INTRODUCTION

Acute encephalopathy sometimes occurs in the early stages of severe infectious diseases with pyrexia, impaired consciousness, convulsions or seizures associated with brain edema (1, 2). The antecedent infection of acute encephalopathy is often viral, mainly influenza virus and HHV-6 (human herpes virus-6) (3, 4), though many other viruses can also be involved, such as adenovirus and Coxsackie virus. According to a nation-wide clinical survey of influenza-associated encephalopathy (IAE) in Japan, IAE patients do not only show brain edema but also develop multi-organ failure, and the condition is associated with high mortality (31.8%) and disability (27.7%) rates (5). Clinical and genetic studies in such IAE patients have demonstrated the presence of high fever-induced disorder of mitochondrial fatty acid β-oxidation caused by heat-inactivation of carnitine palmitoyltransferase II (CPT II)
(EC 2.3.1.21) (MIM#600650), a pivotal component of ATP generation through mitochondrial fatty acid β-oxidation (4, 6-8).

The human CPT II is a homotetramer encoded by a single CPT II gene, which spans 20 kb, contains 5 exons ranging from 81 to 1,305 bp, and is located on chromosome 1p32. CPT II deficiency is the most common inherited disease of energy metabolism and has three distinct clinical forms: a neonatal form, which results in sudden death, and two less severe forms, the early-onset infantile and late-onset adult forms (9-13). Several studies have examined the potential relationships among the genotypes of CPT II deficiency and their clinical phenotypes or their impact on fuel utilization (14, 15).

We reported recently that the majority of fatal and handicapped IAE patients exhibited transiently elevated serum acylcarnitine ratio [(C16 : 0+C18 : 1)/handicapped IAE patients exhibited transiently elevated serum acylcarnitine ratio [(C16 : 0+C18 : 1)/C2)], which results in sudden death, and two less severe forms, the early-onset infantile and late-onset adult forms (9-13). Several studies have examined the potential relationships among the genotypes of CPT II deficiency and their clinical phenotypes or their impact on fuel utilization (14, 15).

We reported recently that the majority of fatal and handicapped IAE patients exhibited transiently elevated serum acylcarnitine ratio [(C16 : 0+C18 : 1)/C2] ≤ 0.048 the upper cutoff value, particularly> 0.09, suggesting secondary CPT II deficiency and impaired energy metabolism during high-grade fever, despite the asymptomatic disorders of CPT II in daily life (6). Analyses of genotype and allele compositions of CPT II in a large number of patients with severe IAE showed the predominance of a thermolabile phenotype of CPT II mutations, [c.1055T>G (p.F352C)], in East Asians, and its compound form [c.1055T>G (p.F352C)] + [c.1102G>A (p.V368I)] (6, 7). The mutations [c.1102G>A (p.V368I)] and [c.1939A>G (p.M647V)] have no effect on enzyme activity and its thermolability (7).

The present investigation is an extension to the above study and was designed to characterize the enzymatic properties of CPT II mutations in a patient with adenovirus-associated encephalopathy and members of his family. To characterize the CPT II phenotypes of the patient and family, CPT2 with various mutations were expressed in COS-7 cells, and the activities, thermal instability and half-lives of CPT II were analyzed and compared with those of the wild type (WT) CPT II.

### MATERIALS AND METHODS

#### Materials

L-[methyl-3H] carnitine, L-[35S] methionine, ECL-Plus detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). BCA reagent was obtained from Pierce (Rockford, IL). ABI DyeDeoxy Terminator Cycle Sequencing Kit was from PE-Applied Biosystems (Foster, CA).

#### Anti-CPT II (H-300, sc-20671) was from Santa Cruz Biotechnology, Inc. Anti-CPT II (H-300, sc-20671) was from Santa Cruz Biotechnology, Inc.

