2004), *NDUFS4* (van den Heuvel *et al.*, 1998), *NDUFS6* (Kirby *et al.*, 2004), *NDUFS7* (Triepels *et al.*, 1999), *NDUFS8* (Loeffen *et al.*, 1998), *NDUFA2* (Hoefs *et al.*, 2008), *NDUFA11* (Berger *et al.*, 2008) and *NDUFA1* (Fernandez-Moreira *et al.*, 2007). Additionally, mutations have been reported in several chaperones required for the proper assembly and stability of complex I [*NDUFAF1* (Dunning *et al.*, 2007), *NDUFAF2* (Ogilvie *et al.*, 2005), *NDUFAF3* (Saada *et al.*, 2009), *NDUFAF4* (Saada *et al.*, 2008), *C8orf38* (Pagliarini *et al.*, 2008) and *C20orf7* (Gerards *et al.*, 2009; Sugiana *et al.*, 2008)]. Most of the nuclear DNA-encoded defects are inherited in an autosomal recessive manner, although X-linked inheritance has been described for *NDUFA1* (Fernandez-Moreira *et al.*, 2007).

In this article, we present a report on a double consanguineous family with exercise intolerance and an isolated complex I deficiency that is responsive to riboflavin treatment. The genetic defect was identified as a pathogenic homozygous amino acid substitution in *ACAD9*. Subsequent analysis of a second, unrelated patient with a comparable phenotype revealed a compound heterozygous amino acid substitution in *ACAD9*. We demonstrate that *ACAD9* is involved in complex I function, making this gene an important new candidate for mutation screening in patients with complex I deficiency.

Materials and methods

Clinical findings

In a large, double consanguineous Dutch family, patients present with easy fatigability and exercise intolerance due to fatigue and pain [CB, VII:11; MJ, VII:6; and JJ, VII:8, (Scholte *et al.*, 1995)]. When these patients are fatigued they have the strong urge to vomit, which gives them some relief and makes it possible to proceed with their activities. They also feel mentally slow for an hour. When they have enough rest and adapt their lifestyle according to their physical capabilities, they can cope with everyday life. There are no other complaints such as headaches or signs of hearing and vision loss. Cardiac function is normal. Onset of symptoms occurred from the age of 4 years onwards. Extensive laboratory screens of Patient VII:11 for metabolic diseases (measurement of serum very-long-chain fatty acids, serum and urinary amino acids, organic acids, acylcarnitine spectrum and purine and pyrimidine levels) were all normal except a lactic acid of 3 mmol/l (controls < 1.9 mmol/l) and a thyroid function compatible with thyrax medication-corrected hypothyroidism. Patients showed subsarcolemmal mitochondrial proliferation. Muscle was predominantly affected, but not the brain, except for a single stroke-like episode in Patients VII:11 and VII:8. The function of other organs appeared normal. Other family members (siblings and parents) were able to exercise normally. Activities of the individual oxidative phosphorylation complexes were determined in muscle biopsies (Scholte *et al.*, 1995) and fibroblasts of patients (Table 1), as described earlier (de Wit *et al.*, 2008), revealing an isolated complex I deficiency. The patients were treated with a high dosage of riboflavin, which restored complex I activity to the lower normal range, but neither normalized blood lactate nor inhibited the proliferation of mitochondria (Scholte *et al.*, 1995). An unrelated patient (C.V.) with a similar phenotype who has previously been described (Scholte *et al.*, 1995) was also included

Table 1 Oxidative phosphorylation complex activities in fibroblasts

Patient	Complex I, %	Complex II, %	Complex III, %	Complex IV, %	Complex V, %	Citrate synthetase, %
VII:11	40	103	ND	117	100	141
VII:8	50	93	88	103	48	ND
VII:6	38	ND	ND	159	ND	107
VII:5	100	ND	ND	200	ND	123

The citrate synthetase activity represents the percentage of the mean control value. The values for complexes I–V represent the percentage of the mean control value relative to citrate synthetase (van den Bosch *et al.*, 2005; Sgobbo *et al.*, 2007; de Wit *et al.*, 2008). ND = not determined.

in the candidate gene analysis, as was her mother for segregation analysis.

Homozygosity mapping

DNA samples from Patients VI:1, VI:2, VII:6, VII:8, VI:3, VI:4, VII:10 and VII:11 were genotyped using the Weber V6 set from the Cooperative Human Linkage Centre as described earlier (Sheffield *et al.*, 1995), followed by the Affymetrix GeneChip Human Mapping 10K 2.0 for fine mapping. Parametric log of odds scores were calculated using the Merlin package (version 1.1.2) (Abecasis *et al.*, 2002) with a recessive disease model. For the Affymetrix CHIPs, samples were processed and labelled according to the instructions of the manufacturer, hybridized in a GeneChip hybridization oven followed by washing and staining with the GeneChip Fluidics Station 450 and scanning with the GeneChip Scanner 3000 (Affymetrix). Genotypes were generated by the GeneChip DNA Analysis Software followed by detection of homozygous regions in patient samples with the Copy Number Analysis Tool (Affymetrix).

