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RESEARCH ARTICLE

Novel Mutations Associated With Carnitine Palmitoyltransferase II Deficiency

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The most common form of carnitine palmitoyltransferase II (CPT II) deficiency occurs in adults and is characterized by muscle pain, stiffness, and myoglobinuria, triggered by exercise, fasting, or other metabolic stress. This study reports the molecular heterogeneity of *CPT2* mutations and their biochemical consequences among a series of 59 individuals who were suspected of having CPT II deficiency based on the decreased CPT activity observed in muscle or leukocytes samples, clinical findings, or referral for mutation analysis from other laboratories. Only19 subjects were considered to be at particularly high risk of CPT II deficiency based on review of their clinical symptoms and residual CPT activity. The samples were initially screened for 11 mutations with allele-specific oligonucleotides (ASO). Extensive sequence analysis was subsequently performed on 14 samples which either had a *CPT2* mutation detected by ASO screening or the residual CPT activity was below that observed in ASO positive samples. Three known (P50H, S113L, and F448L) and three novel mutations were identified among 13 individuals in this study. A single nucleotide polymorphism was also identified 11 bp distal to the *CPT2* polyadenylation site that will be useful for linkage analysis. Two of the new mutations were single nucleotide missense mutations, R503C and G549D, that occurred in highly conserved regions of the CPT isoforms, and the third was a frameshift mutation, 413 delAG, caused by a 2-bp deletion upstream of a previously identified missense mutation, F448L. The 413 delAG mutation was the second most common mutation identified in our study (20% of mutant alleles) and all individuals with the mutation were of Ashkenazi Jewish ancestry suggesting a defined ethnic origin for the mutation. Despite rigorous mutation analysis, six of 13 individuals identified with *CPT2* mutations remained as heterozygotes. We propose that heterozygosity for certain *CPT2* mutations, S113L and R503C, is sufficient to render individuals at risk of clinical symptoms. Hum Mutat 13:210–220, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: **fatty acid metabolism; mitochondrial disease; carnitine palmitoyltransferase deficiency; allele-specific oligonucleotide; mutation(s); muscle disease**

INTRODUCTION

Carnitine palmitoyltransferase (EC 2.3.1.21; CPT) exists as two distinct mitochondrial membrane enzymes (CPT I and CPT II) that have important roles in the transport of long-chain fatty acids into the mitochondrial matrix. Several enzymes are involved in this process (Fig. 1). Acyl-Coenzyme A (CoA) synthetase esterifies long-chain fatty acids on the cytosolic side of the outer membrane, after which CPT I, spanning the outer mitochondrial membrane, transfers the acyl group from CoA to carnitine for transport through the inner membrane via carnitine-acylcarnitine translocase [Bieber, 1988]. CPT II is on the inner mitochondrial membrane and catalyzes the reverse reaction to reform acyl-CoA. Once inside the mitochondrial matrix, the acyl-CoA subsequently enters the β-oxidation pathway and free carnitine recycles to the extramitochondrial compartment [McGarry et al., 1989]. Although there are genetically distinct inherited defects of CPT I, CPT

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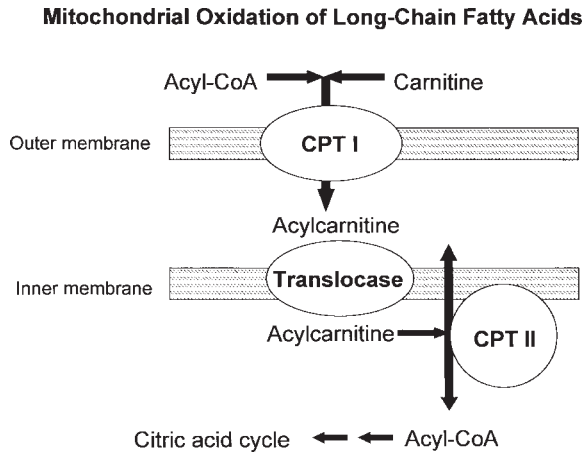

FIGURE 1. **Schematic diagram of the enzymes involved in the transport of long-chain fatty acids into mitochondria.**

II and carnitine-acylcarnitine translocase, they share some common features that necessitate the use of specific biochemical and molecular analyses to distinguish them.

CPT I has the pivotal role in the regulation of fatty acid oxidation through its inhibition by malonyl CoA, the first committed intermediate in the synthesis of fatty acids [McGarry and Brown, 1997]. Liver (L-CPT I) and skeletal muscle (M-CPT I) isoforms exist with differential sensitivity to malonyl CoA (M-CPT I, I_{50} = 0.025 μM; L-CPT I, I_{50} = 1.6 μM) and affinity for carnitine (M-CPT I, K_m = 480 μM; L-CPT I, K_m = 39 μM) [McGarry et al., 1983]. The tissue-specific differences in malonyl CoA sensitivity and substrate specificity account for variations observed in the regulation of fatty acid metabolism in different organs. The L-CPT I gene (CPT1A; MIM# 600528) [Britton et al., 1995] is located at chromosome 11q22–23 [Britton et al., 1997] . The M-CPT I (CPT1B; MIM# 601987) contains 20 exons [Vanderleij et al., 1997; Yamazaki et al., 1997] and is located on chromosome 22qter [Britton et al., 1997; Vanderleij et al., 1997]. CPT I deficiency (MIM# 255120) is a rare infantile disorder with the hallmark of potentially fatal hypoketotic hypoglycemia [Taroni and Uziel, 1996]. To date, no cases of M-CPT I deficiency have been reported and this deficiency may be lethal given the importance of the enzyme for cardiac function [McGarry and Brown, 1997].

