Molecular characterization of L-CPT I deficiency in six patients: insights into function of the native enzyme

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Abstract **Carnitine palmitoyltransferase I (CPT I) catalyzes the formation of acylcarnitine, the first step in the oxidation of long-chain fatty acids in mitochondria. The enzyme exists as liver (L-CPT I) and muscle (M-CPT I) isoforms that are encoded by separate genes. Genetic deficiency of L-CPT I, which has been reported in 16 patients from 13 families, is characterized by episodes of hypoketotic hypoglycemia beginning in early childhood and is usually associated with fasting or illness. To date, only two mutations associated with L-CPT I deficiency have been reported. In the present study we have identified and characterized the mutations underlying L-CPT I deficiency in six patients: five with classic symptoms of L-CPT I deficiency and one with symptoms that have not previously been associated with this disorder (muscle cramps and pain). Transfection of the mutant L-CPT I cDNAs in COS cells resulted in L-CPT I mRNA levels that were comparable to those expressed from the wild-type construct. Western blotting revealed lower levels of each of the mutant proteins, indicating that the low enzyme activity associated with these mutations was due, at least in part, to protein instability. The patient with atypical symptoms had ~20% of normal L-CPT I activity and was homozygous for a mutation (c.1436C→T) that substituted leucine for proline at codon 479. Assays performed with his cultured skin fibroblasts indicated that this mutation confers partial resistance to the inhibitory effects of malonyl-CoA. The demonstration of L-CPT I deficiency in this patient suggests that the spectrum of clinical sequelae associated with loss or alteration of L-CPT I function may be broader than was previously recognized.**—Brown, N. F., R. S. Mullur, I. Subramanian, V. Esser, M. J. Bennett, J-M. Saudubray, A. S. Feigenbaum, J. A. Kobari, P. M. Macleod, J. D. McGarry, and J. C. Cohen. **Molecular characterization of L-CPT I deficiency in six patients: insights into function of the native enzyme.** ***J. Lipid Res.*** **2001.** 42: **1134–1142.**

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The carnitine palmitoyltransferase (CPT) system encompasses enzyme and transporter functions that effect the net transport of long-chain fatty acyl groups from the cytosol into the mitochondrial matrix, the site of fatty acid β-oxidation. The first component of the system, CPT I, an integral mitochondrial outer membrane protein, acts on cytosolic long-chain acyl-CoA, catalyzing the transfer of the acyl group to carnitine. The acyl-carnitine formed is then able to enter the mitochondrial matrix via a specific carnitine/acyl-carnitine carrier. A distinct gene product, CPT II, loosely associated with the matrix face of the inner mitochondrial membrane, then acts to reverse the CPT I reaction, regenerating acyl-CoA in the matrix and releasing carnitine (1).

In addition to performing a transport function, the CPT system is the primary control point for regulation of β-oxidation (1). This is mediated at the level of CPT I, which is potently inhibited by malonyl-CoA, the product of the acetyl-CoA carboxylase (ACC) reaction, and the first committed intermediate in the opposing pathway of de novo fatty acid biosynthesis. Thus, in the liver, during periods of fatty acid synthesis (fed state), ACC is active, malonyl-CoA levels rise, CPT I is inhibited, and the newly synthesized fatty acid is directed toward esterification rather than oxidation. Conversely, during periods of fasting, ACC activity is diminished, malonyl-CoA levels fall, inhibition of CPT I is relieved, and fatty acids can enter the mitochondrial matrix to be oxidized completely as a source of energy or to provide acetyl-CoA for the synthesis of ketone bodies. Although first elucidated in the context of hepatic fatty acid metabolism, it has since become apparent that the same mechanism operates in nonlipogenic tissues. Hence, even in tissues such as heart and skeletal muscle, ACC is present and the levels of malonyl-CoA are

Abbreviations: ACC, acetyl-CoA carboxylase; CPT I, carnitine palmitoyltransferase I; L-CPT I, liver CPT I; M-CPT I, muscle CPT I.

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seen to rise and fall with physiological state, as in the liver (2–4). In these tissues it is thought that synthesis of malonyl-CoA is directed purely toward regulation of CPT I.

Whereas CPT II is the product of a single gene and is expressed as the same protein in all tissues of the body, CPT I is known to exist in at least two isoforms (1, 5, 6). These are denoted L-CPT I (or liver type) and M-CPT I (or skeletal muscle type), because they are apparently the exclusive isoforms associated with those organs, although each is expressed in several tissues (1). The CPT I isoforms differ in certain important respects. Notably, M-CPT I is much more sensitive to inhibition by malonyl-CoA (IC_{50} ~0.03 μM compared with ~3 μM for L-CPT I) and has a higher K_m for the substrate carnitine (~500 μM vs. ~30 μM). The cDNAs for L-CPT I, M-CPT I, and CPT II have each been cloned from rat, human, and other species (1) and the human chromosomal localization of each gene is now known (7–9). L- and M-CPT I are proteins of 773 and 772 amino acid residues, respectively, and are 63% identical (5, 10). CPT II is smaller, containing 658 residues (11). The difference in size is due to an N-terminal extension present only in the CPT I isoenzymes. This N-terminal domain contains two membrane-spanning regions, explaining the stronger membrane association of CPT I. The N terminal is also critical for the unique sensitivity of CPT I to malonyl-CoA (12–16). Several studies have also begun to delineate regions of the molecule that may be involved in catalysis and substrate binding (12, 13, 17–19). CPT I is believed to be oriented in the outer mitochondrial membrane such that the smaller, N-terminal, regulatory domain, and the larger, C-terminal, catalytic domain both reside on the cytosolic aspect (20–22).