Sequence analysis and mutation-specific restriction digestion

Exons and flanking intronic regions of the human *PCCB*, *MRPL3*, *SELB* and *ACAD9* genes were amplified using specific intronic primers (Supplementary Table 1). Polymerase chain reaction (PCR) products were directly sequenced with the PRISM Ready Reaction Sequencing Kit (Perkin-Elmer) on an ABI3730 automatic sequencer (Applied Biosystems).

A mutation-specific restriction assay was developed to screen controls for the c.1594C>T mutation in the *ACAD9* gene. A 10 µl PCR product of exon 16 was digested with AciI for 1 h at 37°C, followed by heat inactivation for 20 min at 65°C. The mutation resulted in the loss of a restriction site presenting a fragment of 198 bp instead of 147 bp, which was visualized on a 3% Metaphor gel (Cambrex). The c.1405C>T mutation resulted in the loss of a MspI restriction site for the PCR product of exons 14–16, presenting a fragment of 376 bp instead of 257 bp. This was visualized on a 2% agarose gel (Invitrogen) after the digestion of 5 µl of PCR product for 1 h at 37°C followed by heat inactivation for 20 min at 65°C. A mutation-specific restriction assay was developed to investigate whether the single nucleotide polymorphism c.379A>C and the mutation c.380G>A were in *cis* or *trans* positions. Either of the mutations leads to the loss of a SmlI restriction site for the PCR product of exon 4, leading to a fragment of 219 bp instead of 146 bp, which was visualized on a 3% agarose gel (Invitrogen).

Structural modelling of the human ACAD9 protein

The three-dimensional (3D) structure of the human ACAD9 protein (residues 28–621) was modelled by template-based modelling techniques, using the Yasara/What if Twinset package. As a template the 1.45-Å coordinate set for the human very-long-chain acyl-CoA dehydrogenase (ACADVL, Protein Data Bank code: 2uxw) was used. A secondary structure was predicted for the target sequence in order to aid in the alignment between target and template. This was performed by running the Basic Local Alignment Search Tool to retrieve homologous sequences, creating a multiple sequence alignment and feeding it to the 'Discrimination of Secondary Structure Class' prediction algorithm (King and Sternberg, 1996). Next, an optimal alignment was made, sequence identity between target and template being 47%. Loops were modelled using a knowledge-based approach and, after side-chain replacement and global optimization, loops were minimized using simulated annealing. A 29-amino acid gap was present in this alignment between A458 and N486, for which a loop was built. A full restrained simulated annealing was run on the entire model and, based on the final *Z*-score of −0.201 and the model was approved for further analysis. *In silico* mutageneses were performed using the Yasara/What if twinset package using standard protocols and molecular dynamics simulations were run for 1.6 ns, for each of the protein variants (wild-type and R532W), to study structural impacts of the mutation. The dynamics parameters used were: pH 7.0, 0.9% NaCl at 298 K in explicit water as a solvent with the AMBER3 force field and inclusion of long-range coulombic forces following the particle-mesh Ewald approximation. The molecular dynamics trajectories were then analysed to obtain root-mean-square fluctuation and energies.

Blue native-polyacrylamide gel electrophoresis

Mitoplasts from patient and control fibroblasts were isolated as previously described (Nijtmans *et al.*, 2002). Isolated mitoplasts (10 µg) were analysed on polyacrylamide gradient gels (4–16%; Invitrogen). Western blot analysis was performed by hybridization with monoclonal antibodies raised against complex I subunit GRIM19 and 70 kDa complex II subunit (Mitosciences).

Complementation assay

Feline immunodeficiency virus-based lentiviral transduction was performed according to the protocol of the manufacturer (GeneCopoeia, version V09031903). For feline immunodeficiency virus production, HEK293T cells were plated 1 day before transfection

in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal bovine serum at 70% confluence. The medium was replaced with Dulbecco's modified Eagle's medium supplemented with 2% foetal bovine serum and the HEK293T cells were co-transfected using Lipofectamine™ LTX and PLUS™ Reagents (Invitrogen), with the packaging plasmids and the expression vector containing the ACAD9 complementary DNA and a neomycin-resistance gene in a 5:1 ratio. The supernatant containing the viral particles were collected 24 and 48 h after transfection, centrifuged at 3000 r.p.m. for 5 min and filtered through a 0.45 μm filter. Patient fibroblasts (60% confluence) were transduced by changing Dulbecco's modified Eagle's medium (Invitrogen, 10% foetal calf serum, Invitrogen, 200 μm uridine, Sigma) with Dulbecco's modified Eagle's medium containing viral particles in a 1:2 dilution. Transduced fibroblasts were selected with G418 (Sigma, 2 μl/ml) for >3 weeks, starting 48 h after transduction. The activities of complex I, citrate synthase and complex IV were measured spectrophotometrically as described by Sgobbo *et al.* (2007) at three independent time points for each cell line.

