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C R Fisher, … , R A Star, D T Chuang

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Research Article

Maple Syrup Urine Disease (MSUD) in Mennonites is associated with homozygosity for a T to A transversion in the E1 alpha gene of the branched-chain alpha-keto acid dehydrogenase complex. This causes a tyrosine to asparagine substitution at position 393 (Y393N). To assess the functional significance of this missense mutation, we have carried out transfection studies using E1 alpha-deficient MSUD lymphoblasts (Lo) as a host. The level of E1 beta subunit is also greatly reduced in Lo cells. Efficient episomal expression in lymphoblasts was achieved using the EBO vector. The inserts employed were chimeric bovine-human cDNAs which encode mitochondrial import competent E1 alpha subunit precursors. Transfection with normal E1 alpha cDNA into Lo cells restored decarboxylation activity of intact cells. Western blotting showed that both E1 alpha and E1 beta subunits were markedly increased. Introduction of Y393N mutant E1 alpha cDNA failed to produce any measurable decarboxylation activity. Mutant E1 alpha subunit was expressed at a normal level, however, the E1 beta subunit was undetectable. These results provide the first evidence that Y393N mutation is the cause of MSUD. Moreover, this mutation impedes the assembly of E1 alpha with E1 beta into a stable alpha 2 beta 2 structure, resulting in the degradation of the free E1 beta subunit.

Maple Syrup Urine Disease in Mennonites

Evidence that the Y393N Mutation in E1α Impedes Assembly of the E1 Component of Branched-chain α-Keto Acid Dehydrogenase Complex

Carolyn R. Fisher,* Jacinta L. Chuang,* Rody P. Cox,‡ Charles W. Fisher,* Robert A. Star, and David T. Chuang*
**Departments of Biochemistry and ‡Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235*

Abstract

Maple Syrup Urine Disease (MSUD) in Mennonites is associated with homozygosity for a T to A transversion in the E1α gene of the branched-chain α-keto acid dehydrogenase complex. This causes a tyrosine to asparagine substitution at position 393 (Y393N). To assess the functional significance of this missense mutation, we have carried out transfection studies using E1α-deficient MSUD lymphoblasts (Lo) as a host. The level of E1β subunit is also greatly reduced in Lo cells. Efficient episomal expression in lymphoblasts was achieved using the EBO vector. The inserts employed were chimeric bovine-human cDNAs which encode mitochondrial import competent E1α subunit precursors. Transfection with normal E1α cDNA into Lo cells restored decarboxylation activity of intact cells. Western blotting showed that both E1α and E1β subunits were markedly increased. Introduction of Y393N mutant E1α cDNA failed to produce any measurable decarboxylation activity. Mutant E1α subunit was expressed at a normal level, however, the E1β subunit was undetectable. These results provide the first evidence that Y393N mutation is the cause of MSUD. Moreover, this mutation impedes the assembly of E1α with E1β into a stable $\alpha_2\beta_2$ structure, resulting in the degradation of the free E1β subunit. (*J. Clin. Invest.* 1991. 88:1034–1037.) Key words: defective protein assembly • $\alpha_2\beta_2$ structure • mitochondrial multienzyme complexes • episomal expression • lymphoblasts

Introduction

Maple syrup urine disease (MSUD)[1] or branched-chain ketonuria is an autosomal recessively inherited deficiency in the mitochondrial branched-chain α-keto acid dehydrogenase (BCKAD) complex. The multienzyme complex degrades the α-keto acids derived from the three branched-chain amino acids leucine, isoleucine, and valine (1). The classical form of the disease is characterized by the rapid onset of severe ketoacidosis at birth, which is associated with seizures, coma, and death if untreated (2). There is a high incidence of mental retardation in survivors. The residual activity of the BCKAD complex in the classical patient is less than 2% of normal. Several milder variant forms of MSUD have also been described, which include intermediate, intermittent, thiamin-responsive, and deficiencies of specific subunits (1).

