0026-895X/06/7005-1602–1611$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 70:1602–1611, 2006

Vol. 70, No. 5
28126/3150735
Printed in U.S.A.

Functional Genetic Diversity in the High-Affinity Carnitine Transporter OCTN2 (*SLC22A5*)

Thomas J. Urban, Renata C. Gallagher, Chaline Brown, Richard A. Castro, Leah L. Lagpacan, Claire M. Brett, Travis R. Taylor, Elaine J. Carlson, Thomas E. Ferrin, Esteban G. Burchard, Seymour Packman, and Kathleen M. Giacomini

Departments of Biopharmaceutical Sciences (T.J.U., C.B., R.A.C., L.L.L., E.G.B., K.M.G.), Pediatrics (R.C.G., S.P.), Anesthesiology (C.M.B.), Genome Core Facility (T.R.T., E.J.C.), and Pharmaceutical Chemistry (T.E.F.), University of California San Francisco, San Francisco, California

Received June 19, 2006; accepted August 24, 2006

ABSTRACT

Systemic carnitine deficiency (SCD) is a rare autosomal recessive disease resulting from defects in the OCTN2 (*SLC22A5*) gene, which encodes the high-affinity plasma membrane carnitine transporter. Although OCTN2 is fairly well studied in its relationship with SCD, little is known about the carrier frequency of disease-causing alleles of OCTN2, or of more common functional polymorphisms in this gene. To address these issues, we screened for genetic variants in the OCTN2 coding region by direct sequencing of the exons and flanking intronic region of OCTN2 in a large sample ($n = 276$) of ethnically diverse subjects. In addition, we established lymphoblastoid cell lines from subjects homozygous for either allele of the previously identified promoter region variant, −207G>C. We found eight amino acid sequence variants of OCTN2, of which three (Phe17Leu, Leu144Phe, and Pro549Ser) were polymorphic in at least one ethnic group. When assayed for functional activity by expression in human embryonic kidney 293 cells, using as probes both the endogenous substrate (L-carnitine) and the organic cation tetraethylammonium, three variants showed functional differences from the reference OCTN2 (Phe17Leu, Tyr449Asp, Val481Phe; $p < 0.05$). Further studies of the Phe17Leu polymorphism showed a reduced V_{max} for L-carnitine transport to approximately 50% of the reference OCTN2. Confocal microscopy studies using an OCTN2-GFP fusion protein showed that Phe17Leu had distinct subcellular localization from the reference OCTN2, with diffuse cytoplasmic retention of Phe17Leu, in contrast to reference OCTN2, which localized specifically to the plasma membrane. Lymphoblasts from subjects homozygous for the −207G allele showed increased L-carnitine transport compared with the −207C/C homozygotes ($p < 0.05$). This study suggests that although loss-of-function mutations in OCTN2 are likely to be rare, common variants of OCTN2 found in healthy populations may contribute to variation in the disposition of carnitine and some clinically used drugs.

Primary systemic carnitine deficiency (SCD) is an early-onset disorder marked by cardiac and skeletal myopathy, hypoketotic hypoglycemia, hyperammonemia, encephalopathy, and, in some cases, acute liver failure (Lahjouji et al., 2001; Tein, 2003). The symptoms are reversible on administration of high doses of oral L-carnitine (Lamhonwah et al., 2002). SCD has been shown to result from mutations in the high-affinity plasma membrane carnitine transporter, OCTN2 (*SLC22A5*) (Nezu et al., 1999; Tang et al., 1999; Wang et al., 1999) (reviewed in (Lahjouji et al., 2001; Tein, 2003)). The primary defect in SCD seems to be a severe reduction in active reabsorption of carnitine in the kidney (Horiuchi et al., 1994), highlighting the importance of OCTN2 in renal salvage of carnitine, and thus maintenance of total body stores of carnitine, an essential cofactor in mitochondrial fatty acid oxidation (Bremer, 1983). Mutations identified in patients with SCD include both missense and nonsense mutations as well as insertions/deletions (Lahjouji et al., 2001; Tein, 2003). With few exceptions, unique OCTN2 mutations have been found in each of the unrelated cases of SCD for which a causative mutation was identified; i.e., they are private mutations. Thus, the allele frequencies of these loss-of-function mutations are not known, but are believed to be very rare. Estimates of the overall carrier frequency have ranged from 1:100 in Akita, Japan(Koizumi et al., 1999) to as

Dr. Urban is a recipient of a PhRMA Foundation Predoctoral Fellowship in Pharmaceutics. This work was supported by grants from the National Institutes of Health (GM61390 and GM36780).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.028126.

ABBREVIATIONS: SCD, systemic carnitine deficiency; OCTN2, novel organic cation transporter 2; TEA, tetraethylammonium; SNP, single-nucleotide polymorphism; GFP, green fluorescent protein; HBSS, Hanks' buffered salt solution; LCL, lymphoblastoid cell line.

low as 1:150–1:480 in an Australian population (Wilcken et al., 2001).

OCTN2 (*SLC22A5*) is a member of the SLC22 family of plasma membrane solute carrier proteins, which includes multispecific transporters of organic cations, anions, and zwitterions. OCTN2 is unique in that it is a bifunctional transporter, facilitating the transport of both organic cations [such as the xenobiotic tetraethylammonium (TEA)] in a pH-dependent and Na^+-independent manner, and of zwitterions (such as the prototypical substrate, L-carnitine) in a Na^+-dependent manner (Wu et al., 1999; Ohashi et al., 2001). OCTN2 is expressed ubiquitously, with high expression in kidney and lower expression in heart, skeletal muscle, and other tissues (Tamai et al., 1998; Wu et al., 1998).

A mouse model of systemic carnitine deficiency exists (the *jvs* mouse), and exhibits a phenotype very similar to human SCD, including cardiomyopathy and fatty infiltration of visceral organs (Kuwajima et al., 1991). The *jvs* mouse, which has a mutation in the mouse ortholog of OCTN2, exhibits decreased renal secretory clearance of TEA and increased renal secretory clearance of carnitine, consistent with dual function of OCTN2 (Ohashi et al., 2001), and suggesting that humans with genetic defects in OCTN2 may exhibit abnormalities in the disposition of carnitine and in the pharmacokinetics of organic cations [such as cardiovascular drugs (Grube et al., 2006)] that are OCTN2 substrates.

Given that complete loss-of-function mutations in OCTN2 cause severe abnormalities in carnitine disposition and fatty acid oxidation (i.e., SCD), and that this syndrome is extremely rare, it is unlikely that null alleles of this gene exist at high frequency in the general population. However, it is possible that more moderate defects in OCTN2 activity may be caused by genetic polymorphisms in the OCTN2 gene and may explain variability in the disposition of carnitine and other OCTN2 substrates. In both humans (Koizumi et al., 1999) and mice (Lahjouji et al., 2002; Xiaofei et al., 2002), heterozygosity for OCTN2 mutations has been shown to produce a moderate carnitine deficiency phenotype, demonstrating that even partial loss of OCTN2 function may be detrimental.

Peltekova et al. (2004) recently identified a single-nucleotide polymorphism (SNP) in the promoter region of OCTN2, −207G>C, that formed, in combination with an amino acid substitution in the paralog OCTN1 (OCTN1-Leu503Phe), a two-point haplotype that was enriched in patients with Crohn's disease in a case-control study of persons of European descent. This SNP was found to disrupt a heat shock element in the OCTN2 promoter, and biochemical assays showed that the −207C allele had reduced responsiveness to heat shock and arachidonic acid treatment. Although a causal role for this polymorphism in the pathogenesis of Crohn's disease remains controversial (Trinh and Rioux, 2005a,b), the biochemical evidence seems to suggest that this SNP may modify the functional expression of OCTN2 and thus may help to explain interindividual variability in carnitine homeostasis as well as drug response.