The carnitine-acylcarnitine translocase gene (CACT; MIM# 212138) [Huizing et al., 1997] is located at chromosome 3p21.1 [Viggiano et al., 1997]. CACT transcripts are found in a variety of human tissues, but they are most prominent in liver, skeletal muscle, and heart [Huizing et al., 1997]. Carnitine-acylcarnitine translocase deficiency is a rare disorder of infants and young children characterized by fasting-induced hypoketotic hypoglycemia, cardiomyopathy, arrhythmia, muscle pain, and weakness. Coma and sudden death may also occur with this disorder [Stanley et al., 1992]. Homozygosity for a 1-bp insertion in the *CACT* gene has been shown in one patient with the disease [Huizing et al., 1997].

CPT II is a single ubiquitously expressed isoform [Roe et al., 1995]. The enzyme is a homotetramer both in rats [Miyazawa et al., 1983] and in humans [Finocchiaro et al., 1990], consisting of 68-kDa monomers [Finocchiaro et al., 1990]. While CPT II has considerable homology with CPT I, it is a smaller protein (658 vs 773 amino acid residues) that does not possess the extensive transmembrane domains found in CPT I and is not inhibited by malonyl CoA. It is also easily solubilized from the inner membrane using detergents and retains enzymatic activity upon disruption of the mitochondrial membrane [McGarry and Brown, 1997]. The human *CPT2* gene [Finocchiaro et al., 1991] (MIM# 600650) spans 20 kb, contains 5 exons ranging from 81 to 1,305 bp [Verderio et al., 1995], and is located at chromosome 1p32 [Gellera et al., 1994]. CPT II deficiency presents as different age-dependent clinical phenotypes: an adult myopathic form (MIM# 255110), a lethal neonatal form (MIM# 600649), and a severe infantile form. The adult form is characterized by muscle pain and stiffness triggered by exercise, fasting, and extremes in temperature or anesthesia; it usually follows an autosomal recessive mode of inheritance [DiMauro and Papadimitriou, 1986]. In severe cases, rhabdomyolysis may lead to myoglobinuria and life-threatening renal failure. The neonatal form is characterized by hypoketotic hypoglycemia, cardiomyopathy, and congenital anomalies [Hug et al., 1991; Witt et al., 1991], while the infantile form has predominantly liver, heart, and skeletal muscle symptoms [Demaugre et al., 1991; Taroni et al., 1992b]. Reports of at least 13 *CPT2* mutations associated with the adult and infantile disorders have appeared (see Table 2) [Gellera et al., 1992; Yang et al., 1998]. One missense mutation, S113L, accounts for approximately 60% of mutant alleles responsible for adult myopathic CPT II deficiency [Taroni et al., 1993]. Three polymorphisms have also been described that by themselves do not cause CPT II deficiency but appear to contribute to further reductions in enzyme activity when com-

bined with *CPT2* mutations [Taroni et al., 1992a; Wataya et al., 1998].

The purpose of the present study was to gain insight into the molecular basis of CPT II deficiency by conducting a retrospective analysis of samples from 59 patients who were originally referred for screening for metabolic muscle disease, including CPT deficiency or were specifically referred for *CPT2* mutation screening. A large proportion of the subjects selected for the study included adults with a relatively wide range of residual muscle CPT activity in order to investigate if some patients could be manifesting carriers or have functionally dominant mutations in *CPT2*. Our data indicate that individuals who are heterozygous for the common S113L mutation or for the R503C mutation, reported in this study, may be predisposed to develop symptoms when exposed to metabolic stress.

MATERIALS AND METHODS

Subjects

CPT2 mutation analysis was performed on skeletal muscle biopsies from 36 patients and leukocytes or lymphoblastoid cultures from 23 patients who were referred for mitochondrial myopathy screens or for CPT II deficiency. The patients ranged from 12 months to 72 years (mean age = 39 ± 17 years). CPT activity in skeletal muscle or in lymphoblasts from these individuals was ≥2 standard deviations (SD) below the established normal reference mean of 77.8 ± 13.3 nmol $min^{-1}g^{-1}$ in muscle [Vladutiu et al., 1992] and 1.54 ± 0.11 nmol $min^{-1}mg^{-1}$ protein in lymphoblasts [Vladutiu et al., 1993]. The residual CPT activity ranged from 10% to 63% of the respective normal reference means. Whole blood from 50 anonymous donors was obtained from the American Red Cross as population controls, to determine whether newly identified *CPT2* mutations were polymorphisms. Informed consent for genetic testing was obtained for all patients following the experimental protocols approved by the Institutional Review Board of Children's Hospital of Buffalo.