The mammalian BCKAD complex is a macromolecule consisting of three catalytic components: i.e., a decarboxylase (E1) comprised of two α (M_r = 47,000) and two β (M_r = 37,000) subunits, a transacylase (E2) core consisting of 24 identical lipoate-bearing subunits (M_r = 46,500) and a dehydrogenase (E3) that exists as a homodimer (monomer M_r = 52,000), and is shared with the pyruvate and α-ketoglutarate dehydrogenase complexes (3). In addition, the mammalian BCKAD complex contains two regulatory enzymes, a specific kinase and a specific phosphatase, that control the activity of the enzyme complex through a phosphorylation/dephosphorylation cycle (4). Thus, there are at least six genetic loci encoding this mitochondrial enzyme complex, and a mutation in any of these loci may produce the MSUD phenotype.

The incidence of the classic form of MSUD in Philadelphia Mennonites is very high. 1 in 176 live births is affected (5). We (6) and others (7) have reported a homozygous missense mutation in the E1α subunit of Mennonites that changes a tyrosine to asparagine at residue 393 (symbol Y393N). This mutation was previously described in a non-Mennonite compound heterozygote (8). The functional significance of this mutation has not been examined to date. In this communication we elucidate the biochemical mechanism of the Y393N mutation by transfection experiments. The results provide evidence that the homozygous Y393N mutation impairs the assembly of the E1α and E1β subunits into a functional and stable $\alpha_2\beta_2$ complex. This is the first protein assembly defect shown to be responsible for the MSUD phenotype.

Methods

Cell lines. Normal lymphoblast cultures were established by standard methods from peripheral blood lymphocytes by infection with EBV (9). Lymphoblasts from an MSUD patient Lo (10) were kindly supplied by Dr. Dean Danner, Emory University, Atlanta GA.

Construction of EBO expression vectors. The EBO-pLPP plasmid (11) was generously provided by Dr. Robert Margolskee, Roche Institute of Molecular Biology, Nutley, NJ. The construction of chimeric bovine-human E1α cDNAs was carried out as follows: the bovine mi-

Address correspondence and reprint requests to Dr. D. T. Chuang, Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235.

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1. *Abbreviations used in this paper:* BCKAD, branched-chain α-keto acid dehydrogenase; E1, branched-chain α-keto acid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoyl dehydrogenase; MSUD, maple syrup urine disease; Y393N, tyrosine 393 → asparagine substitution.

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tochondrial targeting sequence was prepared by digestion of full-length bovine E1α cDNA in Bluescript SK$^-$ with XbaI and EcoRI restriction enzymes (12). The mature portion of human E1α cDNA was obtained by digesting the insert in Bluescript SK$^-$ (Stratagene, La Jolla, CA) with EcoRI and PstI (corresponding to bases 191 to 1286) (10). The 3′ region of human E1α cDNA (bases 995 to 1376) was amplified by PCR from either normal subjects or a Mennonite (P.K.) MSUD patient with T to A mutation at base 1307 (13). The normal and mutant PCR fragments were ligated to KpnI linkers at both ends. The amplified fragments, which contain an internal PstI site at base 1286, were digested with PstI and KpnI. All fragments (XbaI—EcoRI, EcoRI—PstI, and PstI—KpnI) were purified by HPLC as described previously (14). The three fragments were ligated in sequence into EBO vectors, which had previously been digested with XbaI and KpnI. The normal and mutant sequences in PCR-generated fragments were confirmed by nucleotide sequencing.

Culture and transfection. Lymphoblasts were grown in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) containing 15% heat-inactivated fetal calf serum and antibiotics (Kanamycin 0.1 mg/ml, penicillin 50 U/ml, and streptomycin 50 μg/ml). Transfection procedures were carried out as described (11). Cells were at a concentration of 5 $\times$ 10^7/ml in the above medium. EBO plasmid DNA (100 μg/ml) and sheared salmon sperm DNA (400 μg/ml) were added. The cell/DNA mixture was incubated at room temperature for 10 min with several gentle inversions. 1-ml aliquots of cell/DNA mixture were transferred to an electroporation chamber and electroporated at 250 V and 1,180 μF in a Cell-Porator (Bethesda Research Laboratories, Bethesda, MD). After a 10-min incubation at room temperature, the cell/DNA suspension was diluted ninefold in RPMI 1640 medium with 20% heat-inactivated fetal calf serum. After 48 h, hygromycin selection was initiated as previously described (11). Viable cells were separated from nonviable by Ficoll gradient at 15 d. 4 wk after selection with hygromycin at 200 μg/ml the concentration was reduced to 100 μg/ml to allow more rapid growth of the cultures. 2 wk before harvesting hygromycin was increased to 200 μg/ml.