Coding region variants, specifically nonsynonymous variants (i.e., amino acid substitutions), may result in changes in protein function. To systematically investigate functional genetic variation in OCTN2, we resequenced the OCTN2 coding region in a large (n = 270), ethnically diverse sample of healthy volunteers and identified 20 nucleotide substitutions, including 15 previously unreported variants, in this region. We then examined the functional effects in cellular assays of amino acid sequence variants of OCTN2 identified in this screen. Because OCTN2 is a bifunctional transporter, we assessed the activity of OCTN2 variants with respect to both the endogenous substrate L-carnitine and the xenobiotic TEA. We further characterized the subcellular localization of the most common protein sequence variants of OCTN2. In addition, we established immortalized lymphoblasts from subjects homozygous for either allele of the promoter region polymorphism −207G>C, to determine the effects of this polymorphism on L-carnitine transport and OCTN2 mRNA expression ex vivo.

Materials and Methods

Chemicals. [^{3}H]L-Carnitine (80 Ci/mmol) and [^{14}C]tetraethylammonium (55 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Lipofectamine 2000 and pcDNA3 were purchased from Invitrogen (Carlsbad, CA). BCA Protein Assay Kit was purchased from Pierce Biotechnology Inc. (Rockford, IL). Unlabeled L-carnitine and tetraethylammonium bromide were purchased from Sigma (St. Louis, MO). Cell culture supplies were purchased from the Cell Culture Facility (UCSF, San Francisco, CA). All other chemicals were of reagent grade and were commercially available.

Identification of SLC22A5 Variants. Genomic DNA samples were collected from unrelated healthy persons in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. *SLC22A5* variants were identified by direct sequencing of genomic DNA as described previously (Leabman et al., 2003) from an ethnically diverse population of 270 individuals: 80 African Americans, 80 European Americans, 60 Asian Americans (50 Chinese Americans and 10 Japanese Americans), and 50 Mexican Americans. The reference cDNA sequence of *SLC22A5* was obtained from GenBank (accession number NM_003060). Primers were designed manually to span the exons, and 50 to 200 base pairs of flanking intronic sequence per exon. The primer sequences can be found at http://www.pharmgkb.org. Variant positions are relative to the ATG start site and are based on the reference cDNA sequence of *SLC22A5*.

Genetic Analysis of SLC22A5. The neutral parameter (θ), nucleotide diversity (π), and Tajima's D statistic were calculated as described by Tajima (1993). Each parameter was determined for various sites within the coding region of *SLC22A5* (e.g., synonymous and nonsynonymous sites, and sites within transmembrane domains and loops) for the entire population and for each ethnic group. Synonymous and nonsynonymous sites were defined as described by Hartl and Clark (1997). Evolutionarily conserved amino acid residues were defined as residues identical among human, mouse, and rat orthologs of OCTN2. Chemical distance, or the degree of difference between alternative amino acid residues, was taken from the amino acid substitution matrix of Grantham (1974).

Construction of OCTN2 Variants. Human OCTN2 cDNA (GenBank accession number NM_003060) was subcloned into the mammalian expression vector pcDNA3 to obtain OCTN2-reference, which corresponds to the highest-frequency amino acid sequence in all ethnic groups. Variant cDNA clones were constructed by site-directed mutagenesis of the reference clone using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). Variants that showed functional differences from OCTN2 reference were reverted to the reference sequence by site-directed mutagenesis and assayed further, to confirm that the functional effect was due to the intended mutation. In all cases, OCTN2 reference in pcDNA3 was used as the template, except for OCTN2-Phe17Leu-reversed, OCTN2-Tyr449Asp-reversed, and OCTN2-Val481Phe-reversed, for which OCTN2-

Phe17Leu, OCTN2-Tyr449Asp, and OCTN2-Val481Phe were used as templates, respectively. Sequences of variant cDNA clones were confirmed by direct sequencing, and the full cDNA was sequenced to verify that only the intended mutation was introduced.

Cellular Assays of OCTN2 Variants. Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. Cells were seeded onto 24-well, poly-D-lysine–coated plates (BD Biosciences Discovery Labware, Bedford, MA) at a density of 1.5×10^5 cells per well and grown for 24 h. Cells were then transfected with 1 μg of OCTN2 reference or variant DNA and 3 μg of Lipofectamine 2000 in each well according to the manufacturer's protocol. Cells were incubated for 48 h and assayed for activity by measurement of cellular uptake of radiolabeled probe substrates. For studies of transport kinetics, OCTN2-reference and the polymorphic variants (Phe17Leu, Leu144Phe, and Pro549Ser) were subcloned into the expression vector pcDNA5/FRT and used to generate stable cell lines using the Flp-In System (Invitrogen) according to the manufacturer's protocol. This system, which generates single-copy, site-specific integration of target constructs, allows for comparisons among allelic variants in stable cell lines by eliminating concerns regarding gene-dose-dependent or integration site-dependent difference among cell lines. In brief, Flp-In-293 cells were plated at a density of 6×10^5 cells per well in six-well tissue culture treated plates using antibiotic-free media and incubated overnight. Cells reached ~95% confluence at 24 h after seeding, at which point cells were transfected with 0.4 μg of OCTN2 (reference or variant) cDNA, 3.6 μg of pOG44 DNA, and 20 μg of Lipofectamine 2000. Two days after transfection, cells were trypsinized and split 1:4 into new six-well plates and selected for stable transfectants by addition of hygromycin B (75 μg/ml) to the growth media. After 10 to 14 days under selection, colonies were pooled and expanded in 25-cm^2 flasks and used for transport experiments by seeding in 24-well poly-D-lysine coated plates as described above.

Uptake studies were performed by first washing the cells with warm Hanks' buffered salt solution (HBSS) and incubating at 37°C for 15 min, after which the wash buffer was removed and replaced with HBSS containing either [^{3}H]L-carnitine (10 nM [^{3}H]L-carnitine, 1 μM unlabeled L-carnitine) or [^{14}C]tetraethylammonium (10 μM radiolabeled). Cells were returned to 37°C for 10 min, which was determined to be within the linear range of uptake versus time. Uptake was terminated by rapidly removing the extracellular media and washing three times with ice-cold HBSS. Cells were lysed in 1 ml of 0.1 N NaOH/0.1% SDS, and 800 μl of the lysate was added to 3 ml of Ecolite scintillation fluid (MP Biomedicals, Irvine, CA). Intracellular radioactivity was determined by scintillation counting and normalized to per-well protein content as measured using the BCA protein assay. Results were expressed as the percentage activity of the OCTN2-reference wells. Kinetics studies were performed as described above, with varying concentrations of unlabeled substrate (L-carnitine or TEA) added to the uptake buffer. Rates of uptake V, expressed in picomoles per minute per milligram of protein, were fit to the equation $V = V_{max} \times [S]/(K_m + [S])$, or $V = V_{max} \times [S]/(K_m + [S]) + K_o \times [S]$, where K_o represents the first-order rate constant for non–OCTN2-mediated uptake, [S] is the substrate concentration, and V_{max} and K_m are the Michaelis-Menten kinetic parameters.

Statistical differences between variant and reference OCTN2 activity were assessed by paired t-tests using mean values from replicate experiments. $p < 0.05$ was used as the threshold for significance.

Subcellular Localization Studies. Polymorphic OCTN2 variant cDNA clones were used to make GFP fusion constructs to determine their subcellular localization. The terminal codon in OCTN2 was mutated to introduce a BstBI recognition site, and the GFP coding sequence was ligated to the 3′ end of the OCTN2 cDNA in the expression vector pcDNA5/FRT. The resulting clones were used to generate stable cell lines as described above. For localization studies, cells were seeded at 1.5×10^5 cells per well on 12-mm poly-D-lysine coated glass coverslips (BD Discovery Labware, Bedford, MA) in 24-well plates. Cells were stained using the Image-IT Live labeling kit (Invitrogen) and fixed in 4% paraformaldehyde according to the manufacturer's protocol. Coverslips were mounted in Vectashield antifade solution (Vector Laboratories, Inc., Burlingame, CA) on glass microscope slides and visualized by confocal microscopy using a Zeiss 510 laser scanning microscope (Carl Zeiss Inc., Thornwood, NY).

