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Report

Deficiency of the ADP-Forming Succinyl-CoA Synthase Activity Is Associated with Encephalomyopathy and Mitochondrial DNA Depletion

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The mitochondrial DNA (mtDNA) depletion syndrome is a quantitative defect of mtDNA resulting from dysfunction of one of several nuclear-encoded factors responsible for maintenance of mitochondrial deoxyribonucleoside triphosphate (dNTP) pools or replication of mtDNA. Markedly decreased succinyl-CoA synthetase activity due to a deleterious mutation in *SUCLA2*, the gene encoding the β subunit of the ADP-forming succinyl-CoA synthetase ligase, was found in muscle mitochondria of patients with encephalomyopathy and mtDNA depletion. Succinyl-CoA synthetase is invariably in a complex with mitochondrial nucleotide diphosphate kinase; hence, we propose that a defect in the last step of mitochondrial dNTP salvage is a novel cause of the mtDNA depletion syndrome.

The oxidative phosphorylation (OXPHOS) system consists of 87 proteins that are assembled into five enzymatic complexes. Of the 87 subunits, 13 are encoded by mtDNA, which also encodes all mitochondrial tRNA and rRNA transcripts. mtDNA is constantly synthesized throughout the cell cycle, requiring a balanced supply of dNTPs within the mitochondria and a large number of factors, mostly nuclear encoded, that participate in the replication, transcription, and translation processes.

In the past decade, a growing number of patients with OXPHOS dysfunction due to depletion of mtDNA have been reported. The onset of symptoms usually occurs during the first months of life, and most patients die in early childhood. Clinical presentation is heterogeneous and includes progressive muscle weakness, liver failure, encephalopathy, cardiomyopathy, and renal disease, usually accompanied by lactic acidemia (Moraes et al. 1991; Tritschler et al. 1992; Vu et al. 1998; Ducluzeau et al. 1999; reviewed by Elpeleg 2003). Autosomal recessive inheritance was suspected in many cases, and no mutations were identified in the mtDNA molecule, which suggests that mtDNA depletion results from defects in nuclear factors involved in mtDNA replication. Four genes have previously been implicated in the pathogenesis of mtDNA depletion; mutations in the thymidine phosphorylase gene are associated with leukodystrophy and gastrointestinal disorder (Nishino et al. 1999); mutations in the deoxyguanosine kinase and mtDNA polymerase γ genes are associated with encephalopathy and fatal infantile liver disease (Mandel et al. 2001; Naviaux and Nguyen 2004); and mutations in the mitochondrial thymidine kinase gene result in progressive muscle disease with variable brain involvement (Saada et al. 2001).

We identified a small Muslim pedigree (fig. 1) with autosomal recessive encephalomyopathy associated with mtDNA depletion. Patient II-7 is a female, the second child born to consanguineous parents of Muslim origin. Her older sister died of neurodegenerative disease at age 7 mo, and her younger sister and brother are healthy. The pregnancy was uneventful, apart from a markedly elevated level of α-feto protein in maternal blood. The patient was born at term but was small for gestational age (weight 2,250 g, <3rd percentile). In early infancy, the patient was irritable, with inconsolable crying. Psychomotor development was severely retarded, with marked muscle hypotonia and impaired hearing. She had frequent respiratory infections, which were thought to