Specimens and Enzyme Analyses

Skeletal muscle biopsies were collected under local or general anesthesia, promptly flash-frozen in liquid nitrogen and stored at –70°C until shipped to the laboratory on dry ice. Peripheral blood lymphocytes were isolated from whole blood, using dextran sedimentation and transformed with Epstein-Barr virus (EBV) as previously described [Freshney, 1983; Vladutiu et al., 1993]. Muscle samples were homogenized in 9 vol of homogenization buffer (50 mM Tris and 150 mM KCl buffer, pH 7.4; 10% w/v), in an all-glass motor-driven homogenizer (Talboy Engineering Corp., Emerson, NJ). Lymphoblasts and peripheral blood leukocytes were extracted by sonication with a Kontes Micro-Ultrasonic Cell Disruptor (Vineland, NJ) as previously described [Vladutiu et al., 1993]. CPT activity was measured in aliquots of muscle homogenate using the isotope exchange method [Norum, 1964]. The assay measures the reversible incorporation of [methyl-^{14}C]carnitine (New England Nuclear) into a pool of palmitoylcarnitine (palmitoylcarnitine + CoA + [methyl-^{14}C]-carnitine→palmitoyl[methyl-^{14}C]carnitine). Reaction rates are linear over a 10-min period, and enzyme activity is expressed as nmol of palmitoyl-^{14}C-carnitine formed $min^{-1}g^{-1}$ wet weight tissue. The normal reference mean for citrate synthase was 15.7 ± 4.4 μmol^{-1} min^{-1} g and was determined as previously described [Srere, 1969].

CPT2 Mutation Analysis

DNA was isolated following standard procedures or using a Puregene DNA isolation kit from Gentra Systems. Genomic DNA was diluted to 50 ng/μl in water for polymerase chain reaction (PCR) amplification. The sets of primers used to amplify each of the *CPT2* gene exons and the flanking untranslated regions are found in Table 1. PCR amplification reactions were performed in 200-μl reaction tubes under mineral oil in a 96 well thermocycler (Stratagene). The 50-μl reactions contained 200 ng genomic DNA, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5–2.5 mM $MgCl_2$ (Table 1), 250 nM of each primer, 200 μM deoxynucleotide triphosphates, 0.02% gelatin and 1.25 U *Taq* DNA polymerase (Amplitaq Gold, Perkin-Elmer). The reactions were incubated at 95°C for 10 min to inactivate the antibody bound to polymerase, then subjected to 35 cycles of 1 min 95°C, 1 min 62°C, 2 min 72°C, and a final cycle at 72°C for 7 min. PCR reactions were diluted with one-fifth volume of gel loading buffer (50% Ficoll, 50 mM EDTA, 2.0% sodium dodecyl sulfate [SDS], 0.02% bromophenol blue) for analysis by agarose gel electrophoresis or for dot-blot application to nylon membranes (Amersham).

ASO primers were designed for each *CPT2* mutation and the corresponding normal sequence. The strand used for each ASO was selected to result in a strongly destabilized mismatched base pair (A-A, C-C, T-T, C-T, C-A) for the alternative sequence (Table 2). In all instances, the opposite

TABLE 1. **Primers Used for PCR Amplification of the *CPT2* Gene***

Region	Forward primer	Reverse primer	Size	Conditions[a] (mM $MgCl_2$)
5´UT	atccatccactcaggtgagc	ggggagttctggagtctaaaca	621	1.5
Exon 1	cttgtgtttagactccagaactcc	gtcatgagtgactgcagtcaggttg	292	2.5; 4% DMSO
Exon 2	ctgtcagccttacactgaccc	aactctcggggcttggtc	305	2.0
Exon 3	tttagggctatgctgttggg	aggaagggaggatgagacgt	358	2.0
Exon 4	ctctggaggttgatgccatt	acccaagcactgaggacaag	1,472	1.5
Exon 5	tttcctgaggtccttttccatcctg	atgaggaagtgatggtagcttttca	425	2.0
3´UT	cagtgagctgagatcacacca	gcgatcccaccatcactc	458	2.0

*The predicted sizes of the *CPT2* exons are: exon 1 (668 bp), exon 2 (81 bp), exon 3 (107 bp), exon 4 (1,305 bp), and exon 5 (929 bp), as described by Verderio et al. [1995].

strands were used for the respective mutant and wild type sequences. The PCR samples were prepared as dot blots on nylon membranes and were processed for hybridization by sequential 5-min exposure to filter paper saturated with denaturation solution (0.5 M NaOH, 1.5 M NaCl), neutralization solution (1 M Tris-HCl, 3 M NaCl, pH 7.5), 2× SSC (0.03 M trisodium citrate, 0.3 M NaCl). The membranes were dried briefly at room temperature and exposed to ultraviolet (UV) cross-linking conditions (Stratalinker). ASO analysis used 5´-biotinylated primers with subsequent detection by streptavidin-alkaline phosphatase (SAAP) cleavage of fluorescent substrates. Nylon membranes were pre-incubated for 1 hr in hybridization solution (4× SSC, 40 mM sodium phosphate buffer, pH 6.5; 2× Denhardt's, 300 μg/ml sheared salmon testis DNA, 0.1% SDS), followed by overnight incubation at 37°C in the hybridization solution containing 4 pmoles/ml of the respective biotinylated ASO. Unbound ASO was removed by rinsing in 4× SSC at 37°C for 15 min, followed by a stringent washing for 15 min in 2× SSC at 2°C below the calculated T_m for each ASO (Table 2). If control DNA samples containing the particular mutation (positive control) were not available to confirm the ability of an ASO to detect a particular mutation, a control filter was washed at 42°C (nonstringent conditions) to confirm its specificity. The presence of the biotinylated ASO on the blot was detected using the Phototope-Star Detection Kit (New England Biolabs) with exposure of blots to Hyperfilm MP (Amersham) for 30 sec to 5 min.