Isoform-specific CPT I inhibitors have aroused interest as potential pharmacological tools in the treatment of disorders characterized by excessive fatty acid oxidation. An inhibitor directed against L-CPT I could have application in the acute reversal of ketoacidosis in type 1 diabetes or as a hypoglycemic agent in the long-term management of the type 2 disease (23, 24). An inhibitor of M-CPT I could have application in the treatment of ischemic heart disease (25–27).

In humans, a significant number of genetic CPT II deficiencies have been identified and many of these have been characterized at the molecular level (28). The severity of the disease is markedly dependent on the degree of deficiency, ranging from the so-called "muscular" form, often diagnosed in adulthood, to more serious, often fatal conditions presenting in infancy (28). Deficiency of L-CPT I is much rarer and, consequently, much less well characterized. Although the first patient was described in 1981 (29), only 13 families have been reported to date, and from these only 2 mutations have been defined at the molecular level (30, 31). L-CPT I deficiency normally presents in the first year of life and is characterized by episodes of hypoketotic hypoglycemia and Reye syndrome-like manifestations. The clinical presentation, diagnosis, and pathology of CPT II and L-CPT I deficiencies have been reviewed (28). Interestingly, no patients with inherited M-CPT I deficiency have been described, perhaps because of the critical contribution of this enzyme to heart function.

In the current study, we have characterized six additional patients with inherited defects in L-CPT I. One of these was, in fact, the second case of L-CPT I deficiency to be described (32) and five are new to the literature. Through analysis of the patients' fibroblasts, characterization of their cDNAs, and exogenous expression of the variant molecules, we have been able to determine the molecular basis for each mutation. In addition, this "natural mutagenesis" experiment has provided novel insights into the function of the wild-type L-CPT I enzyme.

SUBJECTS AND METHODS

Patient descriptions

Consent for these studies was obtained from the referring physicians. A clinical description of patient 1 has been published previously (32).

Patient 2 was born at term to first cousin consanguineous East Indian parents. She had been healthy with normal development until age 14 months, when she developed a flulike illness, became increasingly encephalopathic over a 2-day period, and was admitted to hospital. Physical examination revealed a very large (9-cm span), soft liver. She was not hypoglycemic but had a metabolic acidosis. She remained stable for 36 h with intravenous dextrose infusion, but subsequently developed profound coma and progressive hepatomegaly (liver span, 16.5 cm) with rising transaminases [aspartate aminotransferase (AST) and alanine transaminase (ALT), both >300 U/l] and ammonia (>100 μM). Plasma cholesterol was normal (4.06 mM) but her plasma triglyceride level was markedly elevated (8.93 mM). Serum creatine phosphokinase was elevated at 10,383 U/l (normal, <390 U/l) but rapidly normalized. Urinary organic acid chromatography showed trace dicarboxylic aciduria but no ketonuria. A two-dimensional echocardiogram and an electrocardiogram were normal. Liver biopsy revealed marked fatty change with extensive storage of neutral lipids on oil red O staining. There was no evidence of necrosis or inflammation. Investigations for a fatty acid oxidation defect revealed mildly elevated plasma total carnitine, high plasma free fatty acid levels (3.02–3.46 mM), and low β-hydroxybutyrate (0.04–0.1 mM). A diagnosis of CPT I deficiency was confirmed by assay of the enzyme in cultured skin fibroblasts, which had 1% of normal activity. CPT II activity was normal.

Patient 3 is a 6-year-old Caucasian girl with a benign perinatal and early infant course who was hospitalized for episodes of vomiting, diarrhea, and dehydration at age 12 months and again at age 17 months. Physical examination was considered normal. Laboratory studies revealed a low normal glucose, no acidosis, negative urinary ketones, and mildly elevated liver transaminases (AST, 199 U/l; ALT, 283 U/l). Plasma free fatty acid concentration was markedly elevated (3.96 mM). She then presented with new onset hepatomegaly with increasing liver enzyme levels over the next several months and was admitted with her third episode of vomiting and dehydration at 20 months of age. Blood glucose was normal (4.4 mM), urinary ketones were negative, and ammonia, total bilirubin, and bicarbonate were all normal. Liver transaminases were markedly abnormal (AST, 966 U/l; and ALT, 803 U/l). She received intravenous hydration and did well except for a possible seizure event. Physical examination revealed normal growth parameters and an enlarged liver palpated 6 cm below the costal margin and extending across the midline. Open liver biopsy done to rule out a glycogen storage disorder revealed a fatty liver. Fibroblast studies for evaluation of a fatty acid oxidation disorder revealed <5% normal CPT I activity.