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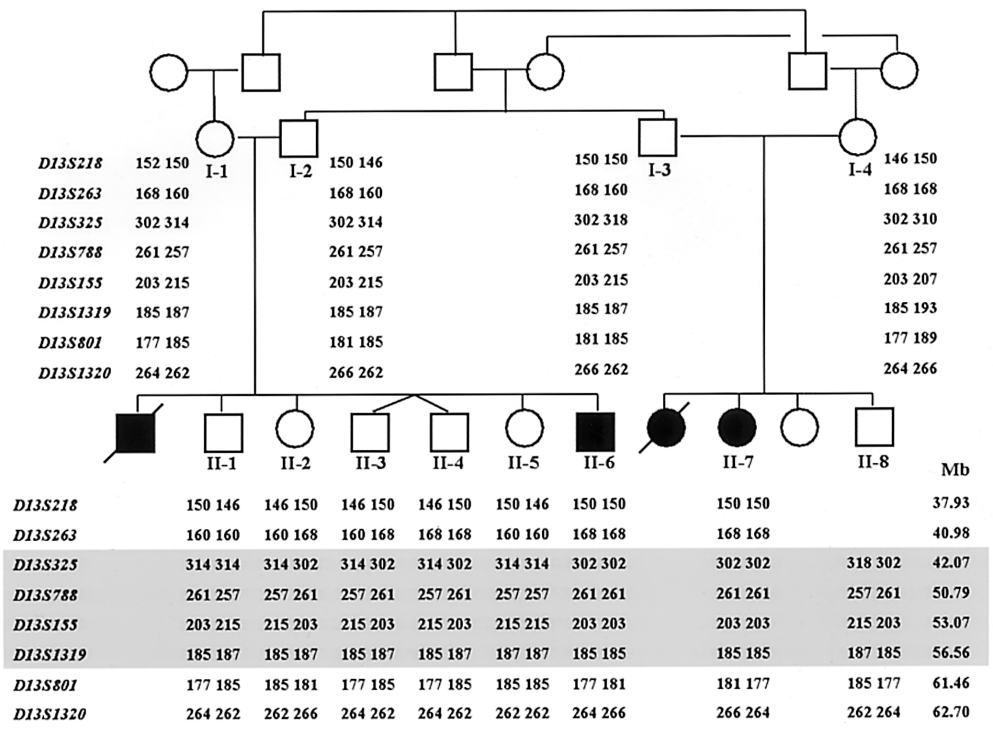

Figure 1 Family tree, with allele sizes of the microsatellite markers in the critical region on chromosome 13. Only those individuals whose DNA was available for analysis were annotated. The region of shared homozygosity is shaded. Markers are listed in the left column, and their physical locations on the chromosome are given (in Mb) in the right column.

be secondary to severe gastroesophageal reflux. Generalized seizures, first noted at age 1 year, were controlled by valproic acid. Brain MRI performed at age 18 mo was suggestive of Leigh syndrome, with high signal T2 intensity in the putamen bilaterally and moderate dilatation of the lateral ventricles and sulci, compatible with generalized atrophy. Physical examination at age 7 years revealed contractures at the knee and hip joints and dystonic posturing of the fingers. The patient was able to roll over but was unable to sit unsupported. At age 7 years, she recognized and greeted her relatives with a smile and cried when being scolded but had neither speech nor gestures. She had mild ptosis, but her vision appeared intact. The patient's hearing was impaired, but this condition was not assessed any further. Chronic microcytic anemia (hemoglobin level [Hb] 9.1 g/dl; mean corpuscular volume [MCV] 65 femtoliters [fl]) and an elevated lactate level in plasma (4.3 mM; normal level is <2.1 mM) were present. Liver and renal function tests, plasma creatine kinase level, abdominal ultrasound, electrocardiogram, and echocardiogram were normal.

Patient II-6 is the cousin of II-7 and is the seventh child born to consanguineous parents. An older brother had a cot death at age 4 mo. The patient was born at term but was small for gestational age (weight 2,180 g, <3rd percentile). At age 2 mo, he was found unresponsive and hypopneic. Electroencephalogram showed generalized slow activity, and CT scan of the brain revealed lacunar infarcts in the basal ganglia. The patient slowly regained consciousness and, after 2 d of assisted ventilation, started breathing spontaneously, but his subsequent development was poor. Echocardiogram was normal. At age 1 year, there was generalized muscle hypotonia, lack of voluntary movements, and bilateral hearing loss. A generalized seizure disorder, first noted at age 3 years, was controlled by clonazepam. At age 5 years, the patient's condition was unchanged, with severe psychomotor retardation and prominent muscle hypotonia. He had microcytic anemia (Hb 11.1 g/dl; MCV 65 fl) and an elevated lactate level in plasma (4–5 mM), but his liver and renal function tests and plasma creatine kinase level were normal.

The enzymatic activities of the OXPHOS complexes I–V in muscle mitochondria were determined by standard spectrophotometric methods (Hoppel and Cooper 1969; Dooijewaard and Slater 1976). The activities of complexes I and IV were significantly decreased, and complexes III and V were less affected, whereas complex II, the only complex that does not contain mtDNA-encoded proteins, was borderline normal (table 1). It is notable that the pathological and histochemical examinations of the muscle tissue from the two patients showed

Table 1

Activity of the Enzymatic Complexes of the Mitochondrial Respiratory Chain in Mitochondria Isolated from Muscle and Fibroblasts