Decarboxylation assays with intact cells. The rates of decarboxylation of [1-^{14}C] α-ketoisovalerate and [1-^{14}C] pyruvate were measured on viable intact cells (30 $\times$ 10^6/ml) as previously described (15). Cell viability was determined by Trypan blue dye exclusion.

Northern and Western blotting. Total RNA was isolated from untransfected and transfected lymphoblasts, and Northern blot analysis carried out as described previously (10). The 1.5-kb human E1α cDNA fragment (hE1α-2) was radiolabeled with [α-^{32}P]dCTP by the random priming method (16) and used as a probe. Western blotting was carried out as described previously using either anti-E1α or anti-E1β antibody as a probe (10). These antibodies were purified by affinity column chromatography (10).

Results

We have shown that Mennonite patients with MSUD are homozygous for the Y393N mutation in the E1α gene that segregates in families as an autosomal recessive trait (6). To assess the functional effects of this mutation on the E1 polypeptide, we carried out transfection experiments. Fig. 1 shows the EBO shuttle vector used in these studies. The insert is a bovine and human chimeric E1α cDNA. The bovine mitochondrial-targeting sequences were necessary, as the human cDNAs isolated to date do not contain the complete mitochondrial targeting region (10). The 5′ sequences encoding residues 1 through 22 of the mature peptide were also bovine. Human and bovine amino acid sequences are identical in this region (10, 12). Thus, during mitochondrial import, removal of the bovine presequence results in a complete mature human E1α sequence. Two chimeric constructs were inserted into the vector. One was the normal E1α cDNA and the other contained the T to A transversion at base 1307 (6) that corresponds to the Y393N mutation (Fig. 1).

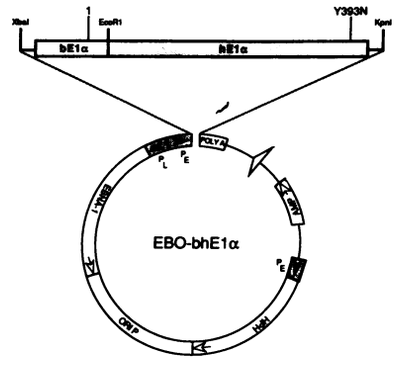

Figure 1. EBO vector carrying the chimeric bovine human E1α cDNA. OriP is the origin of replication and the EBNA-1 sequence encodes the transacting EBV nuclear antigen. The HPH sequence encodes hygromycin phosphotransferase which confers antibiotic resistance. The expression of the insert is driven by the SV40 virus early promoter (P_E). The chimeric insert was constructed by fusing the 5′ region of the bovine E1α cDNA that contains the complete mitochondrial targeting sequence to the mature human E1α cDNA through a common intrinsic EcoRI site. Either the normal or the Y393N mutant constructs were inserted into the vector for transfection experiments.

The host (Lo) lymphoblasts were from an MSUD patient with deficient levels of E1α mRNA and protein (10). The level of E1β mRNA in Lo cells is normal (17), but the amount of E1β subunit is greatly reduced (10). The putative mutation in the E1α locus of Lo cells is unknown. The lymphoblasts were transfected by electroporation with vectors containing either normal or Y393N mutant E1α cDNA, or with the vector without insert. The cultures were subjected to hygromycin selection as the EBO vector contains a hygromycin-resistant gene. Viable transfected cells were separated from nonviable cells by Ficoll gradients.

