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EVIDENCE FOR A STRUCTURAL MUTATION (^{347}Ala TO Thr) IN A GERMAN FAMILY WITH 3-KETOTHIOLASE DEFICIENCY

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Summary: The molecular basis of 3-ketothiolase deficiency (3KTD) was examined in a 3KTD family. Immunochemical analyses showed that mitochondrial acetoacetyl-CoA thiolase (T2) biosynthesized in the patient's fibroblasts (GK06) was unstable and that the parents and brother were obligatory carriers of 3KTD. When sequencing the PCR-amplified patient's T2 cDNA, we noted a G to A replacement which caused ^{347}Ala to Thr substitution of the mature T2 subunit. Transfection analysis revealed that this substitution resulted in an instability of the T2 protein. Analyses of the T2 cDNA and gene of the family indicated that the patient was a compound heterozygote; the allele that derived from the mother had a point mutation (^{347}Ala to Thr) and the other allele from the father has a mutation which would abolish the T2 gene expression. This report is apparently the first definition of a mutant allele for 3KTD, at the gene level.

3-Ketothiolase deficiency (3KTD) (McKusick 20375) is an inborn error of isoleucine and ketone body catabolism caused by a deficiency in mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9). Patients with 3KTD have an episodic severe ketoacidosis and an increased excretion of 2-methyl-acetoacetate, 2-methyl-3-hydroxybutyrate, and tiglylglycine is present in their urine (1,2). Middleton et al. reported that 2-methyl-acetoacetyl-CoA thiolase activity was undetectable in 3KTD fibroblasts and that acetoacetyl-CoA thiolase activity in the mutant cells is not susceptible to activation by K^+, a characteristic property of T2 (3-6).

In immunochemical analyses we found that 3KTD is caused by a defect in T2 biosynthesis, and that there is an heterogeneity in the T2 defects (7,8). We cloned rat and human T2 cDNAs (9,10) and the human T2 gene (submitted) and noted heterogeneity in the T2 mRNA expression.

We describe here the molecular events in a 3KTD patient, at the protein, cDNA and gene levels.

Abbreviations used in this paper: 3KTD, 3-ketothiolase deficiency; T1, mitochondrial 3-ketoacyl-CoA thiolase; T2, mitochondrial acetoacetyl-CoA thiolase; βG, β-glucuronidase; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Patient and cell lines. The patient (GK06) was a German boy, born of nonconsanguineous parents. Although his development was within normal ranges before the first ketoacidotic attack at the age of 6 months, severe retardation then followed. He was diagnosed as having 3KTD, by urinary organic acid analysis during the attack. Fibroblasts from the patient, his brother, and parents were cultured in Eagle's minimum essential medium containing 10% fetal calf serum.

Protein and Northern blot analyses. Immunoblotting, pulse-chase experiments, and Northern blotting were performed as described elsewhere (8,10).

Complementary DNA synthesis and amplification of T2 cDNA and T2 gene. Total cellular RNA was prepared by the AGPC method (11). First strand cDNAs were synthesized from 5 μg of RNA using 0.5 μg of T2 specific 135 primer (see below) and RAV 2 reverse transcriptase (Takara Shuzo), according to the manufacturer's instructions. PCR amplification was done with an aliquot of the above cDNA solution or 1 μg of genomic DNA as a template. Forty cycles of PCR procedure (1 min at 94°C, 2 min at 54°C, and 4 min at 72°C) were performed with a DNA thermal cycler and a gene amp™ DNA amplification reagent kit (Perkin-Elmer Cetus). The two sets of primers were designed based on the sequence of normal T2 cDNA (10) :
-03 (5'-AGTCTACGCCTGTGGAGC-3')/64 (3'-ACTTGTCCTGCGAATACGAT-5')
60 (5'-AGCTGTGCTGAGAATACAGC-3')/135 (3'-CACACTGATGACACCCAGT-5')
The most 5' sense primer -03 was positioned 23-39 bases upstream of the initial ATG codon, and the most 3' antisense primer 135 was 57-76 bases downstream of the TAG stop codon.