Results

Homozygosity mapping of a consanguineous complex I family

In order to determine the chromosomal region carrying the genetic defect in a double consanguineous family (Fig. 1) with an isolated complex I deficiency, we performed a whole-genome scan using polymorphic markers of the Weber V6 set from the Cooperative Human Linkage Centre. A region on chromosome 3 was identified with a maximum two-point log of odds score of 4.5. This region was 26.9 Mb in length, spanning from marker D3S2460 at 119.8 Mb to marker D3S3694 at 146.7 Mb, including 407 transcripts. The region was refined by homozygosity mapping using the Human Mapping 10K v2 Chips on Patients VII:6, VII:8 and VII:11, their parents VI:1, VI:2, VI:3 and VI:4 and an unaffected sibling VII:10. This reduced the homozygosity region to 14.4 Mb (126.2–140.6 Mb) containing 166 genes, of which six were predicted to have a mitochondrial function according to the mitochondrial compendium (Pagliarini *et al.*, 2008), which was based on mass spectrometry, green fluorescent protein targeting and machine learning. Four of these, i.e. *PCCB* (propionyl Coenzyme A carboxylase), *MRPL3* (mitochondrial ribosomal protein L3), *EEFSEC* (eukaryotic elongation factor, selenocysteine-tRNA specific) and *ACAD9* (acyl-CoA dehydrogenase 9), were prioritized for mutation analysis.

Mutation analysis revealed a p.Arg532Trp mutation in *ACAD9*

Conventional sequence analysis of the exons and flanking intron sequences of the first four candidate genes in Patient VII:6 revealed only one homozygous, potentially pathogenic mutation. This was a C-to-T transversion of nucleotide 1594 in *ACAD9*