#### Expression of WT and mutant CPT II s in COS-7 cells

Genomic DNA was purified from whole blood kindly provided by Dr. K Maruyama, from the Central Hospital, Aichi Prefectural Colony. This investigation was approved by the ethics review committee for human genome analysis at our institution. A full-length WT human CPT II cDNA clone (hCPT2 Genbank accession number NM_000098) containing the entire coding region was a kind gift from Dr. V. Esser, from the University of Texas. Plasmid pCMV6-WT was used as a parental vector to generate full-length mutant CPT II cDNA clones, pCMV6-Q216R [c.647A>G (p.Q216R)], pCMV6-Q216R+G249EfsX16 [c.647A>G (p.Q216R) ; c.1102G>A (p.V368I) and c.1939A>G (p.M647V)], pCMV6-Q216R+G249EfsX16 [c.647A>G (p.Q216R) and c.745delG (p.G249EfsX16)] and pCMV6-G249EfsX16 [c.745delG (p.G249EfsX16)] using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). CPT2 exons 1-5 of genomic DNA were amplified by PCR and sequenced as described previously (7). The primers used for mutations induction are listed in Table 1. All substitutions and integrity of the CPT2 cDNAs were confirmed by sequence analysis.

COS-7 cells (2 × 10^5) cultured at 37°C in 5% CO₂ in low-glucose Dulbecco’s modified Eagle’s essential medium (DMEM) (Invitrogen, Grand Island, Delaware).

#### Table 1. Primers used for construction of CPT2 mutants.

<table>
<thead>
<tr>
<th>Region</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q216R</td>
<td>5’-taccccctggatagtcgcccggatattgcgctttc-3’</td>
<td>5’-gaaagccgaatataaggggatcatcaggggata-3’</td>
</tr>
<tr>
<td>G249EfsX16</td>
<td>5’-ctcctgccctaaaggaattttatatatc-3’</td>
<td>5’-aaagatataaaatctttccctagacacgag-3’</td>
</tr>
<tr>
<td>V368I</td>
<td>5’-catgccctactgcctgtagagccacttgccttg-3’</td>
<td>5’-caagagtctgtccatgtgggtagccagtacccagc-3’</td>
</tr>
<tr>
<td>M647V</td>
<td>5’-aagcttgagtcagttgtagctgctagacag-3’</td>
<td>5’-cctctagacacgagctcttagcctgctgtg-3’</td>
</tr>
</tbody>
</table>

Reference sequences are GenBank accession number NC_000001 for human CPT II genomic DNA. GenBank accession number NM_000098 for WT human CPT II. The mutant sites appear bold and underlined.
NY) containing 10% fetal calf serum (FCS) (Roche Molecular Biochemicals, Indianapolis, IN) were used for transfection. WT and mutant CPT2 cDNAs (6 μg each) were transfected into COS-7 cells by FuGENE 6 transfection reagent (Roche Diagnostics Co. Indianapolis, IN) using the instructions provided by the manufacturer. After transfection for 72 h, cells were washed twice with calcium and magnesium-free phosphate-buffered saline [PBS(-)], lysed with 0.5 ml of CPT II reaction buffer (5 mM Tris-HCl buffer, pH 7.4, containing 0.1% Tween 20 and 0.5 M KCl) and then centrifuged at 147,600×g for 1 h at 4°C. pSVβ-Galactosidase control vector (Promega, Madison, WI) was co-transfected into COS-7 cells with various pCMV6-CPT2 plasmids as an internal standard for the monitoring of transfection efficiency. Mock transfection was also carried out as a control.

Western blot analysis of CPT II expression

WT and CPT II mutant proteins overexpressed in COS-7 cells were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, followed by western immunoblotting. Cell lysates were applied onto 10-20% SDS-PAGE gels. After electrophoresis, proteins in the gels were transferred electrophoretically on to PVDF membranes. Excess sites on the membranes were blocked by incubation with 3% (w/v) non-fat dried skimmed milk in TBS (Tris-buffered saline; 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) for 2 h at room temperature. After washing and incubation with 0.3 μg/ml of anti-CPT II antibodies (H-300, sc-20671, Santa Cruz Biotech) and applied onto 10-20% SDS-PAGE gradient gels. After electrophoresis, proteins in the gels were transferred electrophoretically to PVDF membranes. To identify and characterize the expression, we analyzed amino acid sequences of these mutant proteins. N-terminal amino acid sequences of immunoprecipitated CPT II proteins were determined using an Applied Biosystems model 492 gas-phase sequencer/140C system and the instructions provided by the manufacturer.