Tissue and Subject(s)	Citrate Synthase Activity	Activity of Enzymatic Complex[a]					
		I	I+III	II+III	II	IV	V
Muscle mitochondria:							
Patient II-7	1,780	127 (52%)	148 (28%)	202 (72%)	255 (87%)	409 (35%)	501 (83%)
Patient II-6	1,790	107 (43%)	134 (25%)	181 (64%)	211 (72%)	307 (26%)	351 (58%)
Controls ($n = 50$):							
Mean ± SD	1,990 ± 370	274 ± 127	586 ± 234	312 ± 118	327 ± 52	1,326 ± 501	645 ± 203
Range	1,320–2,900	115–503	305–999	192–531	255–442	829–2,300	222–1,002
Fibroblast mitochondria:							
Patient II-7	199	23 (93%)	ND	82 (78%)	44 (59%)	335 (101%)	ND
Controls ($n = 27$):							
Mean ± SD	249 ± 97	31 ± 11	ND	131 ± 44	81 ± 31	412 ± 59	ND
Range	135–415	15–38	ND	63–204	50–101	203–586	ND

[a] Values are given in nmol/min/mg of protein. For patients, the percentage of the control mean normalized for citrate synthase activity is shown in parentheses. ND = not determined.

unremarkable results. The activities of all OXPHOS complexes were normal in mitochondria isolated from fibroblasts (table 1). The findings in muscle mitochondria suggested a defect in the synthesis of the mtDNA-encoded proteins. Sequence analysis of muscle mtDNA excluded mutations in the 22 mitochondrial tRNA genes in patient II-7. Southern blot analysis of muscle DNA, performed as described elsewhere (Saada et al. 2001), revealed a normal-size band of mtDNA in both patients, but the ratio of mtDNA to nuclear DNA, determined by real-time PCR, was reduced to 32% of the mean control value. The mtDNA:nuclear DNA ratio in fibroblasts was 95% of the mean control value.

Genomic DNA was extracted from blood samples from the two patients and 10 unaffected family members, after informed consent was obtained from the subjects or their guardians. To localize the mutated gene in this family, we performed a genomewide screen to search for homozygosity by using polymorphic microsatellite markers with an average intermarker distance of 10 cM (ABI PRISM Linkage Mapping Set MD10, version 2.0 [Applied Biosystems]). Genotypes were obtained for 402 markers, of which 21 were homozygous in both patients. Five of these markers were located successively on an ~50-Mb fragment of chromosome 13, bordered by markers *D13S175* and *D13S156*. Additional markers from other mapping sets and other selected polymorphic dinucleotide repeat markers were genotyped, which enabled us to exclude all other chromosomal regions and to narrow the shared homozygosity region to 20 Mb on chromosome 13, bordered by (but excluding) markers *D13S263* and *D13S801* (fig. 1). Mitochondrial localization was predicted (score >60) in only 3 of 113 ORFs in this region, by use of the MitoP2 prediction program: ORFs encoding the β chain of the ADP-forming succinyl-CoA ligase (*SUCLA2* [MIM 603921, NCBI accession number NM_003850]), a hypothetical protein FLJ25853, and the copper-transporting ATPase 2 (ATP7B) associated with Wilson disease (Andreoli et al. 2004). Sequence analysis of the 12 exons of the *SUCLA2* gene (*Homo sapiens* chromosome 13, nt 47414793–47473463 [NCBI accession number NC_000013.9]) and their exon-intron boundaries (primer sequences available on request) revealed a complex genomic rearrangement at the 3′ end of exon 6, with a homozygous deletion of 43 nt, encompassing the last 14 nt of exon 6 and the first 29 nt of IVS6, and an insertion of 5 nt (32720del43ins5) (fig. 2*A*). Analysis of the *SUCLA2* cDNA by use of primers cF1 (5′-TTCAAAAGATGTCTGATAAAGG-3′) and cR1 (5′-CGCATGATTCCTCCAAAAATGT-3′) resulted in two amplification products of nearly equal abundance, both of which skip exon 6 and one of which also lacks the first 74 bp of exon 7 (fig. 2*B*). Analysis of the genomic PCR products from the other family members, by use of exon/intron 6 primers 6F1 (5′-GTTGATTAGACATATG-CAAACTACCT-3′) and 6R2 (5′-AGGGAAGCATATTA-GAGAAATAAATG-3′), confirmed that all available relatives are heterozygous for the rearrangement, except for individual II-5, who is homozygous for the wild-type sequence. Using the same primer set, we analyzed 105 anonymous Muslim Arabs for the presence of the mutation and found no carriers.