Patient 4 was healthy until age 2 years 10 months, when she became hypoglycemic and suffered a seizure after fasting for 12 h. One month later she developed otitis media and torovirus-positive diarrhea. After 12 h of overnight fasting she was unresponsive and hypoglycemic (plasma glucose <1.1 mM) with no urinary ketones. She recovered rapidly after receiving intravenous glucose. At this presentation weight, height, and head circumference were all at the 50th centile for age. Results of general and systemic examination were normal except for the liver, which was firm and palpable 3.5 cm below the right costal margin. Venous blood gas analysis revealed a pH of 7.28, with a bicarbonate level of 17 mM and an anion gap of 14. Electrolytes, liver and renal functions, and cholesterol and triglycerides were normal. Chromatography of urinary organic acids showed trace amounts of dicarboxylic acids only. A prolonged fast under controlled conditions was extended to 22 h before her blood glucose fell from >3 to 2.4 mM. There was no response to intravenous glucagon at this time. Her plasma free fatty acid and β-hydroxybutyrate concentrations were 2.52 and 0.27 mM, respectively. Total and free plasma carnitine concentrations were 50 and 43 μM, respectively. Urine acylglycine and blood acylcarnitine profiles were normal. Fatty acid oxidation in her cultured skin fibroblasts was 7% of normal for oleate and 37% of normal for myristate. CPT I activity in her fibroblasts was 10% of control levels. Her CPT II activity was normal.

Patient 5 was born at term (7 lb) to apparently healthy parents. He was clinically well until age 9 months, when he was admitted to hospital twice during a 2-week period. On both occasions he had diarrhea, dehydration, and hypoglycemia (2.4 mM). He had several seizures during this period. He responded to volume expansion with increased urine output and correction of blood urea nitrogen. After a subsequent episode of febrile illness at age 10 months he became lethargic, responding only to pain. On examination he was found to have a large liver and abnormal liver enzymes (AST, 59 U/l; ALT, 212 U/l; γ-glutamyl transpeptidase, 96 U/l). Screens for hepatitis A, B, and C were negative, as were urine, blood, and stool cultures. A complete blood count was unremarkable. The child appeared to improve over the next few days, although he remained extremely lethargic. His liver enzymes remained elevated and his liver remained large. Liver biopsy revealed severe fatty degeneration but no fibrosis or inflammation. Metabolic studies revealed markedly reduced oxidation of palmitate and myristate (15% to 20% of control values) in cultured skin fibroblasts, and a 2-fold increase in plasma total and free carnitine levels (94 and 79 μM). These findings suggested CPT I deficiency, which was confirmed by direct assay of the enzyme in cultured fibroblasts (2% of control values). CPT II activity in the patient's fibroblasts was normal.

Patient 6 is a 44-year-old man who was apparently well until the age of 33 years, when he suffered a single episode of muscle cramping following an alcohol binge while logging. He recovered spontaneously within 1 day, and was then completely well for a further 6 years until he began to experience episodes of muscle cramps. Each episode begins with painful cramping of the fingers and calves. He frequently vomits during these episodes, but does not experience nausea. Symptoms then escalate over minutes to hours, such that hospitalization is required. During the past 5 years he has been hospitalized 85 times for this problem. On each occasion, administration of intravenous fluids, narcotics, and antiemetics relieved symptoms within 2 h. Laboratory analysis revealed markedly elevated serum creatine kinase activity (2733 U/l). Elevated creatinine concentrations (380 mM) have also been noted, although these improve with fluid administration. He has lost consciousness twice during episodes when therapy was delayed, but has never had hypoglycemia during episodes. He does not describe confusion, seizure activity, or lower gastrointestinal symptoms during episodes. There are no known exacerbating or relieving factors for these episodes. There is no family history of consanguinity and no family members have similar symptoms. In between episodes he is completely well, does not require narcotics, and is able to exercise without consequence. Muscle biopsy, electromyogram, and ischemic exercise tests were normal, as were liver enzymes. CPT II activity in his cultured skin fibroblasts was normal, but CPT I activity was markedly diminished (15% of normal controls).

Fibroblast culture

Fibroblasts were obtained from skin biopsy of patient and control individuals and cultured in DMEM supplemented with 10% (v/v) FCS in an atmosphere of 5% CO_2. Fibroblasts were between passage 10 and 22 during the course of this study.

COS cell culture and transfection

Simian COS-M6 cells were grown in DMEM supplemented with 5% (v/v) FCS under 5% CO_2. Using this concentration of serum, endogenous CPT I activity is maintained at a low level. Cells were plated at a density of 1.8×10^6 per 15-cm plate and transfection with various L-CPT I expression plasmids was as described (11, 13), with analysis 3 days later. Control plates received empty expression vector or no plasmid.

Analysis of human fibroblast L-CPT I cDNA

RNA was isolated from fibroblasts with a monophasic solution of phenol and guanidine isothiocyanate (TRIzol; GIBCO-BRL, Rockville, MD) and reverse transcribed (SuperScript; GIBCO-BRL). Genomic DNA was isolated from fibroblasts, using a commercially available kit (QIAamp blood kit; Qiagen, Valencia, CA). Genomic DNAs and cDNAs were amplified by PCR and sequenced with an ABI PRISM BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the protocol of the supplier. Sequencing reaction products were analyzed on an ABI model 377 automated sequencer (Perkin-Elmer Applied Biosystems).