Phenotyping of OCTN2 −207G>C Promoter Polymorphism in Immortalized Lymphoblasts. Subjects in the SOPHIE cohort were screened by direct sequencing of the promoter region of OCTN2 to identify persons homozygous for either −207G/G or −207C/C. Because this polymorphism had only been described in persons of European ancestry, this screen was restricted to the European American subset of SOPHIE. Homozygotes were recruited into a clinical study designed to assess differences between subject groups in carnitine renal clearance and in carnitine transport activity and OCTN2 mRNA expression in lymphoblastoid cell lines (LCLs). To establish these cell lines, 10 ml of whole blood was drawn from each subject (−207G/G, $n = 7$; −207C/C, $n = 8$), and the buffy coat was isolated by separation of blood using Ficoll Paque Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK). White blood cells were washed twice in phosphate-buffered saline and transformed with Epstein-Barr virus in the presence of 1 μg/ml cyclosporin A in growth media (RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were incubated in 25-cm^2 flasks at 37°C in 5% CO_2 for 7 to 10 days before feeding. Once colonies were established, cells were expanded by feeding every 2 to 3 days, then transferred to 75-cm^2 flasks. L-Carnitine transport studies were performed using the method described by Tein and Xie (1996). In brief, 6×10^6 cells were incubated in the presence of 5 μM or 5 mM L-carnitine (10 nM [^{3}H]L-carnitine plus unlabeled L-carnitine to achieve the final desired concentration) for 3 h in triplicate 1.5-ml Eppendorf tubes. Cells were then washed three times in ice-cold phosphate-buffered saline and lysed in 1 ml 0.1 N NaOH/0.1% SDS, and 800 μl of cell lysate was used for scintillation counting. Active transport was determined by subtracting nonspecific uptake (at 5 mM) from total uptake (at 5 μM) after correcting for total protein as determined by the BCA protein assay. Transport studies were performed at least twice for each cell line, and average values from replicate experiments were used in the final analysis. To measure OCTN2 mRNA expression, 5×10^6 cells from each line were pelleted. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse-transcribed using the Superscript III Reverse Transcription Kit (Invitrogen) according to the manufacturers' protocols. The resulting cDNA was used as template for real-time quantitative PCR using TaqMan primers and probes specific for human OCTN2 and GAPDH (Applied Biosystems, Foster City, CA). OCTN2 expression was normalized to GAPDH mRNA and expressed as a relative expression versus the lowest-expressing cell line. To determine the effect of heat shock on OCTN2 expression, cells were incubated in water baths in room air at either 42°C or 37°C for 2 h and returned to 37°C in 5% CO_2 incubator. Cells were pelleted for RNA extraction at various time points (0, 1, 2, or 6 h) after termination of heat shock. Differences between treatments (for heat shock studies) were tested for significance using a paired t test. Differences between genotype groups were assessed using an unpaired t test.

Results

Variant Discovery and Population Genetics of OCTN2 Coding Region Variants. We identified 20 nucleotide substitution variants in the survey region, including 10 in coding and 10 in noncoding regions of the OCTN2 gene. Five of these had previously been reported on the NCBI single nucleotide polymorphism database (dbSNP): two high-

frequency synonymous SNPs (c.285C>T and c.807G>A), one amino acid substitution (L144F), one intronic SNP (IVS4 + 13C>T), and one SNP in the 3′-untranslated region. In addition, one rare variant (Tyr449Asp) had been identified previously in a patient with a suspected carnitine transport defect (Amat di San Filippo and Longo, 2004).

Of the variants we identified in noncoding regions, nine were intronic and one was found in the 3′-untranslated region, 47 base pairs downstream of the stop codon. Among coding region variants, two were synonymous (i.e., did not alter the amino acid sequence) and eight were nonsynonymous. Although a total of eight nonsynonymous variants were found, only seven nonsynonymous sites were identified, as one of the sites was triallelic (c.1441G>T, c.1441G>A). The variant identification data have been deposited in the public databases PharmGKB (http://www.pharmgkb.org) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Table 1 summarizes the population genetics statistics for the OCTN2 coding region. It is noteworthy that although only two synonymous variants were found (versus eight nonsynonymous variants), the nucleotide diversity at synonymous sites (π_S) was much higher than that at nonsynonymous sites (π_{NS}). The ratio of π_{NS}/π_S is frequently used as a measure of the extent of selective pressure on a gene, with low π_{NS}/π_S corresponding to a high degree of negative selection (i.e., low tolerance for nonsynonymous substitution versus synonymous substitution); the ratio of 0.04 for OCTN2 is among the lowest of all transporter genes surveyed by our group (Leabman et al., 2003; Urban et al., 2006), suggesting a very low evolutionary tolerance for alterations in OCTN2 protein structure. This is consistent with the observation that a potentially lethal early-onset disorder (SCD) results from null alleles of this gene.

Figure 1 shows the predicted secondary structure of OCTN2 and the location of the nonsynonymous coding region variants. Only one of the eight nonsynonymous variants is predicted to occur in the transmembrane domain (TMD), five in the intracellular loop and two in the extracellular loop regions of the protein. Variants occurring in the loop regions do not disrupt any predicted *N*-glycosylation or phosphorylation motifs. All but one (Met530Val) of the nonsynonymous variants occur in evolutionarily conserved amino acid residues, defined as residues identical among human, mouse, and rat orthologs of OCTN2.

It is noteworthy that one OCTN2 variant, Tyr449Asp, had been identified previously in the heterozygous state in a patient who died of sudden cardiac arrest at 3 months of age (Vockley et al., 2000; Amat di San Filippo and Longo, 2004). This patient was found on autopsy to have moderately reduced carnitine transport activity (to 57% of control) and reduced very long-chain acyl-CoA dehydrogenase (VLCAD) activity (to 46% of control) in cultured fibroblasts. The patient was also heterozygous for a point mutation in the VLCAD gene, and death is presumed to have resulted from synergism between defects in multiple steps in the fatty acid oxidation pathway (see Vockley et al., 2000). This variant occurs at an evolutionarily conserved residue in an intracellular loop that has been shown to influence sodium activation of OCTN2 (Amat di San Filippo and Longo, 2004).

Of the eight nonsynonymous variants identified, five were singletons; that is, they were found on only one chromosome in our sample. Three were polymorphic (defined as ≥1% allele frequency in at least one ethnic group): Phe17Leu, found at an allele frequency of 1.7% in the Asian American sample; Leu144Phe, found at a 7.5% frequency in the African American sample; and Pro549Ser, found at a frequency of 10.0% in the African American sample. The characteristics of the coding region variants and their population-specific allele frequencies are shown in Table 2.

Activity of OCTN2 Protein Sequence Variants in Cellular Assays. We probed OCTN2 activity using 2 compounds: the endogenous substrate L-carnitine, and the synthetic molecule TEA. When expressed in mammalian cells, the majority of nonsynonymous variants of OCTN2 retained

TABLE 1

Population genetics statistics for variation in *SLC22A5*

The neutral parameter (θ), nucleotide diversity (π), and Tajima's *D* statistic were calculated as described by Tajima (1993). π for synonymous and nonsynonymous sites was calculated as described by Hartl and Clark (1997).

Population and Region/Type	Base Pairs Surveyed	No. of Chromosomes	Total Base Pairs Sequenced	No. of Variable Sites	θ ($\times 10^4$)	π ($\times 10^4$)	*D*
Total							
All	2996	552	1653792	18	8.72 ± 2.62	6.47 ± 4.07	−0.64
Coding	1674		924048	9	7.80 ± 2.98	6.58 ± 4.77	−0.26
Noncoding	1322		729744	9	9.88 ± 3.77	6.34 ± 5.00	−0.58
Synonymous	407		224664	2	7.14 ± 5.22	23.93 ± 18.01	1.38
Nonsynonymous	1267		699384	7	8.02 ± 3.38	1.01 ± 1.70	−1.21
AA							
All	2996	160	479360	12	7.09 ± 2.60	8.01 ± 4.84	0.30
Synonymous	407		65120	2	8.71 ± 6.46	23.49 ± 17.87	1.2
Nonsynonymous	1267		202720	4	5.59 ± 3.07	2.73 ± 2.99	−0.58
EA							
All	2996	160	479360	8	4.73 ± 1.98	5.48 ± 3.60	0.29
Synonymous	407		65120	2	8.71 ± 6.46	22.7 ± 17.4	1.14
Nonsynonymous	1267		202720	1	1.40 ± 1.43	0.10 ± 0.51	−0.40
AS							
All	2996	120	359520	11	6.85 ± 2.63	5.24 ± 3.48	−0.54
Synonymous	407		48840	2	9.17 ± 6.85	19.8 ± 16.0	0.87
Nonsynonymous	1267		152040	2	2.94 ± 2.20	0.39 ± 1.04	−0.65
ME							
All	2996	100	299600	9	5.80 ± 2.41	4.32 ± 3.02	−0.53
Synonymous	407		40700	2	9.50 ± 7.11	18.6 ± 15.3	0.74
Nonsynonymous	1267		126700	2	3.05 ± 2.28	0.32 ± 0.93	−0.69