DNA sequence analysis of the *CPT2* gene was performed by cycle sequencing of PCR products with the Thermo-Sequenase ^{33}P-labeled terminator cycle sequencing kit (Amersham). PCR samples were purified using spin columns (High Pure PCR Product Purification Kit; Boehringer). Plasmid clones containing the respective mutant or wild-type sequences present in PCR samples were obtained using the TA cloning vector (Invitrogen). Sequence analysis was performed with the primers used for PCR amplification and internal primers.

RESULTS

We initially screened 47 archival samples (36 muscle samples and 11 lymphoblastoid or whole blood cell samples) for 11 *CPT2* mutations by ASO

TABLE 2. ***CPT2* Allele-Specific Oligonucleotides***

Exon	Mutation	Nucleotide change	Position	Mutant ASO	Wild-type ASO	Reference
1	P50H	CCC→CAC	665	gctcacctgtgcaggctgt	acagcctgcccaggtgagc	Verderio [1995]
3	S113L	TCG→TTG	854	tacctacccaaaatgtagc	gctacatttcgggtaggta	Taroni [1993]
4	E174K	GAA→AAA	1036	ctggagccaaaagtrgttcc	ggaacacttctggctccag	Yamamoto [1996]
4	M214T	ATG→ACG	1157	ccctggatacgtcccagta	tactgggacatatccaggg	Weiser [1997]
4	P227L	CCC→CTC	1196	ctgggtttgagtaaacgagt	ctcgtttacccaaaccag	Taroni [1994]
4	F383Y	TTT→TAT	1664	tgctcagatattttaatga	tcattaaaaaatctgagca	Yamamoto [1996]
4	413 delAG	C[AG]→CAA	1754-5	gtcacggtgcaaactcaac	gttgagtttctgcaccgtg	This study
4	F448L	TTT→CTT	1858	tgcgtccagcttcagagag	ctctctgaaactggacgca	Wieser [1997]
4	Y479F	TAC→TTC	1952	tgcggcagttcgggcagac	gtctgcccgtactgccgca	Wieser [1997]
4	R503C	CGC→TGC	2023	gccgggcagatggtctc	gagaccatccgcccggc	This study
5	G549D	GGC→GAC	2162	tttctcacagaccagggct	agccctggcctgtgagaaa	This study
5	D553N	GAC→AAC	2173	cagggctttaaccgacact	agtrgtcggtcaaagccctg	Verderio [1995]
5	Y628S	TAC→TCC	2399	tctcttcctccccaggccg	cggcctgggtaggaagaga	Bonnefont [1996]
5	R631C	CGC→TGC	2407	gggcattgcagcctgggta	tacccaggccgcaatgccc	Taroni [1992a]

*The site of the mutation is indicated on the predicted *CPT2* transcript described by Verderio et al. [1995].

hybridization (Table 2). Three different mutations (P50H, S113L, and P448L) (Table 3) were identified among seven of the 47 subjects. The respective muscle CPT activities of these samples ranged from 10% to 45% of the normal reference mean. The seven ASO-positive samples together with seven additional ASO-negative samples, which exhibited CPT activity below that found with ASO-positive samples (less than 45% of control mean) were subjected to mutation scanning sequence analysis. The regions examined included portions of the 5′ and 3′ untranslated regions, each of the 5 exons, and a portion of the flanking intron regions, as indicated in Table 1. Three new mutations were identified among the samples as described below. After completion of this phase of the study, we examined 12 additional individuals, suspected of CPT deficiency by referral, for mutations by ASO analysis. Four of the 12 individuals were found to have mutations: two homozygotes and one heterozygote for the S113L mutation and one compound heterozygote for the S113L/F448L mutations. A total of 13 individuals among 59 we examined were found to have CPT2 mutations (Table 4).

413 delAG Frameshift Mutation

Three individuals were found to be compound heterozygotes for the S113L and F448L mutations by initial ASO screening (Table 3). Their muscle CPT activities ranged from 10% to18% of the normal reference mean. Sequence analysis confirmed the presence of both mutations, however, a small deletion proximal to the F448L mutation in exon 4 was also evident in these individuals (Fig. 2). Plasmid clones from one of these compound heterozygous individuals demonstrated a 2-bp deletion (413 delAG; residues 1754–1755) at codon 413 that was 103 bp proximal to the F448L mutation (nt1858) on the same chromosome (Fig. 2). Thus, the 413 delAG and F448L mutations constitute a complex mutation (haplotype). The presence of this mutation was confirmed by ASO analysis in this and three additional individuals. The 413 delAG mutation results in the alteration of six amino acids (414–419) and a termination codon at 420. The resulting truncated polypeptide is approximately two-thirds of normal size (419 vs 658 amino acids) and is missing a portion of the catalytic domain that includes a catalytic triad of histidine/aspartate/serine residues conserved in the carnitine/choline acyltransferase family of proteins [Brown et al., 1994]. The F448L mutation is not included in the truncated polypeptide and therefore does not have any functional significance. No other changes were detected in the three muscle samples containing the 413 delAG, F448L, and S113L mutations.

