Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5186–5190, June 1993
Medical Sciences

Identification of two missense mutations in a dihydrolipoamide dehydrogenase-deficient patient

(inborn error/flavoprotein/compound heterozygosity)

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Communicated by Frederick C. Robbins, March 15, 1993 (received for review November 12, 1992)

ABSTRACT **The molecular basis of dihydrolipoamide dehydrogenase (E_3; dihydrolipoamide:NAD^+ oxidoreductase, EC 1.8.1.4) deficiency in an E_3-deficient patient was studied. Fibroblasts cultured from the patient contained only ≈6% of the E_3 activity of cells from a normal subject. Western and Northern blot analyses indicated that, compared to control cells, the patient's cells had a reduced amount of protein but normal amounts of E_3 mRNA. Direct sequencing of E_3 cDNA derived from the patient's RNA as well as each of the subclones of the cDNA revealed that the patient had two substitution mutations in the E_3 coding region. One mutation changed a single nucleotide from A to G, resulting in substitution of Glu (GAA) for Lys-37 (AAA). The other point mutation was a nucleotide change from C to T, resulting in the substitution of Leu (CTG) for Pro-453 (CCG). These mutations appear to be significant in that they alter the active site and possibly the binding of FAD.**

The mammalian dihydrolipoamide dehydrogenase (E_3; dihydrolipoamide:NAD^+ oxidoreductase, EC 1.8.1.4) is a common component of the three mitochondrial multienzyme α-ketoacid dehydrogenase complexes: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and branched-chain α-ketoacid dehydrogenase complexes (1). E_3 is also a component (referred to as L protein) in the glycine cleavage system in mitochondria (2). E_3 reoxidizes the dihydrolipoyl moiety covalently linked to specific lysine residue(s) in the acyltransferase components of the three α-ketoacid dehydrogenase complexes and the dihydrolipoyl moiety of the hydrogen-carrier protein of the glycine cleavage system (3). The mammalian E_3 is a homodimer with subunit M_r of 51,000; a molecule of FAD is noncovalently bound to each subunit.

E_3 deficiency causes a reduction in activities of the three α-ketoacid dehydrogenase complexes. General clinical symptoms of E_3 deficiency include lactic acidosis, neurological dysfunctions, and increased concentrations of plasma levels of branched-chain amino acids as well as increased urinary levels of α-ketoacids (4–11). As a result of accumulation of the branched-chain ketoacids, E_3 deficiency is classified as a variant form of maple syrup urine disease accompanied by primary lactic acidosis (7). Only a few patients with documented deficiency of E_3 have been reported and characterization of the deficiency has been largely limited to measurements of enzyme activity and immunoidentification of the enzyme (12, 13). Definitive diagnosis of E_3 deficiency has been performed by enzymatic assay of E_3 activity in skin fibroblasts, lymphocytes, or tissue extracts (5). Previously, we reported the isolation and characterization of a full-length human E_3 cDNA (14). The availability of this E_3 cDNA has made it possible to further investigate the nature of mutation(s) in a previously reported E_3-deficient patient (15). The present study demonstrates the presence of two substitution mutations in the coding region of the E_3 gene.

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MATERIALS AND METHODS

Subject. The clinical features of the patient investigated in this study were reported elsewhere (15). In the first week of life, the patient's blood leucine was found to be 5 mg/dl but the patient was left on a normal diet. At the age of 6 months, the patient exhibited hypotonia and minimum dystonic movements of the upper extremities (15). He had elevated plasma levels of pyruvate, α-ketoglutarate, and branched-chain amino acids and α-ketoacids (15). Urinary excretion of these ketoacids and their metabolites were also elevated (9). At 7 months of age, the patient was placed on a branched-chain amino acid-restricted formula, resulting in improvement of some biochemical and developmental parameters (15). The patient died at the age of 21 months, following a ketoacidotic episode. The total pyruvate dehydrogenase complex activity of the patient's cells was 10–30% that of control cells. Pyruvate dehydrogenase (E_1) and dihydrolipoamide acetyltransferase (E_2) activities were normal in the patient's muscle, but E_3 activity was not detectable in either muscle or liver specimens obtained at autopsy (12).

