

CPT1c Is Localized in Endoplasmic Reticulum of Neurons and Has Carnitine Palmitoyltransferase Activity*

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CPT1c is a carnitine palmitoyltransferase 1 (CPT1) isoform that is expressed only in the brain. The enzyme has recently been localized in neuron mitochondria. Although it has high sequence identity with the other two CPT1 isoenzymes (a and b), no CPT activity has been detected to date. Our results indicate that CPT1c is expressed in neurons but not in astrocytes of mouse brain sections. Overexpression of CPT1c fused to the green fluorescent protein in cultured cells demonstrates that CPT1c is localized in the endoplasmic reticulum rather than mitochondria and that the N-terminal region of CPT1c is responsible for endoplasmic reticulum protein localization. Western blot experiments with cell fractions from adult mouse brain corroborate these results. In addition, overexpression studies demonstrate that CPT1c does not participate in mitochondrial fatty acid oxidation, as would be expected from its subcellular localization. To identify the substrate of CPT1c enzyme, rat cDNA was overexpressed in neuronal PC-12 cells, and the levels of acylcarnitines were measured by high-performance liquid chromatography-mass spectrometry. Palmitoyl-carnitine was the only acylcarnitine to increase in transfected cells, which indicates that palmitoyl-CoA is the enzyme substrate and that CPT1c has CPT1 activity. Microsomal fractions of PC-12 and HEK293T cells overexpressing CPT1c protein showed a significant increase in CPT1 activity of 0.57 and 0.13 nmol·mg⁻¹·min⁻¹, respectively, which is ~50% higher than endogenous CPT1 activity. Kinetic studies demonstrate that CPT1c has similar affinity to CPT1a for both substrates but 20–300 times lower catalytic efficiency.

Carnitine palmitoyltransferase 1 (CPT1)⁴ catalyzes the conversion of long chain fatty acyl-CoAs into acylcarnitines, the

first step in the transport of long chain fatty acids from the cytoplasm to the mitochondrial matrix, where they undergo β -oxidation. This reaction is not only central to the control of fatty acid oxidation, but it also determines the availability of long chain acyl-CoA for other processes, notably the synthesis of complex lipids.

There are three different CPT1 isozymes: CPT1a (also called L-CPT1) encoded by *CPT1a*, CPT1b (also called M-CPT1) encoded by *CPT1b*, and the recently described CPT1c (also called CPT1-C) encoded by *CPT1c*. *CPT1a* and *CPT1b* have been extensively studied since they were cloned for the first time, in 1993 and 1995, respectively (1, 2). CPT1a is the most ubiquitous expressed isoform and is found not only in liver but also in pancreas, kidney, brain, blood, and embryonic tissues. CPT1b is expressed only in brown adipose tissue, muscle, and heart. Both isozymes present significantly different kinetic and regulatory properties: CPT1a displays higher affinity for its substrate carnitine and a lower affinity for the physiological inhibitor malonyl-CoA than the muscle isoform (3). In addition, the amino acid residues that are critical for catalytic activity or malonyl-CoA sensitivity have been identified for both enzymes, and three-dimensional structures have been predicted based on the carnitine acetyl transferase, carnitine octanoyl transferase, and carnitine palmitoyltransferase II crystals (4). CPT1a and CPT1b are localized in the outer mitochondrial membrane with the N and C termini facing to the cytosolic side. Western blotting and activity characterization suggested that CPT1a is also localized in microsomes, but expression studies with EGFP fused to the C terminus of CPT1a showed that CPT1a is targeted only to mitochondria and that previous detection of CPT1a in microsomes was probably derived from membrane contact sites between ER and mitochondria (5). CPT1a and CPT1b have a critical role in the heart, liver, and pancreatic β -cells and are potential targets for the treatment of metabolic disorders, including diabetes and coronary heart disease.

Less is known about CPT1c. Although the protein sequence is highly similar to that of the other two isozymes, CPT1c expressed in yeast or HEK293T cells displays no catalytic activity with common acyl-CoA esters as substrates (6, 7). One

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⁴ The abbreviations used are: CPT1, carnitine palmitoyltransferase 1; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; PBS,

phosphate-buffered saline; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; LC, liquid chromatography; ESI, electrospray ionization; MS/MS, tandem mass spectrometry.

explanation is that palmitoyl-CoA is not a substrate for CPT1c and that another brain-specific acyl-CoA might be its natural substrate. Expression studies indicate that CPT1c is localized exclusively in the central nervous system, with homogeneous distribution in all areas (hippocampus, cortex, hypothalamus, and others). The pattern resembles that of FAS, acetyl-CoA carboxylase- α (enzymes related to biosynthesis) rather than CPT1a or ACC- β (enzymes related to oxidation) (6, 8). The capacity of CPT1c to bind malonyl-CoA has been demonstrated, and it has been suggested that CPT1c regulates malonyl-CoA availability in the brain cell.

It has recently been reported that knock-out mice for CPT1c ingest less food and have a lower body weight when fed a standard diet. When these animals are fed high fat chow, body weight increases more than control animals, and they become resistant to insulin, suggesting that CPT1c is involved in energy homeostasis and control of body weight (7). Moreover, ectopic overexpression of CPT1c by stereotactic hypothalamic injection of a CPT1c adenoviral vector protects mice from adverse weight gain caused by high fat diet (9).

Herein we report that CPT1c is localized in neurons but not in astrocytes of adult brain. We also demonstrate that CPT1c is localized in the ER of the cells and not in mitochondria, and that CPT1c shows carnitine palmitoyltransferase activity.

EXPERIMENTAL PROCEDURES

Culture of PC-12, SHSY5Y, Fibroblasts, and HEK293T Cells

The human neuroblastoma cell line, SHSY5Y, the human embryonic kidney-derived cell line, HEK293T, and human fibroblasts cells were grown at 37 °C in the presence of 5% CO₂ in Dulbecco's modified Eagle's medium with high glucose containing 2 mM glutamine, 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). Medium for PC-12 cells was Dulbecco's modified Eagle's medium with high glucose containing 2 mM glutamine, sodium pyruvate, 5% fetal calf serum, 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml).