Assay of CPT II activity and thermal instability

CPT II activities in the lysates of COS-7 cells transfected with WT and mutant CPT2 cDNAs were measured at 30°C for 2 h, by detecting palmitoyl-L-[methyl-1H] carnitine formed from 200 μM L-[methyl-1H] carnitine and 50 μM palmitoyl-CoA as described previously (17). The formed palmitoyl-L-[methyl-1H] carnitine was extracted with 1-butanol and the radioactivity was counted by liquid Scintillation Counter (model LS 6500, Beckman, Fullerton, CA). To analyze the heat stability of WT and CPT II mutants, 100 μl of the cell lysates were preincubated at 41°C or 30°C for 2 h, and the enzyme activities were then measured by the addition of 200 μM L-[methyl-1H] carnitine followed by incubation at 30°C for 2 h. Protein concentrations in the cell lysate were measured using bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL).

Half-life of WT and mutant CPT IIs

Half-life of WT and CPT II mutants in transfected COS-7 cells was analyzed as described previously (7). COS-7 cells (2×10⁵) in 35 mm dishes were transfected with CPT2 WT and mutant cDNAs expression plasmids (1 μg each). After 48 h, the cells were washed and incubated with serum- and methionine-free medium for 30 min, followed by a pulse labeling with 370 MBq ³⁵S-methionine (specific activity 1000 Ci/mM) for 2 h. After labeling, cells were washed twice with PBS (-) at 37°C and then incubated with low-glucose DMEM containing 10% FCS and 2 mM methionine to chase for 0, 1, 3, 6, 12 and 18 h. At various chase intervals, the cells were lysed with 0.5 ml RIPA buffer (10 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40, 0.05% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 150 mM NaCl, and protease inhibitors cocktail) and were immunoprecipitated with 3 μg anti-CPT II and mutant cDNAs expression plasmids. After 72 h, the cell lysates were immunoprecipitated with 3 μg anti-CPT II antibody (H-300, sc-20671, Santa Cruz Biotech) and applied onto 10-20% SDS-PAGE gradient gels. After electrophoresis, proteins in the gels were transferred electrophoretically to PVDF membranes. To identify and characterize the expression, we analyzed amino acid sequences of these mutant proteins. N-terminal amino acid sequences of immunoprecipitated CPT II proteins were determined using an Applied Biosystems model 492 gas-phase sequencer/140C system and the instructions provided by the manufacturer.

Immunoprecipitate and amino acid sequence analysis

COS-7 cells were transfected with CPT2 WT and mutant cDNAs expression plasmids. After 72 h, the cell lysates were immunoprecipitated with 3 μg anti-CPT II antibody (H-300, sc-20671, Santa Cruz Biotech) and applied onto 10-20% SDS-PAGE gradient gels. After electrophoresis, proteins in the gels were transferred electrophoretically on to PVDF membranes. To identify and characterize the expression, we analyzed amino acid sequences of these mutant proteins. N-terminal amino acid sequences of immunoprecipitated CPT II proteins were determined using an Applied Biosystems model 492 gas-phase sequencer/140C system and the instructions provided by the manufacturer.
antibody (H-300, sc-20671, Santa Cruz Biotech) coupled to rec-Protein G-Sepharose 4B (Zymed Laboratories, San Francisco, CA) by incubation overnight at 4°C under constant shaking. Immunoprecipitates were separated by 10-20% SDS-PAGE under reducing conditions and the dried gels were analyzed by autoradiography.