Construction of human L-CPT I expression plasmids and in vitro mutagenesis

We have previously isolated partial human L-CPT I cDNA clones (8). A segment of one of these clones, encoding 1,200 bases of the 3′ end of the coding region, was labeled by the hexamer priming method and used to screen a λgt10 human heart cDNA library. A 4.2-kb cDNA clone was isolated, excised with restriction enzyme *Eco*RI, and ligated into pBluescriptSK(+). A 3.3-kb fragment of this clone encompassing the coding region, as well as ~70 bp of 5′ untranslated region and ~900 bp of 3′ untranslated region, was removed with *Eco*RI (5′) and *Hin*dIII (3′). The fragment was ligated into plasmid pCMV6 to generate pCMV6-rL-CPT I for expression in COS cells and into M13mp18 for site-directed mutagenesis. Mutagenesis was performed with a Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (17). Mutagenic oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Mutated cDNAs were identified by DNA sequencing and subcloned into pCMV6, as described above.

CPT assay

For fibroblasts, one confluent 15-cm plate was washed with 10 ml of ice-cold phosphate-buffered saline and scraped in 5 ml of the same, using a rubber policeman. The cells were sedimented by centrifugation at 2,000 *g* for 10 min at 4°C and resuspended in 1 ml of 150 mM KCl-5 mM Tris-HCl, pH 7.2 (buffer A). The cells were broken by 10 cycles in a glass homogenizer fitted with a tight pestle and then centrifuged at 16,000 *g*, 4°C for 5 min.

Final resuspension of the membrane pellet was in 800 μl of buffer A. Direct assay of this material measures CPT I (see Discussion). For assay of CPT II, a portion (250 μl) of this was made 1% with respect to the detergent octyl glucoside (OG), by addition of 12.5 μl of a 20% (w/v) solution, and the mixture was placed on ice for 30 min prior to assay. Under the detergent-solubilized conditions, CPT I is inactivated and CPT II is released from the matrix in active form (33). Harvest of COS cells was similar except that two plates of cells were combined with a final resuspension in 1 ml of buffer A. A 100-μl portion was then diluted with 150 μl of buffer A before addition of OG, as described above.

CPT activity was assayed in the direction of palmitoylcarnitine formation, as described (6). Reaction times were 15 and 6 min for fibroblasts and COS cells, respectively. Protein concentrations were determined by the method of Lowry et al. (34).

Immunoblot analysis

For immunoblot, one 15-cm plate of cells was washed, harvested, and homogenized as described above and resuspended in a final volume of 1 ml of buffer A. A 50 μl portion was taken for protein assay. Fifty microliters of 20% (w/v) Tween 20 was added, giving a final concentration of 1%. After 30 min on ice, this was centrifuged at 16,000 *g* for 5 min at 4°C. The supernatants (containing CPT II) were removed and the pellets (containing CPT I) were frozen in liquid nitrogen and stored at −70°C. For SDS-PAGE, the pellets were thawed and resuspended in buffer A. Equivalent portions were then prepared for electrophoresis on 8% gels. (Detergents interfere with the Lowry protein assay and so, for convenience, we assumed that the percentage recovery during the extraction procedure was the same for each sample, and based the sample equalization on protein content before detergent addition.) Electrotransfer to Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) and immunoblotting were performed as described (13), using a polyclonal rabbit anti-rat L-CPT I antibody.

Northern blot analysis

Poly(A)$^+$ RNA was prepared from each fibroblast line, using a QuickPrep Micro mRNA purification kit (Pharmacia, Piscataway, NJ). Total RNA was prepared from transfected COS cells by the TRIzol method. Portions of the RNA [approximately 2–3 μg of fibroblast poly(A)$^+$ RNA or 20 μg of COS cell total RNA] were electrophoresed on 1% agarose gels and Northern blotting was performed as described (35). Probes for human L-CPT I and CPT II were as detailed (35). The human actin probe corresponded to bases 153–537 of the published sequence (36).

RESULTS

For five of the six patients under investigation, initial interpretation of the clinical data suggested that there might be a deficiency of L-CPT I, and this diagnosis was confirmed by assay of CPT I activity in fibroblasts harvested from the patients. Low L-CPT I was discovered in the sixth patient during a test for CPT II deficiency. Because these diagnostic assays were performed in more than one clinical laboratory, and by slightly varying protocols, we performed a detailed analysis of the CPT activities in each of the patient fibroblast cell lines under standardized conditions. For comparison, three fibroblast lines obtained from healthy individuals were analyzed in parallel. The results are shown in **Table 1**. Assays for CPT I were performed with cell homogenates in which the mitochondria were deemed to be largely intact. Under these conditions, control cells displayed a mean CPT I activity of 1.34 nmol/min per mg protein, of which 98% was inhibited in the presence of 100 μM malonyl-CoA. The low level of residual activity probably represents some malonyl-CoA-insensitive CPT II that is released from the matrix because of mitochondrial breakage. In contrast, in each of the patient fibroblast lines, CPT I activity was markedly reduced. In the case of patients 1, 2, 3, and 5, the low level of apparent activity was not affected by malonyl-CoA and was similar to the residual activity observed in control cells. Essentially, therefore, CPT I activity was undetectable in those patients. Patient 4 exhibited a CPT I activity that was 9% compared with control levels, and was inhibited by malonyl-CoA similarly to controls. CPT I activity in fibroblasts from patient 6 was approximately 22% of control levels. However, 100 μM malonyl-CoA inhibited CPT I by only ~68%, leaving a residual activity three to four times higher than was observed with the other lines. After disruption of the mitochondria with detergent, levels of CPT II activity were also determined and found to be similar in all cases. Under the assay conditions used, the ratio of CPT II activity to CPT I was approximately 0.53 in control fibroblasts.