AA, African American; EA, European American; AS, Asian American; ME, Mexican American.

function, having activity approximately equal to OCTN2-reference (Fig. 2A). None of the variants tested showed complete loss of function; however, one rare variant (Val481Phe) and one polymorphism (Phe17Leu) showed reduced function. Val481Phe exhibited reduced activity to approximately 71% of OCTN2-reference activity with respect to L-carnitine transport (after subtracting background; i.e., uptake in mock-transfected cells), and 53% activity with respect to transport of TEA when averaged over triplicate experiments ($p < 0.05$). It is noteworthy that this variant occurred at a triallelic site: two rare substitutions (Val481Phe and Val481Ile) were found at the same amino acid position, but only Val481Phe exhibited a functional difference from the reference protein. The more common Phe17Leu variant showed a greater decrease in function, reduced to 51% of OCTN2-reference activity toward L-carnitine ($p < 0.05$) and to only 20% activity toward TEA ($p < 0.01$).

The Tyr449Asp variant, previously identified in a patient heterozygous for a carnitine transport defect, showed increased preference for TEA versus L-carnitine: 150% of OCTN2-reference activity toward TEA ($p < 0.0001$), compared with a reduction in L-carnitine transport to 62% of the reference clone ($p < 0.05$). The kinetic constant (K_m) of Tyr449Asp toward L-carnitine was not different from the reference OCTN2; however, sodium activation kinetic studies showed reduced sodium stimulation of L-carnitine transport by Tyr449Asp compared with the reference OCTN2 (data not shown), consistent with previous findings (Amat di San Filippo and Longo, 2004).

To confirm that the differences in functional activity exhibited by these variants were due to the intended mutations and not to unidentified mutations introduced through site-directed mutagenesis in generating the variant cDNAs, we reversed these variant cDNAs back to the reference sequence and assayed the resulting cDNA clones for activity. In all cases, reversing the mutation back to the reference sequence restored wild-type function (Fig. 2B), demonstrating that the functional differences observed were specifically caused by the intended nonsynonymous variants.

Detailed Phenotyping of OCTN2 Protein Sequence Polymorphisms. No obvious functional differences were identified in the initial screen among the most common OCTN2 variants, Leu144Phe and Pro549Ser. However, the Phe17Leu polymorphism showed significant reduction in activity toward both substrates. To determine whether more subtle quantitative differences might exist among the Leu144Phe and Pro549Ser variants and to further characterize the alterations in activity of the Phe17Leu variant, concentration dependence of L-carnitine and TEA transport was examined. Kinetics of both L-carnitine and TEA transport were nearly identical between OCTN2-reference and both Leu144Phe and Pro549Ser (Fig. 3). For the Phe17Leu variant, the decrease in L-carnitine transport was explained by a reduction in V_{max} to approximately 50% of OCTN2-reference (446 ± 18 versus 861 ± 18 pmol/min/mg of protein for OCTN2-reference) with no effect on K_m (12.5 ± 0.5 versus 13.5 ± 0.8 μM for OCTN2 reference). Phe17Leu was also shown to lower V_{max} of TEA transport by OCTN2 (470 ± 74 versus 720 ± 47 pmol/min/mg of protein for OCTN2-refer-

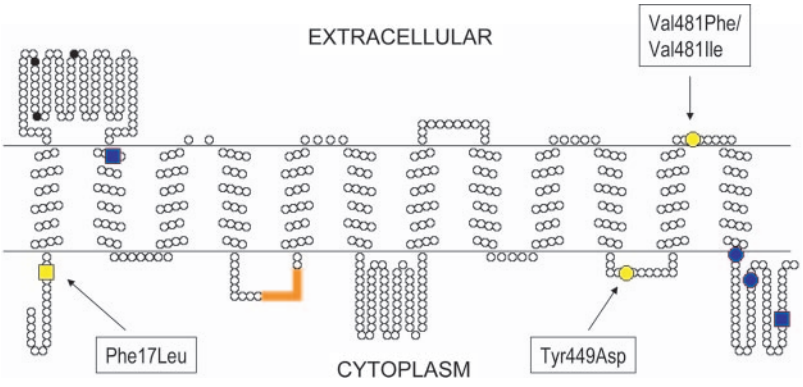

Fig. 1. Predicted secondary structure of OCTN2 showing the position of the eight nonsynonymous coding region variants. The transmembrane topology diagram was rendered using TOPO2 transmembrane protein display software (http://www.sacs.ucsf.edu/TOPO/topo.html). Polymorphic variant sites (allele frequency >1%) are shown as squares; rare variants are shown as circles. Variants with significant functional differences from the reference OCTN2 are shown in yellow, all other variants are shown in blue, putative *N*-glycosylation sites are shown in black, and an ATP-binding motif is boxed in orange. The three functionally significant nonsynonymous variants are indicated by arrows.

TABLE 2

Coding region variants of OCTN2

CDS position is given in reference to the ATG start site (where position + 1 is the "A" in ATG). Grantham score is shown as a measure of chemical distance between alternative residues; a high Grantham score corresponds to large chemical changes (Grantham, 1974). Evolutionarily conserved (EC) residues are defined as residues identical among human, mouse, and rat orthologs of OCTN2; all other residues were classified as evolutionarily unconserved (EU). Population-specific allele frequencies are given. Variant information has been deposited in PharmGKB (http://www.pharmgkb.org) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

dbSNP ID#	CDS Position	Nucleotide Change	Amino Acid Position	Amino Acid Change	Grantham Score	EC/EU	Allele Frequency				
							AA (n = 160)	EA (n = 160)	AS (n = 120)	ME (n = 100)	Total (n = 540)
rs11568520	51	C→G	17	Phe→Leu	22	EC	0	0	1.7	0	0.4
rs2631365	285	T→C	95	Syn		EC	38.7	35.6	70.8	25.0	42.8
rs10040427	430	C→T	144	Leu→Phe	22	EC	7.5	0	0	1.0	2.4
rs274558	807	A→G	269	Syn		EC	38.7	35.6	70.8	25.0	42.8
rs11568514	1345	T→G	449	Tyr→Asp	160	EC	0.6	0	0	0	0.2
rs11568513	1441	G→T	481	Val→Phe	50	EC	0	0.6	0	0	0.2
rs11568513	1441	G→A	481	Val→Ile	29	EC	0.6	0	0	0	0.2
rs11568521	1522	T→C	508	Phe→Leu	22	EC	0	0	0	1.0	0.2
rs11568524	1588	A→G	530	Met→Val	21	EU	0	0	0.8	0	0.2
rs11568525	1645	C→T	549	Pro→Ser	74	EC	10.0	0	0	0	2.9

AA, African American; EA, European American; AS, Asian American; ME, Mexican American.

ence), as well as increase K_m (1.09 ± 0.28 versus 0.53 ± 0.07 mM for OCTN2-reference).

To further address the mechanism for reduced activity of the Phe17Leu polymorphism, GFP fusion proteins were constructed and expressed by stable transfection in Flp-In-293 cells. It was determined by confocal microscopy that although OCTN2 reference (as well as Leu144Phe and Pro549Ser) showed strict localization to the plasma membrane, Phe17Leu showed a more diffuse pattern of localization, with some plasma membrane staining as well as marked cytosolic retention of the variant protein (Fig. 4).