Enzymatic Analysis. Both patient and control skin fibroblasts were grown in a minimum essential medium supplemented with 20% fetal calf serum, streptomycin (100 μg/ml), penicillin (60 μg/ml), and amphotericin B (1.5 μg/ml) (16). The cells were harvested in phosphate-buffered saline containing 0.5% Triton X-100. Sonicated cell lysates were used for enzyme assays. Studies with cultured skin fibroblasts were performed according to protocols approved by the Institutional Review Board of University Hospitals of Cleveland.

E_3 activity was assayed spectrophotometrically by following the reduction of NAD^+ at 340 nm at 37°C (17). Citrate synthase activity was assayed by measuring the production of mercaptide ion at 412 nm at 37°C (18). One unit of enzyme activity was defined as 1 μmol of NADH or mercaptide ion produced per min per mg of protein. Protein concentration was determined by the method of Lowry *et al.* (19) using bovine serum albumin as the standard.

Western Blot Analysis. For Western blot analyses, cells were harvested and resuspended in 10 mM Tris·HCl (pH 7.4) containing 250 mM sucrose and 1 mM 2-mercaptoethanol. After addition of phenylmethylsulfonyl fluoride (0.5 mM) and leupeptin (25 μg/ml) as protease inhibitors, the sample was homogenized and then centrifuged at 500 × *g* to remove cell

Abbreviations: E_3, dihydrolipoamide dehydrogenase; E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase.

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debris. The supernatant fraction was then centrifuged at 10,000 × *g* for 15 min to obtain mitochondrial pellets for SDS/PAGE (20). After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane in transfer buffer (25 mM Tris·HCl/192 mM glycine, pH 8.3) at 75 mA for 18 h. After transfer, the membrane was washed with Tris-buffered saline and incubated with 5% bovine serum albumin in Tris-buffered saline at room temperature overnight and then reacted with an epitope-selected rabbit anti-porcine heart E_3 antibody for 3 h. Another membrane was incubated with a combined preparation of antisera, which were raised individually against bovine kidney E_1, bovine heart E_2, and porcine heart E_3. The proteins were detected by the enhanced chemiluminescence Western blotting detection system (Amersham). Levels of protein were quantitated with a scanning densitometer (United States Biochemical).

Northern Blot Analysis. Total RNA was extracted from cultured skin fibroblast cells using the guanidine thiocyanate method (21). About 25 μg of total RNA was separated on a 0.8% agarose gel containing 50% formaldehyde (22) and subsequently transferred to a GeneScreen membrane. The membrane was prehybridized at 42°C overnight in a solution of 20 mM Pipes, pH 6.4/2 mM EDTA/0.8 M NaCl/50% formaldehyde/100 μg of salmon sperm DNA per ml. The prehybridized membrane was hybridized at 65°C for 30 min and then at 42°C for a further 48 h, with a 1.1-kb fragment of E_3 cDNA located at the 5′ end of the previously reported E_3 cDNA sequence (14). This probe was labeled prior to hybridization by the random-primer reaction (23) to a specific activity of 100 cpm/pg with [α-^{32}P]dCTP. After hybridization, the membrane was washed at 50°C first with 2× standard saline citrate (SSC) buffer containing 1% SDS, then with 1× SSC buffer containing 0.1% SDS, and finally with 0.1× SSC buffer containing 0.1% SDS. The 1× SSC solution contained 150 mM NaCl and 15 mM sodium citrate (pH 7.0). The washed membrane was air-dried and autoradiographed.