RESULTS

CPT II mutations in a patient with adenovirus-associated encephalopathy and family members

Genotype analysis by the five exons PCR and sequence check of \textit{CPT2} in the patient with adenovirus-associated encephalopathy showed heterozygous compound mutations; heterozygous mutation [c.647A>G (p.Q216R)] on one allele and heterozygous mutation [c.745delG (p.G249EfsX16)] on another allele, which caused a frame shift resulting in a premature termination codon (p.G249EfsX16). The mother had homozygous mutation [c.647A>G (p.Q216R)], while the father had heterozygous mutation [c.745delG (p.G249EfsX16)] on one allele and heterozygous mutations [c.1102G>A (p.V368I)+c.1939A>G (p.M647V)] on the other allele, and the sister had heterozygous mutation [c.647A>G (p.Q216R)] on one allele and heterozygous mutations [c.1102G>A (p.V368I)+c.1939A>G (p.M647V)] on the other allele (Table 2). Among these mutations, [c.647A>G (p.Q216R)] and [c.745delG (p.G249EfsX16)] were novel missense mutations and did not match more than 25 mutations reported thus far in CPT II deficiency of late-onset muscular, infantile/juvenile hepatic and severe neonatal phenotypes (15, 18-26).

\textit{In vitro expression and western blot analysis}

To characterize these \textit{CPT2} mutations, we expressed WT and CPT II mutants by transfection in COS-7 cells: pCMV6-WT, single mutants pCMV6-Q216R and pCMV6-G249EfsX16, and double compound mutant pCMV6-Q216R+G249EfsX16. This was based on the location of V368I and M647V near the acylcarnitine binding sites, though have little effects on \textit{Km} values (7). The triple compound mutant, pCMV6-Q216R+V368I+M647V, was prepared to analyze the effects of mutations of V368I and M647V on the novel mutant Q216R. Western immunoblotting analysis of the overexpressed pCMV6-WT, pCMV6-Q216R and pCMV6-Q216R+V368I+M647V which encoded 658 amino acids, showed a single protein band with a molecular mass of 67 kDa. On the other hand, the overexpressed pCMV6-Q216R+G249EfsX16 and pCMV6-G249EfsX16 encoded 264 amino acids, showed a faint single protein band with 27 kDa (Fig. 1). The amounts of CPT II proteins expressed in the cell lysates were normalized to that of the internal control (β-actin).

<table>
<thead>
<tr>
<th>Kindred</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Mutation type</th>
<th>Exon</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mother, sister, patient</td>
<td>c.647A&gt;G</td>
<td>p.Q216R</td>
<td>Missense</td>
<td>4</td>
<td>current study</td>
</tr>
<tr>
<td>father, patient</td>
<td>c.745delG</td>
<td>p.G249EfsX16</td>
<td>Frameshift</td>
<td>4</td>
<td>current study</td>
</tr>
<tr>
<td>father, sister</td>
<td>c.1102G&gt;A</td>
<td>p.V368I</td>
<td>Missense</td>
<td>4</td>
<td>6, 7, 13</td>
</tr>
<tr>
<td>father, sister</td>
<td>c.1939A&gt;G</td>
<td>p.M647V</td>
<td>Missense</td>
<td>5</td>
<td>6, 7, 13</td>
</tr>
</tbody>
</table>

Fig. 1. Expression of WT and mutant CPT IIs in COS-7 cells. The expression levels of WT and mutant CPT IIs of the patient and his family in COS-7 cells after transfection for 72 h was analyzed by western immunoblotting with anti-CPT II antibody. The amount of CPT II protein in the cell lysate was normalized to the amount of β-actin as an internal control. Data are representative results of three separate experiments.
compound form pCMV6-Q216R+G249EfsX16 were immunoprecipitated with anti-CPT II antibody. The resulting immunoprecipitates were applied onto 10-20% SDS-PAGE gradient gel under reducing conditions and the proteins in the gels were transferred electrophoretically onto PVDF membranes (Fig. 2). The N-terminal 10 residues of immunoprecipitated CPT II protein bands with a molecular mass of 27 kDa were analyzed. Both 27 kDa protein bands from pCMV6-G249EfsX16 and pCMV6-Q216R+G249EfsX16 showed the sequence alignment, KPLLNDGQFR, although the N-terminal sequences of the 67 kDa mature CPT II proteins from WT, pCMV6-Q216R and pCMV6-Q216R+V368I+M647V showed SAGSGPGQYL (GenBank accession number NP_000089). These results suggest that the N-terminal 43 residues of these deletion mutant CPT II proteins were removed additionally in COS-7 cells, probably during the course of proteolytic maturation of CPT II.