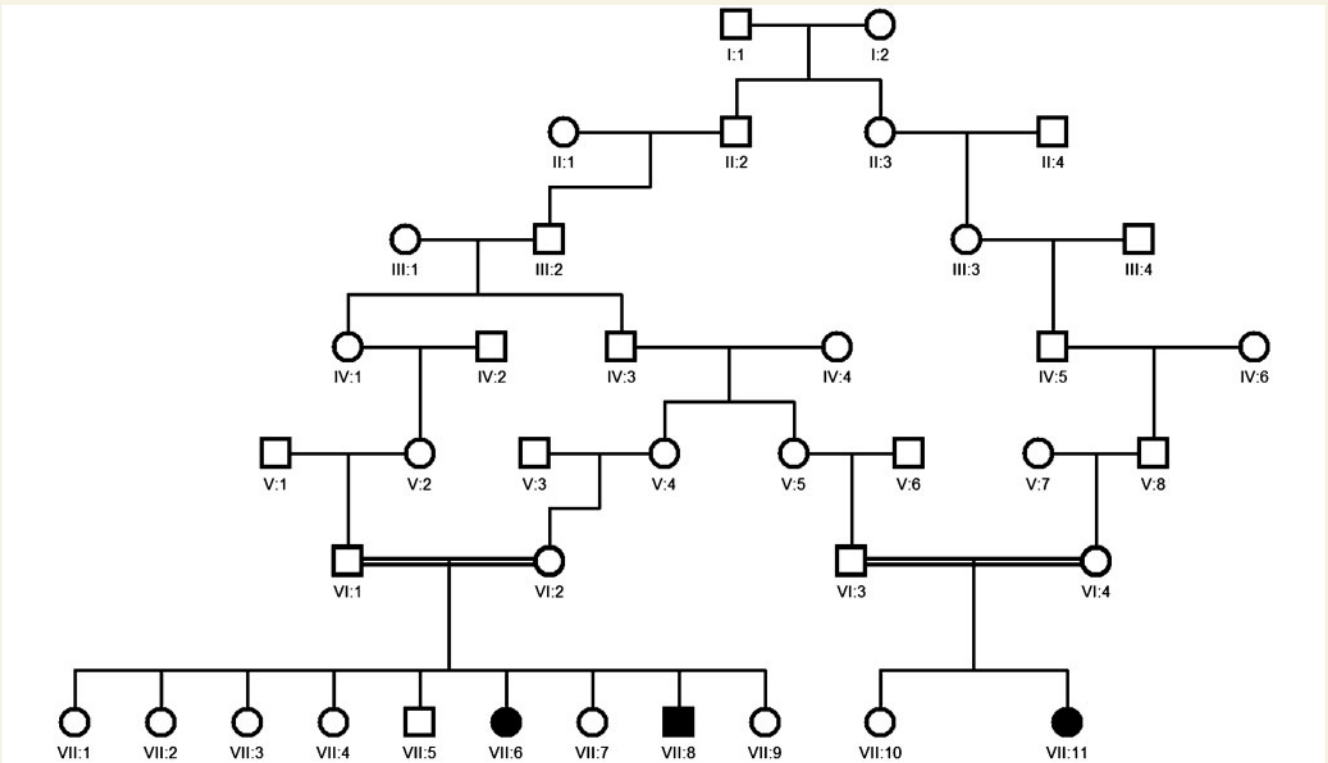

Figure 1 Dutch double consanguineous family with exercise intolerance, vomiting and complex I deficiency. Circles represent females and squares represent males. Filled symbols indicate patients, open symbols indicate unaffected individuals. Double lines indicate consanguinity.

(NM_014049.4) leading to the substitution of arginine by tryptophan at position 532. The two other patients (Patients VII:8 and VII:11) were also homozygous for this mutation, the parents were heterozygous and the other siblings were either heterozygous or homozygous wild-type. A mutation-specific restriction assay revealed the absence of the mutation in 376 Dutch control alleles, further supporting a pathogenic role. The p.Arg532Trp mutation occurred within a region that is highly conserved in *ACAD9* variants from human to zebrafish (Fig. 2A). Moreover, among a set of homologous proteins retrieved via a Basic Local Alignment Search Tool from the SWISS Prot and UNIPROT KB public databases, amino acid 532 is completely conserved (Fig. 2B). Because ACAD9 is 47% identical in amino acid sequence with ACADVL, we used the crystal structure 2uxw.A to model the effect of the missense mutation on intra-molecular forces (Fig. 3A and B). Overall model quality was checked using What_Check and Yasara, employing knowledge-based potentials for validation. The final overall *Z*-score of −0.021 indicates good overall model quality. In the loop region between A458 and N486, caution is to be taken with respect to the local structure. The structure of the

Figure 2 Conservation of the relevant part of the ACAD9 protein with the three amino acid substitutions. (**A**) Cross-species comparison of part of ACAD9 (amino acid 111–146 and 458–543). Arginine 127 and 532 in ACAD9 are conserved from man to zebrafish (ClustalW). Position 469 is less conserved at the amino acid level. However, the polarity is conserved at that residue from man to zebrafish. (**B**) Sequence alignment of different ACAD proteins among different species (amino acid 520–544), which shows that arginine 532 is also highly conserved between different types of ACAD.

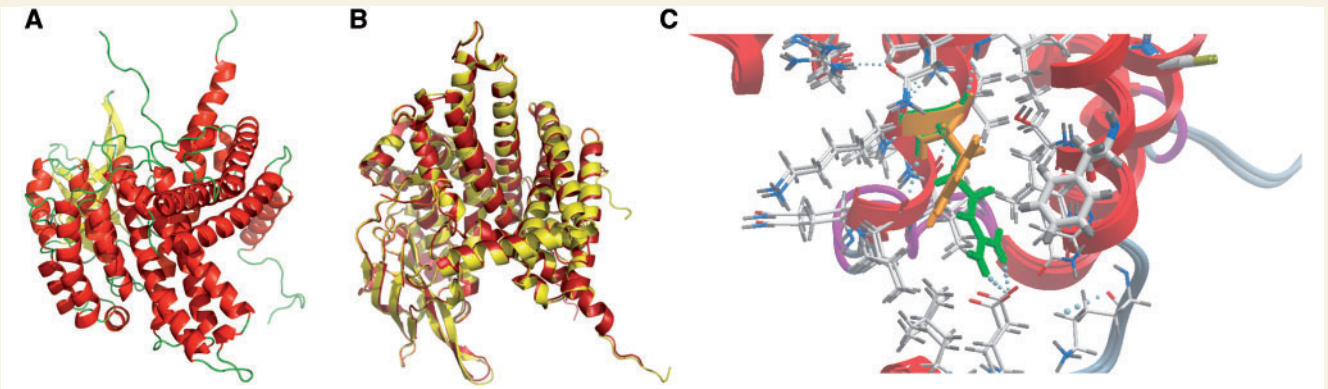

Figure 3 The 3D structure of normal and mutated ACAD9. (**A**) Model of human ACAD9 with crystal structure 2uxw.A as model. (**B**) Overlay of model of human ACAD9 (red) and template 2uxw.A (yellow). (**C**) Overlay of wild-type and mutant ACAD9 with focus on Arg532 (green), which establishes a double hydrogen bond with Glu592. This double hydrogen bond is lost when arginine is changed into tryptophan (orange).