Preparation of E_3 cDNAs from Patient's Fibroblasts and DNA Sequencing. E_3 cDNA fragments were obtained from the patient's total RNA by reverse transcription and PCR (24). Three pairs of sequence-specific primers (A5-7/A3-521, B5-417/B3-1088, and C5-1034/C3-1671; Table 1) were used for PCR as sense and antisense primers. These three fragments overlapped each other and covered the entire coding region of E_3 cDNA. The reverse transcription reaction was performed at 42°C for 45 min with 1 μg of total RNA in 20 μl of reaction mixture containing 10 mM Tris·HCl (pH 8.4), 2.5 mM $MgCl_2$, 5 pmol of the 3′ primer, 200 μM each dNTP (dATP, dCTP, dGTP, and dTTP), 200 μg of gelatin per ml, and 2 units of Moloney murine leukemia virus reverse transcriptase. The newly generated E_3 cDNAs were further amplified for 35 cycles with *Thermus aquaticus* DNA polymerase (Perkin–Elmer/Cetus) and 20 pmol of the 5′ and 3′ primers. Each cycle was performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The amplified cDNA fragments were purified by electrophoresing the DNA in low melting temperature agarose and excising the amplified product. The purified fragments were then either used for asymmetric PCR (25) or subcloned into Bluescript plasmid (digested with *Sma* I) using blunt-end ligation. Plasmids used for sequencing were purified by the protocol described by Maniatis *et al.* (26).

Asymmetric PCR was performed to obtain single-stranded DNA for direct DNA sequencing. The amplification conditions for the asymmetric PCR were the same as described above except the molar ratio of the two primers was 100:1 (50 pmol/0.5 pmol). DNA sequencing of the amplified single-stranded DNA and the Bluescript E_3 cDNA subclones were performed with appropriate primers (as internal primers shown in Table 1) and Sequenase, as suggested by the manufacturer (United States Biochemical).

Table 1. Primers for PCR amplification and sequencing

Primer*	Sequence	Remark
A5-7	AGCGGAGGTGAAAGTATTGGCGGA	Sense
A3-521	AGTTATCTTTCTATATCCATTGAC	Antisense
B5-417	ATGATGGAGCAGAAGAGTACTGCA	Sense
B3-1088	TTTAGTTTGAAATCTGGTATTGAC	Antisense
C5-1034	AATTGAACTAGATCCCAGAGGTAG	Sense
C3-1671	TCTTGGAGCTGTGAGAATATCCT	Antisense
E3-274	CACTTGGTGGTACCTCCTTGAATGTTG	IP sense
E3-365	ATCTTTTCCATGGGCCATAT	IP antisense
E3-768	CAGCAGTTGAACGTTTAGGTCATG	IP sense
E3-835	GAAAGTTTTTAGATATCTCC	IP antisense
E3-1282	TCTTTCAACTGCTCTTCTGA	IP antisense
E3-1462	TGGAATATGGAGCATCCTGT	IP sense
E3-1522	TAAGGTCGGCTGTGCATGA	IP antisense

IP, internal primer.
*Number represents location of the 5′ end of the primer, which corresponds to the E_3 cDNA numbering system published by Pons *et al.* (14).

RESULTS

Enzymatic Analysis. E_3 and citrate synthase activities were determined (Fig. 1). The patient's cells had 6% of the E_3 activity of control cells while the citrate synthase activity of the patient's cells was comparable to that of control cells. The low E_3 and normal citrate synthase activities in the patient's cells indicated that the E_3 deficiency of the patient resulted from a defect specific to E_3 rather than generalized defects of mitochondrial function.

Western and Northern Blot Analyses. Western blot analysis using an epitope-selected anti-porcine heart E_3 antibody (Fig. 2*A*) indicated an ≈40% reduction in E_3 protein in the patient's cells compared to control cells. Another blot was analyzed for the presence of E_1, E_2, and E_3 component proteins of the pyruvate dehydrogenase complex using a combined rabbit antisera raised individually against bovine kidney E_1, bovine heart E_2, and porcine heart E_3 (Fig. 2*B*). The intensity of the bands was determined with a densitometer. The patient's ratios of E_3 to E_2, $E_1\alpha$, and $E_1\beta$ compared to control cells were approximately 65%, 63%, and 67%, respectively. These results are consistent with the reduction in E_3 protein observed in Fig. 2*A* and indicate a qualitative change in the amount of E_3 protein in patient's fibroblasts. Northern blot