Cells cultured in 24-wells plates were transfected with 0.8 μ g of plasmid (purified with the Qiagen Maxi Prep Kit) using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. Transfection efficiency was ~30–50%.

Plasmid Constructions

For pCPT1c-EGFP and pCPT1a-EGFP, rat CPT1c cDNA was obtained by reverse transcription-PCR performed with 2 μ g of total rat brain RNA. The 2700-bp fragment amplified was cloned in pBluescript and sequenced. pEGFP-N3 vector (from Clontech, BD Biosciences) was used to clone the coding region of CPT1c or CPT1a, to create pCPT1c-EGFP and pCPT1a-EGFP, respectively. pCPT1c-EGFP and pCPT1a-EGFP plasmids encode CPT1c and CPT1a proteins fused to the N-terminal region of EGFP, respectively.

Chimera Constructions

pCPT1ac-EGFP—460 bp of the 5' coding sequence of rat CPT1a gene was PCR-amplified with primers that created an HindIII site and an HpaI site at the ends of the amplified frag-

ment. This PCR product was cloned into a pCPT1c-EGFP plasmid previously digested by HindIII and HpaI (which deleted the 460 bp of the 5' terminus of CPT1c coding sequence). The resulting plasmid encodes a fused protein constituted by the N terminus and the two transmembrane domains of CPT1a, the catalytic domain of CPT1c, and EGFP.

pCPTca-EGFP—A segment of the first 462 bp of rat CPT1c gene was PCR-amplified with two primers that created a HindIII site a PpuMI site at the ends of the amplified fragment. This PCR product was digested and cloned into a pCPT1a-EGFP plasmid, previously digested by HindIII and PpuMI (which deleted the 460 bp of the 5' terminus of CPT1c coding sequence). The resulting vector contained the N terminus, the two transmembrane domains of CPT1c, and the catalytic domain of CPT1a fused to EGFP.

pIRES-CPT1a and pIRES-CPT1c—The coding regions of rat CPT1a and CPT1c were cloned in vector pIRES2-EGFP (Clontech, BD Biosciences), which permits both the gene of interest and the EGFP gene to be translated from a single bicistronic mRNA.

Co-localization Studies in Brain Sections

For co-localization studies we performed combined *in situ* hybridization/immunocytochemistry or double immunofluorescence, using standard protocols.

For combined *in situ* hybridization, coronal sections (30 μ m) from adult mouse forebrains were used. Processed sections were hybridized overnight at 56 °C, with *cpt1c* Riboprobes (full rat cDNA) labeled with digoxigenin-d-UTP (Roche Applied Science) at a concentration of 500 ng/ml. After stringent washing, sections were incubated at 4 °C overnight with an anti-DIG antibody (1/2000) conjugated to alkaline phosphatase (Roche Applied Science) and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Invitrogen). Tissue sections were mounted on gelatinized slides with Mowiol. Those sections that were hybridized with control sense Riboprobes did not give any hybridization signal.

After *in situ* hybridization, some slices were collected and processed by immunofluorescence. The primary antibody was mouse anti-NeuN (1:75, Chemicon). The secondary antibody was biotinylated (Vector Laboratories, Inc., Burlingame, CA). The streptavidin-horseradish peroxidase complex was from Amersham Biosciences. Sections were developed with 0.03% diaminobenzidine and 0.003% hydrogen peroxide, mounted onto slides, and dehydrated, and coverslips were added with synthetic resin.

In double immunofluorescence experiments, sections obtained as indicated above were incubated with primary antibodies against glial fibrillary acidic protein (1/500, Chemicon MAB360) and CPT1c (1/100) overnight at 4 °C in the same blocking solution. The sections were washed three times in PBS (0.1 M) and incubated for 2 h with secondary antibodies coupled to fluorochromes Alexa 488 (for green fluorescence) and Alexa 568 (for red fluorescence) at a dilution of 1/500. Sections were mounted with Mowiol and observed using a confocal Leica TCS SP2 microscope (Leica Lasertechnik GmbH, Mannheim, Germany). Images were saved in TIFF format and analyzed using Adobe Photoshop 3.0.

CPT1c Location and Activity

Co-localization Studies in Culture Cells

Cultured cells were grown on lysine-treated coverslips in 24-well plates. Co-localization studies were performed 48 h after transfection with plasmids containing *CPT1c* or *CPT1a* fused to the 5'-end of EGFP. To visualize the ER, cells were washed twice in PBS (10 mM), fixed with 3% paraformaldehyde in 100 mM phosphate buffer and 60 mM sucrose for 15 min at room temperature, and then washed twice in PBS. Cells were permeabilized with 1% (w/v) of Triton X-100 in PBS and 20 mM glycine for 10 min at room temperature and then washed twice in PBS. Nonspecific binding of antibody was blocked by incubation with 1% (w/v) BSA in PBS with glycine 20 mM at room temperature for 30 min. Cells were then incubated with mouse anti-calnexin polyclonal antibody (BD Biosciences, 1:50 in 1% (w/v) BSA/PBS/20 mM glycine/0.2% Triton X-100) for 1 h at 37 °C. After washing twice in PBS/20 mM glycine, cells were incubated with goat anti-mouse Alexafluor 546 (Molecular Probes, 1:500 in 1% (w/v) BSA/PBS/20 mM glycine/0.2% Triton X-100) for 1 h at 37 °C, and then washed twice in PBS. Coverslips were mounted on glass slides with Mowiol. Mitochondria were visualized by incubating cells with 500 nM MitoTracker Orange CM-H2TMRos (Molecular Probes) in complete medium for 30 min, followed by 30 min in complete medium without MitoTracker, after which they were fixed as mentioned above.

Fluorescent staining patterns were visualized by using a fluorescence microscope (Leica). The captured images were processed using Adobe Photoshop 5.0.

