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Newly Identified Forms of Electron Transfer Flavoprotein Deficiency in Two Patients with Glutaric Aciduria Type II

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ABSTRACT. Newly identified forms of electron transfer flavoprotein (ETF) deficiency in two patients with glutaric aciduria type II (GA II) were described. GA II has been attributed to a defect of either ETF or ETF dehydrogenase, resulting in multiple acyl-CoA dehydrogenation deficiency. ETF is a mitochondrial flavoprotein consisting of an α-subunit, α-ETF, and a β-subunit, β-ETF. We used pulse-chase experiments to examine the biosynthesis of ETF in fibroblasts from two patients with GA II. Patient 1 was a boy with the neonatal onset form, but without congenital anomalies, who is living at age 2 y. A defect of β-ETF biosynthesis was noted in this patient. Patient 2 was a boy with the neonatal onset form with congenital anomalies who died on the 3rd postnatal day. He presented with a peculiar face and polycystic kidneys. In patient 2, both α- and β-ETF were synthesized, but both the subunits were rapidly degraded. The lability of ETF was considered to be the cause of GA II in this patient. These two cases appear to be new forms of ETF deficiency in GA II. (*Pediatr Res* 29: 60–63, 1991)

Abbreviations

GA II, glutaric aciduria type II
ETF, electron transfer flavoprotein
α-ETF, α-subunit of ETF
β-ETF, β-subunit of ETF
pα-ETF, precursor of α-ETF
ETF-DH, electron transfer flavoprotein dehydrogenase
GC/MS, gas chromatography and mass spectrometry
R6G, rhodamine 6G

GA II, first described in 1976 (1), is an inherited disorder of organic acid metabolism caused by metabolic blocks at several steps of mitochondrial flavin-containing acyl-CoA dehydrogenases: short-chain acyl-CoA dehydrogenase, medium-chain acyl-CoA dehydrogenase, or long-chain acyl-CoA dehydrogenase involved in fatty acid β-oxidation; isovaleryl-CoA dehydrogenase, isobutyryl-CoA dehydrogenase, and methylbutyryl-CoA dehydrogenase involved in branched-chain amino acid catabolism; glutaryl-CoA dehydrogenase in lysine, hydroxylysine, or tryptophan catabolism; and sarcosine dehydrogenase (2). Many patients with GA II present with hypotonia, tachypnea, hypoglycemia, and, often, neonatal death, or Reye's syndrome-like illness. Urinary organic acid analysis by GC/MS shows an increased excretion of characteristic compounds such as adipate, suberate, sebacate, glutarate, 2-hydroxyglutarate, ethylmalonate, or isovalerylglycine, which are the corresponding metabolites derived from defective steps (2). The etiology of this disorder was postulated to be due to deficiency of a protein component common to acyl-CoA dehydrogenation (2–11).

A genetic deficiency of ETF or ETF-DH in GA II was demonstrated by enzyme assay, immunoblotting, or pulse-labeling techniques (12–14). ETF is a heterodimeric flavoprotein consisting of two subunits, an α-subunit (α-ETF) and a β-subunit (β-ETF), localized in the mitochondrial matrix (15–17). We now describe two new forms of ETF deficiency in patients with GA II, identified in pulse-chase experiments. In one patient there seems to be a defect in β-ETF biosynthesis, and in the other, lability of the ETF subunits seems to be the cause.

MATERIALS AND METHODS

Fibroblasts. Skin fibroblasts obtained from two Japanese children with GA II, patients 1 and 2, were cultured in Eagle's minimal essential medium containing 10% FCS (vol/vol) and antibiotics (standard medium).