Despite the low level of CPT I expression in human fibroblasts, immunoblot analysis using an antibody directed against the rat L-CPT I protein detected a band of the appropriate size in membrane extracts from two control

TABLE 1. CPT activity in patient fibroblasts

	CPT I			CPT II		
	− Malonyl-CoA	+ Malonyl-CoA				
	nmol/min/mg		%	*nmol/min/mg*	%	*n*
Control 1	1.23 ± 0.13	0.021 ± 0.003		0.666 ± 0.079		3
Control 2	1.57 ± 0.16	0.029 ± 0.010		0.778 ± 0.086		3
Control 3	1.22 ± 0.12	0.019 ± 0.011		0.684 ± 0.067		6
Mean control	1.34	0.023	100.0	0.709	100.0	
Patient 1	0.039 ± 0.017	0.035 ± 0.006	2.9	0.777 ± 0.095	109.6	3
Patient 2	0.035 ± 0.013	0.025 ± 0.010	2.6	0.819 ± 0.106	115.5	3
Patient 3	0.025 ± 0.012	0.030 ± 0.010	1.9	0.733 ± 0.047	103.4	4
Patient 4	0.120 ± 0.009	0.019 ± 0.004	9.0	0.828 ± 0.061	116.8	3
Patient 5	0.026 ± 0.012	0.010 ± 0.004	1.9	0.692 ± 0.066	97.6	4
Patient 6	0.290 ± 0.043	0.094 ± 0.022	21.6	0.544 ± 0.031	76.7	3

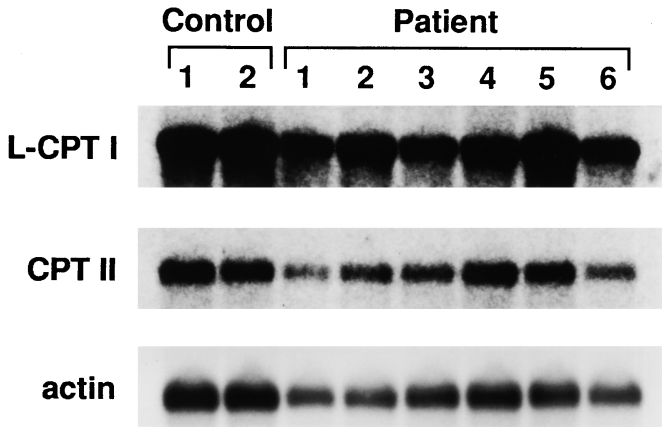

Fig. 1. Northern blot analysis of poly(A)$^+$ RNA from patient fibroblasts. See Subjects and Methods for details. Results are shown for two cell lines from control individuals and for the six patient lines. Membranes were probed sequentially for human CPT II, L-CPT I, and β-actin.

fibroblast lines (not shown). No such band was observed in fibroblast membranes from patients 1 through 5 although a signal was observed with patient 6. **Figure 1** shows Northern blot analysis of mRNA isolated from the control and patient cell lines. The blot membrane was probed sequentially for CPT II, L-CPT I, and actin (as loading control). The levels of the L-CPT I transcript are similar in all cases. As expected, the same was also true for CPT II mRNA.

To determine whether mutations were present, the L-CPT I cDNA for each patient was sequenced. This revealed seven mutations among the six patients. Six of the seven mutations were single-nucleotide substitutions and one was a three-nucleotide deletion (**Table 2**). Two patients (cases 1 and 3) had two different mutations. Mutation c.1069C ›T was present in two patients (cases 1 and 2). The mutations were distributed throughout the open reading frame (**Fig. 2**).

To determine the effects of these mutations on L-CPT I function, we expressed each of the mutant cDNAs in COS cells. **Table 3** summarizes the data obtained from the transfection experiments. Control cells, transfected with the pCMV6 vector, displayed a CPT I activity of 0.35 nmol/min per mg protein, similar to previously reported values (6, 13). CPT II activity in these cells was 3.92 nmol/min per mg protein, giving a CPT II:CPT I ratio of ~11. At first analysis, in control cells, malonyl-CoA appears to inhibit CPT I activity by only 74%. However, the high residual activity is probably the result of the high level of CPT II in these cells (see Discussion). Transfection with plasmid containing the wild-type human L-CPT I cDNA resulted in a 10-fold increase in CPT I activity. There was also an apparent increase in CPT II activity, as measured in detergent solution, a phenomenon that we have reported in previous studies (13).

To allow for direct comparison of the CPT I activities induced by transfection with the various plasmids, the CPT activity present in null-transfected cells has been subtracted from each and the result expressed as a percentage of the activity conferred by the wild-type cDNA. Transfection with plasmids encoding mutations R357W, L484P, R123C, and P479L resulted in induced CPT I activities of 14.5%, 23.9%, 71.8%, and 53.0% of control, respectively. Mutation C304W and deletion of Arg-395 resulted in no significant induction of CPT I activity. In contrast, the cDNA containing mutation A275T resulted in activity similar to that of the wild-type sequence.

