The liver isoform of carnitine palmitoyltransferase 1 is not targeted to the endoplasmic reticulum

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Liver microsomal fractions contain a malonyl-CoA-inhibitable carnitine acyltransferase (CAT) activity. It has been proposed [Fraser, Corstorphine, Price and Zammit (1999) FEBS Lett. **446**, 69–74] that this microsomal CAT activity is due to the liver form of carnitine palmitoyltransferase 1 (L-CPT_1) being targeted to the endoplasmic reticulum (ER) membrane as well as to mitochondria, possibly by an N-terminal signal sequence [Cohen, Guillerault, Girard and Prip-Buus (2001) J. Biol. Chem. **276**, 5403–5411]. COS-1 cells were transiently transfected to express a fusion protein in which enhanced green fluorescent protein was fused to the C-terminus of L-CPT_1. Confocal microscopy showed that this fusion protein was localized to mitochondria, and possibly to peroxisomes, but not to the ER. cDNAs corresponding to truncated (amino acids 1–328) or full-length L-CPT_1 were transcribed and translated in the presence of canine pancreatic microsomes. However, there was no evidence of authentic insertion of CPT_1 into the ER membrane. Rat liver microsomal fractions purified by sucrose-density-gradient centrifugation contained an 88 kDa protein (p88) which was recognized by an anti-L-CPT_1 antibody and by 2,4-dinitrophenol-etomoxiryl-CoA, a covalent inhibitor of L-CPT_1. Abundance of p88 and malonyl-CoA-inhibitable CAT activity were increased approx. 3-fold by starvation for 24 h. Deoxycholate solubilized p88 and malonyl-CoA-inhibitable CAT activity from microsomes to approximately the same extent. The microsomal fraction contained porin, which, relative to total protein, was as abundant as in crude mitochondrial outer membranes fractions. It is concluded that L-CPT_1 is not targeted to the ER membrane and that malonyl-CoA CAT in microsomal fractions is L-CPT_1 that is derived from mitochondria, possibly from membrane contact sites.

Key words: carnitine acyltransferase, malonyl-CoA, mitochondria, peroxisomes.

INTRODUCTION

Carnitine acyltransferases (CATs) catalysing interconversion of acyl-CoA and acylcarnitine esters have been described in liver mitochondrial, peroxisomal and microsomal fractions [1]. In each fraction/organelle there is a membrane-associated CAT which is inhibited by malonyl-CoA and a soluble or loosely membrane-associated CAT in the lumen/matrix of the organelle, which is unaffected by physiological concentrations of malonyl-CoA.

The extensively investigated CAT system of mitochondria provides a means of transporting long-chain fatty acids to the matrix for β-oxidation [1]. It consists of carnitine palmitoyltransferase 1 (CPT_1), a carnitine:acylcarnitine carrier (CAC) and carnitine palmitoyltransferase 2 (CPT_2), which is associated with the matrix face of the mitochondrial inner membrane. The malonyl-CoA-inhibitable enzyme CPT_1, an 88 kDa protein, is associated with the mitochondrial outer membrane and is also found at contact sites where the former closely approaches the inner membrane [2–5]. Liver (L-) and muscle (M-) isoforms of CPT_1 are found.

Liver peroxisomes also contain a CAT/CAC system which could allow shuttling of fatty acyl units between the cytosol and the peroxisomal matrix. CAT from the peroxisomal matrix, which is distinct from CPT_2, has been purified and cloned [6,7], and a protein, immunologically similar to and of similar molecular size to the mitochondrial CAC, is present [8]. The malonyl-CoA-inhibitable CAT of the peroxisomal membrane, which is distinct from the matrix enzyme [9], has not been characterized in detail, although liver peroxisomes contain an 88 kDa protein which cross-reacts with mitochondrial CPT_1 and which is labelled by inhibitors of CPT_1 [10,11].