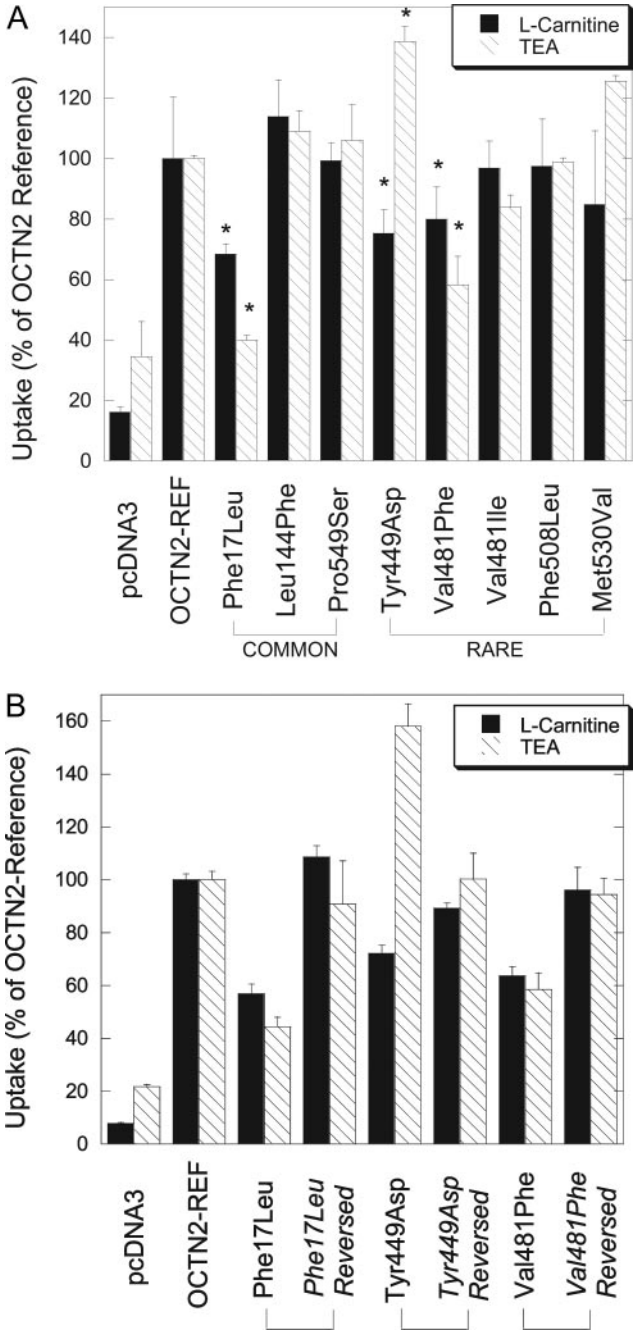

Fig. 2. Functional screen of protein-altering variants of OCTN2. Variants of OCTN2 were expressed by transient transfection in human embryonic kidney 293 cells and assayed for activity by measurement of uptake of radiolabeled probe substrates (1 μM [^{3}H]L-carnitine or 10 μM [^{14}C]-TEA) at 10 min. A, functional activity of all nonsynonymous variants was assessed. B, variant cDNAs for those variants found to have altered function were reversed to the reference sequence by site-directed mutagenesis and assayed for restoration of wild-type activity. Mock-transfected cells (pcDNA3) serve as the negative control. Results are expressed as a percentage of the reference sequence clone (OCTN2-REF) after correcting for per-well protein content. Values represent mean ± S.D. from triplicate wells in a representative experiment. *, $p < 0.05$ versus OCTN2-Reference.

Ex Vivo Phenotyping of −207G>C Promoter Polymorphism. To determine the effect of the −207G>C polymorphism in the promoter of the OCTN2 gene, lymphoblastoid cell lines (LCLs) were derived from subjects homozygous for either −207G/G or −207C/C and assayed for L-carnitine uptake and OCTN2 mRNA expression. As shown in Fig. 5A, cell lines from −207C/C subjects had significantly lower total and specific (i.e., saturable) transport of L-carnitine compared with −207G/G subjects ($p < 0.05$). When OCTN2 mRNA levels were compared between groups, −207C/C subjects showed a trend toward reduced mRNA expression in LCLs. This difference was not significant ($p = 0.20$), although the lack of statistical significance might be explained by a single −207C/C cell line, which showed the highest OCTN2 mRNA expression among all cell lines. When this cell line was excluded from the analysis, −207C/C subjects also showed reduced OCTN2 mRNA expression compared with −207G/G subjects ($p < 0.05$). There was, however, a significant correlation between L-carnitine transport activity and OCTN2 mRNA level when all cell lines were included (Fig. 5B, $p < 0.05$). No effect of heat shock on OCTN2 mRNA

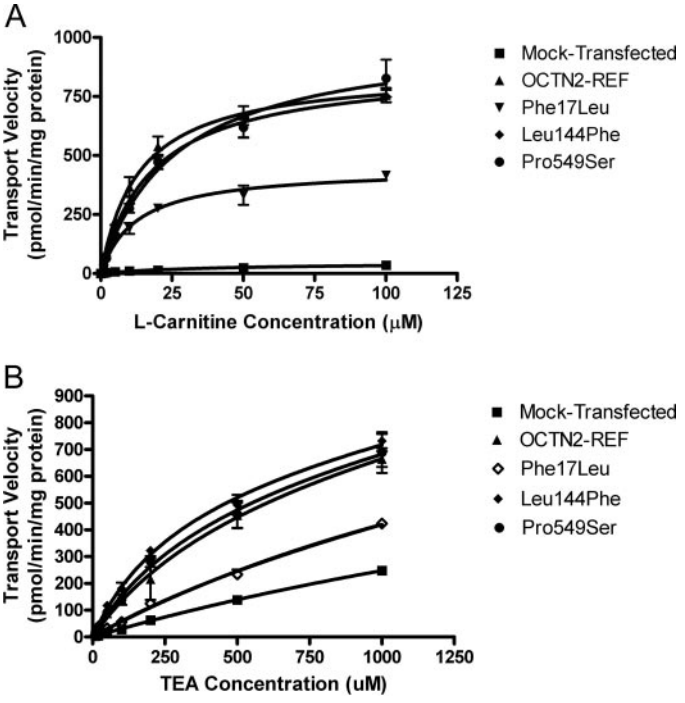

Fig. 3. Concentration-dependence of L-carnitine and TEA transport by common OCTN2 protein sequence variants. OCTN2-reference, Phe17Leu, Leu144Phe, and Pro549Ser were expressed in stably transfected Flp-In-293 cells. Kinetics of L-carnitine (A) and TEA (B) transport were measured by incubation with radiolabeled probe compounds (10 nM [^{3}H]L-carnitine or 10 μM [^{14}C]TEA) and varying concentrations of unlabeled substrate for 10 min. Data for TEA transport were fit to a modified Michaelis-Menten equation that included a first-order nonsaturable component. The nonsaturable uptake rate constant was 0.195. Mock-transfected cells served as a negative control. Results are expressed as picomoles per minute per 10^6 cells. Values represent mean ± S.D. from triplicate wells.

expression was observed in either genotype group at any of the time points tested (0, 1, 2, or 6 h after heat shock; data not shown).