Patient 1 was a boy born to unrelated parents, and the family history was noncontributory. He was diagnosed with GA II at the age of 10 mo, based on the results of the urinary organic acids analyzed by GC/MS. Urinary organic acids such as dicarboxylic acids (adipate, suberate, and sebacate), 2-hydroxy-glutarate, isovalerylglycine, and ethylmalonate were elevated. He had transient respiratory distress in the neonatal period and a mild gross motor retardation was evident in early infancy. At 5 mo of age, hypotonia, tachypnea, vomiting, hypoglycemia, and metabolic acidosis occurred after an infection. Subsequently, similar episodes occurred several times after an infection or a bout of diarrhea.

Patient 2 was a boy born to unrelated parents. His brother had died of unknown causes 12 h after delivery. This patient died on the 3rd postnatal day, with apnea, hypotonia, lethargy, vomiting, and a peculiar body odor. Laboratory test revealed hypoglycemia, metabolic acidosis, and hyperammonemia. Urinary organic acids analysis by GC/MS revealed an increased excretion of adipate, sebacate, glutarate, ethylmalonate, 2-hydroxy-glutarate, and isovalerylglycine. An enlarged head and anterior fontanel were also

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noted, and polycystic kidney and fatty infiltration of the liver were revealed at autopsy.

Materials. Tran [^{35}S]-Label (>1000 Ci/mmol) as [^{35}S]methionine was from ICN Radiochemicals, Irvine, CA; EN^{3}HANCE was from Du Pont/NEN Research Products, Boston, MA; the fixed *Staphylococcus aureus* cells were from Sanraku Co., Tokyo, Japan; protease inhibitors were from the Peptide Institute, Osaka, Japan; anti-[human fibronectin]IgG (goat) was from CAPPEL Laboratories, West Chester, PA; and the immunoblotting system was from Promega Biotec, Madison, WI.

Preparation of ETF and the antibody. ETF was purified from rat liver, and the antibody against rat ETF was raised in rabbits and partially purified as described (15).

Immunoblot analysis. Fibroblast extracts were applied to SDS/PAGE, and immunoblotting was then done as described (18) using the system recommended in the supplier's instructions.

Pulse-labeling and chase experiment. Fibroblasts were grown in 6-cm plastic dishes. Pulse-labeling was performed as described (18) using 200 μCi of [^{35}S]methionine per each dish. When R6G, an inhibitor of transport of the precursors of the mitochondrial enzymes and their processing (19) was used, it was added to the medium to give a final concentration of 5μg/mL 30 min after start of the preincubation, and to the labeling medium, as described (20, 21). In pulse-chase experiments, the labeling medium was replaced with 3 mL of the standard medium after a 1-h pulse-labeling, then the cells were chased for 6 h, 24 h, and 72 h, respectively, as described (18).

Immunoprecipitation and fluorography. The labeled and solubilized cells were treated as described (18), and immunoprecipitated with anti-[rat ETF]IgG. For competition experiment, about 5μg of purified rat ETF was added before incubating with the antibody. The immune complexes were recovered with the *S. aureus* cell suspension, and then subjected to SDS/PAGE using 10% gels according to the method of Laemmli (22). The gels were stained with Coomassie brilliant blue, treated with EN^{3}HANCE, dried, and flurographed according to the supplier's instructions.

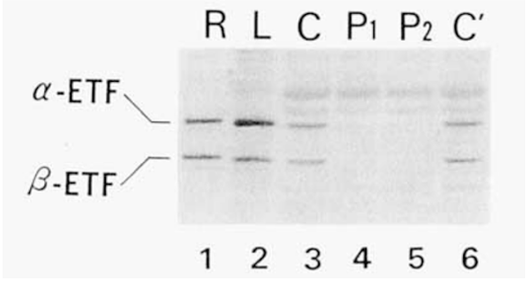

Fig. 1. Immunoblot analysis of ETF. *Lane 1*, purified rat ETF (5 ng of protein applied); *lanes 2* and *7*, human liver (10 μg each of protein); *lanes 3* and *6*, control fibroblasts (50 μg each of protein); *lanes 4* and *5*, fibroblasts of patient 1 and patient 2, respectively (50 μg each of protein). *R*, rat ETF; *L*, human liver; *C* and *C'*, two different control fibroblast lines; P_1 and P_2, fibroblasts from patient 1 and patient 2, respectively.