Liver microsomal fractions contain two physically separable CATs which have distinct properties [12,13]. One of these, described as a luminal malonyl-CoA-insensitive CAT, is an approx. 54 kDa protein that is claimed to be immunologically distinct from other CATs [14,15]. The other microsomal CAT activity is membrane-bound and is overt in microsomal vesicles [12,13]. Several of its properties resemble those of the CPT_1 of liver mitochondrial outer membranes, i.e. it is inhibited reversibly by malonyl-CoA and by sulphonylurea drugs and irreversibly by CoA esters of 2-oxirane compounds [11–13,16], and it shows altered sensitivity to malonyl-CoA with fasting, changed temperature or altered membrane environment [17]. However, there are some kinetic differences between this microsomal CAT and CPT_1 [13] and, in particular, the relative ease with which the

Abbreviations used: AADA, arylacetamide deacetylase; CAC, carnitine:acylcarnitine carrier; CAT, carnitine acyltransferase; CPT_1, the overt carnitine palmitoyltransferase of mitochondria (the prefixes L- and M- refer to the liver and muscle isoforms respectively); CPT_2, the latent carnitine palmitoyltransferase of mitochondria; DNP-etomoxir, the 2,4-dinitrophenol derivative of 2-[6-(4-chlorophenoxy)hexyl]oxirane carboxylic acid; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; EST, expressed sequence tag; NCS, the N-terminal sequence which is highly conserved between L- and M- isoforms of CPT_1; NEM, *N*-ethylmaleimide; TBS, Tris-buffered saline; TMD, transmembrane domain; TRITC, tetramethylrhodamine β-isothiocyanate.

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microsomal malonyl-CoA-inhibitable CAT is solubilized in stable form by deoxycholate is a property that is not shared by the CPT_1 from a mitochondrial outer membrane fraction [13]. Despite these apparent differences, liver microsomes contain a polypeptide of identical size with L-CPT_1 (88 kDa) which cross-reacts with antibodies against L-CPT_1 and which is covalently labelled by DNP-etomoxiryl-CoA (where DNP-etomoxir is the 2,4-dinitrophenol derivative of 2-[6-(4-chlorophenoxy)hexyl]-oxiranecarboxylic acid) [11,18], leading to the possible conclusion that L-CPT_1 can be targeted to the endoplasmic reticulum (ER) as well as to mitochondria (and peroxisomes). This has important implications for recently advanced models where acylcarnitines are suggested to provide substrates for lipidation processes in the lumen of the ER [18–21]. The present study has addressed the possibility that L-CPT_1 might be targeted to ER membranes, but concludes that contamination of the microsomal fraction with fragments of mitochondria-related membranes is the most probable source of malonyl-CoA-inhibitable CAT within the microsomal fraction.

MATERIALS AND METHODS

Materials

Standard laboratory reagents, enzyme assay substrates and animals were as described previously [13,17]. The CoA ester of DNP-etomoxir (from Dr H. P. O. Wolf, Projekt-Entwicklung GmbH, Allensbach, Germany) was synthesized via the acid chloride. Rat liver poly(A)$^+$ RNA, plasmid pEGFP-N1 and rabbit anti-GFP antibody were from Clontech. The first strand cDNA synthesis kit was from Pharmacia. Restriction enzymes were from either New England Biolabs or Gibco BRL. Vent DNA polymerase was from New England BioLabs. Pfu DNA polymerase and plasmid pCR-Script-Amp were from Stratagene. Oligonucleotides were from VH Bio or Pharmacia. All other molecular biology and *in vitro* translation reagents were from Promega. A CPT_1 anti-peptide antibody (raised against residues 428–441 of rat L-CPT_1) [22] was a gift from Dr. V. Zammit, Hannah Research Institute, Ayr, U.K. An anti-catalase polyclonal antibody was a gift from Professor C. Danpure, Department of Biology, University College London, U.K. The anti-DNP antibody (rabbit), anti-guinea pig IgG–biotin conjugate and anti-rabbit IgG–FITC conjugate were from Sigma. Rabbit anti-goat IgG–peroxidase conjugate, goat anti-rabbit IgG–peroxidase conjugate, goat anti-mouse IgG–peroxidase conjugate and anti-porin monoclonal antibody (clone mAb4) were from Calbiochem. Anti-calnexin antibody was from StressGen, British Columbia, Canada. Avidin–FITC conjugate was from Zymed. Mitotracker Red CMX Ros was from Molecular Probes. Tissue culture reagents were from either Sigma or Gibco BRL.