To determine whether the 413 delAG and the F448L mutations were always associated as a complex mutant haplotype, we analyzed blots containing the exon 4 PCR samples with the wild-type and mutant ASOs (Fig. 3). All three samples that were heterozygous for the F448L mutation also were heterozygous for the 413 delAG mutation. One additional compound heterozygote for the S113L/413 delAG-F448L mutations was identified among the 12 additional patients tested after the initial screen (Table 4). Among the 59 patients screened for 14 mutations with ASOs, the complex mutant allele

TABLE 3. **Comparison of CPT and Citrate Synthase Activities Among Patients With Identified *CPT2* Mutations***

Patient No.	*CPT2* mutation	CPT[a,c,d]	Citrate synthase[b,c,d]	Clinical findings
1000	S113L/413 delAG-F448L	8.1 (10)	13.7 (87)	Exercise intolerance, pain, cramps, myoglobinuria
1001	S113L/413 delAG-F448L	9.1 (12)	13.0 (83)	Exercise intolerance, pain, stiffness, myoglobinuria
1002	S113L/413 delAG-F448L	13.8 (18)	13.8 (87)	Exercise intolerance, pain, myoglobinuria
1614	P50H/G549D	10.1 (13)	17.2 (109)	Exercise intolerance, pain, weakness, myoglobinuria
1605	S113L	9.9 (13)	ND	Exercise intolerance, pain, weakness, myglobinuria
1003	S113L	30.1 (39)	17.5 (111)	Exercise intolerance, pain
1004	S133L	35.3 (45)	13.1 (83)	Exercise intolerance, weakness, cramps
1607	R503C	9.9 (13)	5.8 (36)[e]	Exercise intolerance, severe progressive weakness
912	R503C	ND	ND	Malignant hyperthermia-like episode
Range		[10–45]	[83–111]	

CPT, carnitine palmitoyltransferase; ND, not determined.
*Four additional patients with *CPT2* mutations were referred for mutation analysis alone without the measurement of CPT activity in leukocytes or skeletal muscle (Table 4).
[a]CPT normal reference mean activity: 77.8 ± 13.3 $nmol^{-1}min^{-1}g$.
[b]Citrate synthase normal reference mean activity: 15.7 ± 4.4 $\mu mol^{-1}min^{-1}g$.
[c]Numbers in parentheses represent the percentage of normal reference mean.
[d]Numbers in brackets represent the range of percentages of normal reference mean.
[e]Several additional mitochondrial enzyme activities were reduced to ~33% of normal reference mean, while non-mitrochondrial enzymes were within normal reference range.

TABLE 4. ***CPT2* Mutations Identified and Their Relative Frequencies Among Patients**

No. of Patients	P50H	S113L	413 delAG-F448L	R503C	G549D
2		+/+			
4		+/–			
4		+/–	+/–		
2				+/–	
1	+/–				+/–
Relative Frequency	0.05	0.6	0.2	0.1	0.05

+/+, homozygous for the mutation; +/–, heterozygous for the mutation.

(413 delAG-F448L) occurred in four of 20 mutant alleles (20%), second in frequency only to the S113L mutation which occurred in 12 of 20 mutant alleles (60%) (Table 4). The 413 delAG and F448L mutations were not identified among 50 individuals in the general population.

R503C

An R-to-C substitution at residue 503 was identified by sequence analysis in an individual who exhibited 13% of normal reference mean CPT activity in muscle and who was negative for the other 13 mutations detected by ASO analysis (patient 1607; Table 3). Sequence analysis revealed a single nucleotide substitution (CGC→TGC) at nucleotide 2023. No other alterations were identified despite extensive screening of the *CPT2* gene. The R503C mutant allele was also detected in the 24-year-old son of patient 1607, who has had myopathic symptoms of CPT II deficiency similar to, but not as severe as, those of his mother since the age of 9 years. Both he and his mother were found to have approximately 47% of normal reference mean CPT activity in lymphoblasts in an earlier study [Vladutiu et al., 1993]. No muscle biopsy sample was available for the son. One additional heterozygote for this mutation was detected (patient 912) upon screening of all 59 patients (Fig. 3, Table 3). This individual had 58% of normal reference mean CPT activity in lymphoblasts as reported previously in a study of individuals with malignant hyperthermia [Vladutiu et al., 1993]. The 503R residue is highly conserved among both CPT I and CPT II isoforms (Fig. 4A). This mutation was not identified in 50 individuals from the general population.

G549D

The G549D mutation was identified by sequence analysis in an individual who was originally identified by ASO hybridization to have a P50H