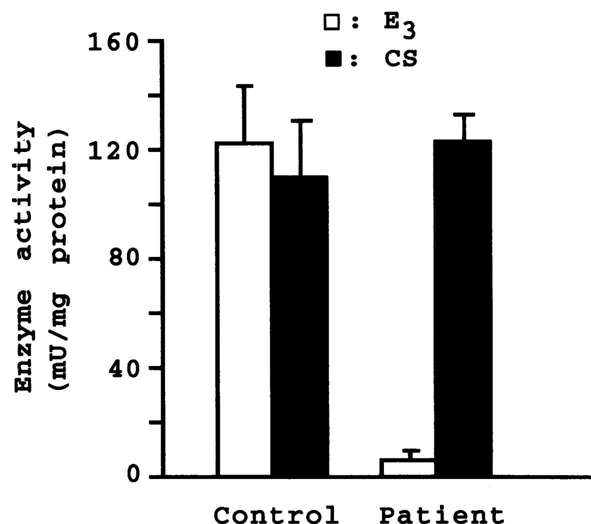

FIG. 1. Activities of E_3 and citrate synthase (CS) in fibroblasts from a control subject and the E_3-deficient patient. Results are means ± SE of six culture dishes.

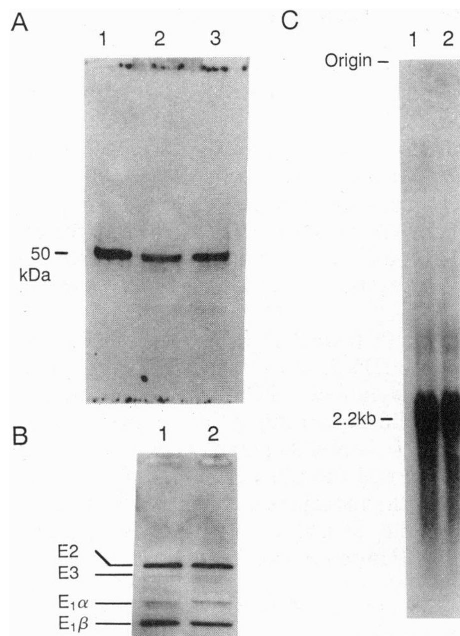

FIG. 2. Western blot analyses (*A* and *B*) of the mitochondrial fraction prepared from cultured skin fibroblasts (75 μg of protein) of control and patient. (*A*) Lanes: 1, 4 ng of purified porcine E_3; 2, patient; 3, control. This membrane was reacted with an epitope-selected anti-porcine heart E_3 antibody. (*B*) Lanes: 1, control; 2, patient. $E_1\alpha$, $E_1\beta$, E_2, and E_3 proteins were identified by using a combined antibody preparation. (*C*) Northern blot analysis of total RNA (25 μg) from cultured skin fibroblasts of the control (lane 1) and patient (lane 2).

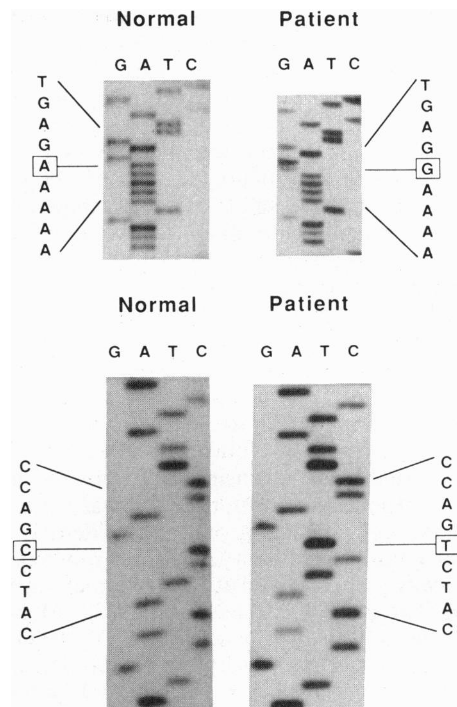

FIG. 3. DNA sequencing of the patient's specific E_3 cDNA. (*Upper*) 5′ normal and mutant sequence of subcloned cDNAs. (*Lower*) 3′ normal and mutant sequences of subcloned cDNAs.

analysis using a 5′ E_3 cDNA-specific fragment (≈1.1 kb) (Fig. 2*C*) indicated that E_3 mRNA was present in normal amounts in the patient's cells. These results indicate that the E_3 deficiency of the patient did not result from a defect in transcription of the E_3 gene. The observed moderate reduction in E_3 protein level could not account for a near absence of enzyme activity. Therefore, it was considered likely that the mutation(s) would be located within the coding region(s) of the E_3 gene, resulting in inactivity of the E_3 protein.