Isolation and treatment of subcellular fractions

Membranes were isolated from livers of fed or 24 h-starved male Sprague–Dawley rats. A crude mitochondrial outer membrane fraction was separated from mitoplasts following a cycle of swelling and shrinkage of mitochondria [23]. This was used to investigate the distribution of porin (see below). A fraction described as 'purified mitochondrial outer membranes' was obtained by sucrose-density centrifugation of this crude fraction [23] and was used in Western-blotting studies (see below). Purified rough microsomal membranes were isolated by sucrose-density-gradient centrifugation [24]. Microsomal membranes depleted of luminal proteins were prepared as described previously [13]. DNP-etomoxiryl-CoA-labelled microsomes were prepared by incubating membranes (2 mg of protein/ml) in 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.2) containing 20 % (v/v) glycerol, bestatin/pepstatin/ leupeptin (20 μg of each/ml) and 1 mM PMSF with 10 μM DNP-etomoxiryl-CoA at approx. 20 °C for 20 min after which the membranes were diluted with 2 vol. of the ice-cold potassium phosphate/glycerol buffer containing BSA (3 mg/ml). The membranes were then centrifuged (100000 ***g*** for 30 min) and the pellet was resuspended in potassium phosphate/glycerol buffer at approx. 2 mg of protein/ml.

COS-1 cells were grown in 100 mm diameter culture dishes, washed with ice-cold PBS, scraped into ice-cold PBS and sedimented by centrifugation (500 ***g***, 3 min, 4 °C). The cell pellet was homogenized and then fractionated to yield a 10000 ***g*** mitochondrial pellet, as described previously [13].

CAT assays

CAT activity was assayed spectrophotometrically [13] using palmitoyl-CoA as the substrate. Malonyl-CoA-inhibitable CAT activity was the activity that was inhibited by 100 μM malonyl-CoA. 1 m-unit of CAT activity represents 1 nmol/min at 25 °C.

Western-blotting

Proteins were separated by SDS/PAGE and blotted on to nitrocellulose membranes. Generally, the membrane was blocked with 3 % (w/v) BSA in Tris-buffered saline (TBS: 20 mM Tris/HCl buffer [pH 7.4] containing 500 mM NaCl) prior to incubating with the appropriate primary antibody in 1 % (w/v) BSA/TBS. After washing with TBS, the blot was incubated with the appropriate anti-IgG–peroxidase conjugate in 1 % (w/v) BSA/TBS. Bands were visualized using 4-chloronaphthol/H_2O_2 or enhanced chemiluminescence (ECL®). With the anti-CPT_1 antibody, membranes were blocked with 2 % (w/v) non-fat milk powder in PBS prior to incubating with the CPT_1 anti-peptide antibody in 0.2 % (w/v) milk powder/PBS. After washing in PBS, the blot was incubated with rabbit anti-goat IgG–peroxidase conjugate in 0.2 % (w/v) milk powder/PBS. Blots were quantitated by densitometry. In the case of porin, samples of 5, 10 and 20 μg of membrane protein were analysed. The densitometry signal was proportional to the amount of protein applied to SDS/PAGE and therefore the relative abundance of porin in each membrane fraction was calculated from the gradient of the best-fit straight line relating densitometry signal to amount of protein.

Molecular biological methods

Oligonucleotides used are shown in Table 1. Site-directed CPT_1 mutants were created using the Gene Editor Site-Directed Mutagenesis Kit (Promega). First-strand L-CPT_1 cDNA was synthesized from rat liver poly(A)$^+$ RNA using the sequence-specific primer CD1 (corresponding to nucleotides 2540–2560). The coding sequence (103–2423) of this cDNA was subsequently amplified by PCR (Pfu) as two fragments which overlapped at a unique *Ava*I site (1169). Fragment F1 (nucleotides 9–1204) was amplified using primers NB1 and NB2. Fragment F2 (1141–2508) was amplified using primers NB3 and NB4. Aliquots of each first-round PCR were re-amplified with nested primers, which introduced restriction sites as described in Table 1. Fragment F1 was re-amplified using primers NB2 and NB5 to give fragment F1a (78–1204). F2 was re-amplified using primers NB3 and NB6 to give fragment F2a (1141–2487). Fragments F1a and F2a were gel-purified and cloned into the vector pCR-Script-Amp. Positive clones of F1a and F2a were excised as *Eco*RI/*Ava*I and

Table 1 Oligonucleotide primers used in the present study

Restriction sites introduced by the primers are underlined and mismatched bases are italicized. Locations of restriction sites in L-CPT$_1$ cDNA sequence are shown in parentheses. Start and stop codons are shown in bold. f, forward primer; r, reverse primer.