Sequence analysis of PCR amplified fragments. Amplified fragments subcloned into pTZ vector (U.S. Biochemical Corp.) were sequenced with a Sequenase Kit (U.S. Biochemical Corp.). For direct sequencing, the purified PCR fragments (about 25-50 ng) were subjected to a second PCR with only one of the original primers, as described (12). After polyethyleneglycol precipitation, the second-PCR products were sequenced with Taq DNA polymerase (Promega), and ^{32}P end-labeled opposite strand primers, as described (13).

RESULTS AND DISCUSSION

Protein analyses. When immunoblot analysis was done using both anti-(T2)IgG and anti-(mitochondrial 3-ketoacyl-CoA thiolase (T1))IgG, the signal for T2 in tissue from the patient (Fig. 1, lane P) was undetectable and signals in his father (lane F), mother (lane M) and brother (lane B) were reduced to less than 50 % of the controls' (lanes C). In contrast, the signal for T1, another thiolase, in tissues from all family members was much the same as noted in the controls. Thus, his parents and brother were obligatory carriers for 3KTD. In pulse-chase experiments (Fig. 4A), the mutant T2 in the patient was detected at the 1-hr pulse but disappeared after a 24-hr chase, thus, the mutant T2 was unstable.

Based on these results, we presumed that the molecular basis for 3KTD in the patient was due to point mutation(s) which made the T2 protein labile.

Identification of a point mutation in the patient's T2 mRNA. When the PCR products were subcloned and sequenced, a G to A substitution was found at position 1138 (nucleotide number starts from the first residue of the initiator ATG triplet), in all four products tested from the primer pair 60-135. This mutation causes an amino acid change from ^{347}Ala to Thr in the mature T2 subunit. Direct sequencing revealed that the G to A substitution was homogeneous in T2 cDNA from the patient (Fig. 2). No other mutation was detected in the T2 cDNA, using these methods.

Alu I restriction analyses of the mutation site. The Alu I (AGCT) site present in the normal cDNA sequence was abolished by the transition from G to A (AACT) in the mutant sequence. Hence, the 60-135 fragments of the patient and his family were subjected to Alu I restriction assay. As shown

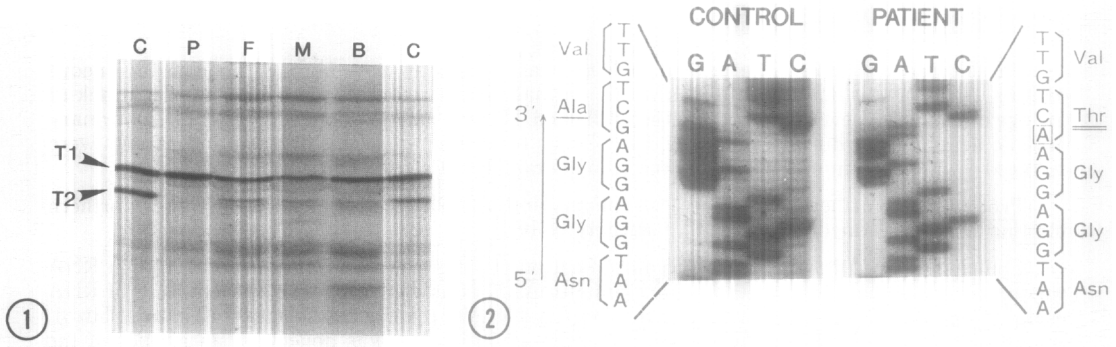

Figure 1. Western blot of T2 extracted from cultured fibroblasts. Forty μg of fibroblasts' protein was subjected to SDS-PAGE followed by immunoblotting. First antibody was a mixture of anti-(rat T2) IgG and anti-(rat T1) IgG. Abbreviations: C, control, P, patient; F, father; M, mother; B, brother.

Figure 2. Partial nucleotide sequences of the normal and the patient's T2 cDNA. Autoradiograms of the direct sequencing encompassing the mutated region are shown. A single base substitution of A for G at position 1138 results in replacement of Alanine (GCT) with Threonine (ACT).

in Fig. 3A, the patient's fibroblasts expressed only the mutant T2 mRNA (lane 3), his brother (lane 2) and father (lane 4) had a normal T2 mRNA while his mother (lane 1) had both.

