Evidence for Both a Regulatory Mutation and a Structural Mutation in a Family with Maple Syrup Urine Disease

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Abstract

Maple syrup urine disease (MSUD) results from a deficiency of branched chain α-ketoacid dehydrogenase (BCKDH). We have studied the etiology of MSUD by determining the enzyme activity, protein, and mRNA levels of BCKDH in fibroblasts from a classic MSUD patient and his parents. By enzymatic amplification of the patient's mRNA followed by cloning and DNA sequencing, we have identified a T to A transversion that alters a tyrosine to an asparagine at residue 394 of the E1α subunit. Amplification of both mRNA and genomic DNA, in combination with allele-specific oligonucleotide hybridization, demonstrated that the father was heterozygous for this mutant allele. The mother was homozygous for the allele encoding the normal Tyr_{394}, but expressed only about half of the normal level of mRNA and protein. The patient was genetically heterozygous for this altered allele, although only the abnormal allele was expressed as mRNA. We conclude that the patient was a compound heterozygote, inheriting an allele encoding an abnormal E1α from the father, and an allele from the mother containing a *cis*-acting defect in regulation which abolished the expression of one of the E1α alleles. Our results revealed for the first time that a case of MSUD was caused by structural and regulatory mutations involving the E1α subunit.

Introduction

Branched chain α-ketoacid dehydrogenase (BCKDH)[1] catalyzes the rate-limiting step in the catabolism of the branched chain amino acids (1, 2). Covalent modification via phosphorylation by a specific kinase and dephosphorylation by a specific phosphatase has been established as an important mechanism for regulation of BCKDH activity (3–5). The enzyme complex consists of 2-oxoisovalerate dehydrogenase (E1, composed of E1α and E1β, EC 1.2.4.4.), dihydrolipoamide acyltransferase (E2, no EC number), and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4.). E1α is probably the catalytic subunit, and it contains the phosphorylation sites (two serine residues) responsible for regulation of the enzyme by covalent modification (6).

Maple syrup urine disease (MSUD), inherited in an autosomal recessive fashion, is caused by a deficiency of BCKDH (7). The disease is characterized by ketoacidosis and mental retardation, with the molecular defects most often (8) but not exclusively (9, 10) in the E1 component. Four clinically different forms of this disease have been described: classic, thiamin-responsive, intermediate, and intermittent (7). To gain insight into the structure and mechanism of regulation of BCKDH complex and to define the genetic defects of MSUD, we cloned cDNAs encoding BCKDH E1α subunit from both rat and human liver (11, 12). We report here the first determination at the DNA and RNA level of the mutations causing a case of classic MSUD: two different mutant alleles of the E1α gene.

Methods

Radioisotopes and chemicals. α-Keto[1-^{14}C]isovaleric acid ([1-^{14}C]KIV) was produced from [1-^{14}C]valine obtained from Research Products International Corp. (Mount Prospect, IL) (13). Most molecular cloning reagents and enzymes were from Bethesda Research Laboratories (Gaithersburg, MD). Taq DNA polymerase was from Perkin Elmer Cetus (Norwalk, CT). The nick-translation kit was from Amersham Corp. (Arlington Heights, IL), and radiolabeled nucleotides were from Du Pont-New England Research Products (Boston, MA).

Cell strains and culture. Fibroblasts derived from a patient with classic MSUD (GM649) and his father (GM650) and mother (GM651) were obtained from NIGMS Human Genetic Mutant Cell Repository. Normal human fibroblast cell line was obtained from American Type Culture Collection (Rockville, MD). Fibroblasts were grown in monolayer culture in DME (Gibco, Grand Island, NY) containing 10% (vol/vol) fetal calf serum (FCS, Sigma Chemical Co., St. Louis, MO) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml) at 37°C in 5% CO_2 atmosphere. The cells were grown to confluency and harvested by trypsinization 3–6 d after the last medium change.

Enzyme assay. Cells were collected by trypsinization, washed with PBS containing 10% FCS, and incubated with 1 mM α-chloroisocaprate in the culture medium at 37°C for 15 min to activate BCKDH complex by dephosphorylation. Extracts of the cells were prepared by freeze-thawing in complete Krebs-Ringer buffer. BCKDH total activity was measured radiochemically at 37°C with [1-^{14}C]KIV as substrate (14).