The *SUCLA2* gene spans nearly 60 kb and encodes the 463 amino acids of the β subunit of the ADP-forming succinyl-CoA synthetase (SCS-A) (Allen and Ottaway 1986; Weitzman et al. 1986). This mitochondrial matrix enzyme catalyzes the formation of succinate and ATP from succinyl-CoA and ADP in the tricarboxylic acid (TCA) cycle in a reversible manner. SCS-A is a heterodimer that shares its α subunit with another mitochondrial matrix succinyl-CoA synthetase, SCS-G, which uses GDP (Sanadi et al. 1954). Thus, the two enzymes, SCS-G and SCS-A, catalyze a similar reaction by using different phosphate donors, with their β subunits deter-

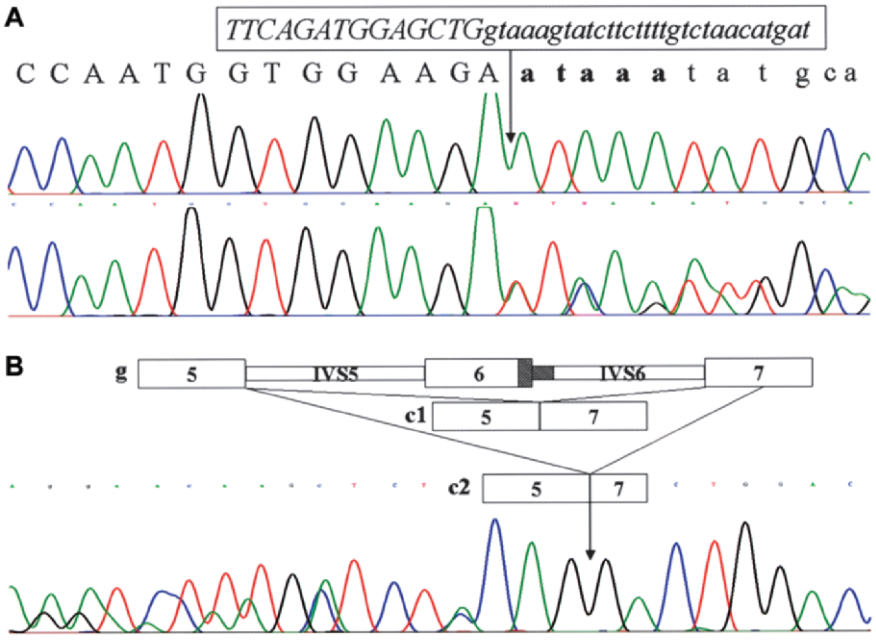

Figure 2 *A,* Genomic sequence of the exon 6–IVS6 junction of the *SUCLA2* gene in patient II-7 and her mother, I-4 (upper and lower chromatograms, respectively). The deleted 43 nt are shown in italics, and the 14 exonic nucleotides are also capitalized. The deletion start site is indicated by an arrow. The inserted 5 nt are shown in bold and are followed by the normal IVS6 sequence. *B, SUCLA2* cDNA sequence of patient II-7. A schematic representation (g) shows the exon-intron organization around the mutation site, which is shown in black. With the use of primers cF1 and cR1, two amplification products (c1 and c2) were seen, both of which skip exon 6, and transcript c2 also lacks the first 74 bp of exon 7. The splicing site of exon 5 with mid-exon 7 is indicated by an arrow.

mining the nucleotide specificity (Johnson et al. 1998*a*, 1998*b*). Notably, the activity of both SCS-G and SCS-A is considerable in mitochondria from rat liver, kidney, and heart, but SCS-A, the ADP-forming enzyme, predominates in the brain (Lambeth et al. 2004).

Exon 6 of the *SUCLA2* gene is composed of 139 nt and encodes 47 amino acids (residues 221–267) that form the ATP grasp domain of the protein. Skipping exon 6 is likely to result in the synthesis of the first 221 amino acids, followed by 50 residues of a different reading frame. Since the CoA-ligase domain is created by residues 305–441, truncated polypeptide chains are likely to adversely affect the catalytic activity. The shorter transcript, lacking exon 6 as well as the first part of exon 7, remains in-frame and is predicted to lead to the absence of 71 residues, starting at amino acid 221 but preserving the CoA-ligase domain. Accordingly, SCS-A activity in muscle mitochondria from patient II-6, determined in the direction of succinyl-CoA formation (Cha and Parks 1964), was decreased to 0.038 U per mg of mitochondrial protein (U/mg) (the average control value from six healthy children who underwent orthopedic surgery was 0.210 U/mg; range 0.18–0.25 U/mg). In contrast, the activity of SCS-G in the patient's muscle mitochondria was 0.408 U/mg (average control value 0.386 U/mg; range 0.31–0.41 U/mg; $n = 6$). Thus, the ratio of SCS-A to SCS-G activity in mitochondria isolated from the patient muscle was reduced to 17% of the control value. The activity of SCS-A was normal in mitochondria isolated from muscle of a patient with markedly decreased activities of complexes I and IV due to a high load of the A3243G mutation in the tRNALeu(UUR) gene, indicating that impaired OXPHOS activity does not perturb the function of SCS-A.