RNA Extraction and Real-time PCR Conditions

RNA was extracted from cells by the TRIzol method (Invitrogen) and quantified spectrophotometrically. 2 μ g of total RNA was incubated with DNase and reverse transcribed by Superscript III (Invitrogen) following the manufacturer's conditions. 2 μ l of reaction was used in the real-time PCR amplification with TaqMan and primers designed by Applied Biosystems, following the manufacturer's conditions. An 18 S expression assay was used to normalize the samples.

Lipid Extraction

Cells were washed in cold PBS buffer and gently collected with a pipette. They were then centrifuged at $700 \times g$ for 5 min at 4 °C and washed in PBS. 20 μ l of samples was taken for Bradford protein assay. After that, 200 μ l of 0.2 M NaCl was added to the pellet, and the mixture was immediately frozen in liquid N₂. To separate aqueous and lipid phases, 750 μ l of Folch reagent (chloroform:methanol, 2:1) and 50 μ l of 0.1 M KOH were added, and, after vigorous vortex mixing, the phases were separated by 15-min centrifugation at $2000 \times g$ at 4 °C. The top aqueous phase was removed, and the lipid phase was washed in 200 μ l of methanol/water/chloroform (48:47:3). After vortex mixing, centrifugation was performed at $700 \times g$ for 5 min at 4 °C, and the lower phase (lipid extract) was dried.

Quantification of Acylcarnitines by HPLC

Acylcarnitines were analyzed via an LC-ESI-MS/MS System (API 3000 PE Sciex) in positive ionization mode as described in

a previous study (10). Quantification was done through multiple reaction monitoring experiments using the isotope dilution method with deuterated palmitoylcarnitine as internal standard (200 ng·ml⁻¹). 10 μ l of sample was injected in the LC-ESI-MS/MS system. Multiple reaction monitoring transitions were as follows: 400.4/85.2 for quantification of palmitoylcarnitine, 400.4/341.4 for confirmation of palmitoylcarnitine, and 403.4/85.2 for quantification of *d*₂-palmitoylcarnitine. The method was linear over the range from 2 to 2000 ng·ml⁻¹. The limit of detection and the limit of quantification were 0.14 ng·ml⁻¹ (0.35 nmol·liter⁻¹) and 0.48 ng·ml⁻¹ (1.2 nmol·liter⁻¹), respectively (in standard solutions).

Microsome Purification

Cells were recovered by centrifugation at $1200 \times g$ for 5 min at 4 °C, washed in 1.5 ml of PBS, and re-suspended in 2 ml of lysis buffer (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, supplemented with 1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 10 ng/ml leupeptin, and 100 ng/ml pepstatin). Cells were disrupted by Dounce homogenization (30 pulses with loose pestle and 30 pulses with tight pestle). Homogenates were centrifuged at $2,000 \times g$ for 3 min at 4 °C to remove cell debris. This crude extract was further centrifuged at $10,000 \times g$ for 30 min at 4 °C to remove the mitochondrial fraction. Supernatant was centrifuged at $10,000 \times g$ for 1 h at 4 °C to sediment the microsomal fraction. Pellets were immediately used in the carnitine palmitoyltransferase activity assay.

CPT1 Activity

Radiometric Method—Carnitine acyltransferase activity was determined by the radiometric method as previously described (11). The substrates were palmitoyl-CoA and L-[methyl-³H]carnitine. Enzyme activity was assayed for 4 min at 30 °C in a total volume of 200 μ l. The protein sample, 40 μ l (20 μ g), was preincubated for 1 min, and then 160 μ l of the reaction mixture was added. The final concentrations were 105 mM Tris-HCl (pH 7.2), 2 mM KCN, 15 mM KCl, 4 mM MgCl₂, 4 mM ATP, 250 μ M reduced glutathione, 50 μ M palmitoyl-CoA, 400 μ M L-[methyl-³H]carnitine (0.3 μ Ci), and 0.1% defatted bovine albumin. Reactions were stopped by the addition of 200 μ l of HCl 1.2 N, and the product acyl-L-[methyl-³H]carnitine was extracted with water-saturated *n*-butanol. Values were estimated by analyzing the data from three experiments performed in triplicate. All protein concentrations were determined using the Bio-Rad protein assay with bovine albumin as standard.

Chromatographic Method—The same procedure used previously (11) was followed except that all carnitine used was cold (not radioactive). In addition, acylcarnitines extracted with water-saturated *n*-butanol were analyzed by an LC-ESI-MS/MS system, as described above.

Western Blot Experiments

A polyclonal rabbit antibody against the last 15 residues (796–810) of mouse CPT1c was developed following the indications in a previous study (7), by Sigma-Genosys. The specificity of the antibody was determined by enzyme-linked immunosorbent assay and Western blot experiments. For CPT1a detection, a polyclonal antibody against amino acids 317–430

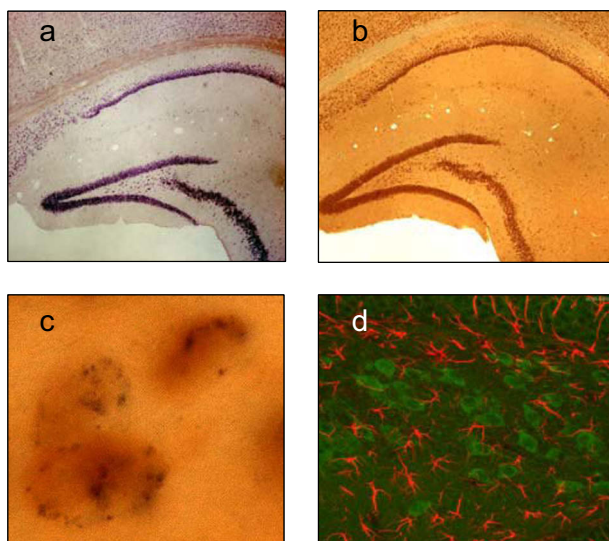


FIGURE 1. Co-localization studies of CPT1c mRNA with NeuN and glial fibrillary acidic protein in brain sections. Brain sections were processed using *in situ* hybridization with CPT1c antisense Riboprobe (a) or immunocytochemistry with NeuN primary antibodies and biotinylated secondary antibodies (b) or both methods (c). Mouse adult brain sections were processed by double immunocytochemistry with CPT1c antibodies (green stain) and glial fibrillary acidic protein (molecular marker of astrocytes, red stain) (d).

of rat-CPT1a (12) was used. Generally, 60 μ g of protein extracts was subjected to SDS-PAGE. A 1:2000 dilution of anti-CPT1c was used as primary antibody. The secondary antibody was used at 1:5000 dilution. The blots were developed with the ECL Western blotting system from Amersham Biosciences.