Western blot. Crude mitochondrial pellets from the fibroblasts were prepared by treating cells with 1% digitonin (15) in buffer containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), and a

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1. *Abbreviations used in this paper:* BCKDH, branched chain α-ketoacid dehydrogenase; E1, decarboxylase component of BCKDH; E1α, α subunit of E1; E1β, β subunit of E1; E2, acyl-transferase component of BCKDH; E3, dihydrolipoyl dehydrogenase; KIV, α-keto-isovaleric acid; MSUD, maple syrup urine disease; PCR, polymerase chain reaction.

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spectrum of protease inhibitors (1 mM N-α-tosyl-L-lysylchloromethane, 0.1 mg/ml trypsin inhibitor from egg white, 0.5 μM each of leupeptin, aprotinin, and pepstatin A) and stored at −70°C. The frozen pellets were then resuspended in ice-cold buffer containing 30 mM KP_i (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1% Triton, and the protease inhibitors as described above. The samples were sonicated three times for 10 s each with 30-s intervals on ice. A soluble fraction was obtained by centrifugation for 5 min at 14,000 *g* and protein concentration of the samples was determined by the bicinchoninic acid method (16). 100 μg of mitochondrial protein was then subjected to electrophoretic analysis on 10% polyacrylamide gels with 5% stacking gels (17). The protein was transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) by electroblotting. The membrane was incubated with polyclonal antibodies against E1 and E2 subunits of the BCKDH complex. The bound primary antibodies were then detected with the AuroProbe BLplus kit (Janssen Life Science Products, Olen, Belgium) following the manufacturer's instructions. The amounts of immunoreactive E1α, E1β, and E2 proteins were quantitated with a GS300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA) and analyzed with an Apple IIe computer.

Northern blot. Total cellular RNA (20 μg) prepared from fibroblasts using the guanidinium isothiocyanate method (18) was electrophoresed in a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to a Hybond membrane (Amersham Corp.), and probed with nick-translated human liver E1α cDNA. The hybridization was carried out in 50% formamide, 50% Thomas solution A (19), and 0.5% SDS at 42°C overnight. The blot was washed three times in 2 × SSC/0.1% SDS at room temperature (15 min each) and 2 times in 0.2 × SSC/0.1% SDS at 60°C (30 min each) followed by autoradiography with Kodak XAR-5 film and two intensifying screens at −70°C for 48 h. The blot was scanned with an AMBIS β-scanner (Applied Microbiological Supplies, Inc., San Diego) to quantitate radioactivities associated with signals.

Polymerase chain reaction (PCR). First-strand cDNAs were generated from 30 μg of total RNA using MMLV reverse transcriptase (Bethesda Research Laboratories) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, and 0.5 mM dNTPs (dATP, dCTP, dGTP, and dTTP) with specific antisense oligonucleotides or oligo (dT) as primers. The cDNAs were then subjected to 30–40 cycles of enzymatic amplification (20). The five sets of sense/antisense oligonucleotides designed based on the normal human and rat cDNA sequences were:

5′-CCCCAGCAGGCAGCAGCAACAG-3′/

TCTTGTAGAGCTTCAGCACCTT;

ATCCAGCCCAACGTCATCTCTG/

TTGCCATAGCACTGGGCCATGA;

AGGTGTGCTGATGTATCGGGAC/

ATACCCGGGGCCTCGTGCTGCA;

CTTCTGCCGGAACAATGGCTAC/

GACTGCTTCCTCCAGGCCTTCT;

GCACTATCTGCTGAGCCAAGGC/

CCTTAGAGTGGGGCTACCTCTC.