Activity and thermal instability of CPT II mutations

WT and four mutant CPT2 cDNAs found in the patient and his family were transfected into COS-7 cells: pCMV6-WT, pCMV6-Q216R, pCMV6-Q216R+V368I+M647V, pCMV6-G249EfsX16 and pCMV6-Q216R+G249EfsX16. The specific activities of pCMV6-WT and pCMV6-Q216R, pCMV6-Q216R+V368I+M647V, pCMV6-Q216R+G249EfsX16, and pCMV6-G249EfsX16 were immunoprecipitated with anti-CPT II antibody. The N-terminal amino acid sequences were determined using an Applied Biosystems model 492 gas-phase sequencer/140C system.

We have reported previously a thermolabile mutation of F352C CPT II in patients with severe IAE (6). To evaluate the thermal instability of the mutations identified in the patient’s family, lysates of COS-7 cells expressing these CPT II mutants were pre-incubated under high temperature of 41°C for 2 h as well as under stable condition at 30°C, and then CPT II activities were measured at 30°C by the addition of substrates L-carnitine and palmitoyl-CoA. Mild thermal instability of pCMV6-Q216R was detected at 41°C and the instability was markedly enhanced at 41°C by the additional compound mutations of V368I and M647V, although that of WT was well maintained at above 90% under the same conditions. No enzyme activities were observed in the CPT II mutants of pCMV6-G249EfsX16 and pCMV6-Q216R+G249EfsX16 in comparison of the enzyme activities of cells transfected with the vector alone.

Half-lives of CPT II mutants

To analyze the intracellular stability of the CPT II mutants, COS-7 cells transfected with WT and four mutant CPT2 cDNAs were metabolically labeled with L-[35S] methionine and the half-lives of mutants and WT CPT II were analyzed in a pulse-chase protocol (Fig. 4). There was no significant difference in the protein labeling efficiency among WT CPT II, Q216R CPT II and Q216R+V368I+M647V CPT II, suggesting similar rates of protein synthesis in WT and these CPT II mutants in COS-7 cells. However, the labeling efficiencies of CPT II mutants, Q216R+G249EfsX16 CPT II and G249EfsX16 CPT II, were markedly
lower than those of WT, suggesting that mRNAs of these mutants are unstable and the rates of synthesis of CPT II are low. Pulse-labeling and chase experiments demonstrated the longest half-life in WT CPT II (T1/2 17 h), compared with 11 h and 3.5 h for pCMV6-Q216R and pCMV6-Q216R+V368I+M647V mutants, respectively. Furthermore, the half-lives of the deletion mutants of pCMV6-Q216R+G249EfsX16 and pCMV6-G249EfsX16 were the shortest with a T1/2 < 1 h.

DISCUSSION

Acute encephalopathy is caused by various viruses and often accompanied by febrile convulsions or seizures with severe brain edema. There is no specific relationship between the viruses and the type of encephalopathy; the pathogenesis of acute encephalopathy is probably mediated primarily by host factors. The mechanisms underlying encephalopathy and multiple organ failure are vascular hyperpermeability and the subsequent severe edema affecting the brain and various organs (27). The brain capillary endothelium is characterized by a greater density of mitochondria than that of peripheral capillaries (28) and fatty acid oxidation in mitochondria is the major energy source at about 70% of the ATP generation particularly in the endothelial cells (29). These findings well explain that a large proportion of patients with fatal and handicapped IAE have high fever-related disorder of mitochondrial fatty acid β-oxidation caused by heat-inactivation of CPT II, an important component of ATP generation through mitochondrial fatty acid β-oxidation (6-8).