RESULTS

Immunoblot analysis of ETF. Figure 1 summarizes the results of immunoblot analysis of ETF. The bands for α- and β-ETF were clearly seen in the human liver tissue (Fig. 1, *lane 2*) and the control fibroblasts (Fig. 1, *lanes 3* and *6*). In both patients, bands for α- and β-ETF were not detected (Fig. 1, *lanes 4* and *5*, respectively). Other fainter bands may be due to nonspecific reactions.

Pulse-labeling of precursor and mature subunits of ETF. Cultured fibroblasts were incubated for 1 h with [^{35}S]methionine in the presence and the absence of R6G, an inhibitor of mitochondrial import of the precursor proteins. Figure 2 summarizes the results. In the control fibroblasts, the fluorographic bands for the mature form of α-ETF and β-ETF were apparent in the absence of R6G (Fig. 2, *lane 3*). In the presence of R6G, pα-ETF was observed migrating slightly slower than the corresponding mature counterpart. The β-ETF precursor migrated at the same position as the mature subunit detected in the absence of R6G (Fig. 2, *lane 2*). The competition experiment with purified rat ETF gave no band (Fig. 2, *lane 1*) or a fainter band (Fig. 2, *lane 4*). After fibroblasts from patient 1 were pulse-labeled, the signals for the pα-ETF and α-ETF were clearly seen in the presence and the absence of R6G, respectively, but no bands for β-ETF were detected in either lane (Fig. 2, *lanes 6* and *7*). In fibroblasts from patient 2, signals for pα-ETF, α-ETF, and β-ETF were obtained (Fig. 2, *lanes 10* and *11*), as in the normal control. These findings suggest that patient 1 has a defect in the biosynthesis of β-ETF. However, in patient 2, synthesis of both α- and β-ETF seemed normal.

Pulse-chase experiments. Chase experiments were performed for 6 h, 24 h, and 72 h after pulse-labeling for 1 h to examine the stability of synthesized subunits of ETF. Figure 4 summarizes the results. In the normal control, the signals for both α- and β-ETF were observed after a 72-h chase (Fig. 3*A* and *B*, *lanes 1–4*). In patient 1, α-ETF band was visible after a 6-h chase (Fig. 3*A*, *lanes 5* and *6*) but could not be detected after a longer chase (Fig. 3*A*, *lanes 7* and *8*). No band for the β-ETF was present in any lane (Fig. 3*A*, *lanes 5–8*). For patient 2, the bands for α- and β-ETF were seen at a 6-h chase, but both bands disappeared after a 24-h chase (Fig. 3*B*, *lanes 5–8*). Fluorograms of mitochondrial