model starts at Arg28 by extension of the sequence alignment by 10 residues. Analysis of the 3D model revealed that Arg532 (Arg567 in ACADVL) establishes a double hydrogen bond with Glu592 (Fig. 3C) located 2.1 and 1.9 Å from each other (distance between atom oe1 from Glu592 and hh12 and hh22 of Arg532). This interaction contributes to the stabilization of the α-helix network, orientation of the C-terminal helix and is localized in a region not far from the flavine adenine dinucleotide (FAD) binding pocket. The loss of a stabilizing hydrogen bond enhances a local flexibility gain, as evidenced from the analysis from molecular dynamics simulations (Fig. 4). Major conformational backbone changes are not observed due to the mutation; however, changes in flexibility are observed for Lys588, Glu592 and Leu594, which are at a distance <5 Å from Arg/Trp532, in the hinge region connecting the two C-terminal α-helices. This helix as a whole appears to shift due to the mutation. The bulkier tryptophan side chain induces a number of smaller side-chain conformational changes and may engage in π–π bonding with the nearby (<6 Å) Phe581, as indicated by the overall slightly lower flexibility in helix III in the Arg532Trp mutant. Together this may result in reorientation of α-helices, altering FAD binding. In the wild-type protein, the linker region between helices I and II (Fig. 4) is more flexible than in the mutant Arg532Trp. ACAD9 has previously been reported to occur in a homodimer, like ACADVL (Ensenauer *et al.*, 2005). However, Arg532 is not located near the residues responsible for the homodimer formation, making it unlikely that Arg532Trp affects the homodimer formation.

Transduction with wild-type *ACAD9* restores complex I activity

To investigate whether the *ACAD9* c.1594C>T mutation caused the observed complex I deficiency, we performed a complementation assay with wild-type and mutant *ACAD9* in fibroblasts from Patients VII:6, VII:7 and VII:9. These fibroblasts were stably transduced with lentiviral clones containing wild-type *ACAD9* and *ACAD9* containing the c.1594C>T mutation. The deficient complex I activity in the mutant cell line was restored by wild-type *ACAD9* to control values, but not with mutant *ACAD9* (Fig. 5, Supplementary Table 2). Remarkably, the complex I activity in the heterozygous cell lines transduced with mutant *ACAD9* was decreased to the same level as the homozygous mutant cells.

Blue native-polyacrylamide gel electrophoresis does not show an effect of defective *ACAD9* on assembly of complex I

Blue native-polyacrylamide gel electrophoresis was performed to test if the complex I deficiency was due to an assembly defect. No difference in the total amount or pattern of complex I was observed (data not shown). In addition, although expected by the reported function of ACAD9, no primary defect in β-oxidation was observed, based on metabolites in serum and urine (data not shown).

ACAD9 mutation in a patient with a similar phenotype

An unrelated patient with a similar phenotype as the patients of the consanguineous family was screened for mutations in the *ACAD9* gene. Mutation analysis revealed that the patient was compound heterozygous for the c.380G>A and c.1405C>T mutation. Additionally, the patient was heterozygous for the common single nucleotide polymorphism c.379A>C, which is located in the same codon as the c.380G>A. The presence of the single-nucleotide polymorphism in *cis* or *trans* has implications for the nature of the amino acid substitution. Restriction digestion of the PCR product for exon 4 showed that these mutations were