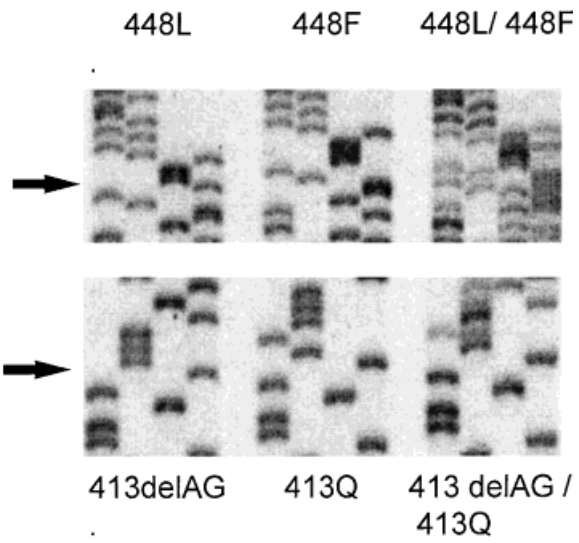

FIGURE 2. **DNA sequence analysis of the haplotypes from a double heterozygote for the 413 delAG frameshift and 448L mutations. DNA fragments (1472 bp) containing exon 4 were obtained by PCR amplification of genomic DNA and plasmid clones were obtained using the TA cloning vector (see Methods). DNA sequencing was performed on the genomic PCR sample containing both 413 delAG and 448L mutations and the plasmid clones derived from it using a primer proximal to the 413 delAG mutation (sense orientation). Two regions of the sequencing gel are shown; one surrounding the 413 delAG mutation (lower panel) and the other surrounding the F448L mutation (upper panel; TTT CTT) as indicated by the arrows. Two types of plasmid clones were obtained; one containing both mutations (413 delAG-448L; left) and the other containing the respective normal sequencies (center). The genomic PCR sample (right) revealed a complicated sequencing pattern due to the 2 bp deletion at codon 413 (delAG) located 103 nucleotides proximal to the T→C substitution at codon 448. Thus this heterozygous individual had two haplotypes, one with both mutations (413 delAG-448L; Left) and the other with the normal sequences (413Q-448F; center).**

mutation. The residual CPT activity in muscle was 13% of the normal reference mean (Table 3). A single nucleotide substitution was found at codon 549 (GGC→GAC). Analysis of this region revealed that the 549G residue was strongly conserved among both CPT I and CPT II isoforms (Fig. 4B). ASO screening did not identify additional individuals with this mutation, nor was it identified in 50 individuals from the general population.

Polymorphism in the 3′ Untranslated Region

A single nucleotide polymorphism (T→C) was identified 11 bp distal to the polyadenylation site at nucleotide 3101. Allele frequencies of T = 0.57 and C = 0.43, respectively, were observed among seven individuals analyzed. No association of the respective alleles with CPT2 activity was noted among the samples examined, although there

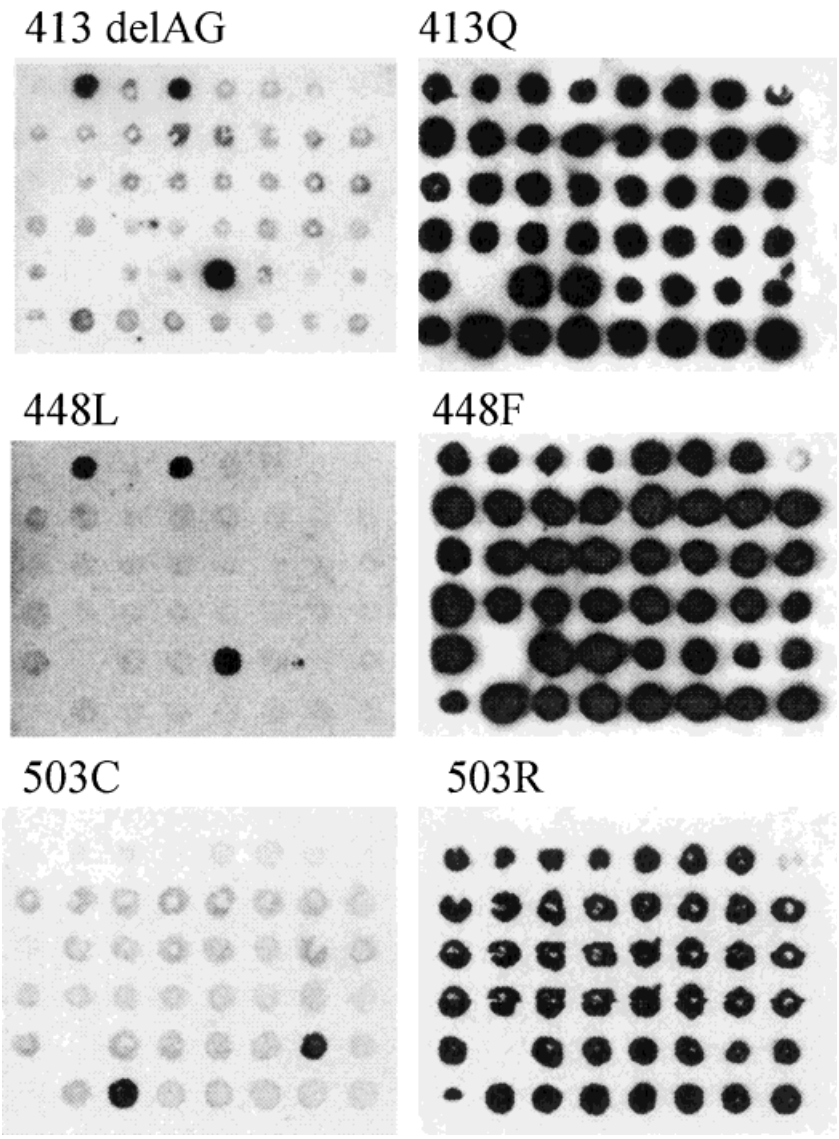

FIGURE 3. **Detection of *CPT2* exon 4 mutations and normal sequences at codons 413,448 and 503 in 48 DNA samples by ASO hybridization. DNA samples containing exon 4 and portion of the flanking introns (1472 bp) were obtained by PCR amplification of genomic DNA, transferred to nylon membrane, denatured and hybridized with the 413 delAG, 413 Q, 448L, 448F, 503C and 503R ASOs. Nylon blots were hybridized overnight with individual ASOs, washed in 2X SSC at a temperature of 2-4 °C below the T_m as described in Methods. One position contained a weak PCR sample (upper right corner) and another position contained a PCR negative sample (second column fifth row). Three samples were heterozygous for the 413 delAG-448L haplotype described in Figure 3.**

could conceiveably by an effect on the polyadenylation of transcripts by decreasing the number of mature transcripts of the gene. As a polymorphism, it will be useful for linkage and segregation analysis of *CPT2* mutations.