In the present report, we characterized CPT II mutations found in adenovirus-associated encephalopathy in a patient who had repeated attacks of encephalopathy several times a year from 9 months to
4-year and 9 months of age. Genomic CPT2 analysis of the patient and his family revealed two new mutations: the first was heterozygous mutation [c.647A>G (p.Q216R)], which caused mild thermal instability, and the second heterozygous mutation was deletion [c.745delG (p.G249EfsX16)], which caused a frame shift resulting in a premature termination codon and silencing of enzyme activity. The father had another missense mutation of V386I and M647V. The sister had mutation of Q216R on one allele and V368I and M647V on the other allele. In this regard, a previous study showed that the V386I and/or M647V mutation lead to further reduction in enzyme activity in conjunction with the F352C mutation, although these mutants by themselves only slightly reduced the activity (7). In this study, we analyzed the negative effects the V386I and/or M647V mutation on the novel Q216R CPT II mutant, and showed that additional compound mutations of V386I and M647V to Q216R enhanced thermal instability of CPT II with Q216R (Fig. 3). Furthermore, we also analyzed the additional effect of Q216R mutation on the half-life of deletion mutant [c.745delG (p.G249EfsX16)] (Fig. 4).

CPT II protein is a homotetramer encoded by a single gene, CPT2. CPT II activities in the leukocytes of the patient and his father with [c.745delG (p.G249EfsX16)] mutation on one allele, was about half or less than half of WT CPT II activities (Yamaguchi M, manuscript submitted for publication), probably because the full length CPT II protein of 67 Kda from one allele, but not CPT II protein of 27 Kda, contributes to the enzyme activity. The significantly lower labeling efficiency with L-[35S] Met of 27 kDa CPT II fragment protein than those of full-length 67 kDa mutant CPT IIs at 0-time in the chase experiments (Fig. 4) indicates that the rate of 27 kDa CPT II protein synthesis was significantly lower than that of 67 kDa CPT IIs. This might be due to accelerated degradation of mutant RNAs of minigene, as reported previously by Gong et al. (30). Furthermore, the N-terminal 43 residues of the newly synthesized 27 kDa CPT II were removed and showed the sequence K69PLLN DGQFR78. The results also suggest potential instability of the mutant protein in COS-7 cells. The half-lives of these deletion mutants of pCMV6-Q216R+G249EfsX16 and pCMV6-G249EfsX16 were the shortest with a T1/2< 1 h. There was no significant effect of Q216R mutation on the half-life of deletion mutant [c.745delG (p.G249EfsX16)] (Fig. 4).

CPT II deficiency is one of the most common inborn errors of fatty acid oxidation and CPT II is an important enzyme for ATP production (31, 32). Depletion of ATP in endothelial cells weakens interactions between tight junction proteins, such as occluding and ZO-1, and the intracellular cytoskeleton, which control the permeability of the blood-brain barrier (33, 34). The phenotypic manifestations of ATP depletion appear suddenly when ATP levels fall below a threshold value (35). The clinical manifestations of sudden-onset brain edema in patients with virus-associated encephalopathy support the hypothesis of an ATP threshold in the etiology of that edema. The thermal instability and short T1/2 of the novel CPT II mutations identified in our study might play important roles in reducing ATP levels below the phenotypic threshold in brain endothelial cells of patients with virus-induced brain encephalopathy, particularly those with hyperpyrexia.

**DISCLOSURE**

The authors report no conflicts on interest.

**ACKNOWLEDGMENT**

This study was supported by a Grant-in-Aid for Promotion of Fundamental Studies in Health of NIBIO, CLUSTER Project of MEXT, and Brain Science (H12-Brain-015) from the Ministry of Health, Labor and Welfare of Japan.

**REFERENCES**

25. Yang BZ, Ding JH, Dewese T, Roe D, He G,