Palmitate Oxidation

Palmitate oxidation to CO_2 and acid-soluble products were measured in PC-12 cells 48 h after transfection. On the day of the assay, cells were washed in Krebs-Ringer bicarbonate/Hepes buffer (KRBH)/0.1% BSA, preincubated at 37 $^\circ\text{C}$ for 30 min in KRBH/1% BSA, and washed again. Cells were incubated for 2 h at 37 $^\circ\text{C}$ with fresh KRBH containing 2.5 mM glucose, 0.8 mM carnitine, 0.25 mM palmitate, and 1 $\mu\text{Ci/ml}$ [$1\text{-}^{14}\text{C}$]palmitate bound to 1% BSA. Oxidation measurements were performed as previously described (13).

RESULTS

CPT1c Cell Type Localization

To identify the types of brain cell in which CPT1c is expressed, co-localization studies with NeuN (a nuclear neuronal marker), or glial fibrillary acidic protein (an astrocyte marker), antibodies were performed in adult mouse brain sections. Fig. 1 shows co-labeling of CPT1c mRNA, as revealed by *in situ* hybridization studies, with NeuN. This pattern confirms that CPT1c is expressed mainly in neurons. In addition, no co-localization was detected between CPT1c and glial fibrillary acidic protein (double immunohistochemistry) (Fig. 1d), indicating that CPT1c is not present in brain astrocytes.

CPT1c Subcellular Localization

CPT1c Is Localized in ER of Cultured Cells—To study the intracellular localization of CPT1c, fibroblasts were transiently transfected with pCPT1a-EGFP or pCPT1c-EGFP, which

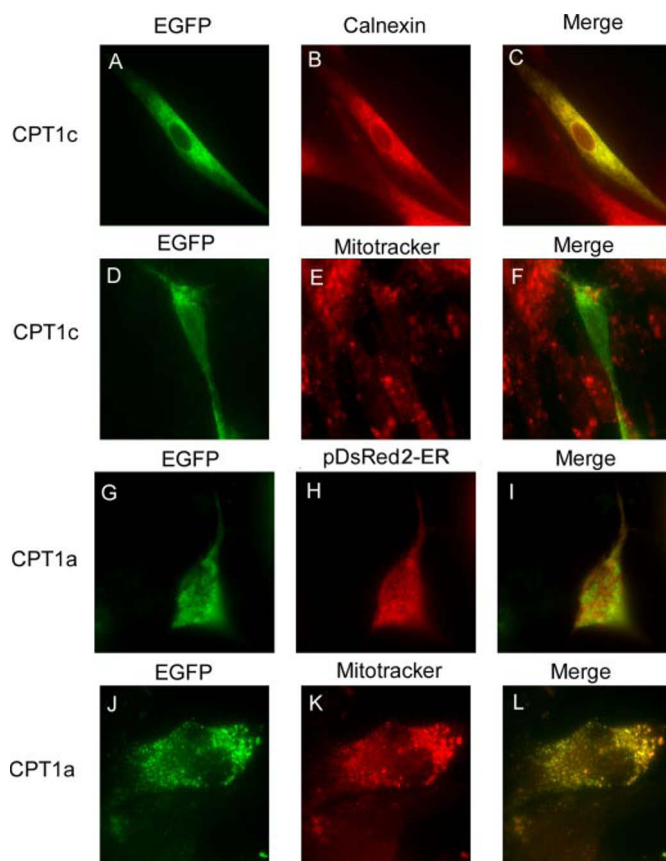


FIGURE 2. Co-localization studies of CPT1c in mitochondria and ER. Fibroblasts were transfected with pCPT1c-EGFP (A–F) or pCPT1a-EGFP (G–L) and incubated with anti-calnexin as primary antibody (B) or stained by MitoTracker (E and K) or co-transfected with pDsRed2-ER (H). Images were taken by confocal microscopy with a filter to see green emission, red emission, or the merged image (C, F, I, and L).

encode CPT1a or CPT1c proteins, respectively, fused at their C-terminal end to EGFP. 48–52 h after transfection, the fluorescence pattern shown by CPT1a-EGFP (which was expressed in a punctuate manner) was different from that of CPT1c-EGFP (which was expressed in a reticular manner). Co-localization studies were performed with MitoTracker, a potential-sensitive dye that accumulates in mitochondria, and with anti-calnexin, an ER integral protein. In some experiments cells were co-transfected with pDsRed2-ER (Clontech, Takara BioEurope, SAS), a subcellular localization vector that stains the ER red. Fig. 2 clearly shows that CPT1c is localized in the ER membrane, but not in mitochondria. In contrast, CPT1a is localized in mitochondria, as previously described in other cells (5). The slight co-localization of CPT1a with the product of pDsRed2-ER may be due to the contacts between the ER membrane and the mitochondrial outer membrane, labeled as mitochondrial-associated membranes. To assess whether either isoform is localized in peroxisomes, other organelles implicated in fatty acid oxidation, co-localization studies were performed with anti-PMP70, a peroxisomal membrane protein. No major co-localization was observed between PMP70 and CPT1c or CPT1a. The slight co-localization of CPT1c with PM70 may be due to a residual localization of this protein in peroxisomes (Fig. 3). The same experiments were performed with SH-SY5Y cells, PC-12 cells, and HEK293T cells with same results.