The reaction was carried out in 100-μl mixtures containing 10 μl of the above reverse transcription mixture and 50 mM Tris-Cl (pH 8.7), 6 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 10% DMSO, 200 μg/ml BSA, 10 mM β-mercaptoethanol, 100 pmol of each primer, 250 μM each of the four dNTPs, and 2.5 U of Taq DNA polymerase. Each cycle consisted of 1 min at 93°C, 1 min at 50°C, and 1–3 min at 63°C. The amplified cDNAs were subcloned into M13, and four independent clones of each amplified cDNA segment were sequenced using Sequenase (United States Biochemical Corp., Cleveland, OH). Genomic DNA was purified from the fibroblast cells (21) and amplified by PCR as described above. The sense/antisense primers for amplification of genomic DNA were:

5′-TCTCTGGCCCGCCACCTGC-3′/

TGGGCTGAGCAGGTCTCACT.

Allele-specific oligonucleotide hybridization. The amplified cDNA and genomic DNA were slot blotted onto ZetaProbe membranes (Bio-Rad Laboratories, Richmond, CA). The allele-specific probes were: GAGCACTACCCACTG (normal), and GAGCACAACCCACTG (mutant). The probes were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The hybridization and washing were carried out at 45 and 50°C, respectively, in the solutions described (22). The filters were first probed with the normal allele-specific oligonucleotide. The same filters were boiled to remove the probe and then hybridized to the mutant oligonucleotide. The filters were autoradiographed at −70°C for 1–12 h.

Results

BCKDH activity and protein level in cultured fibroblasts from a normal human and the MSUD family. As indicated in Table I, total BCKDH activity (4) in fibroblast extracts from the patient (GM649) is < 10% of normal; the father (GM650) and mother (GM651) each have about half of the normal activity. Earlier kinetic studies showed that E1 was the component deficient in GM649 (8). Western blots (Fig. 1) were performed to measure the protein level of BCKDH complex in the family. The results demonstrated that the patient, mother, and father had 12%, 55%, and 59%, respectively, of the normal amount of immunoreactive E1α protein. The reduction in the amount of E1α was closely paralleled by the reduction of E1β in each family member when compared to the normal control. All samples contained nearly identical amounts of E2.

Measurement of E1α mRNA level. Northern blots of total RNA probed with human liver E1α cDNA (12) demonstrated a single mRNA band of 1.8 kb (Fig. 2). The radioactivity of this band was quantitated and a ratio of 2.1:1.1:2.2:1.0 was obtained for normal/patient/father/mother. The higher level of BCKDH E1α mRNA in the normal control and the father than in the patient and the mother was confirmed by reprobing the filters for β-actin mRNA to normalize the amount of total RNA loaded on the gels (data not shown).

Table I. Total Activity of BCKDH in Cultured Fibroblasts from a Normal Human and the MSUD Family

Cell line	BCKDH activity
	nmol/min per mg protein
Normal	0.202±0.046
GM649 (patient)	0.015±0.015
GM650 (father)	0.114±0.044
GM651 (mother)	0.096±0.010

Cells were treated with α-chloroisocaprate and disrupted by freeze-thawing. BCKDH activity was assayed radiochemically. Each value represents the mean±SD for at least six determinations. The total activity of BCKDH of the normal fibroblasts agrees well with that reported previously (14).

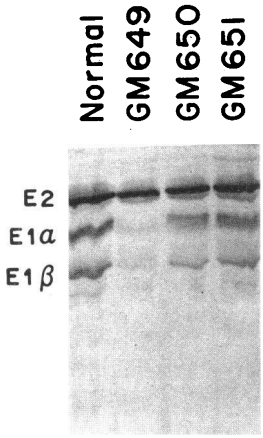

Figure 1. Western blot of BCKDH extracted from cultured fibroblasts. Mitochondrial protein was subjected to SDS–PAGE followed by immunoblotting analysis. The positions of E1α, E1β, and E2 subunits are indicated by the labels.

Identification of Tyr to Asn mutation. To define the mutations at the DNA and RNA level, we cloned and sequenced the cDNA from the patient by reverse transcription of RNA followed by PCR to amplify specifically BCKDH E1α cDNA. Five sets of sense–antisense primers (each defining a 300–400-bp region of the mRNA) were designed based on the normal human E1α cDNA sequence (12). To obtain sequence information for the NH_2-terminal 20 residues of the mature human protein, which had not been previously reported, PCR was performed using a sense primer based on the leader peptide sequence of the rat E1α cDNA (11) (Fig. 3). Thus, the complete coding region of the mature E1α was amplified in overlapping segments and subcloned into M13 for DNA sequencing. Only one single base substitution was found: TAC encoding tyrosine at residue 394 was changed to AAC encoding asparagine. To demonstrate that this change was not an amplification or cloning artifact, four independent amplified fragments containing residue 394 were sequenced from both directions; the Tyr (TAC) to Asn (AAC) substitution was found in all cases.