Alu I digestion of amplified genomic fragments (Fig. 3B) showed that the patient (lane 6) and his mother (lane 2) carried both the mutant and normal alleles, and his brother (lane 4) and father (lane 8) had only the normal allele.

These results suggest that the other mutant allele inherited from the father is not expressed. Accordingly, it is expected that the amount of the T2 mRNA of the patient, his father, and his brother decreased to about 50% of controls'. It was difficult to detect a 50% decrease in the amount of T2 mRNA by Northern blotting (data not shown). Southern blot analysis with the full-length T2 cDNA showed no obvious abnormalities of the T2 genes of this family (data not shown), thereby suggesting that the abnormality in the other allele does not involve a large deletion or a rearrangement. Based on these results, we propose that the patient is a compound heterozygote; one allele inherited from his mother has a point mutation from G to A (^{347}Ala to Thr) while the other allele from his father has a mutation which abolishes the expression of T2 mRNA.

Characterization of the ^{347}Ala to Thr substitution. We attempted to confirm the significance of this amino acid change by transfection assay of T2 cDNA, using COS cells. It was difficult to distinguish the thiolase activity derived from the transfected cDNA from the intrinsic T2 activity of COS cells. Accordingly, we carried out pulse-chase experiments on the COS cells transfected with the expression plasmid of the normal and mutant T2 (Fig. 4B). A human β-glucuronidase (βG) expression plasmid, pSVLβG, was co-transfected as an internal standard, and the intrinsic T1 was used to standardize the cell numbers. Expression of the normal cDNA was efficient and the signal intensity of the T2 protein remained unchanged during the 24-hr chase (lanes 3 and 4). On the other hand, the mutant cDNA directed the synthesis of T2 (mutant) protein at a significant level (compare lanes 1 and 5; also see the signals of βG), but, after a 24-hr chase, the signal intensity was reduced to one

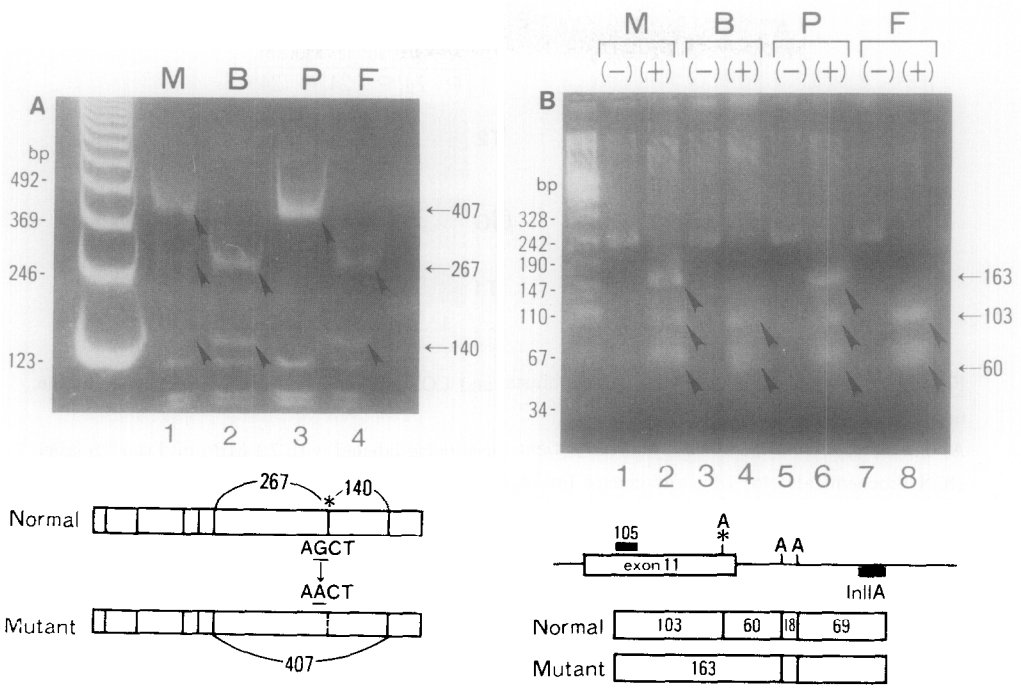

Figure 3. Alu I restriction assay for the site of an Ala to Thr mutation. In the lower panels, an asterisk indicates the Alu I site which is deleted in the mutant sequence. The fragments indicated by arrowheads are those relevant to the mutation site.