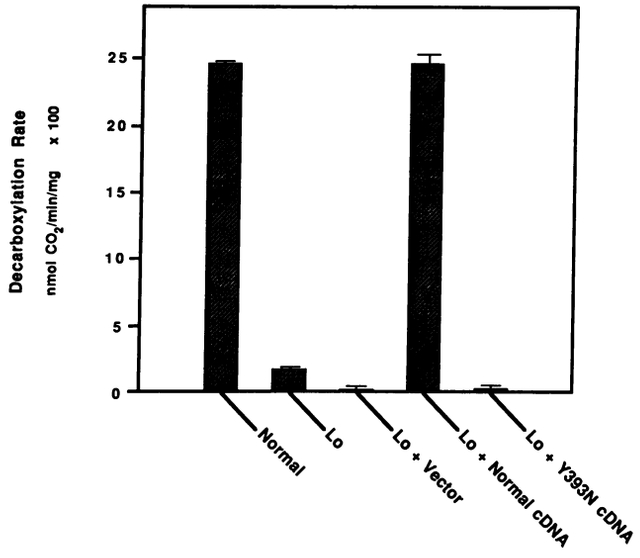

Figure 2. Decarboxylation rates of intact normal and E1α-deficient lymphoblasts (Lo) transfected with EBO vectors containing normal and mutant Y393N E1α cDNA. The vector alone without insert serves as a control. The activities of untransfected normal and Lo cells are in the two left bars. Results are expressed as means±SEM (n = 6). The rates of decarboxylation of [1-^{14}C]pyruvate were normal in both untransfected and transfected cells (data not shown).

Fig. 2 shows the results of [1-C^{14}]α-ketoisovaleric acid decarboxylation by intact untransfected and transfected lymphoblasts. Untransfected Lo cells have greatly reduced activity when compared with normal lymphoblasts. Transfection of Lo cells with EBO vector without an insert did not increase the rate of decarboxylation. The decrease in residual specific activity is probably the result of overexpression of vector proteins of viral origin. Transfected cells have 1.5- to 2-fold more protein per cell than nontransfected lymphoblasts. Transfection of Lo lymphoblasts with EBO vector containing normal E1α cDNA restores the rate of decarboxylation to that observed in normal cells. In contrast, Lo cells transfected with EBO vector containing the Y393N mutant insert exhibit low residual activity similar to that observed with vector alone. These results establish unequivocally that the Y393N is responsible for classic MSUD in Mennonites homozygous for this mutation.

Fig. 3 shows the results of Northern blot analysis using total RNA from untransfected and transfected lymphoblasts. Lo cells were previously shown to have markedly reduced E1α mRNA compared with normal (10). Transfection with vector alone did not change the level of E1α mRNA. Transfection of Lo cells with vector containing either chimeric normal or Y393N mutant inserts results in overexpression of E1α mRNA (Fig. 3).

To determine the protein content of E1α and E1β subunits, lysates were prepared from untransfected and transfected lymphoblasts. Western blotting was carried out using E1α or E1β antibody as a probe. As shown in Fig. 4 (*upper panel*), E1α content of Lo is low compared with normal lymphoblasts. Transfection of Lo cells with vector alone did not increase the E1α subunit content. Transfection of Lo with vector containing either normal or Y393N E1α insert increased the levels of this subunit. The overexpressed normal and mutant E1α peptides were in excess and degraded as indicated by the presence of the smaller immunoreactive species below the mature E1α (Fig. 4). The faint upper bands may be precursors of the E1α subunit. The lower panel in Fig. 4 shows that E1β is present in trace amounts in Lo lymphoblasts untransfected or transfected with the vector alone. When Lo cells are transfected with vector

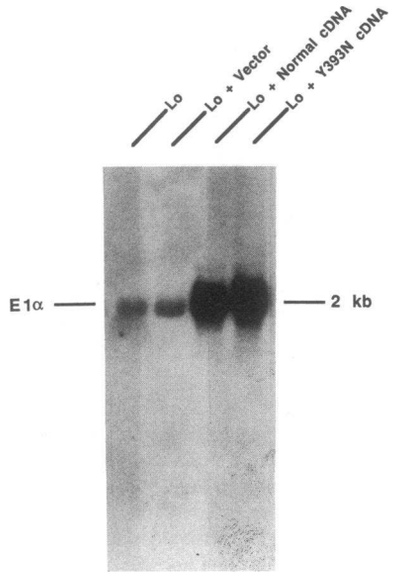

Figure 3. Northern blot analysis of total RNA from untransfected and EBO vector-transfected Lo cells. The 1.5-kb human E1α cDNA (hE1α-2) (10) was radiolabeled by the random-priming method with [α-^{32}P]-dCTP, and used as a probe to hybridize with total RNA (30 μg/lane).