Discussion

Although rare null mutations in OCTN2 have been identified in patients with primary systemic carnitine deficiency, this study represents a systematic survey of the coding region of OCTN2 for functionally significant genetic polymorphisms in an ethnically diverse sample. Others have previously surveyed the entire OCTN2 gene for novel polymorphisms; however, the samples used for SNP identification were restricted to 48 individuals from a single ethnic group (Japanese) and did not identify any amino acid sequence variants (Saito et al., 2002). Another group used an "extreme phenotype" approach to estimate the carrier frequency of loss-of-function mutations in OCTN2; however, this study was restricted to persons in the Akita prefecture of Japan (Koizumi et al., 1999). We did not identify any of the variants reported by Koizumi et al. (1999) in our sample. Based on the results of a newborn screening program to

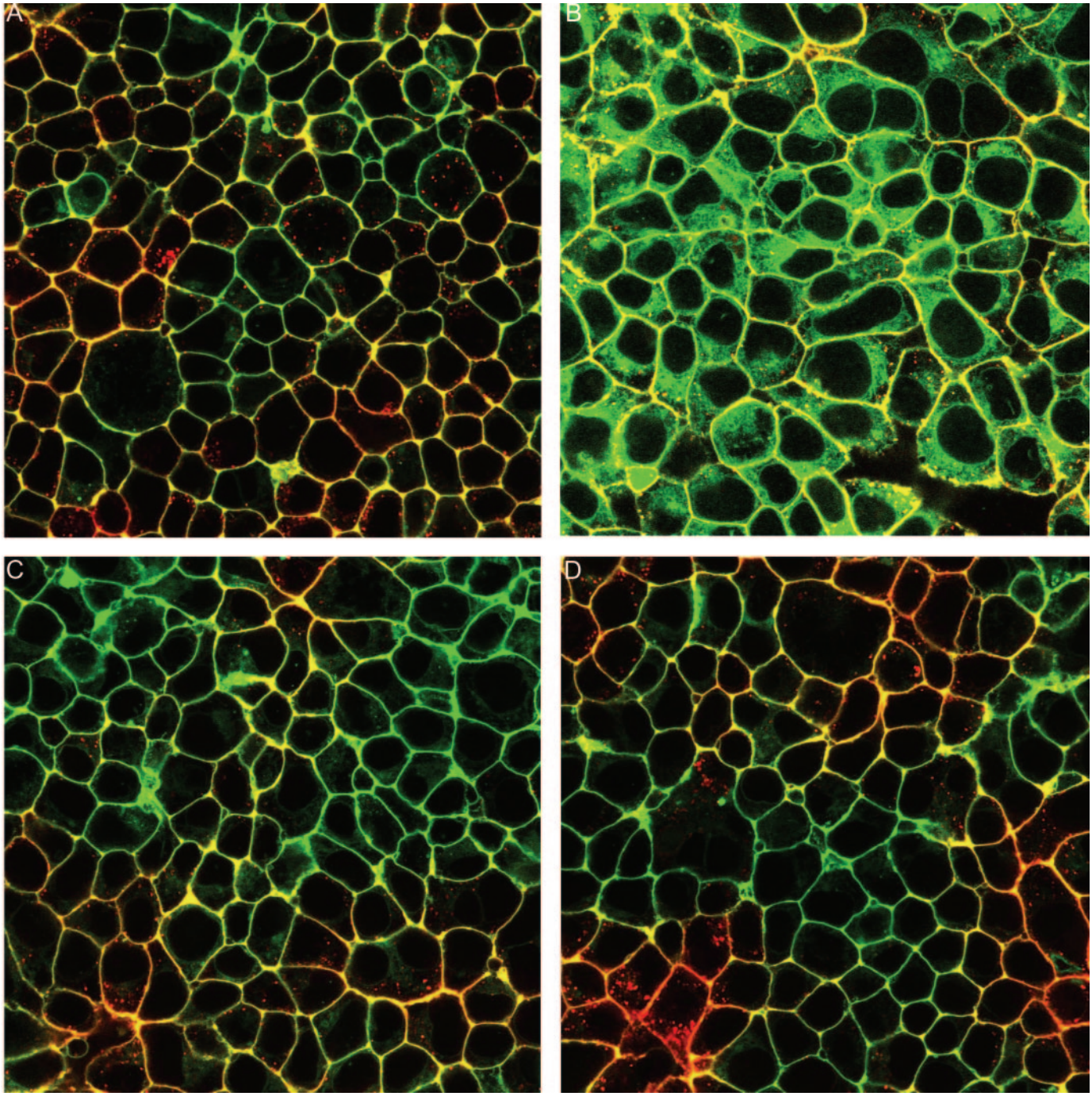

Fig. 4. Subcellular localization of polymorphic OCTN2 variants. GFP fusion constructs were generated for the OCTN2 variants with population-specific allele frequencies >1% and used to generate stable cell lines using Flp-In-293 cells. The plasma membrane was stained using AlexaFluor594 WGA and cells were visualized by confocal microscopy. A, OCTN2-reference; B, Phe17Leu; C, Leu144Phe; D, Pro549Ser.

detect SCD and other fatty acid oxidation disorders in New South Wales, Wilcken et al. (2001) anticipate a carrier frequency of 1:240 (<0.5%) for defective alleles of OCTN2 in their study population.

We did not identify any complete loss-of-function mutations in our cohort of healthy volunteers, which suggests that the carrier frequency for loss-of-function alleles of OCTN2 is lower in our study populations than can be reliably estimated in a sample of this size (i.e., less than 1% in each ethnic group included in our sample); however, several variants exhibited significant alterations in function compared with the reference sequence protein. We found two variants with significantly decreased function in heterologous expression systems: Phe17Leu and Val481Phe (Fig. 2). Persons who carry these variants may be at risk for toxicity related to insufficient renal tubular reabsorption of carnitine or to reduced clearance of substrate drugs. Recent studies have shown that heterozygous *jvs* mice, for example, have significantly reduced free carnitine and increased triglycerides in the liver (Lahjouji et al., 2002) and are at increased risk for age-associated cardiomyopathy (Xiaofei et al., 2002). Humans who are heterozygous for loss-of-function alleles of OCTN2 have also been shown to be at increased risk for late-onset cardiac hypertrophy (Koizumi et al., 1999). Thus, even partial loss of OCTN2 function may produce clinical symptoms of carnitine insufficiency. In addition, there is growing evidence for the concept of synergistic heterozygosity, in which heterozygosity for a reduced-function allele of one gene may produce a severe phenotype in the context of heterozygosity for a mutation in another gene in the same pathway (Vockley et al., 2000). This phenomenon has already been described for mutations in OCTN2 and for other genes involved in fatty acid metabolism (Vockley et al., 2000; Schuler et al., 2005). Thus, persons carrying the reduced-function OCTN2 alleles identified here may be at risk for defects in fatty acid oxidation as a result of these mutations, per se, or may be at increased risk of fatty acid oxidation defects resulting from synergism of OCTN2 mutations with mutations in other genes involved in mitochondrial fatty acid oxidation.

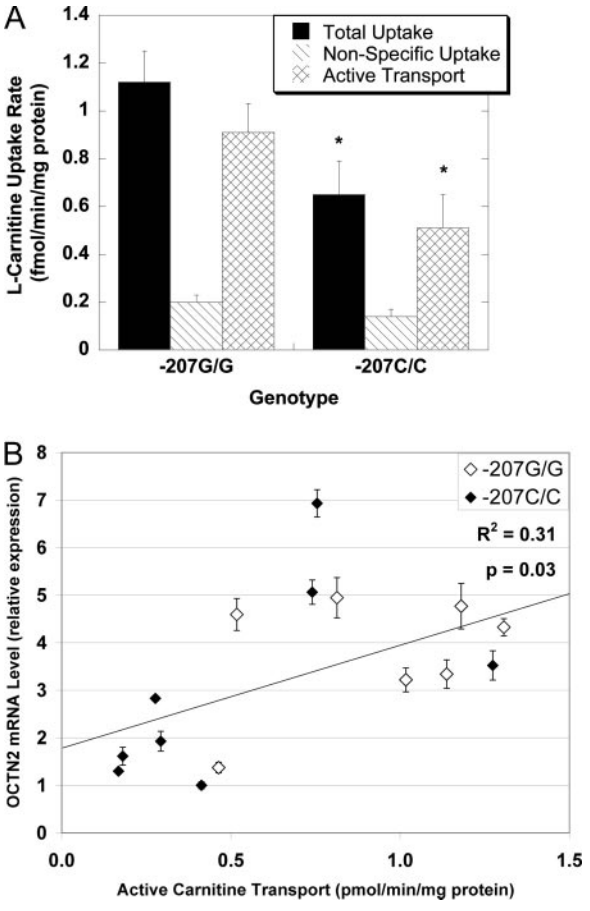

Fig. 5. Effect of OCTN2 −207G>C promoter polymorphism on L-carnitine transport and OCTN2 mRNA expression in lymphoblastoid cell lines. LCLs were derived from healthy human subjects homozygous for −207G/G or −207C/C and assayed for L-carnitine transport activity and OCTN2 mRNA expression. A, subjects homozygous for −207C/C showed significantly reduced total and specific L-carnitine transport (*, $p < 0.05$ versus −207G/G). Transport experiments were performed twice for each cell line, and the average values over two experiments were used in the final analysis. Data shown here represent the mean and standard deviation for all cell lines in each genotype group. B, correlation between OCTN2 mRNA level and transport activity in LCLs. open symbols, −207G/G; closed symbols, −207C/C.