A) Amplified 60-135 cDNA fragments were electrophoresed on a 5% polyacrylamide gel after digestion with Alu I. The cDNA samples were derived from the mother, brother, the patient, and the father (lanes 1, 2, 3, and 4, respectively). Normal cDNA fragment was digested to 267 and 140 bp fragments together with smaller fragments, while the mutated one was digested to the 407 bp fragment.

B) PCR amplification of genomic region encompassing the mutation site was carried out with a pair of 105 (5'-GATATTGCAATGTGGGAAG-3') and In11A (5'-GCCTTATGAAAGATGGGTAGG-3') primers. The amplified genomic fragments were electrophoresed on 5 % NuSieve agarose gel (FMC BioProducts) before (lanes 1, 3, 5, and 7) and after (lanes 2, 4, 6, and 8) Alu I digestion. Lanes 1 and 2, fragments from his mother; lanes 3 and 4, brother; lanes 5 and 6, the patient; and lanes 7 and 8, father. "A" in the lower panel indicates Alu I sites .

third that of the 1-hr pulse (lanes 5 and 6). The residual signal seemed to be largely due to the intrinsic T2 (compare lanes 2 and 6 ; also see the signals of T1). Thus, in COS cells, the mutant T2 protein carrying the Ala to Thr substitution is degraded much more rapidly than the normal T2. These results coincide with the result of the pulse-chase experiment of the patient's fibroblasts (Fig. 4A, patient) and indicate that the mutation (^{347}Ala to Thr) is responsible for 3KTD.

The primary structures in nine molecular species of thiolases have been reported (9,10,15-17). The ^{347}Ala to Thr mutation is located in a highly conserved region, as we reported (9). The sequence in all the thiolases can be summarized as Gly-Gly-Ala-X-X-Leu-Gly-His-Pro-X-Gly. As the Ala residue is conserved from E. coli to humans, irrespective of substrate specificities, it seems to be indispensable for thiolase function. Our report seems to be the first of a mutant allele identified at the gene level.

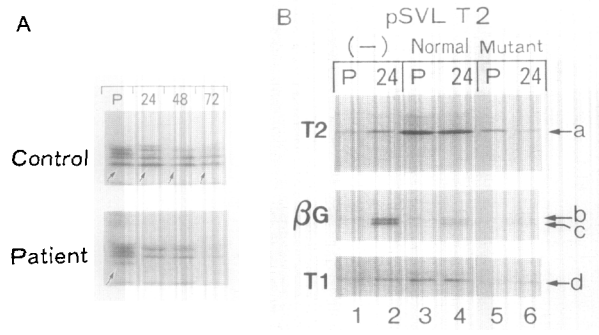

Figure 4. Pulse-chase experiments of fibroblasts and COS cells which were transfected with the normal and the mutant T2 cDNA.

A) Fibroblasts from the control and the patient were pulse-labeled with 7.4 MBq of Tran ^{35}S-label (ICN Biochemicals) for 1 hr, and chased for 24, 48, and 72 hrs. Arrows indicate T2 proteins.

B) The normal and the mutated T2 cDNA were subcloned to the pSVL expression vector (Pharmacia). pSVL expressing human β-glucuronidase (pSVLβG) (14) were cotransfected to standardize the efficiency of transfection. Ten μg of pSVLT2s and 2.5 μg of pSVLβG were transfected to COS cells by the DEAE-dextran/chloroquine method (14). The cells were then divided equally into two dishes. Pulse-chase experiments were performed as in A. The cell extracts were immunoprecipitated with antibodies against rat T2, human βG, and rat T1. Lanes 1 and 2 : pSVLβG alone was transfected. Lanes 3 and 4 : pSVLT2normal and pSVLβG were transfected. Lanes 5 and 6 : pSVLT2mutant and pSVLβG were transfected. P and 24 indicate 1-hr pulse and 24-hr chase, respectively. The positions of the mature subunit of T2, precursor of βG, mature βG, and mature subunit of T1, are indicated by a, b, c, and d, respectively.

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