CPT1c Location and Activity

CPT1c Is Localized in Microsomal Fraction of Adult Mouse Brain—To eliminate the possibility that overexpression experiments in cultured cells could modify the subcellular localization of CPT1c, we performed Western blot experiments with different cellular fractions of some adult mouse tissues. CPT1c was only present in brain tissue and absent in any other tissues analyzed (Fig. 4). In addition, CPT1c was localized in the microsomal fraction of brain (Fig. 4). Only some levels of CPT1c protein were present in brain mitochondria, probably by residual contamination from microsomes. The same membranes, once de-hybridized, were used with CPT1a antibodies, as a positive control for mitochondria. CPT1a was present at high levels in mitochondria from liver and kidney, and some residual levels were found in the microsomal fraction of all tissues examined.

The N-terminal Region of the Protein Is Responsible for CPT1c-specific Subcellular Localization—We aimed to test whether the N-terminal end of CPT1c was responsible for the ER localization. We made new chimeric plasmid constructions in which 460 bp of the 5' end of *CPT1a* gene (which encodes the two trans-membrane domains) and the mitochondrial import signal described by the Prip-Buus group (14) was replaced by the 5' end of *CPT1c*, and *vice versa* (see scheme in Fig. 5). The recombinant plasmids were called pCPT1c-EGFP and pCPT1a-EGFP, respectively. SY-SH5Y cells transiently transfected with those constructions showed that CPT1c-EGFP

was localized in ER, and that CPT1a-EGFP was localized in mitochondria, indicating that exchange of N-terminal ends between the two CPT1 isoforms swapped the intracellular localization of recombinant chimeric proteins (Fig. 5). These results demonstrate that the N-terminal end of CPT1c lacks the mitochondrial import signal present in CPT1a and contains a putative microsomal targeting signal responsible for ER localization.

CPT1c Does Not Participate in Fatty Acid Oxidation—To examine whether CPT1c participates in mitochondrial fatty acid oxidation, we measured increases in CO₂ in PC-12 cells overexpressing CPT1c. As expected by its subcellular localization, CPT1c did not increase fatty acid oxidation, whereas CPT1a did (Table 1).

CPT1c Substrate Identification

To identify the substrate of CPT1c, we overexpressed the enzyme in PC-12 cells and attempted to identify any increased acylcarnitine species present in the lipid cell extract, 48 h after transient transfection. PC-12 cells were easily transfected with Lipofectamine (Invitrogen) or Metafecten (Biontex, Germany) with transfection efficiencies of ~40–70% of total PC-12 cells, as measured by the fluorescence in a cell-counter FACS Scan. PC-12 cells were transfected with pIRES-CPT1c, pIRES-CPT1a, or empty pIRES. Western blot experiments showed a 5- to 10-fold increase in CPT1c and CPT1a levels in transfected cells. The lipid fraction of transfected cells was extracted, and the levels of acylcarnitines were measured. To quantify acylcarnitines, we used a new HPLC-MS/MS method where no derivatization or ionic-pair chromatography is needed (10). Precursor ion scan of *m/z* 85 experiment allows the identification of all acylcarnitines present in the sample. Areas below chromatographic peaks (chromatograms acquired in multiple reaction monitoring mode) were measured for all acylcarnitines detected. Fig. 6 shows relative areas from chromatographic peaks present in overexpressing cells compared with control (cells transfected with empty expression vector). Cells transfected with pIRES-CPT1c showed an increase of >2-fold in palmitoylcarnitine levels (Fig. 6). No other acylcarnitine was significantly increased. Cells transfected with pIRES-CPT1a (positive control) showed a 5-fold increase in palmitoylcarnitine levels and a 2- to 3-fold increase in other long chain acylcarnitines. The Wilcoxon statistic test (a non-parametric test for two paired samples) between CPT1c-transfected cells and control cells indicated that only palmitoylcarnitine levels increased significantly in CPT1c-transfected cells. These results indicate that CPT1c has carnitine palmitoyltransferase activity and that palmitoyl-CoA is a substrate for the CPT1c isoenzyme.

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CPT1c Activity

Once palmitoyl-CoA had been identified as a CPT1c substrate, we compared CPT1 activity in isolated microsomal fractions of PC-12 and

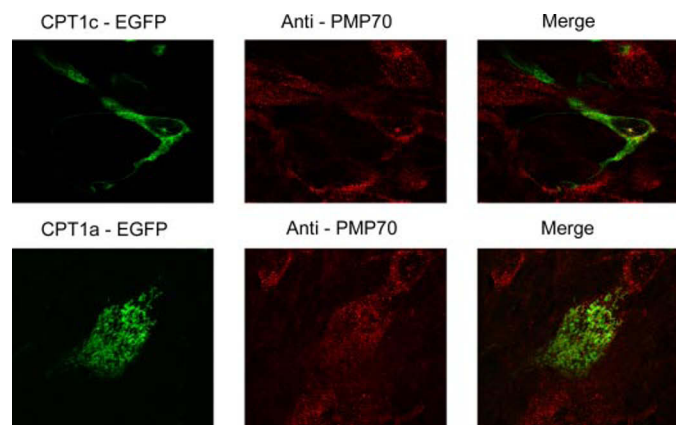


FIGURE 3. Co-localization studies of CPT1c in peroxisomes. HEK293T cells transfected by pCPT1c-EGFP or pCPT1a-EGFP were incubated with anti-PMP70 as primary antibody. Images were taken by confocal microscopy with a filter to see green emission, red emission, or the merged image.

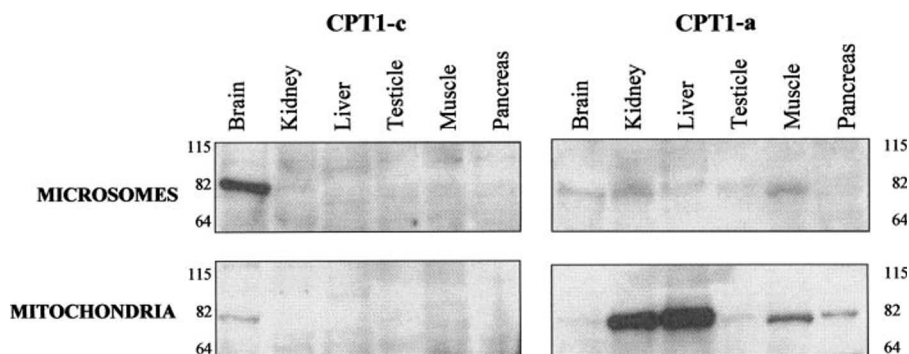


FIGURE 4. Western blot of CPT1c in mitochondrial and ER cell fractions from different tissues of adult mouse. 60 μ g of protein cell fraction was run in each line. The same membranes were incubated with anti-CPT1c and anti-CPT1a antibodies.