DISCUSSION

Three novel *CPT2* mutations were found in this study, two missense substitutions in otherwise highly conserved regions of the CPT isoforms (R503C and G549D) and a frameshift mutation (413 delAG) that encodes a truncated protein missing more than one-third of the enzyme molecule. The 413 delAG and G549D mutations were identified in compound heterozygotes in combination with well-characterized *CPT2* mutations and therefore appear to be recessive alleles. The 413 delAG mutation encodes a truncated protein that is likely to be unstable and enzymatically inactive. Because this null allele was only observed in compound heterozygotes, and not in simple heterozygotes, it is likely a recessive mutation whereupon the presence of a single copy is insufficient to cause clinical symptoms. The 413 delAG mutation was the seco'nd most common mutation identified in this study (20% of mutations). Evidence that this mutation results from a single ancestral mutational event was found with the observation that all four individuals with the 413 delAG-F448L haplotype were of Ashkenazi Jewish descent. It remains to be demonstrated if those individuals originally reported by Wieser et al. [1997] to have the F448L mutation also have the 413 delAG mutation.

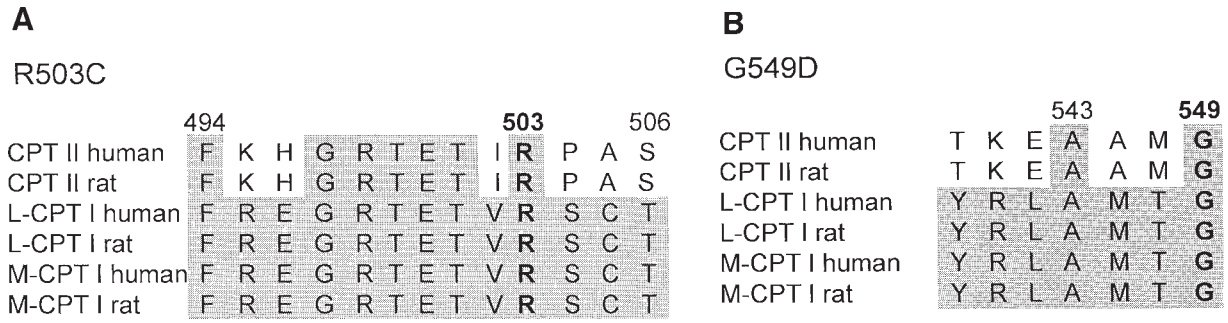

FIGURE 4. **(A) Amino acid sequence of conserved region containing the R503C substitution. Residue 503 is located within a highly conserved region among CPT I and CPT II isoforms (McGarry & Brown, 1997). (B) Amino acid sequence of conserved region containing G549D substitution. Residue 549 is located within a region that is highly conserved among CPT I and CPT II isoforms (McGarry & Brown, 1997).**

In sharp contrast to the 413 delAG and G549D mutations, the R503C mutation was identified in a mother, her son, and another unrelated individual, all of whom appeared to be simple heterozygotes. All three of these individuals are negative for the other 13 CPT2 mutations detected by our ASO screening. It is possible that these individuals may have a second mutation in a region of the *CPT2* gene that we have not yet identified although detailed sequence analysis of one of these individuals was negative. In addition, this explanation is less likely for the son of patient 1607, as there is no consanguinity in the family and the father has no history of myopathic symptoms. Alternatively, the R503C substitution could be a dominant mutation, since the substitution of the disulfide donor cysteine residue for the basic polar arginine residue is expected to be quite disruptive of this strongly conserved region of the CPT isoforms [McGarry and Brown, 1997]. The CPT II enzyme molecules exist in 5 different tetrameric forms at a molar ratio of 1:4:6:4:1 in a heterozygous individual. It could be postulated that, the majority of tetramers in CPT II from an individual with the 503C substitution may contain one or more subunits with the substitution if, in fact, the aberrant subunits are incorporated into the tetramer. In a heterozygote, the substitution could alter protein folding, disulfide formation or subunit polymerization with a marked reduction in enzyme activity or instability of most enzyme molecules. Dominant mutations have been described in several multimeric enzymes, as recently illustrated by mutations in the subunits of galactose-1-phosphate uridyltransferase [Wang et al., 1998]. It will be useful in future studies to investigate the stability of CPT II enzyme molecules in patients with the 503C mutation using the COS cell in vitro expression system [Taroni et al., 1993; Wataya et al., 1998].