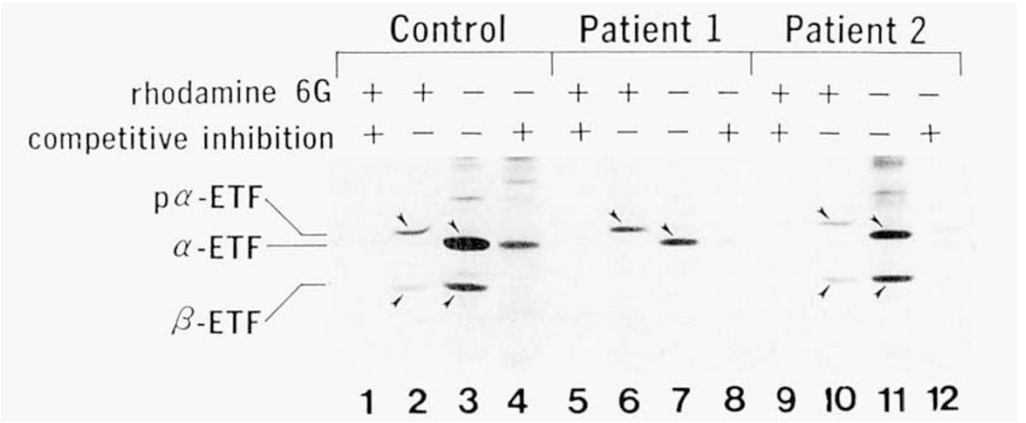

Fig. 2. Pulse-labeling of ETF in fibroblasts in the presence and absence of R6G. *Lanes 1–4*, fibroblasts from the control; *lanes 5–8*, patient 1; *lanes 9–12*, patient 2. Among them, *lanes 2*, *6*, and *10* are in the presence of R6G; *lanes 3*, *7*, and *11* are in the absence of R6G; *lanes 1*, *5*, and *9* are competition experiments in the presence of R6G; *lanes 4*, *8*, and *12* are competition experiments in the absence of R6G. About 5 μg of unlabeled rat ETF was added before immunoprecipitation for the competition experiment (*lanes 1*, *4*, *5*, *8*, and *12*). *Arrowheads* indicate the signals for pα-ETF and α-ETF (*upper*) and β-ETF (*lower*).

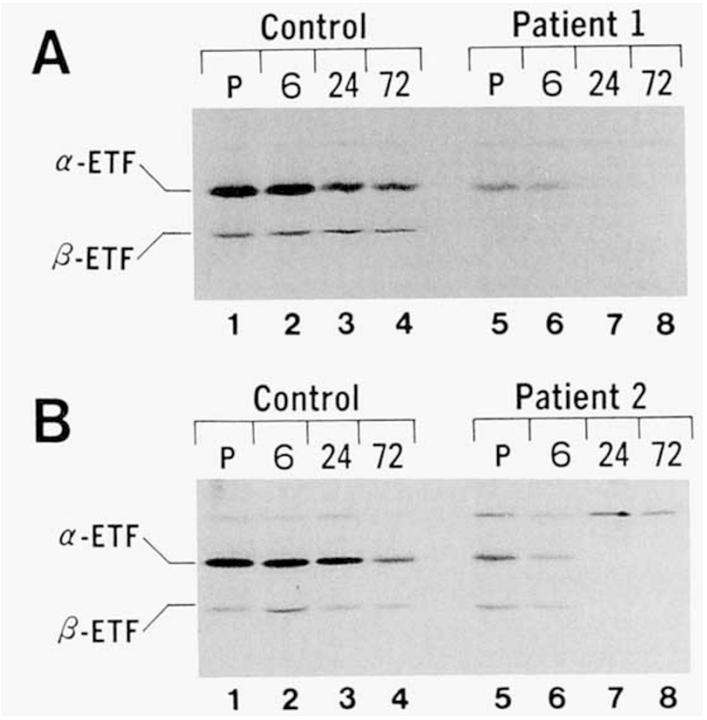

Fig. 3. Pulse-chase experiments of ETF. *Panel A*, patient 1 (*lanes 5* and *6*) and normal control (*lanes 1–4*); *panel B*, patient 2 (*lanes 5–8*) and normal control (*lanes 1–4*). *Lanes 1* and *5*, pulse-labeling with 200 μCi of [^{35}S]methionine for 1 h; *lanes 2* and *6*, chase for 6 h; *lanes 3* and *7*, chase for 24 h; *lanes 4* and *8*, chase for 72 h. *P*, pulse-labeled for 1 h; *6*, *24*, and *72*, chased for 6, 24, and 72 h, respectively, after 1 h pulse-labeling.