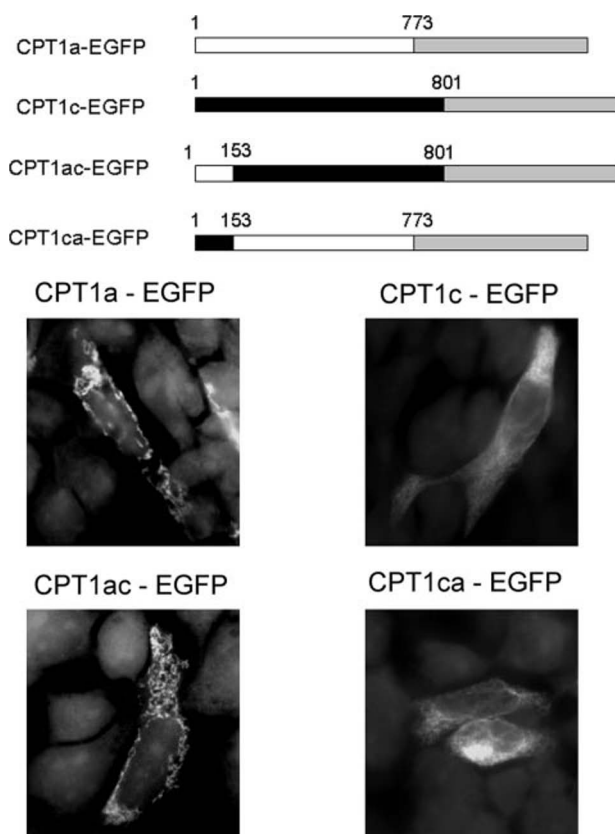


FIGURE 5. Subcellular localization of fused proteins CPT1a-EGFP, CPT1c-EGFP, CPT1ac-EGFP, and CPT1ca-EGFP in cultured cells. Top, schematic representation of fusion proteins. CPT1a coding region is represented in white, CPT1c coding region in black, and EGFP coding region in gray. Bottom, SH-SY5Y human neuroblastoma cells were transfected with recombinant plasmids. 48 h after transfection, cells were visualized in a fluorescence microscope using a 100 \times objective. CPT1a-EGFP and CPT1ac-EGFP have mitochondrial localization (punctate pattern). CPT1c-EGFP and CPT1ca-EGFP present an ER localization (reticular pattern).

TABLE 1

Palmitate oxidation in PC-12 cells overexpressing CPT1c

48 h after transfection of cultured cells with pIRES-CPT1c, pIRES-CPT1a, or empty pIRES, cells were incubated for 2 h with [14 C]palmitate. Palmitate oxidation to CO_2 was determined. Data are presented as the mean \pm S.E. of three independent experiments. Data for CPT1a are significantly different from control cells ($p < 0.05$).

	[14 C] CO_2 production
	nmol/mg/h
Empty pIRES	6.1 \pm 0.9
pIRES-CPT1c	5.7 \pm 0.7
pIRES-CPT1a	9.2 \pm 2.1

HEK293T cells transfected with pIRES-CPT1c with the activity in fractions transfected with empty pIRES vector. CPT1c was overexpressed >10 -fold, and the protein was found mainly in the microsomal fraction (Fig. 7A). Western blot membrane was reprobed with mouse anti-CPT1a antibodies to determine the residual CPT1a protein present in the microsomal fraction of PC-12 cells (Fig. 7B), which is responsible for the endogenous activity in microsomes of control cells. The same antibodies could not be used in HEK293T cells, because they do not recognize the human CPT1a protein. Palmitoylcarnitine formed in the CPT1 assay was measured by the same HPLC-MS/MS method used to identify the substrate (10). Microsomes from CPT1c-transfected cells showed a 50% increase in CPT1 activ-

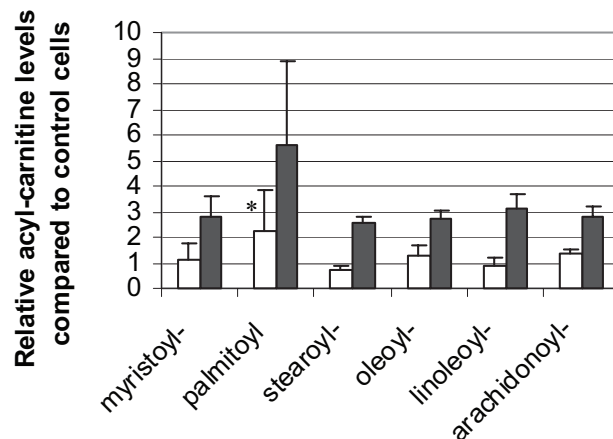


FIGURE 6. Relative levels of different acyl-carnitines in PC12 cells. PC-12 cells were transfected with empty pIRES vector (control cells), or pIRES-CPT1c (white columns), or pIRES-CPT1a (black columns). 48 h after transfection lipid extracts were obtained, and acylcarnitines were determined by HPLC-mass spectrum chromatography. The y-axis represents the area below the chromatographic peak compared with control cells. These values represent the mean of three independent experiments except for palmitoylcarnitine, which represents the mean of six independent experiments. *, $p < 0.05$ versus control cells. The amounts of palmitoylcarnitine, myristoylcarnitine, and arachidonoylcarnitine in control cells were 0.5 ± 0.2 , 0.2 ± 0.1 , and 0.02 ± 0.01 nmol/mg, respectively. For oleoylcarnitine and linoleoylcarnitine, only the chromatographic peak was measured.