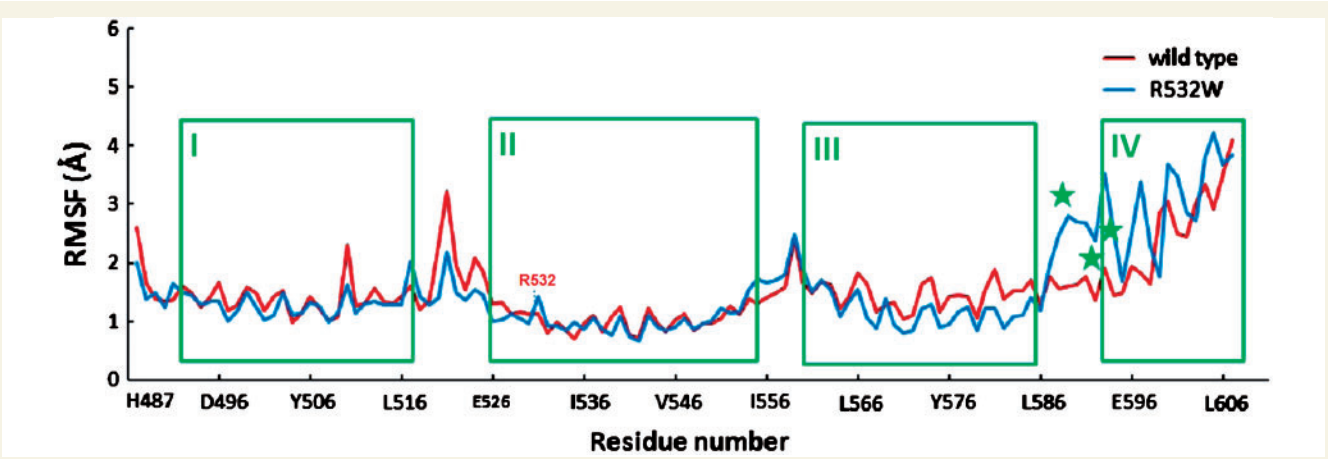

Figure 4 Flexibility analysis of a molecular dynamics simulation of normal and mutated ACAD9. Indicated are root-mean-square fluctuations (RMSFs) per residue for the C-terminal part of the protein for His487–Leu606. Residues 607–621 have been left out because of the intrinsic flexibility of the C-terminus. The C-terminal α-helices I–IV are indicated by green boxes, the residues Lys588, Glu592 and Leu594, positioned <5 Å from Arg532Trp (R532W) are indicated by green stars. Helix I: Leu494–Phe519, helix II: Gln527–Ile556, helix III: His561–Leu586 and helix IV: Asn593–Lys608.

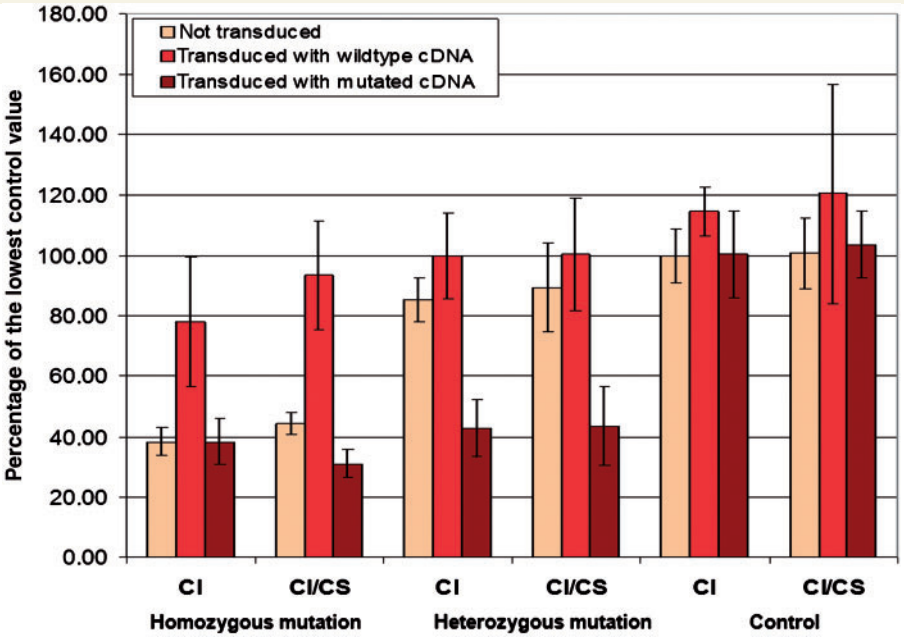

Figure 5 Transduction of mutant fibroblasts with wild-type *ACAD9* restores complex I activity. Fibroblasts of patients homozygous for the Arg532Trp mutation, heterozygotes and wild-type were not transduced (orange bar), transduced with wild-type *ACAD9* (red bar) or mutated *ACAD9* complementary DNA (cDNA) (brown bar). Transduction with wild-type *ACAD9* restores complex I activity in the homozygous mutant, but the mutant does not. Transduction of the heterozygote with mutant *ACAD9* reduces complex I activity to the levels of the homozygous mutant, while complex I activity in homozygous wild-type fibroblasts is unaffected by transduction of either wild-type or mutant *ACAD9*. Complex I activity is indicated relative to protein (CI) or citrate synthase (CS). Error bars represent the standard deviation of the complex I activity, which was measured at three independent time points for one transduction experiment.

in *cis*, resulting in the change of arginine at position 127 into glutamine. The c.1405C>T mutation leads to the substitution of arginine at position 469 with tryptophan. The substitution of Arg127 with glutamine is non-conservative and changes the charge of the amino acid, probably changing structure or function in this region. Arg127 is highly conserved from zebrafish to humans. Analysis of the local structure shows that, in the wild-type protein, Arg127 is not involved in hydrogen bonding, nor is the surface-exposed guanidium group involved in electrostatic interactions.