Sequencing of Patient's Specific E_3 cDNA. Two substitution mutations were identified by amplifying three E_3 cDNA fragments covering the entire coding region of the E_3 cDNA (from base pairs 7–521, 417–1088, and 1034–1671 of the E_3 cDNA). These two mutations were initially identified by using three independent asymmetrical amplifications of the appropriate cDNA fragments followed by direct sequencing. In these experiments, both normal and mutant nucleotide sequences were identified simultaneously (results not shown). To confirm these findings, these two regions were amplified (two independent amplifications for each region) and the cDNA fragments were subcloned into Bluescript for sequencing. One mutation changed a single nucleotide (nt 246) from A to G, resulting in the substitution of Glu (GAA) for Lys-37 (AAA) (Fig. 3 *Upper*). The other mutation replaced C with T (nt 1513), resulting in the substitution of Leu (CTG) for Pro-453 (CCG) (Fig. 3 *Lower*). For the Lys-37 to Glu-37 mutation, a total of 12 subclones derived from two separate amplifications were sequenced, and 10 mutant and 2 normal sequences were found. For the Pro-453 to Leu-453 mutation, a total of 8 subclones derived from two separate amplifications were sequenced and 5 mutant and 3 normal sequences were found.

DISCUSSION

Enzymatic, Western, and Northern blot analyses indicated that the very low E_3 activity of the patient likely resulted from defects in the E_3 protein itself. Results from Western blot analysis (Fig. 2 *A* and *B*) differ from the results previously reported for this patient by Matuda *et al.* (12), who used Ouchterlony double-diffusion analysis to detect E_3 protein in liver and muscle. Since they could not detect any E_3 precipitation band by anti-rat E_3 antibody, it was concluded that the E_3 deficiency was due to the absence of E_3 protein in the patient's cells. In contrast, we detected a reduced amount of the E_3 protein in skin fibroblasts by Western blot analysis using anti-porcine heart E_3 antibody. The double-diffusion method recognizes nondenatured E_3 protein and requires larger amounts of protein to form precipitates while Western blot analysis detects denatured E_3 protein and is able to detect small quantities of antigen. This difference in the detection methods may be responsible for the opposing results. Alternatively, the mutant E_3 in the autopsy specimens (12) may have been too unstable to be detected.

Two substitution mutations were identified by sequencing E_3 cDNA fragments generated from the total RNA of the patient's cells via reverse transcription and PCR. One mutation substituted Glu (GAA) for Lys-37 (AAA). Lys-37 of human E_3 is conserved in E_3s from several sources (human, porcine, yeast, *Azobacter vinelandii* and *Staphylococcus aureus*) and is conservatively substituted by arginine in *Escherichia coli* E_3 (Table 2). *Pseudomonas putida* (valine) and *P. putida* (3) E_3s are the only exception without a match at amino acid 37 by sequence comparison. Based on both the three-dimensional structure of human glutathione reductase (37), *A. vinelandii* E_3 (38), and *P. putida* (valine) E_3 (39) and extensive structural comparison of E_3s from several species (40), Lys-37 is suggested to be located in the FAD-binding region of human E_3. Its main-chain nitrogen atom is expected to form a hydrogen bond to the adenine moiety of FAD (38). The modification of Lys-37 to Glu may alter the charge distribution of the local environment around Lys-37 in human E_3, inasmuch as the positively charged lysine side chain is replaced by a negatively charged glutamic acid side chain. The actual effect of this substitution on human E_3 function

Table 2. Comparison of E_3 amino acid sequence from various sources around mutated residues Lys-37 and Pro-453 in human E_3