Immunoblot analysis of COS cells transfected with the variant human L-CPT I plasmids is shown in **Fig. 3**. Consistent with the many-fold induction of activity, a strong band of the appropriate size was seen in cells transfected with wild-type human L-CPT I, whereas none was observed in cells transfected with plasmid alone. Bands of similar intensity were observed with plasmids encoding A275T and P479L. Mutations R357W, L484P, C304W, and delR395 also generated immunoreactive protein, but in lesser amounts. In the case of mutation R123C, two immunoreactive bands of slightly lower mobility than the wild-type protein were observed. In view of the differences in signal observed by immunoblot, Northern blot analysis was also performed to determine whether there were any significant differences in mRNA levels. As can be seen from **Fig. 4**, no signal for human L-CPT I was observed in untransfected COS cells or in cells transfected with the empty vec-

TABLE 2. L-CPT I mutations

Patient No.	Nucleotide(s)	Amino Acid	Effect
1	c.1069C →T	R357W	Decreased stability
	c.1451T →C	L484P	Decreased stability
2	c.1069C →T	R357W	Decreased stability
3	c.823G →A	A275T	None
	c.912C →G	C304W	Decreased stability, inactive
4	c.367C →T	R123C	Aberrant mobility (processing?)
5	c.1183–1185delCGT	delR395	Decreased stability, inactive
6	c.1436C →T	P479L	Decreased activity Decreased malonyl-CoA sensitivity

Inspection of the sequence electropherogram indicated that patient 1 was heterozygous for both mutations. The other five patients, including patient 3, appeared to be homozygotes.

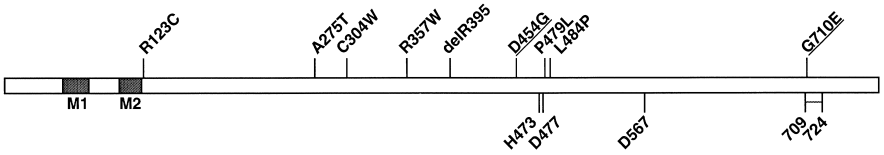

Fig. 2. Schematic representation of human L-CPT I. M1 and M2, membrane-spanning domains; natural mutants identified to date (two underlined are previously published, see text) are indicated above the bar; residues implicated in putative reaction mechanism are listed below the bar; amino acids 709–724 are implicated in the interaction with the substrate, carnitine. The diagram is to a linear scale.

tor. High levels of human L-CPT I message were detected in cells transfected with the wild-type and mutant cDNAs. It should be noted that the exposure time for the L-CPT I panel in Fig. 4 using 20 μg of total COS cell RNA was 1 h, whereas to obtain a similar signal using 2 μg of poly(A)$^+$ fibroblast mRNA the membrane was exposed for 72 h.

DISCUSSION

As a key regulator of mammalian fatty acid oxidation, the CPT system occupies a central position in the control of fuel metabolism and has been the object of intense study. Central to this effort has been a focus on elucidating the structure-function and regulatory properties of CPT I at a molecular level. Whereas this initiative has been greatly aided by the development of nonmammalian expression and reconstitution systems, notably yeast based (12, 14–16, 19, 37–41), these studies do have inherent limitations. In particular, they may not provide insight into those properties of the enzyme specific to a mammalian cell environment, for example, processing and targeting. Analysis of patients with a deficiency of L-CPT I has the potential to provide novel information about both the metabolic role of the enzyme and these subtler aspects of its structure and function. To date only two naturally occurring mutations in human L-CPT I have been characterized (30, 31). In the present study, we have identified and characterized the underlying mutations in six additional patients with L-CPT I deficiency.

Five of the patients in this study presented at an early age with classic symptoms of CPT I deficiency. The age at onset (adult) and presenting symptoms (muscle pain and cramping) of patient 6 have not previously been associated with low L-CPT I activity and are more commonly associated with mutations in CPT II. In each of these patients, however, definitive diagnosis of L-CPT I deficiency was achieved by assay of CPT I in cultured skin fibroblasts, which are known to express exclusively the liver form of this enzyme (8). To facilitate direct comparisons, we performed a detailed analysis of the CPT activities present in skin fibroblasts from each patient and in control fibroblasts. Certain observations in the control samples are noteworthy. First, the fibroblast CPT II:CPT I ratio of ~0.5 is lower than the values for other cells and tissues, which generally range from 1 to 14 (6). Second, when CPT is measured under conditions in which the mitochondria remain largely intact, only the outer membrane form of the enzyme, CPT I, should be available for catalysis. However, if there is some mitochondrial breakage, a portion of the malonyl-CoA-insensitive CPT II is exposed and this may result in a reduced apparent maximal inhibition by malonyl-CoA (6). Table 1 shows that CPT I activity in the control cell homogenates was 98% inhibited by 100 μM malonyl-CoA. This efficient inhibition suggests that the homogenized cell preparation does not cause significant mitochondrial damage, but may also be due, in part, to the low level of CPT II present in these mitochondria, such that any disruption of the organelle would reveal relatively little of the malonyl-CoA-insensitive enzyme. In the case of the patient cell lines, four displayed no CPT I activity, whereas the other two had markedly reduced levels. Taken in conjunction with the absence of detectable immunoreactive protein in five of the six cases, it is apparent that several of the deficiencies result from a diminished level of enzyme protein. However, Northern blot analysis showed that the L-CPT I message levels were normal in each case, suggesting