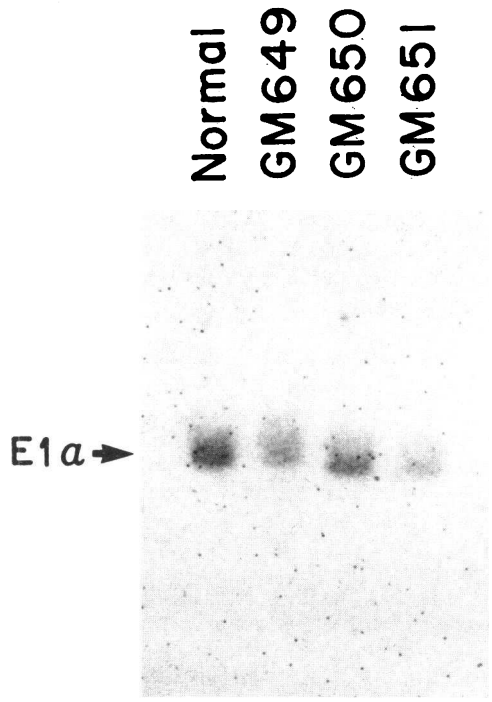

Figure 2. mRNA level of BCKDH E1α in fibroblast cell lines. Total RNA from the fibroblasts was fractionated by electrophoresis and blotted to Hybond membrane. The membrane was then probed with labeled human E1α cDNA, washed, and subjected to autoradiography.

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5' CAG TTT TCA TCT CTG GAT GAC AAG CCC CAG TTC CCA
       Phe Ser Ser Leu Asp Asp Lys Pro Gln Phe Pro

GGG GCC TCG GCG GAG TTT ATA GAT AAG TTG GAA TTC  3'
Gly Ala Ser Ala Glu Phe Ile Asp Lys Leu Glu Phe
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Figure 3. Nucleotide and deduced NH_2-terminal amino acid sequence of the mature human BCKDH E1α protein.

Allele-specific oligonucleotide hybridization. To confirm the point mutation, sequences flanking codon 394 were amplified from the RNA of the patient and his parents. Two allele-specific oligonucleotides centered at codon 394 (one containing TAC and one with AAC) were synthesized and used to probe slot blots of the amplified RNA segments. The amplified RNA from the patient hybridized only to the mutant allele-specific probe and that from the father hybridized to both probes (Fig. 4 *A*). The mother's RNA, however, hybridized only to the normal allele-specific probe, indicating that the mother expressed only the normal allele.

These allele-specific oligonucleotides were also used to probe the amplified genomic DNA segments. The DNA from the father and the mother followed the same pattern as that of the mRNA: the father was heterozygous and the mother homozygous for the normal allele (Fig. 4 *B*). However, the DNA from the patient hybridized to both probes, indicating that he was heterozygous at the gene level with regard to the point mutation, although he expressed only the mutant mRNA. These experiments have been replicated with three independent amplifications of RNA and genomic DNA, to rule out artifacts.

Discussion

We have studied the molecular basis for MSUD in a family with the classic form of the disease by analyzing the enzyme

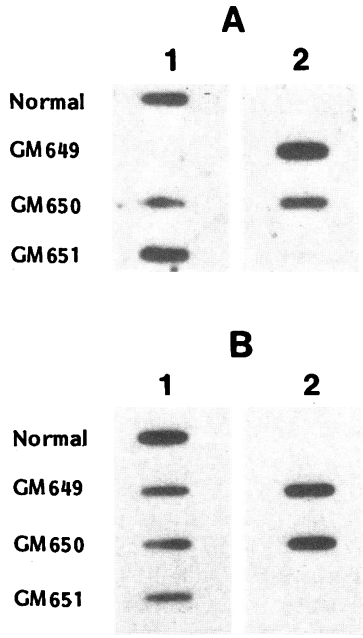

Figure 4. Allele-specific oligonucleotide hybridization analysis of the (*A*) amplified cDNA and (*B*) genomic DNA. Reverse transcription and PCR were carried out as described in Methods. The slot blots were first hybridized with normal allele-specific oligonucleotide probe (lane *1*). After removal of the normal probe, the blots were hybridized with mutant allele-specific probe (lane *2*).