Source	Amino acid sequence	Ref.
E. coli	VERYN . . . HAHPTLHES	27
Pseudomonas fluorescens	IEKYI . . . FAHPTLSEA	28
A. vinelandii	IEKYK . . . FAHPALSEA	29
Staphylococcus aureus	VEKGN . . . HAHPTLGEM	30
Bacillus subtilis	VEKAT . . . HAHPTLGEI	31
P. putida (valine)	VEGQA . . . HAHPTLGEA	32
P. putida (glucose)	IEKYT . . . HAHPTLSEA	33
P. putida (3)	VEGRS . . . HAHPTRSEA	34
Yeast	VEKRG . . . HAHPTLSEA	35
Pig	IEKNE . . . HAHPTLSEA	36
Human	IEKNE . . . HAHPTLSEA	14, 36
E_3 patient	IEENE (37) . . . HAHLTLSEA (453)	This study

cannot be exactly predicted. However, the fact that this residue is conserved in most E_3s (Table 2) suggests that positively charged amino acid residues at this position in E_3 may play a critical role in the structure and/or function of E_3.

The other mutation is the substitution of Leu (CTG) for Pro-453 (CCG). Pro-453 is highly conserved in E_3s (Table 2) and other homologous enzymes such as glutathione reductase and trypanothione reductase (41). Pro-453 is adjacent to the active site His-452 in human E_3. His-452 is a suggested proton acceptor/donor in catalysis by human E_3 (42). Based on the three-dimensional structure of human glutathionine reductase (37), *A. vinelandii* E_3 (38), and *P. putida* (valine) E_3 (39), His-452 is predicted to form a hydrogen bond to Glu-457. These two residues are also highly conserved in E_3, glutathione reductase, and trypanothione reductase. The change of His-452 to Gln in the recombinant human E_3 by site-directed mutagenesis resulted in dramatic decreases in both E_3 activity (0.18% of wild-type activity) and binding affinity of dihydrolipoamide to the enzyme (42). The site-directed mutation of Glu-457 to Gln results in moderate decreases in both E_3 activity (28% of wild-type activity) and binding affinity of dihydrolipoamide to the enzyme (42). X-ray crystallographic studies of human glutathione reductase (37), *A. vinelandii* E_3 (38), and *P. putida* (valine) E_3 (39) indicated that a bend may be expected at Pro-453 in human E_3. This bend is crucial to the formation of a hydrogen bond between His-452 and Glu-457. Therefore, Pro-453 may be important in maintaining the local structure around active site residues His-452 and Glu-457 in human E_3. Substitution of Ala for Pro-451 (corresponding to Pro-453 in human E_3) at the active site of the *A. vinelandii* E_3 by site-directed mutagenesis causes almost complete loss of enzyme activity (43), further indicating the importance of this residue in E_3 function. Based on modeling studies on the crystal structure of *A. vinelandii* E_3, it is suggested that the mutation at the site of proline would lead to a change in the backbone structure in or near the active site (38).

Direct sequencing of the patient's specific E_3 cDNA showed two bands corresponding to both normal and mutant sequences at the same position on the sequencing gel (results not shown). Furthermore, some patient cDNA subclones contained the normal sequence, while others contained the mutant sequence (Fig. 3). The two mutations in the patient's E_3 gene are likely to be located on different alleles. The human E_3 gene is located on chromosome 7 (44). Furthermore, E_3 activities in cultured skin fibroblasts from the parents of an E_3-deficient patient were ≈30% and ≈42% of control values, indicating a possible heterozygous state (45). In our patient, presumably each of the E_3 mutations is derived from one of the parents, which together are responsible for the compound heterozygous state of the patient. Unfortunately, cells from the parents are not available to confirm this assumption (the parents have been separated and are not available for clinical analyses).

This study indicates that two substitution mutations in the E_3 coding region are consistent with the existence of a state of compound heterozygosity in an E_3-deficient patient and documents specific mutations in this protein. The identification of specific mutations adds to the increasing understanding of structure–function relationships of the E_3 protein.

We thank Drs. Douglas S. Kerr, Isaiah D. Wexler, Gary Johanning, and Mark Johnson for their valuable suggestions and critical reading of the manuscript. This work was supported in part by Public Health Service Grant DK42885.

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