TABLE 3. CPT activities in COS cells transfected with various expression plasmids

	CPT I				
	− Malonyl-CoA	+ Malonyl-CoA	CPT II	Induced CPT I	n
	nmol/min/mg		*nmol/min/mg*	%	
pCMV6 vector	0.35 ± 0.06	0.126 ± 0.041	3.92 ± 0.65		7
Wild-type L-CPT I	3.30 ± 0.92	0.262 ± 0.164	5.55 ± 1.57	100.0	7
R357W	0.83 ± 0.13	0.206 ± 0.074	4.01 ± 1.49	14.5 ± 5.6	4
L484P	0.96 ± 0.31	0.116 ± 0.021	2.90 ± 0.52	23.9 ± 3.2	3
A275T	3.63 ± 0.64	0.404 ± 0.210	5.71 ± 1.21	109.9 ± 41.0	6
C304W	0.42 ± 0.11	0.148 ± 0.052	4.43 ± 0.94	1.7 ± 2.2	4
R123C	2.39 ± 0.55	0.160 ± 0.074	3.91 ± 1.13	71.8 ± 20.0	7
delR395	0.35 ± 0.03	0.101 ± 0.005	3.64 ± 0.25	0.1 ± .1.7	3
P479L	2.17 ± 0.32	1.037 ± 0.255	4.13 ± 0.93	53.0 ± 5.5	4

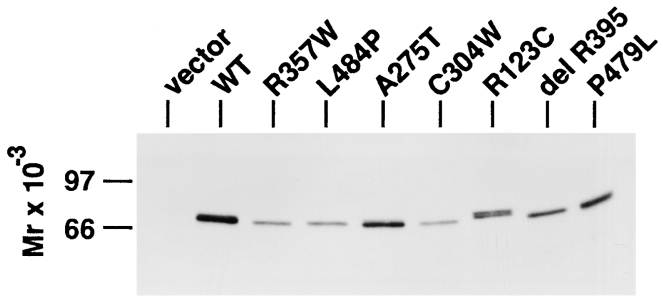

Fig. 3. Immunoblot of COS cells expressing human L-CPT I variants. See Subjects and Methods for details. Vector, cells transfected with empty pCMV6 vector; WT, cells transfected with the wild-type human L-CPT I cDNA; other lanes, cells transfected with mutant cDNAs, as indicated.

that the disease-causing mutations might be contained within the open reading frames.

Sequencing of the L-CPT I cDNA from the patients revealed candidate mutations in each case. To determine the effects of these mutations on the properties of the L-CPT I molecule we generated pCMV6 plasmids encoding each L-CPT I cDNA variant and expressed the wild-type and mutant enzymes in COS cells in tissue culture. This expression system has several advantages. First, the COS cell provides a mammalian environment for transcription, translation, and processing of the foreign protein. Second, it possesses the appropriate subcellular organelles and targeting machinery. Third, the powerful cytomegalovirus (CMV) promoter ensures high levels of transcription. This means that it is often possible to achieve measurable steady-state levels of the enzyme, even if it is relatively unstable. The one disadvantage of COS cell is the presence of endogenous CPT I activity. However, under the culture conditions used (5% FCS), this is maintained at a low level. As a result, the CPT II:CPT I ratio in untransfected COS cells was ~11. This is reflected in a relatively poor apparent inhibition of CPT I by malonyl-CoA in control cells, that is, a small degree of mitochondrial breakage releases a relatively large amount of malonyl-CoA-insensitive CPT II. However, this phenomenon does not affect interpretation of the data for CPT I activity induced during the transfection experiments. The activity, immunoblot, and

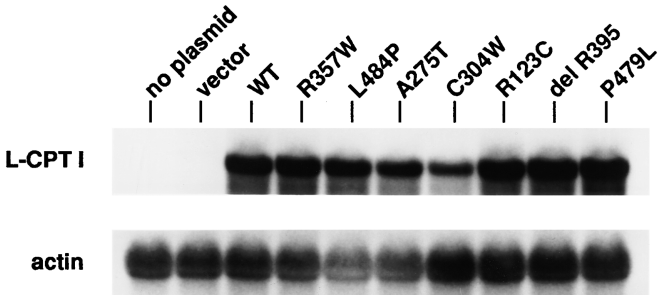

Fig. 4. Northern blot analysis of total RNA from COS cells transfected with human L-CPT I variants. See Subjects and Methods for details. No plasmid, mock-transfected cells; other lanes as detailed in legend to Fig. 3. The membrane was probed sequentially for human L-CPT I and β-actin.

Northern blot data obtained with the COS cell system permitted an explanation for each of the cases of deficiency and revealed some important new information about the function of the wild-type enzyme.

Two mutations, R357W and L484P, were identified in cDNA from patient 1; R357W was also seen in patient 2. Both of these mutations were associated with reduced enzyme activity, and lower levels of enzyme protein, suggestive of protein instability rather than impaired catalytic activity. Given the lower native expression level of L-CPT I in fibroblasts compared with that obtained under the CMV promoter in the heterologous system, it is not surprising that this lack of stability resulted in an essentially complete absence of active protein in the patient cell lines. The R357W mutation lies in a region of intermediate conservation between the CPT proteins. However, it represents an extremely unconservative substitution (large aromatic for positively charged). The second mutation in this patient, L484P, lies in a highly conserved region including residues considered likely to be involved at the active site (17).