Of particular interest in this regard is the Phe17Leu polymorphism, which was found at an allele frequency of 1.7% in the Asian American subset of our sample and showed the most marked reduction in transport activity. The impairment in L-carnitine transport activity by Phe17Leu was explained by a reduction in V_{max}, which suggests a reduction in functional expression of this variant (as contrasted with a reduced affinity for L-carnitine). Indeed, subcellular localization studies confirmed that the expression of Phe17Leu differed from OCTN2-reference, with a lower fraction of the total protein localizing to the plasma membrane and diffuse intracellular retention of the variant protein. Thus, Phe17Leu disrupts, but does not completely abolish, the normal trafficking of OCTN2 to the plasma membrane. These results suggest that Phe17 may be important for proper folding of the OCTN2 protein or for optimal interaction with proteins involved in membrane trafficking. Defects in plasma membrane sorting have recently been shown for disease-causing mutations of OCTN2 (Amat di San Filippo et al., 2006). With regard to TEA transport, Phe17Leu showed an even greater deficiency, to only 20% of OCTN2 reference activity at nonsaturating concentrations. This was explained by a reduced V_{max} for TEA transport (presumably also a result of reduced surface expression) as well as a ~2-fold increase in K_m. Thus, in addition to its effects on protein localization, this variant seems to also affect interactions of the mature transporter with particular substrates. Because this allele is particularly common in persons of Asian ancestry, Phe17Leu may contribute significantly to population variation in carnitine and organic cation transport and may be an important modifier of fatty acid oxidation disorders in this population.

We also identified one variant, Tyr449Asp, that had been previously identified in a patient suspected to be heterozygous for a carnitine transport defect and offered as an example of synergistic heterozygosity (Vockley et al., 2000). Although this mutation had previously been identified in only a single patient, the fact that the same variant was found in an unrelated individual in our cohort of healthy subjects suggests that this variant may be more common than other risk alleles for systemic carnitine deficiency. We show that Tyr449Asp influenced the substrate selectivity of OCTN2; that is, Tyr449Asp showed increased preference for TEA and

reduced activity toward L-carnitine (Fig. 2). A reduction in function with respect to L-carnitine transport is most easily explained by the fact that Tyr449 seems to be involved in sodium stimulation of L-carnitine transport (Amat di San Filippo and Longo, 2004). The degree of impairment in carnitine transport activity was less severe in our study compared with the previous report [previously, carnitine transport activity by Tyr449Asp was found to be reduced to 18% of reference OCTN2, versus 62% in the current study (Amat di San Filippo and Longo, 2004)]. Although the reduction in L-carnitine transport activity was not as great as that for Phe17Leu, even a mild reduction in OCTN2-mediated L-carnitine transport in the kidney may have physiological consequences. Because tubular reabsorption of carnitine is normally a very efficient process (>95% of filtered carnitine is reabsorbed in a single pass), even a small reduction in reabsorption could lead to significant increases in renal clearance (and thus reduced total body and plasma carnitine levels). Whereas OCTN2-mediated L-carnitine is Na^+-dependent, transport of the synthetic organic cation TEA by OCTN2 occurs via a pH-dependent, Na^+-independent mechanism. Our finding that the uptake of TEA is stimulated by Tyr449Asp suggests that Tyr449 is also important for TEA transport by OCTN2.

For variants that showed alterations in function, the degree of change in function was greater for the synthetic substrate (TEA) compared with the physiologic substrate (L-carnitine). This was true for both of the reduced-function SNPs (Phe17Leu and Val481Phe) and the selectivity variant, Tyr449Asp (Fig. 2). These findings are consistent with previous studies demonstrating that the OCTN2 protein has distinct recognition sites for TEA versus L-carnitine (Seth et al., 1999; Ohashi et al., 2002; Amat di San Filippo et al., 2003, 2004; Inano et al., 2004). This also suggests that for genes with dual functions (i.e., a physiological and a pharmacological role), mutations that disrupt the essential physiological function may be less tolerated than those that affect less essential functions, such as interactions with drugs or other xenobiotics.

Peltekova et al. (2004) demonstrated that an SNP in the promoter region of OCTN2 (−207G>C) causes reduced promoter activity as measured by response to heat-shock or treatment with arachidonic acid in reporter and gel-shift assays. This SNP occurs at particularly high allele frequency (approximately 50%) in persons of European descent. In this study, we found that this SNP predicts basal L-carnitine transport ex vivo in lymphoblastoid cell lines, in the absence of heat shock (Fig. 5). We also found that OCTN2 mRNA expression in LCLs derived from −207C/C homozygotes tended to be lower than that in −207G/G cell lines, consistent with the supposition that the effect of this SNP on transport activity is related to transcriptional activity at the OCTN2 promoter. In contrast to the previous study, we found that heat shock had no effect on mRNA expression of OCTN2 or of a positive control, MDR1, in LCLs of either genotype. The reasons for this discrepancy are not clear, though one possibility is that the functional elements required for heat shock-inducible expression of OCTN2 (and of MDR1) are constitutively active in our cell system, but not in that used by Peltekova et al. (2004) (HeLa cells and GM10665 fibroblasts). On the other hand, it is possible that the elements required for heat-shock pathways are not active in this cell system, but the −207G>C polymorphism affects basal expression of OCTN2 in the absence of heat shock. Others have investigated the effect of the −207G>C polymorphism in human cardiac muscle and have found that OCTN2 expression in this tissue was not affected by genotype (Grube et al., 2006). Thus, we conclude that the −207G>C polymorphism in the OCTN2 promoter may have variable effects on OCTN2 expression depending on cell system, tissue type, and experimental conditions. This SNP is in linkage disequilibrium with SNPs in OCTN1, a paralog of OCTN2 that may also be involved in carnitine disposition and has overlapping substrate specificity with OCTN2 (Peltekova et al., 2004; Urban et al., 2005). Thus, multipoint haplotypes comprising polymorphisms in both OCTN1 and OCTN2 may have additive or synergistic effects, as has been suggested for the OCTN1-Leu503Phe/OCTN2(-207)G>C haplotype. Persons who harbor these haplotypes together with reduced function variants identified in this study may be at risk for pathological phenotypes associated with carnitine insufficiency. Recent advances in prospective newborn screening may contribute additional information on common reduced-function alleles of OCTN2, as has been shown for other genes (Andresen et al., 2001).

Acknowledgments

We thank Dr. Xiaowei Fu for her assistance in work related to this manuscript.

References

Amat di San Filippo C, and Longo N (2004) Tyrosine residues affecting sodium stimulation of carnitine transport in the OCTN2 carnitine/organic cation transporter. *J Biol Chem* **279:**7247–7253.

Amat di San Filippo C, Pasquali M, and Longo N (2006) Pharmacological rescue of carnitine transport in primary carnitine deficiency. *Hum Mutat* **27:**513–523.

Amat di San Filippo C, Wang Y, and Longo N (2003) Functional domains in the carnitine transporter OCTN2, defective in primary carnitine deficiency. *J Biol Chem* **278:**47776–47784.

Andresen BS, Dobrowolski SF, O'Reilly L, Muenzer J, McCandless SE, Frazier DM, Udvari S, Bross P, Knudsen I, Banas R, et al. (2001) Medium-chain acyl-CoA dehydrogenase (MCAD) mutations identified by MS/MS-based prospective screening of newborns differ from those observed in patients with clinical symptoms: identification and characterization of a new, prevalent mutation that results in mild MCAD deficiency. *Am J Hum Genet* **68:**1408–1418.

Bremer J (1983) Carnitine–metabolism and functions. *Physiol Rev* **63:**1420–1480.

Grantham R (1974) Amino acid difference formula to help explain protein evolution. *Science (Wash DC)* **185:**862–864.