Primer	Sequence	Restriction site(s)
CD1 (r)	5′-GAGTGACGGTAAACCTTCTGG-3′	
NB1 (f)	5′-GACTCCGAGCTCAGTGAGGACC-3′	
NB2 (r)	5′-CCAGGATCTGCTGCATCTGCTGC-3′	
NB3 (f)	5′-CTCTACCACGATGGGAGGCTGC-3′	
NB4 (r)	5′-CTGCCTGTCCTATGGTCAGG-3′	
NB5 (f)	5′-GCAGAG*G*AATT*C*GTCCCCACTC-3′	*Eco*RI (84)
NB6 (r)	5′-CAACATCTATTCAT*AAGCTT*TGTATC*TCGAG* GGTCCGTTTTCTT*CCG**C**G**G***GGCTCAGG-3′	*Sac*II (2437), *Xho*I (2460), *Hin*dIII (2471)
STO (r)	5′-CCGCCCTCTGTGGTA*C TCGAG*AATGTGCCTGC TGTC**CTA***GATATGTTGGATGG-3′	*Xho*I (1108)
Nglyc1 (r)	5′-CATCAGTGGCCTTACAGA*CTCGAG*GTACCTGCT CACA*T*TATCTTTGACAGCTGGGAC-3′	*Xho*I (661)
Nglyc2 (r)	5′-CTTCTTCTTCCAGGAGTG*GAGACC*CGACAG *CG*AGAT*AT*TTTGAGGGCTTCGTGGCTCAG-3′	*Bsa*I (210)
ΔNCS (r)	5′-CTTCCAGGAGTG*GAGACC*CGACAGGCAGATCT GTTTCTCTGCCATCTTGAGTGGGGACG-3′	*Bsa*I (210)
ΔTM1 (r)	5′-GATCTTTGCGATCATGC*CCGCGG*AGGGGAGGG GTCCACTTTAGTGATGATGCCATTCTTGAACCG-3′	*Sac*II (342)
ΔTM2 (r)	5′-CACCTTCAGCGAGTAGCGCATGGTCATCTTCGT CTGGCTTGACATGCGGCCAGTG-3′	
FLO (r)	5′-GGCTCAGGGGTTTACTTTT*GGGCCC*TGATGGT GAGGCCAAACAAGG-3′	*Apa*I (2413)
MCD1 (r)	5′-GGTCTCAGAACTGGGCACGTG-3′	
FD1 (f)	5′-GAGCTGAGCTCACTAAACCCAGG-3′	
INV (r)	5′-GGTAGACAGCCACGT*GGATCC*TCTCTT**TCA**GA GGTGCTGTAGC-3′	*Bam*HI (1021)

*Ava*I/*Hin*dIII fragments respectively, gel-purified and subsequently ligated with T4 DNA ligase to give fragment F3 (84–2437). F3 was ligated, as a *Eco*RI/*Hin*dIII fragment, into the multiple cloning site of pGEM3Zf(−) (Promega) which was then transformed into *Escherichia coli* JM109. The identity of full-length recombinant clones was confirmed by sequencing. One clone encoded a S315P mutation which destroyed a potential N-glycosylation site (the sequence of this L-CPT$_1$ clone was otherwise identical with that described in [1]). A premature stop codon (using primer STO) was introduced into this clone by site-directed mutagenesis to give pL-CPT$_1$-T. Potential N-glycosylation sites were introduced by site-directed mutagenesis (primers Nglyc1 and Nglyc2) at residues 30 and 182 of pL-CPT$_1$-T. In addition, primers ΔNCS, ΔTM1 and ΔTM2 were used to delete amino acids 4–28 (inclusive), trans-membrane domain 1 (amino acids 53–75) and trans-membrane domain 2 (amino acids 103–122) respectively. All full-length L-CPT$_1$ constructs were derived from a clone pL-CPT$_1$-FL that encoded a protein of identical sequence to that described in [1]. Primer FLO was used to introduce an in-frame *Apa*I site (2413) into the coding sequence of L-CPT$_1$-FL. The coding sequence was excised as an *Eco*RI/*Apa*I fragment and sub-cloned into pEGFP-N1 (Clontech) to produce plasmid pL-CPT$_1$-EGPF, which encodes a fusion protein (L-CPT$_1$-EGFP) in which enhanced green fluorescent protein (EGFP) is fused to the C-terminus of rat L-CPT$_1$.