The cDNA from patient 3 also contained two sequence variants. The A275T variant displayed normal activity and enzyme protein was found at levels similar to those in controls. We suggest that the A275T substitution represents a functionally neutral polymorphism. Indeed, the corresponding residue is threonine in the rat L- and M-CPT I sequences and in rat and human CPT II (1). In contrast, C304W resulted in a diminished level of enzyme protein and a complete lack of activity. It is likely, therefore, that the mutant protein was both inactive and unstable. The cysteine at position 304 is conserved in rat and human L- and M-CPT I (1).

Patient 5 was homozygous for the deletion of Arg-395. This mutation resulted in decreased protein levels and no detectable CPT activity, indicating a lack of catalytic competence combined with protein instability. Arg-395 is completely conserved in rat and human L-CPT I, M-CPT I, and CPT II.

Each of the above-described mutations rendered the resulting enzyme unstable (and in some cases inactive) when expressed in COS cells, a phenotype consistent with the observations in the patient fibroblast lines and with the clinical presentations. The protein phenotype is also similar to that of the only L-CPT I mutation that has been described in detail previously, a missense mutation (D454G) that also results in an unstable protein (30). The other two mutations identified in the present study were associated with more complex phenotypes. Fibroblasts from patient 4, possessing the R123C mutation, retained L-CPT I activity at about 9% of that of control cells, although protein levels were too low to detect. However, when the same variant was expressed in the COS cell system, activity was ~72% of control. A possible explanation for this apparent discrepancy lies in the immunoblot analysis of the exogenously expressed enzyme. It is clear that the immunoreactive protein obtained from the R123C expression construct consisted of two bands, both of which ran with lower mobility (i.e., higher apparent molecular weight)

than the wild-type enzyme, suggesting that the mutant protein was processed incorrectly. Like most mitochondrial outer membrane proteins, L-CPT I is not proteolytically cleaved on mitochondrial import (20). However, it has been reported that the N-terminal 150 amino acid residues contain the necessary information for mitochondrial targeting (15). The R123C mutation lies within this region, immediately distal to the second transmembrane segment. It is likely, therefore, that the substitution of cysteine for arginine at position 123 disrupts the structure of that portion of the nascent molecule, causing mistargeting of the enzyme. Clearly, if the protein were misdirected toward the endoplasmic reticulum/Golgi, it could result in aberrant electrophoretic mobility. At normal expression levels, the misdirected protein would be predicted to be degraded, resulting in reduced activity in patient cells. Once again, however, the high rate of synthesis in the pCMV6/COS cell system generates sufficient protein to observe the malformed intermediates at steady state. This result emphasizes the usefulness of using a mammalian expression context.

The clinical features of patient 6 (muscle pain and cramping) were clearly distinct from those of the other patients in this study and have not been reported previously in L-CPT I-deficient patients. Because L-CPT I does not appear to be expressed to any significant extent in muscle, it is possible that the clinical symptoms observed in this patient are unrelated to the molecular defect observed. Without studies of additional family members with the same phenotype, this possibility cannot be excluded. Interestingly, markedly elevated plasma levels of creatine kinase have been reported in two previously described families with L-CPT I deficiency, indicating that deficiency of the enzyme can be associated with muscle damage. Further clinical studies will be required to resolve this question.

Fibroblasts from patient 6 exhibited the least severe deficiency (22% of normal L-CPT I), and when the P479L variant was expressed in COS cells, it resulted in activity approximately 50% of control values. Intriguingly, however, both in the primary fibroblasts and in the COS cell environment, the mutant L-CPT I had a markedly diminished response to malonyl-CoA. Although the nature of the malonyl-CoA/CPT I interaction is incompletely understood, it appears that the inhibitor exerts its primary effect by binding at an allosteric site distinct from the catalytic center [although an additional minor inhibition probably does occur by direct competition with the acyl-CoA substrate (42)]. As mentioned above, the N-terminal domain is crucial for the primary response to malonyl-CoA. However, whether or not malonyl-CoA interacts directly with that domain and how the N terminus exerts an effect on the catalytic site are unknown. The P479L mutation lies in a highly conserved region believed to contain active site residues and the data strongly point to a role for Pro-479 in mediating the malonyl-CoA effect. Notably, the residue is conserved in rat and human L- and M-CPT I (malonyl-CoA sensitive), but is substituted by valine in the CPT II proteins (malonyl-CoA insensitive). Ironically, the diminished sensitivity of the P479L variant to malonyl-CoA might partially ameliorate the decreased catalytic capacity in patent 6.

Through analysis of these "natural" mutants, therefore, several novel points have emerged. Clearly, the new mutations identified in this study exert their effects on several different aspects of CPT I function. Specifically, we have identified residues that are not only important for overall enzyme folding and stability, but may also be elements crucial for mitochondrial targeting and sensitivity to the physiological inhibitor, malonyl-CoA. In addition, patient 6 raises the possibility that a partial loss of L-CPT I activity may result in a novel phenotype. As more mutations in L-CPT I are identified and characterized, it is possible that clinically distinct syndromes dependent on the degree of enzyme deficit will delineated, as has been demonstrated for CPT II deficiency.

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