How does a mutation in the *SUCLA2* gene cause mtDNA depletion? The gene product of the *LSC1* gene, which is the yeast homologue of the *SUCLA1* gene, is a component of the yeast mtDNA nucleoid (Chen et al. 2005). Defects in some nucleoid proteins in humans, such as the mtDNA polymerase, the mitochondrial single-stranded DNA-binding protein, and the adenine nucleotide translocator 1, were associated with mtDNA depletion or multiple mtDNA deletions. However, the gene product of *LSC2,* the yeast homologue of *SUCLA2,* was not shown in nucleoids, and none of the succinyl-CoA synthase subunits was identified in the mtDNA nucleoids in higher eukaryotes (Bogenhagen et al. 2003).

A plausible explanation is offered by the finding that SCS-A copurifies and is tightly associated in a complex with nucleoside diphosphate kinase (NDPK) in both prokaryotes and eukaryotes (Kadrmas et al. 1991; Kavanaugh-Black et al. 1994; Kowluru et al. 2002). NDPKs are ubiquitous protein kinases that also catalyze the exchange of terminal phosphates between tri- and diphosphoribonucleosides and are crucial for maintain-

ing the homeostasis of ribonucleotides and deoxyribonucleotides (Parks and Agarwal 1973). Specifically, mitochondrial deoxyribonucleotide diphosphates are phosphorylated by a mitochondrial NDPK, NM23-H4 (Milon et al. 2000). The importance of this salvage pathway step for mtDNA synthesis is underscored by the lack of de novo synthesis of dNTPs in this organelle and by the constant need for building blocks for mtDNA synthesis throughout the whole cell cycle (Bogenhagen and Clayton 1976; reviewed by Saada 2004).

Whereas the physical proximity of succinyl-CoA synthetase (SCS) and NDPK is indisputable, the functional significance of their coexistence in a complex is less clear. It was proposed that this proximity signals the involvement of NDPK in energy metabolism, possibly by directly activating the SCS by phosphorylation (Kowluru et al. 2002). Our data suggest that SCS activity is essential for mtDNA synthesis and that SCS deficiency results in mtDNA depletion, possibly because of secondary NDPK dysfunction. Whether such an interference involves the nucleotide triphosphate donation or a conformational change induced by the SCS protein is presently unknown. Regardless of the precise mechanism, the brain seems to be the most vulnerable tissue, not only because all of its SCS activity can be attributed to the SCS-A enzyme, but also because the mitochondrial NDPK is hardly expressed in the human brain (Milon et al. 1997) and because of the rate-limiting role of NDPK in mtDNA replication in postmitotic tissues (Bradshaw and Samuels 2005). The fact that other tissues, such as liver, kidney, and heart, are not affected in our patients suggests that the nature of the interaction between SCS and NDPK is not influenced by the specific phosphate donor.

Genetic defects of the TCA cycle are uncommon in humans, and the most severe defects are probably lethal in early gestation (Rustin et al. 1997). Deficiency of SCS-A in our patients is compatible with life, likely because of the presence of SCS-G, an enzyme with an overlapping activity. For some of the TCA cycle proteins, additional cellular roles beyond their catalytic activities in the cycle have been proposed (Jeffery 1999). For example, fumarase and succinate dehydrogenase act as tumor suppressors in humans (Baysal et al. 2000; Tomlinson et al. 2002). Our finding that SCS-A deficiency is associated with mtDNA depletion in humans parallels the essential role of aconitase in mtDNA maintenance in yeast (Chen et al. 2005), and both highlight yet another pathway in which TCA cycle enzymes are involved.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

MitoP2, http://ihg.gsf.de/mitop2/start.jsp

NCBI, http://www.ncbi.nlm.nih.gov/ (for *Homo sapiens* chromosome 13 [accession number NC_000013.9], nt 47414793–47473463 [complement], and *Homo sapiens* succinate-CoA ligase, ADP-forming, β subunit [*SUCLA2*] mRNA [accession number NM_003850])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *SUCLA2*)

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