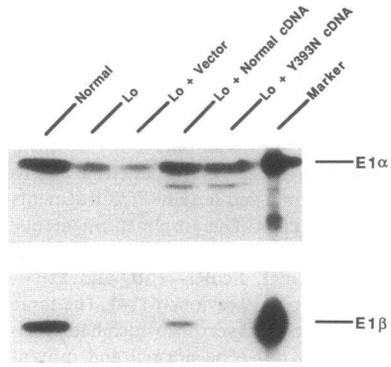

Figure 4. Western blot analysis of E1α and E1β in lysates from normal and Lo cells untransfected and transfected with EBO vectors containing normal or Y393N E1α cDNA. Each lane contains 200 μg of protein. After SDS polyacrylamide electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes. The filters were probed with either E1α (*upper panel*) or E1β (*lower panel*) antibodies radiolabeled by coupling with ^{125}I-protein A.

containing normal E1α cDNA, the level of the E1β subunit is also markedly increased. However, the intensity of the E1β is less in transfected cells compared with normal. As described above, transfected cells have much more protein per cell. Because the same amount of total protein (200 μg/lane) was applied to each lane, this results in reduced cell numbers in transfected lanes. Therefore the E1β observed in transfected cells is expected to be less than in the normal lane. The increase in immunoreactive E1β when normal E1α subunit is synthesized is consistent with expression of full decarboxylation activity by these cells (Fig. 2). When Lo lymphoblasts are transfected with vector containing the Y393N mutant E1α cDNA, the E1β subunit is undetectable. The inability of the mutant E1α peptide to restore E1β explains the failure of these transfected cells to decarboxylate α-ketoisovaleric acid.

Discussion

This communication provides direct evidence that the Y393N mutation in E1α causes the MSUD phenotype. Our study also suggests that this mutation may cause the dysfunction of human BCKAD complex by a novel mechanism. The Y393N mutation in E1α is of unusual significance because of its homozygosity and high frequency in Philadelphia Mennonites (5) and its occurrence in compound heterozygotes of non-Mennonite lineage. The E1β mRNA level and the cDNA sequence are normal in Mennonite MSUD patients (7, 17).

The steps leading to the expression of a functional BCKAD complex are many, as the E1α subunit alone has no demonstrable enzyme activity. The E1α precursor synthesized in the cytoplasm must be imported into mitochondria where the mature E1α assembles with mature E1β to form an active E1 component. E1 and E3 then bind with the E2 core in the inner membrane of the mitochondria to constitute a functional BCKAD complex. In this study, we transfected a lymphoblast cell line (Lo) deficient in E1α subunit with an EBO vector containing either the normal or Y393N mutant E1α cDNA. This vector is ideal for efficient expression in lymphoblasts because it contains OriP replication origin and encodes a transacting nuclear antigen (EBNA-1) (Fig. 1). Both factors are required for and allow stable episomal replication of this vector in EB virus transformed lymphocytes (11).

It has been proposed that E1β is unstable and degraded in cells unless it assembles with E1α subunit to form the stable

$\alpha_2\beta_2$ structure as demonstrated in the BCKAD complex (10, 19) and the related pyruvate dehydrogenase complex (18). This accounts for only trace amounts of E1β by Western blotting in Lo cells where the E1α subunit is markedly reduced (10). The significant increase in E1β by expression of normal E1α peptide in transfected Lo cells supports the contention that the E1β is being normally synthesized. The complete restoration of decarboxylation activity by transfecting with normal E1α cDNA also confirms that the mutation in Lo cells is in the E1α locus, and E1β is unaffected.

In contrast, when Lo cells were transfected with vector carrying the E1α Y393N insert, the mutant E1α peptide was expressed at levels comparable to that observed in cells transfected with the normal E1α subunit. However, the E1β peptide was undetectable. This result suggests that the Y393N mutant E1α peptide is unable to assemble with the E1β subunit, and the free E1β subunit is degraded. Thus, this study provides the first evidence for a protein assembly defect in MSUD that is caused by the Y393N mutation in E1α.

The crystal structure of the E1 component has not been solved. However, our results indicate that the carboxy-terminal region of the human E1α subunit, where the tyrosine 393 resides, is involved in the assembly of E1 component. This region of E1α is highly hydrophilic as determined by the hydropathic plot of Kyte and Doolittle (20), and is rich in charged residues (10, 12, 21), suggesting a possible role in binding the hydrophilic amino-terminal region of E1β through ionic interactions. This process apparently is disrupted by the single Y393N substitution in E1α. This information will facilitate further structural and functional studies of the E1 component. Moreover, this study demonstrates that mutations which produce human disease provide unexpected insights into protein-protein interactions and macromolecular organization.

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