Grube M, Meyer zu Schwabedissen HE, Prager D, Haney J, Moritz KU, Meissner K, Rosskopf D, Eckel L, Bohm M, Jedlitschky G, et al. (2006) Uptake of cardiovascular drugs into the human heart: expression, regulation, and function of the carnitine transporter OCTN2 (SLC22A5). *Circulation* **113:**1114–1122.

Hartl DL and Clark AG (1997) *Principles of Population Genetics,* Sinauer Associates, Sunderland, MA.

Horiuchi M, Kobayashi K, Yamaguchi S, Shimizu N, Koizumi T, Nikaido H, Hayakawa J, Kuwajima M, and Saheki T (1994) Primary defect of juvenile visceral steatosis (jvs) mouse with systemic carnitine deficiency is probably in renal carnitine transport system. *Biochim Biophys Acta* **1226:**25–30.

Inano A, Sai Y, Kato Y, Tamai I, Ishiguro M, and Tsuji A (2004) Functional regions of organic cation/carnitine transporter OCTN2 (SLC22A5): roles in carnitine recognition. *Drug Metab Pharmacokinet* **19:**180–189.

Koizumi A, Nozaki J, Ohura T, Kayo T, Wada Y, Nezu J, Ohashi R, Tamai I, Shoji Y, Takada G, et al. (1999) Genetic epidemiology of the carnitine transporter OCTN2 gene in a Japanese population and phenotypic characterization in Japanese pedigrees with primary systemic carnitine deficiency. *Hum Mol Genet* **8:**2247–2254.

Kuwajima M, Kono N, Horiuchi M, Imamura Y, Ono A, Inui Y, Kawata S, Koizumi T, Hayakawa J, Saheki T, et al. (1991) Animal model of systemic carnitine deficiency: analysis in C3H-H-2 degrees strain of mouse associated with juvenile visceral steatosis. *Biochem Biophys Res Commun* **174:**1090–1094.

Lahjouji K, Elimrani I, Wu J, Mitchell GA, and Qureshi IA (2002) A heterozygote phenotype is present in the jvs +/- mutant mouse livers. *Mol Genet Metab* **76:**76–80.

Lahjouji K, Mitchell GA, and Qureshi IA (2001) Carnitine transport by organic cation transporters and systemic carnitine deficiency. *Mol Genet Metab* **73:**287–297.

Lamhonwah AM, Olpin SE, Pollitt RJ, Vianey-Saban C, Divry P, Guffon N, Besley GT, Onizuka R, De Meirleir LJ, Cvitanovic-Sojat L, et al. (2002) Novel OCTN2 mutations: no genotype-phenotype correlations: early carnitine therapy prevents cardiomyopathy. *Am J Med Genet* **111:**271–284.

Leabman MK, Huang CC, DeYoung J, Carlson EJ, Taylor TR, de la Cruz M, Johns SJ, Stryke D, Kawamoto M, Urban TJ, et al. (2003) Natural variation in human membrane transporter genes reveals evolutionary and functional constraints. *Proc Natl Acad Sci USA* **100:**5896–5901.
Nezu J, Tamai I, Oku A, Ohashi R, Yabuuchi H, Hashimoto N, Nikaido H, Sai Y, Koizumi A, Shoji Y, et al. (1999) Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. *Nat Genet* **21:**91–94.
Ohashi R, Tamai I, Inano A, Katsura M, Sai Y, Nezu J, and Tsuji A (2002) Studies on functional sites of organic cation/carnitine transporter OCTN2 (SLC22A5) using a Ser467Cys mutant protein. *J Pharmacol Exp Ther* **302:**1286–1294.
Ohashi R, Tamai I, Nezu Ji J, Nikaido H, Hashimoto N, Oku A, Sai Y, Shimane M, and Tsuji A (2001) Molecular and physiological evidence for multifunctionality of carnitine/organic cation transporter OCTN2. *Mol Pharmacol* **59:**358–366.
Peltekova VD, Wintle RF, Rubin LA, Amos CI, Huang Q, Gu X, Newman B, Van Oene M, Cescon D, Greenberg G, et al. (2004) Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat Genet* **36:**471–475.
Saito S, Iida A, Sekine A, Ogawa C, Kawauchi S, Higuchi S, and Nakamura Y (2002) Catalog of 238 variations among six human genes encoding solute carriers (hSLCs) in the Japanese population. *J Hum Genet* **47:**576–584.
Schuler AM, Gower BA, Matern D, Rinaldo P, Vockley J, and Wood PA (2005) Synergistic heterozygosity in mice with inherited enzyme deficiencies of mitochondrial fatty acid beta-oxidation. *Mol Genet Metab* **85:**7–11.
Seth P, Wu X, Huang W, Leibach FH, and Ganapathy V (1999) Mutations in novel organic cation transporter (OCTN2), an organic cation/carnitine transporter, with differential effects on the organic cation transport function and the carnitine transport function. *J Biol Chem* **274:**33388–33392.
Tajima F (1993) Statistical analysis of DNA polymorphism. *Jpn J Genet* **68:**567–595.
Tamai I, Ohashi R, Nezu J, Yabuuchi H, Oku A, Shimane M, Sai Y, and Tsuji A (1998) Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *J Biol Chem* **273:**20378–20382.
Tang NL, Ganapathy V, Wu X, Hui J, Seth P, Yuen PM, Wanders RJ, Fok TF, and Hjelm NM (1999) Mutations of OCTN2, an organic cation/carnitine transporter, lead to deficient cellular carnitine uptake in primary carnitine deficiency. *Hum Mol Genet* **8:**655–660.
Tein I (2003) Carnitine transport: pathophysiology and metabolism of known molecular defects. *J Inherit Metab Dis* **26:**147–169.
Tein I and Xie ZW (1996) The human plasmalemmal carnitine transporter defect is expressed in cultured lymphoblasts: a new non-invasive method for diagnosis. *Clin Chim Acta* **252:**201–204.
Trinh TT and Rioux JD (2005a) The promise and perils of interpreting genetic associations in Crohn's disease. *Gut* **54:**1354–1357.
Trinh TT and Rioux JD (2005b) Understanding association and causality in the genetic studies of inflammatory bowel disease. *Gastroenterology* **129:**2106–2110.
Urban TJ, Giacomini KM, and Risch N (2005) Haplotype structure and ethnic-specific allele frequencies at the OCTN locus: implications for the genetics of Crohn's disease. *Inflamm Bowel Dis* **11:**78–79.
Urban TJ, Sebro R, Hurowitz EH, Leabman MK, Badagnani I, Lagpacan LL, Risch N, and Giacomini KM (2006) Functional genomics of membrane transporters in human populations. *Genome Res* **16:**223–230.
Vockley J, Rinaldo P, Bennett MJ, Matern D, and Vladutiu GD (2000) Synergistic heterozygosity: disease resulting from multiple partial defects in one or more metabolic pathways. *Mol Genet Metab* **71:**10–18.
Wang Y, Ye J, Ganapathy V, and Longo N (1999) Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency. *Proc Natl Acad Sci USA* **96:**2356–2360.
Wilcken B, Wiley V, Sim KG, and Carpenter K (2001) Carnitine transporter defect diagnosed by newborn screening with electrospray tandem mass spectrometry. *J Pediatr* **138:**581–584.
Wu X, Huang W, Prasad PD, Seth P, Rajan DP, Leibach FH, Chen J, Conway SJ, and Ganapathy V (1999) Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. *J Pharmacol Exp Ther* **290:**1482–1492.
Wu X, Prasad PD, Leibach FH, and Ganapathy V (1998) cDNA sequence, transport function, and genomic organization of human OCTN2, a new member of the organic cation transporter family. *Biochem Biophys Res Commun* **246:**589–595.
Xiaofei E, Wada Y, Dakeishi M, Hirasawa F, Murata K, Masuda H, Sugiyama T, Nikaido H, and Koizumi A (2002) Age-associated cardiomyopathy in heterozygous carrier mice of a pathological mutation of carnitine transporter gene, OCTN2. *J Gerontol A Biol Sci Med Sci* **57:**B270–B278.

Address correspondence to: Dr. Kathleen M. Giacomini, Department of Biopharmaceutical Sciences, 1550 4th Street, RH584, Box 2911, University of California, San Francisco, CA 94143-2911. E-mail: kmg@itsa.ucsf.edu