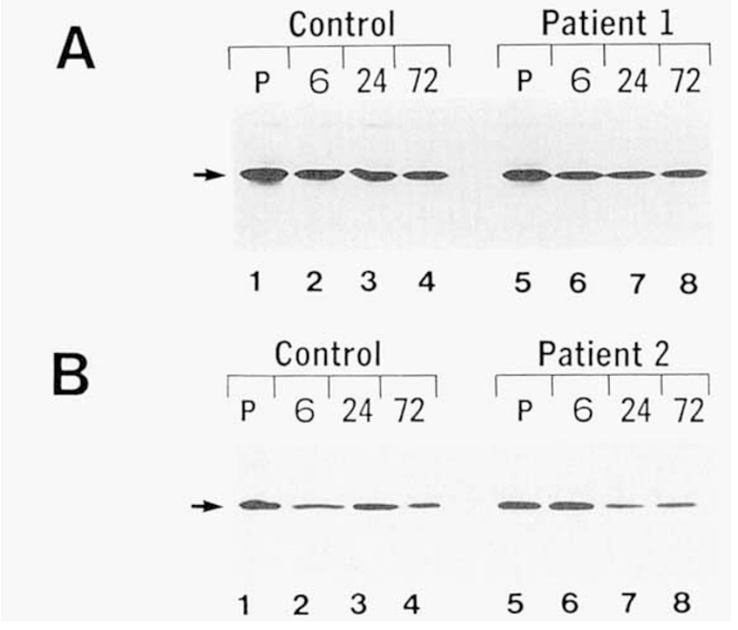

Fig. 4. Pulse-chase experiments of mitochondrial 3-ketoacyl-CoA thiolase performed as the positive control of the mitochondrial enzyme protein. *Panel A*, patient 1 (*lanes 5–8*) and normal control (*lanes 1–4*); *panel B*, patient 2 (*lanes 5–8*) and normal control (*lanes 1–4*). Abbreviations are the same as those in Figure 3. *Arrows* indicate the position of the thiolase.

3-ketoacyl-CoA thiolase were prepared as the positive control of the mitochondrial enzyme protein, using the same samples. As shown in Figure 4, signals were clearly observed after a 72-h chase, the intensity being the same for fibroblasts from the control and the patients. These findings indicate that the primary defect in patient 1 is β-ETF biosynthesis. The β-ETF deficiency may secondarily cause lability of α-ETF, as assembly of the ETF complex in the mitochondria would not occur. In patient 2, both α- and β-ETF were synthesized, but were unstable. This lack of stability is likely to cause GA II.

DISCUSSION

GA II, also known as multiple acyl-CoA dehydrogenation deficiency, is characterized by severe acidosis, hypoglycemia, or Reye's syndrome-like illness (2). GA II may be caused by a defect in either ETF or ETF-DH involved in mitochondrial acyl-CoA dehydrogenases (2, 12, 13). ETF-DH deficiency has been described by enzyme assay and immunoblot analysis (12, 14), and an ETF deficiency was also shown by immunoblotting (12, 14), pulse-labeling (13), or measurement of ETF activity (23).

ETF is a heterodimer consisting of an α- and a β-subunit, which are encoded by different nuclear genes, translated on cytosolic polyribosomes as precursors, and translocated into mitochondria: functional ETF is formed by assembly of α- and β-ETF in the mitochondrial matrix (15–17), as with many other mitochondrial matrix enzymes (24–26). Human α-ETF is translated as a precursor of the 35 kD, which is 3 kD larger than the mature α-ETF. cDNA for human α-ETF precursor was cloned by Finocchiaro *et al.* (27). β-ETF, synthesized in the cytosol (27 kD), is indistinguishable in size from the mature form (13).