The region where the Arg469Trp mutation occurred is less conserved at the amino acid level, but the polarity of the amino acid at position 469 is highly conserved, indicating an important function of the polarity of this residue for ACAD9 structure or function (Fig. 2A). Potential structural effects of the Arg469Trp mutation were analysed. However, given its position in the 29 amino acid loop, which was modelled onto the protein backbone for which a structural template was not available, any detailed analysis of interaction partners of Arg469 is subject to a high degree of uncertainty. The pathogenic role for both substitutions was further supported by the absence of the c.380G>A and c.1405C>T mutations in 306 and 466 control alleles, respectively. Unfortunately, only maternal DNA was available for segregation analysis. However, as the maternal DNA was negative for the c.1405C>T mutation and heterozygous for the c.380G>A mutation, it is most likely that the c.1405C>T allele was inherited from the father and the c.380G>A allele from the mother. Although it cannot be completely excluded that the wild-type alleles would be inherited from the mother and both mutations in *cis* from the father, this is extremely unlikely, based on the very low allele frequencies.

Discussion

Mitochondrial complex I deficiency is the most prevalent oxidative phosphorylation disorder, with a broad variety of phenotypic manifestations, including diabetes mellitus, Leber's hereditary optic neuropathy and Leigh disease. Mutations causing complex I deficiency have been detected in both mitochondrial and nuclear-encoded complex I subunits. Additionally, mutations have been reported in several factors involved in complex I assembly and stability. Although novel factors in the assembly and function of complex I have been discovered in the last few years, the picture is still not complete. Using homozygosity mapping in a family with complex I deficiency, we identified the first missense mutation in *ACAD9* that changes a highly conserved arginine at position 532 to tryptophan. Protein modelling using ACADVL as a template shows the loss of a hydrogen bond between Arg532 and Leu528, which contributes to the stabilization of an α-helix. The substitution of this arginine by tryptophan was predicted to cause a local conformational change influencing the activity of ACAD9. Transduction of patient fibroblasts with wild-type *ACAD9* restored complex I activity,

whereas transduction with mutant *ACAD9* activity failed to rescue complex I activity. Although complex I activity was impaired, blue native-polyacrylamide gel electrophoresis did not reveal a difference in the total amount of complex I in the consanguineous family, indicating that the complex I defect in these patients was not due to a decreased assembly. Our data provide evidence for a new role for *ACAD9* in complex I function and reveal *ACAD9* as a new important candidate gene for complex I deficiency. This is of particular importance as this type of complex I deficiency responds to riboflavin treatment, improving both exercise intolerance and complex I activity (Scholte *et al.*, 1995).

Previous reports proposed a role for *ACAD9* in β-oxidation of long-chain fatty acids based on *in vitro* experiments with purified recombinant ACAD9 (Ensenauer *et al.*, 2005). He *et al.* (2007) identified defects in *ACAD9* messenger RNA and ACAD9 protein in β-oxidation-deficient patients. However, it was striking that mutations causing the messenger RNA defects could not be identified in the *ACAD9* gene itself. This would have confirmed the genetic basis for ACAD9-related β-oxidation pathology. Our data point to another role for *ACAD9*. The patients with an *ACAD9* mutation did not display a defect in β-oxidation, but in activity of complex I (Scholte *et al.*, 1995). Complex I activity was decreased in Patients VII:8 and VII:11 to 32 and 6% in muscle (Scholte *et al.*, 1995) and 50 and 40% in fibroblasts (Table 1), respectively, compared with control values. Muscle was not available for Patient VII:6, but in fibroblasts a complex I decrease was observed (Table 1). The higher complex I activity in fibroblast cultures could be explained by culturing conditions. Riboflavin was one of the additives in the medium and this factor has been demonstrated in these patients to improve complex I activity (Scholte *et al.* 1995). Transduction of the fibroblasts of Patient VII:6 with wild-type *ACAD9* in a lentiviral assay restored the complex I activity to control values. Transduction of a heterozygote with mutant *ACAD9* caused a complex I deficiency comparable with that of the patients. Most likely, the mutant protein is highly over-expressed in these cells, creating a situation where mutant protein prevails and the amount of wild-type protein is insufficient for proper complex I function, resulting in complex I deficient cells. This could also imply that mutant *ACAD9* could be a competitive inhibitor of wild-type *ACAD9* above a certain threshold. Apparently, the levels of mutant protein in the wild-type cell line transduced with mutant *ACAD9* are below this threshold preventing a complex I deficiency from occurring. Our data convincingly demonstrated that the *ACAD9* defect caused the complex I deficiency. This is further supported by a second patient with a compound heterozygous *ACAD9* mutation and a similar phenotype with complex I deficiency. Although no complementation has been done in the cells of this patient, it is probable, based on the nature of the amino acid substitutions, the conservation, the polarity change and the absence of these mutations in a large control population, that these mutations are pathogenic as well.