Thirteen of 59 individuals in our study were found to have *CPT2* mutations. This relatively low incidence of mutations probably results from the the rather liberal inclusion of subjects based on relatively high levels of residual CPT activity (as much as 63% of normal reference mean). It is also possible that *CPT2* mutations were not identified that may reside within the intron regions not examined. Among the 13 individuals with mutations, only 20 of the 26 expected mutant alleles (77%) were identified under the assumption that CPT II deficiency is a recessive disorder. Six of 13 individuals had a single mutation, two with the R503C mutation and 4 with the S113L mutation (Table 4). The fact that two individuals who were heterozygous for the S113L mutation (Table 3) had muscle CPT activity that was 39% and 45% of the normal reference range suggests that a single copy of this mutant allele is sufficient to predispose an individual to clinical symptoms. Previous studies have reported a larger than expected proportion of affected individuals who were heterozygous for the S113L mutation [Taroni et al., 1993; Zierz et al., 1994; Kaufmann et al., 1997] (Table 5). While the simplest explanation for these findings is the failure to detect a second mutation, it is also possible that a single S113L mutation, combined with epigenetic factors, may be sufficient to cause clinical symptoms. By comparison, the present study reported the lowest number of simple heterozygotes (46%) and the highest number of mutant alleles identified (77% of expected) when screening for 13 mutations. Interestingly, only slightly higher numbers of heterozygotes were detected in two of three studies in which only one or two mutations were used for screening (Table 5)[Taroni et al.,

TABLE 5. **Comparison of CPT2 Mutant Allele Frequencies Found in Four CPT-Deficient Patient Groups***

Reference and no. of patients studied	No. of different mutations detected	No. of mutant alleles identified	No. of heterozygotes identified
Taroni et al. [1993] 25 patients	2	30/50 (60)	12/25 (48)
Zierz et al. [1994] 22 patients	1	28/44 (64)	12/22 (54)
Kaufmann et al. [1997] 20 patients	4	24/40 (60)	14/20 (70)
Present study 13 patients	13	20/26 (77)	6/13 (46)

*The frequency of mutations identified is calculated under the assumption of autosomal recessive inheritance and that each symptomatic individual should have two mutations. The frequency of apparent heterozygotes is based upon the total number of individuals with CPT deficiency who were heterozygous for a *CPT2* mutation. Numbers of parentheses represent the percentage of total.

1993; Zierz et al., 1994]. Despite the differences in the rigor of mutation analysis, our data and those of others suggest that the presence of a single S113L mutation in an individual combined with clinical symptoms should be considered as strong evidence for CPT II deficiency and should be investigated further.

It has been suggested that the magnitude of residual CPT II enzyme activity is correlated with the clinical phenotype [Bonnefont et al., 1996; Demaugre et al., 1991]. It its evident that differences exist in the severity of clinical symptoms exhibited by family members with the same mutations [Handig et al., 1996; Kelly et al., 1989]. This may be due to variations in the duration and the degree of metabolic stress. An additional complexity exists among compound heterozygotes who possess mutations originally identified with the infantile or adult phenotypes. A compound heterozygote for the S113L (adult myopathic form) and R631C (infantile form) mutations was reported who had a myopathic phenotype [Taroni et al., 1993], while a compound heterozygote for the E174K (infantile form) and F383Y (adult myopathic form) mutations had an infantile hepatic phenotype [Wataya et al., 1998]. Despite these complexities, it is evident that the S113L mutation renders an individual at risk of the adult phenotype, as shown in studies describing genotype-phenotype associations for various mutations and by examination in vitro of residual CPT activity in transfected COS cells [Taroni et al., 1993; Verderio et al., 1995; Wataya et al., 1998].

Another complexity associated with CPT II deficiency involves the three amino acid polymorphisms that, by themselves, do not cause clinical symptoms but appear to modify the severity of enzyme dysfunction caused by *CPT2* mutations. Verderio et al. [1993] and Taroni et al. [1993] originally described the finding that the V368I and M647V polymorphisms, when combined with the S113L mutation, contributed to a further reduction in CPT II activity. The third polymorphism (F352C), originally considered a pathogenic mutation [Hamajima et al., 1994], has been found only in Japanese [Wataya et al., 1998]. Numerous molecular configurations of the CPT II isoforms are theoretically possible when the polymorphisms exist together with pathogenic mutations, however, the impact on the tetrameric subunits is unknown, as the crystallographic structure of CPT II has not been determined [McGarry and Brown, 1997]. It is apparent that the clinical phenotype of adult myopathic CPT II deficiency is affected by several factors including the nature of triggering events, the type of pathogenic mutations present, and their interaction with intragenic polymorphisms. We also believe that other clinical indications, such as malignant hyperthermia (patient 912), ankylosing spondylitis (patient 1003) or defects in oxidative phosphorylation with the preservation of nonmitochondrial enzyme activities (patient 1607, data not shown), may bring patients to medical attention subsequently revealing an underlying CPT II deficiency.

Our study has provided several lines of evidence that each of the three novel mutations we describe are pathogenic. The mutations either eliminate a substantial portion of the CPT II molecule or result in missense substitutions in highly conserved domains of the enzyme. These mutations were not found among 50 individuals from the general population and therefore are unlikely to be polymorphisms. The presence of the mutations alone or in combination with other mutations predicatably was associated with correspondingly reduced CPT activity. Our study also suggests that certain mutations (S113L or R503C) may lead to clinical symptoms of CPT II deficiency when present in the simple heterozygous state; i.e., they may be functionally dominant mutations. Future studies using an in vitro expression system may prove use-

ful to better understand the nature of enzyme dysfunction caused by these mutations.

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ELECTRONIC DATABASE INFORMATION

URLs for data in this article are as follows:

1. Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim/
2. GenBank NIH genetic sequence database, http://www.ncbi.nlm.nih.gov/Entrez/
3. Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff, University of Wales College of Medicine: http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html

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