First strand M-CPT$_1$ cDNA was synthesized from rat muscle poly(A)$^+$ RNA using the sequence-specific primer MCD1 (corresponding to nucleotides 2435–2456). A truncated coding sequence (27–1013; note that the full-length coding sequence is 27–2346) using primers FD1 (3–26) and INV (1001–1041; this mutagenic primer introduces a stop codon at 1014) was amplified by PCR using Pfu DNA polymerase. The gel-purified PCR product was cloned into the vector pCR-Script-Amp and was designated as pM-CPT$_1$.

A cDNA clone encoding human arylacetamide deacetylase (AADA) was constructed by PCR amplification of Expressed Sequence Tag (EST) cDNA clones from the Medical Research Council Human Genome Mapping Project (Hinxton, Cambridge, U.K.). The 5′ oligonucleotide was mismatched to introduce a *Nco*I site spanning the initiating ATG codon and the 3′ oligonucleotide was mismatched to introduce a *Sal*I site immediately downstream of the termination codon. The cDNA was fused via the *Nco*I site to the 5′-untranslated region of human beta globin, ligated into the vector Bluescript, and was transcribed with T7 RNA polymerase following linearization at the *Sal*I site. It was previously shown that this construct adopts a Type II conformation in microsomes and is N-glycosylated at either or both of two canonical sites [25].

In vitro transcription/translation of CPT$_1$ constructs

Transcripts were synthesized using T7 RNA polymerase (90 min, 37 °C) using approx. 3 μg of DNA template per 25 μl of reaction. *In vitro* translations (25 μl) were performed using nuclease-treated rabbit reticulocyte lysate (Promega) with [^{35}S]methionine, with or without 3 μl of canine pancreatic microsomes (Promega), according to the supplier's instructions. After 60 min at 30 °C, carrier rat liver microsomes (10 μg) were added followed by 100 μl of ice-cold iso-osmotic buffer [0.25 M sucrose, 10 mM Tris/HCl (pH 7.4)] and centrifugation (4 °C) at 35000 ***g***. The pellet was resuspended in 100 μl of 0.1 M Na_2CO_3 (pH 11.5), kept on ice for 60 min, and then re-centrifuged as above. Aliquots of the sucrose supernatant, Na_2CO_3 supernatant and Na_2CO_3 pellet were analysed by SDS/PAGE. Gels were treated with Amplify (Amersham) and visualized by fluorography. Alternatively, *in vitro* translations were performed in the presence of *N*-ethylmaleimide (NEM)-treated microsomes. Microsomes (1 mg of protein/ml) in 10 mM Tris/HCl buffer (pH 7.4) containing 0.25 M sucrose were pre-treated with NEM (200 μM) for 30 min on ice and then washed 3 times by centrifugation and resuspension in 10 mM Tris/HCl buffer (pH 7.4) containing 0.25 mM sucrose. NEM-treated microsomes did not carry out glycosylation/processing of yeast α-mating factor or β-lactamase.

In some experiments, translation products were fractionated by sucrose-density-gradient centrifugation. For this, *in vitro* translations (50 μl) with or without pancreatic microsomes were carried out as above for 90 min at 30 °C followed by dialysis of the reaction mixtures for 3 h against 1 litre of PBS. Samples of dialysed translation mixtures (approx. 80 μl) were diluted with

an equal volume of 0.25 M sucrose and then placed on top of a sucrose gradient followed by an additional 250 μl of PBS. The sucrose density gradient consisted of nine equal steps (each of 100 μl) ranging from 0.25 M to 1.4 M sucrose in 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM EDTA on top of 1 ml of 1.8 M sucrose. After centrifugation in a Beckman TLS-55 swing-out rotor at 50000 rev./min for 15 min at 10 °C, the gradients were unloaded from the top. The first and second fractions collected were of volumes 0.25 ml and 0.15 ml respectively, followed by 10 fractions each of 100 μl which were analysed by SDS/PAGE in Bis-Tris 4–12 % polyacrylamide NuPAGE gradient gels (Novex) in Mops buffer (pH 7.7) at 200 V for 50 min. Gels were soaked in methanol/acetic acid [each 10 % (v/v) in water] overnight and then in Amplify (Amersham) for 30 min. After drying (72 °C for 2 h), gels were visualized by fluorography.