This is the first defect in a presumed β-oxidation gene leading to overt complex I deficiency. A physiological relationship between oxidative phosphorylation and β-oxidation has been known for some time. Gene expression profiles and proteomic data from mouse mitochondria revealed more than 90 co-regulated genes involved in oxidative phosphorylation, β-oxidation and the tricarboxylic acid cycle (Mootha *et al.*, 2003). This is also the case for *ACAD9* expression, regulated by *NRF1* and *CREB*, which are important regulators of most respiratory chain enzyme subunits (He *et al.*, 2007). In addition, various enzymes involved in β-oxidation are associated with respiratory chain supercomplexes. These supercomplexes have been shown to support more rapid electron transfer rates in mitochondria, most likely resulting from more efficient substrate channelling, stabilization of the protein complexes or increased capacity (Vockley, 2008). Studies of rat liver and skeletal muscle mitochondria showed a direct physical interaction between β-oxidation and oxidative phosphorylation (Vockley, 2008). Although, the role of *ACAD9* in complex I could not be fully determined from our data, it is likely that *ACAD9* plays a role in the supercomplexes, of which complex I is an integral part. A role for *ACAD9* as an acyl carrier, required for proper complex I assembly, comparable with acyl carrier protein (Feng *et al.*, 2009), is not supported by the results of the blue native-polyacrylamide gel electrophoresis experiments, which did not show a decrease in assembled complex I. It is evident that further experiments will be required to draw a definite conclusion on the effects of *ACAD9* mutations on metabolic channelling, supercomplex stability and complex I and to explain the functional interaction between *ACAD9* and oxidative phosphorylation in full detail.

Importantly, the patients discussed in this article were riboflavin responsive (Scholte *et al.*, 1995). The patients received riboflavin at a dose of 300 mg/day, which increased the creatine phosphate resynthesis after exercise in muscle by ^{31}P magnetic resonance spectroscopy (Patients VII:11, VII:8) and showed improvement in the patients clinical condition (Bakker *et al.*, 1994). After riboflavin treatment, complex I activity increased from 17 to 47% in Patient VII:8. Riboflavin treatment has not extensively been documented. Previous reports showed clinical and biochemical improvements in small groups of patients with complex I deficiency (Penn *et al.*, 1992; Bernsen *et al.*, 1993), but not in a larger study of 16 patients (Mathews *et al.*, 1993). Until now, riboflavin-responsive complex I deficiency has only been described in patients with predominantly myopathic presentations. It is therefore unlikely that riboflavin will be beneficial for all patients with complex I deficiencies, but it may be for a well-defined subgroup. Riboflavin supplements were likely to increase intra-mitochondrial FAD concentration favouring FAD binding, which is important for the catalytic activity of flavoproteins as well as for their folding, assembly and/or stability (Nagao and Tanaka, 1992; Saijo and Tanaka, 1995). It has been suggested that raising the intra-mitochondrial FAD concentration may compensate for a decreased folding capacity of mutant flavoproteins (Olsen *et al.*, 2007). Probably, ACAD9 folding or its catalytic activity is disturbed by the Arg532Trp mutation, which is predicted to influence the FAD binding pocket of ACAD9. The resulting reduction in FAD binding might be compensated by the increase in FAD binding due to riboflavin supplementation. An alternative or additional effect of riboflavin treatment in our patients might be the enhancement of the assembly of complex I and complex IV (Grad and Lemire, 2006), which, together with complex III, are part of the respiratory chain supercomplexes. Enhancement of supercomplex formation could be hypothesized to complement

the proposed negative effect of *ACAD9* mutations on the function of the supercomplexes.

In conclusion, we identified a pathogenic mutation in *ACAD9* in a Dutch consanguineous family and in an unrelated patient with riboflavin-responsive complex I deficiency. Our results reveal a new function for *ACAD9* in the activity of complex I as demonstrated by complementation of the complex I deficiency in fibroblasts of patients. These findings make *ACAD9* an important new candidate for mutation screening in complex I patients, in which riboflavin might be used to ameliorate clinical symptoms and complex I function.

Acknowledgements

We would like to warmly acknowledge the late neurologist Prof. Er. Herman F.M. Busch (21 August 1929 to 21 January 2005; Erasmus MC Rotterdam, The Netherlands), who discovered this huge family in the 1994–1995 and who would have been extremely happy that we finally, after 15 years, solved the genetic cause. And the same holds for the paediatrician Dr Henk D. Bakker (AMC, Amsterdam, The Netherlands), who discovered Patient CV. This patient was described in 1994 as having the same disorder as the affected family members on clinical and biochemical grounds, which was eventually confirmed by the studies reported in this article. We thank the patients for their participation in this study.

Funding

Our work was supported by a EU grant to the MitoCircle project (Sixth Framework Program, contr. no. 005260). H.P. was supported by the Impulse and Networking Fund of the Helmholtz Association in the framework of the Helmholtz Alliance for Mental Health in an Ageing Society (HA-215), the German Network for Mitochondrial Disorders (mitoNET #01GM0862 and 01GM0867) and Systems Biology of Metabotypes (SysMBo #0315494A).

Supplementary material

Supplementary material is available at *Brain* online.

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