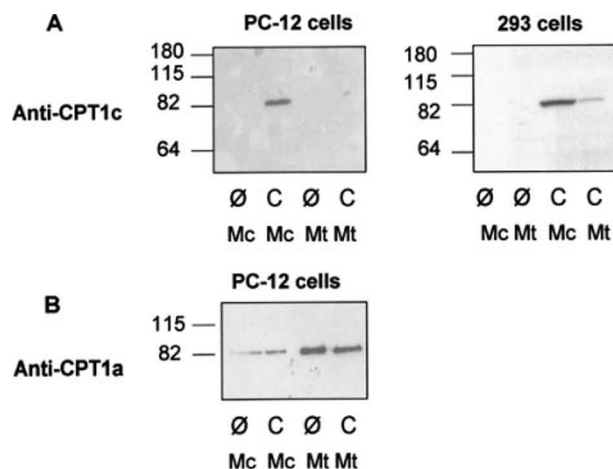


FIGURE 7. Western blot of transfected PC-12 and HEK293T cells. Cells were transfected with pIRES-CPT1c (C), or empty pIRES (Ø). 40 μ g of microsomes (mc) or mitochondria (mt) were run in each lane of SDS-acrylamide gel. A, anti-ratCPT1c antibody; B, anti-rat-CPT1a antibody.

ity compared with control cells (endogenous activity) (Table 2). K_m and V_{max} values for both substrates were calculated (Fig. 8 and Table 3). K_m values were similar to those of CPT1a (25), whereas V_{max} values were 66 times lower than those of CPT1a (25). For example, CPT1c catalytic efficiencies for palmitoyl-CoA and carnitine were 320 and 25 times lower, respectively, than those of CPT1a. CPT1 sensitivity to malonyl-CoA was not measured in cultured transfected cells, because CPT1c activity was too low and the microsomal fraction always retained residual CPT1a activity that masked any inhibitory effect of malonyl-CoA.

DISCUSSION

The presence of a third CPT1 isoform, CPT1c, in the mammalian brain is intriguing. It might show specific expression

TABLE 2

Carnitine palmitoyl transferase activity in PC-12 and HEK293T cells

Cells were transfected with pIRES-CPT1c or empty vector pIRES (control cells). 48 h after transfection, cells were collected and 40 mg of microsomal fraction was assayed for CPT1 activity. The palmitoylcarnitine formed in the assay was determined by HPLC-mass chromatography. Activity is presented as the mean \pm S.E. Wilcoxon test for non-parametric paired samples was used. n = number of experiments. Absolute and percent increases in CPT1c activity are compared to control cells.

Cells	Plasmid transfection	n	Activity	p	Absolute increase	Percent increase
			nmol palmitoylcarnitine/ mg/min			%
PC12	Control	7	1.37 \pm 0.81	<0.05		
	CPT1c	7	1.94 \pm 0.96	<0.05	0.57	41.6
293	Control	9	0.22 \pm 0.11	<0.05		
	CPT1c	9	0.35 \pm 0.18	<0.05	0.13	59

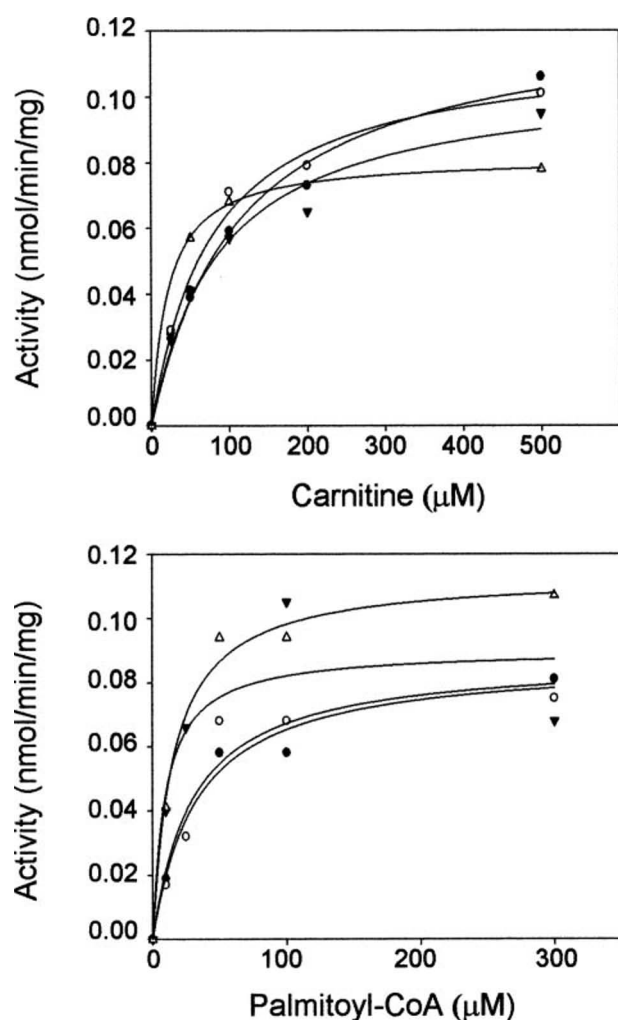


FIGURE 8. Kinetic analysis of CPT1c overexpressed in PC-12 cells. 20 μ g of microsomes was incubated at increasing concentrations of carnitine (upper) and palmitoyl-CoA (lower), and CPT1 activity was measured.

patterns, cellular localization, or biochemical properties that would make it different enough from the other two isoforms to explain its occurrence. The data we report here on the peculiarities of CPT1c may provide clues to its cellular function.

CPT1c is expressed only in the mammalian brain. The other CPT1 isoforms are expressed in other tissues and are present in other organisms like birds, fishes, reptiles, amphibians, or insects. This suggests that CPT1c has a specific function in

TABLE 3

Kinetic parameters of CPT1c overexpressed in PC-12 cells

Microsomes of PC-12 cells overexpressing CPT1c were assayed for activity with different palmitoyl-CoA and carnitine concentrations to calculate K_m and V_{max} values for both substrates. The results are the mean \pm S.D. of three experiments. CPT1a kinetic parameters were obtained from a previous study (25).