Frerman and Goodman (12) analyzed six cell lines from GA II patients by determining the ETF-DH activity and immunoblot analysis of ETF and ETF-DH. They concluded that four of the six lines were related to an ETF-DH deficiency and the other two had an ETF deficiency. The latter two cell lines were reported to have small amounts of an abnormal α-ETF and migration was faster on SDS/PAGE than it was for the normal α-ETF. One cell line (their 1441) had a normal amount of β-ETF but another (their 1196) had no β-ETF. Ikeda *et al.* (13) also studied the biosynthesis of ETF in eight cell lines from GA II patients, using pulse-labeling techniques. They showed that three lines had defects in α-ETF biosynthesis, and that one (Ikeda's 605; Frerman's 1196) from a patient reported by Przyrembel *et al.* (1) had normal β-ETF but no α-ETF. Frerman and Goodman (12) reported that the cell line (their 1196) had abnormal α-ETF and no β-ETF, as determined by immunoblot analysis. Although the reason for this discrepancy is unclear, a possible primary defect of α-ETF biosynthesis in this cell line might have been identified in pulse-labeling experiments. Although immunoblotting is a simpler method for the detection of the ETF deficiency, it reveals only the antigen peptide at the steady state. On the other hand, pulse-chase experiments examine both polypeptide synthesis and stability. In our study, no ETF subunits were detected for either patient by immunoblotting, yet the results of pulse-chase experiments showed that either or both of the subunits of ETF were translated normally.

A genetic deficiency of either ETF or ETF-DH deficiency has been identified in GA II patients (12–14). Cases of α-ETF deficiency were described by Frerman and Goodman (12) using immunoblotting, and by Ikeda *et al.* (13), using pulse-labeling. Immunoblotting was used by Loehr *et al.* (14) to identify β-ETF deficiency. Patient 1 in this report is apparently the first reported case of a defect in β-ETF biosynthesis to be demonstrated by pulse-chase experiments. As described above, in this patient, the lability of α-ETF may also be caused by β-ETF deficiency. Degradation of α-ETF in this patient appears to be occurring inside the mitochondria, given that the transit peptide cleavage occurred in experiments using R6G, an inhibitor of transport of the precursor of mitochondrial enzymes. On the other hand, in patient 2, both α- and β-ETF were synthesized but both the subunits were degraded rapidly. In patient 2, mitochondrial degradation of ETF subunits also probably occurred, given the normal processing of the precursors, as seen in patient 1. The

lability of ETF is likely to cause GA II in this patient. Although cause of the lability is not clear, the following possibilities can be considered: *1*) An ETF complex cannot be formed in the mitochondrial matrix due to abnormality in the peptides of either α- or β-ETF, and the defect results in the lability of both subunits, or *2*) a defect of a component required for assembly or stabilization of the ETF leads to instability of the subunits of ETF. Thus, the two above cases are new forms of ETF deficiency in GA II.

A considerable degree of heterogeneity of expression in GA II patients has been reported. Clinically, GA II has been classified into the three groups (2): *1*) neonatal onset form with congenital anomalies, such as macrocephaly, facial dysmorphism, rocker bottom feet, or polycystic kidney, *2*) neonatal onset form without anomalies, and *3*) mild or late onset form. Most patients with the neonatal onset form, with or without congenital anomalies, have a severe illness and often die within the 1 wk of life, or early in infancy. Other patients who survive longer can have a Reye's syndrome-like illness. On the other hand, some patients with the mild form have intermittent episodes of vomiting or hypoglycemia, and others may have no symptoms during childhood, presenting with the episodic illness in adult life (4). Loehr *et al.* (28) reported that five patients with an ETF-DH deficiency were cases of a neonatal onset with congenital anomalies, and that findings in two cell lines from ethylmalonic adipic aciduria (a mild form of GA II) patients were also due to a partial deficiency of ETF-DH (12, 28). Ikeda *et al.* (13) described that all three patients showing α-ETF deficiency had the typically severe form. On the other hand, mild variants with α-ETF deficiency were also reported (14). In patient 1 with the β-ETF deficiency, the neonatal onset form without anomalies is expected to occur, and patient 2 will likely have the neonatal onset form with congenital anomalies. According to recent reviews of the phenotype in GA II, deficiencies of ETF and ETF-DH have been noted in patients with either the severe or mild form (14, 23, 29). On the other hand, patients with congenital anomalies such as facial dysmorphism, large head or polycystic kidney can have a deficiency of ETF, but it is less extensive than the ETF-DH deficiency (14, 29). Thus, correlation between clinical severity and defective sites remains ambiguous.

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