Cell culture and transfection

COS-1 cells were grown at 37 °C in the presence of 5 % CO_2 in Dulbecco's modified Eagle's medium with Glutamax-1 containing sodium pyruvate (1.25 mM), glucose (5.6 mM), 10 % (w/v) foetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and amphotericin (0.25 μg/ml). Cells were transfected with plasmid pL-CPT_1-EGFP (purified with the Qiagen Endo-Free Maxi Prep kit) using the Tfx-20 reagent (Promega) at a Tfx-20:DNA ratio of 3:1 (w/w) according to the manufacturer's instructions.

Cell staining

Cells were grown on coverslips in 24-well plates and prepared for microscopy 48 h after transfection. To visualize the ER, cells were washed twice with PBS, fixed with methanol/acetone (1:1, v/v) at −20 °C for 15 min and then washed twice with PBS. Non-specific binding of antibody was blocked by incubation with 3 % (w/v) BSA in PBS for 1 h. Cells were then incubated with rabbit anti-calnexin polyclonal antibody [1:200 in 1 % (w/v) BSA/PBS] for 1 h. After extensive washing with PBS, the cells were incubated with goat anti-rabbit IgG-tetramethylrhodamine β-isothiocyanate (TRITC) conjugate for 40 min, after which the cells were washed with PBS and the coverslips mounted on glass slides with VectaShield. Mitochondria were visualized by incubating cells with 1 μM Mitotracker CMX Ros (Molecular Probes) in complete medium for 30 min, followed by 30 min in complete medium lacking MitoTracker, after which they were fixed with methonol/acetone for 15 min at −20 °C prior to mounting the coverslip with VectaShield. To visualize peroxisomes, cells were fixed with 3 % (v/v) paraformaldehyde for 15 min at approx. 20 °C. The cells were then incubated with guinea-pig anti-catalase polyclonal antibody [1:250 in 1 % (w/v) BSA/PBS for 60 min], washed with PBS and then incubated with goat anti-guinea-pig–biotin conjugate [1:200 in 1 % (w/v) BSA/PBS] for 30 min. Finally, after washing with PBS, cells were incubated with avidin–TRITC conjugate for 30 min and then processed as described above.

Confocal microscopy

Fluorescent staining patterns were viewed in a confocal laser-scanning microscope (Leica TCS-NT system). The captured images were processed using Adobe Photoshop software (Adobe Systems Inc.).

RESULTS AND DISCUSSION

Investigation by confocal microscopy of L-CPT_1 targeting *in vivo*: subcellular localization of L-CPT_1-EGFP in transiently transfected COS-1 cells

The cells were transfected with a construct encoding a fusion protein in which EGFP was fused to the C-terminus of L-CPT_1 (L-CPT_1-EGFP). Western-blotting of a 10000 ***g*** particulate fraction with an anti-EGFP antibody detected a polypeptide of approx. 115 kDa (Figure 1), the predicted molecular mass of the L-CPT_1-EGFP fusion protein. L-CPT_1-EGFP was expressed in a punctate manner (Figures 2A, 2D and 3A) and there was clear co-localization of this with Mitotracker (Figures 2B and 2C), a potential-sensitive dye which accumulates in mitochondria. In cells fixed with methanol/acetone (to allow visualization of ER), the distribution of the ER integral protein calnexin was much more diffuse (Figure 2E) and, within the limits of the methodology, we could find no suggestion of an extramitochondrial localization of L-CPT_1-EGFP (Figure 2F). However, it was previously observed that an 88 kDa protein of liver peroxisomes cross-reacts with anti-L-CPT_1 antibodies [10,11]. In view of this, some transfected cells were fixed with paraformaldehyde (which preserves peroxisomal morphology) and stained with an anti-catalase antibody (Figure 3B). Some co-distribution was observed of L-CPT_1-EGFP fluorescence with the fluorescence expected from catalase in punctate structures (Figure 3C). However, these structures differed in morphology from normal peroxisomes, as seen in adjacent untransfected cells (Figure 3B). It is possible that some L-CPT_1 is also delivered to peroxisomes, although further studies will be required to confirm this.