Parameter	Isoform	
	CPT1c	CPT1a
K_m palmitoyl-CoA (mM)	25.35 \pm 7.77	4.9 \pm 0.3
V_{max} palmitoyl-CoA (nmol/min/mg)	0.095 \pm 0.012	6.3 \pm 0.4
Catalytic efficiency palmitoyl-CoA (V_{max}/K_m)	0.004	1.28
K_m carnitine (mM)	58.53 \pm 21.31	127.0 \pm 4.5
V_{max} carnitine (nmol/min/mg)	0.090 \pm 0.010	6.6 \pm 0.8
Catalytic efficiency carnitine (V_{max}/K_m)	0.002	0.05

more evolved brains. Price *et al.* (6) showed that CPT1c is expressed in all regions of brain, in a similar pattern to that shown by neurons. Dai *et al.*, have recently demonstrated that CPT1c is localized to neurons of the central nervous system (9). Our results confirm these findings and demonstrate that CPT1c is not expressed in astrocytes, suggesting that CPT1c function is specific to neurons.

The notion that CPT1c is localized in mitochondria stems from an observation of CPT1c protein in mitochondrial fraction of cells (6) and from co-localization studies with MitoTracker in GT1-7 hypothalamic cells (9). In the first study (6), CPT1c was also found in the microsomal fraction, as revealed by Western blot experiments, although the authors attributed this to contamination problems in cellular fractioning process. In the second study (9) the authors conclude that CPT1c co-localizes with MitoTracker, although the images did not show perfect matching and co-localization studies were not performed with any ER marker. In contrast, subcellular localization studies performed by our group in cultured cells and also in adult brain clearly demonstrate that CPT1c is localized in the ER, not in mitochondria. These results indicate that CPT1c has a different metabolic function than CPT1a or CPT1b, which is other than facilitating the import of long chain fatty acid into mitochondria or peroxisomes to undergo β -oxidation, as demonstrated in palmitate oxidation experiments. Localization of CPT1c in the ER implicates it in a biosynthetic rather than a catabolic pathway.

Intracellular localization experiments with chimeric proteins indicate that the N-terminal region of CPT1c, which includes the two transmembrane domains, is responsible for ER-specific localization. These results complement previous studies in CPT1a protein (14). Prip-Buus and colleagues demonstrate that a region just downstream of the second transmembrane domain (residues 123–147) is important for mitochondrial transport of CPT1a. Amino acid sequence comparison between CPT1a and CPT1c demonstrates that the putative mitochondrial transport sequence is partially altered in CPT1c, with fewer positively charged amino acids (one charged residue *versus* four). In addition, the second transmembrane domain is longer in CPT1c than in the other two isoforms, which may enable it to sort proteins to the ER rather than to mitochondrial outer membrane (15).

Previous studies (6, 7) had shown that CPT1c had no enzyme activity in yeast or HEK293T cells with palmitoyl-CoA or other acyl-CoA molecules as substrate. This indicated that the CPT1c

substrate could be a rare acyl-CoA specific to the brain. We thus attempted to measure variations in all acylcarnitine levels in neural cells overexpressing CPT1c. We found that palmitoylcarnitine was the only product that was increased, indicating that palmitoyl-CoA is the preferred acyl-CoA substrate for CPT1c. Activity measurements in microsomal fractions from PC-12 and HKE293T cells confirmed that CPT1c has carnitine palmitoyltransferase activity. The failure of other authors (6, 7) to detect CPT1c activity has two possible explanations: 1) they used mitochondrial fractions instead of microsomal and 2) they used a radiometric assay instead of a chromatographic method. The HPLC-MS/MS method produces reliable and accurate measurements of palmitoylcarnitine concentrations in biological samples with a sensitivity limit of 0.48 ng/ml, which corresponds to a specific activity of $0.0045 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ in our CPT1 assay conditions (10). The limit of sensitivity of the radiometric assay, calculated as the standard deviation of ten blank points with a signal-to-noise ratio of 3, corresponds to specific activity of $0.4 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. This indicates that the chromatographic method is 100 times more sensitive than the radiometric, as described elsewhere (10). Recently, other authors have also measured CPT1 activity by a tandem mass spectrometry method because of its accuracy and sensitivity (16).

CPT1c has 100 times lower specific activity than CPT1a and CPT1b. One explanation is that CPT1c participates in a biosynthetic pathway, facilitating the constant transport of palmitate across the ER membrane, rather than in a highly active catabolic pathway such as fatty acid oxidation. Another explanation is that CPT1c acts as a metabolic sensor. CPT1c may have low activity in standard or optimal conditions (assay conditions), but its activity increases in certain situations (stress, presence of signal molecules, and others).

Lane and co-workers (7) conclude that hypothalamic CPT1c has a role in energy homeostasis and the control of food ingestion. In addition to this localized function, the wide distribution of the protein in the brain suggests a more general, ubiquitous function, perhaps related with the equilibrium between acyl-CoA pools in the cytosol and the ER lumen. Although it is not known whether CPT2 is present in ER of neurons, we hypothesize that CPT1c facilitates the entry of palmitoyl-CoA to the ER lumen. It has been reported that palmitoyl-CoA cannot cross the ER membrane, although palmitoylcarnitine can (17–20). CPT1a or CPT1b, probably localized in mitochondria-endoplasmic reticulum connections (mitochondrial-associated membrane) (21) may facilitate the entry of palmitoyl-CoA to the reticulum. In the brain, however, fatty acids are not usually oxidized, and levels of CPT1a or CPT1b are low or nonexistent. Thus the occurrence of a specific CPT1c localized in the ER membrane may ensure the entry of palmitoyl-CoA to the lumen of ER. Another possibility is that CPT1c modulates the palmitoyl-CoA pool associated with the ER, thus regulating the synthesis of ceramide and sphingolipids, which are impor-

tant for signal transduction, modification of neuronal membranes, and brain plasticity (22–24).

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