activity, protein, and mRNA levels. The patient had a quite low level of BCKDH total activity. The parents each have about half of the normal enzyme activity, as expected for the obligatory carriers of this autosomal recessive genetic disease.

As shown by Western and Northern blot analysis, the mRNA level and the amount of immunoreactive E1 protein were each half of the normal values in the mother, suggesting the existence of a regulatory mutation which reduced the steady state level of E1α mRNA. In contrast, the father had a normal level of E1α mRNA, but only 59% of the normal level of E1α protein, suggesting a structural mutation which resulted in either destabilization of the E1 protein or inefficient translation of the E1α mRNA. The patient had half of the normal E1α mRNA level, and only 12% of the normal immunoreactive E1α protein, consistent with the hypothesis that there were different mutations in the two parents: a *cis*-acting regulatory mutation in the mother that greatly reduced the expression of one of the E1α alleles, and a structural mutation in the father that produced an altered and unstable protein.

Since the reduction of the level of E1α mRNA correlates with the reduction in the amount of E1α protein in the patient and his mother, the concomitant reduced level of E1β shown in the Western blot (Fig. 1) is best explained by proteolysis of E1β subunits which cannot be incorporated into the stable ($\alpha_2\beta_2$) complex form. Studies of the pyruvate dehydrogenase complex, which has structural similarities to BCKDH complex including an E1 component composed of E1α and E1β subunits, suggest that the presence of the E1α subunit is necessary for the stability of the E1β subunit (23).

In order to define the mutations causing the disease in this family, we sequenced the cDNA from the patient encoding the entire mature protein of the E1α subunit by reverse transcription of RNA followed by PCR and DNA sequencing. Only one single-base mutation (T to A) that caused a Tyr to Asn substitution at codon 394 was identified. The mutation was confirmed by allele-specific oligonucleotide hybridization of the patient's amplified RNA under the conditions which differentiate single-base mismatch (Fig. 4). The allele-specific oligonucleotide hybridization experiment was also carried out using amplified RNA and genomic DNA from the patient's father and mother. The father's RNA and genomic DNA hybridized to both normal and mutant probes, indicating that he was heterozygous for this mutation, and expressed both the normal and mutant alleles. The mother's RNA and DNA, however, hybridized only to the normal allele-specific probe, indicating that she was homozygous normal with regard to the point mutation.

These data indicate that the affected child was a compound heterozygote. He inherited a mutant allele from his father which contained a Tyr(394) to Asn substitution. Since the father is heterozygous for this mutation and has the normal E1α mRNA level but reduced amount of E1 protein, we propose that this mutation affects either the translation efficiency of the mRNA or, more likely, the stability of the protein. The mother is homozygous for the normal codon 394, but expresses only half as much E1α mRNA; she must carry a *cis*-acting mutation in one of her E1α alleles that essentially abolishes its expression as mRNA. The child inherited this nonexpressed allele (which had the normal Tyr 394) from his mother and the mutant allele (which contained Asn 394) from his father. This explains how the patient was genetically heterozygous for the Tyr to Asn substitution, yet expressed only the allele which contained the point mutation. Southern blots of genomic DNA from the members of this family probed with human E1α cDNA were identical to those of the normal individuals, suggesting that there was no major gene deletion or rearrangement in either mother or patient. MSUD in this patient is thus due to two different mutant E1α alleles: a *cis*-acting mutation which nearly eliminates expression of one allele and a point mutation which results in reduced translation efficiency and/or in an unstable E1α protein.

In conclusion, our results delineate the molecular basis of MSUD in a family with the classic form of the disease at DNA and RNA level. The application of the methods established in this study should greatly facilitate the understanding of the molecular defects of this clinically heterogeneous disease.

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