Investigation of L-CPT_1 targeting *in vitro*: transcription/translation of CPT_1 constructs with dog pancreatic microsomes

The *in vivo* studies above gave no support to the notion that L-CPT_1 was localized in the ER. However, it could be argued that some L-CPT_1-EGFP had been targeted to the ER but had then been rapidly degraded (e.g. as occurs for mis-folded proteins in the ER [26]) with a resulting loss of EGFP fluorescence. We therefore investigated the possibility that L-CPT_1 might insert into microsomal membranes in coupled *in vitro* transcription/translation assays, in which protein degradation is known to be minimal [27]. The DNA constructs are listed in Table 2. Initial experiments were performed with the pL-CPT_1-T construct, which was truncated at amino acid 328 but still contained the hydrophobic N-terminal portion (NCS), which is conserved between L- and M- isoforms of CPT_1 [1], the two putative transmembrane domains TMD_1 and TMD_2 [1], a putative microsomal targeting signal [28] and the information required for mito-

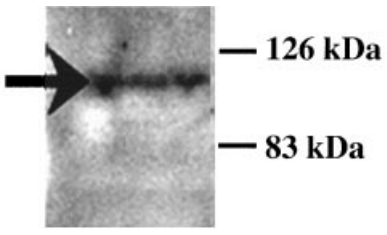

Figure 1 Western blot of L-CPT_1-EGFP fusion protein in a 10000 *g* particulate fraction from transfected COS-1 cells

The blot was probed with antibody against EGFP. The position of the L-CPT_1-EGFP fusion protein is indicated by the arrow. The bars indicate positions of molecular mass markers (kDa).

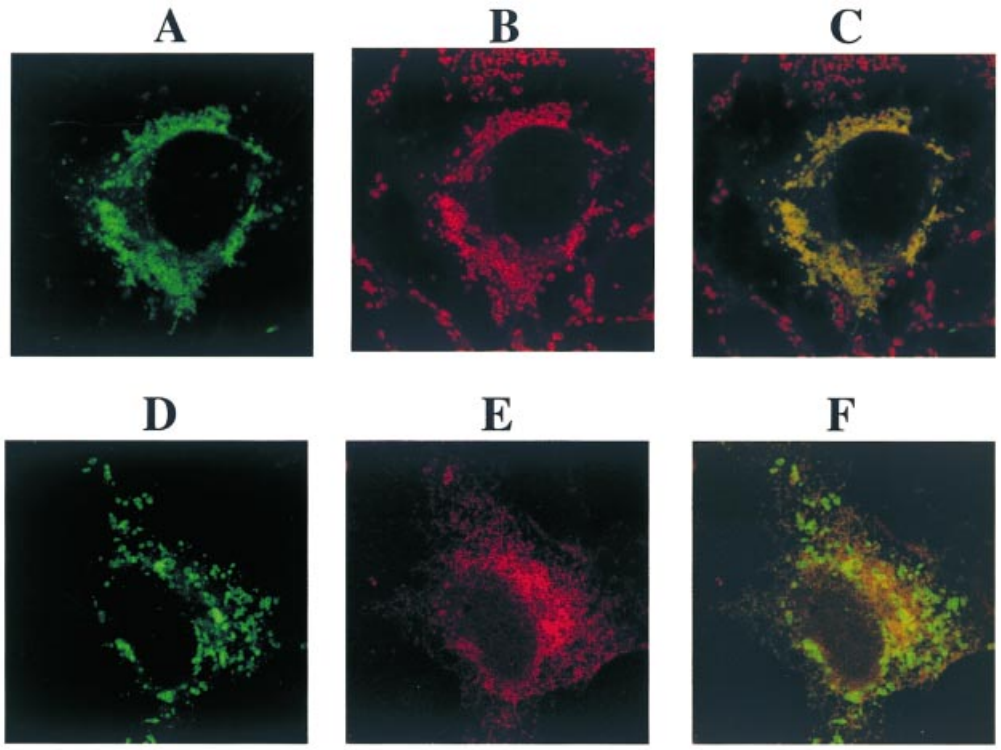

Figure 2 Confocal microscopy of mitochondria and ER in COS-1 cells expressing the L-CPT$_1$-EGFP fusion protein

Cells were fixed with methanol/acetone. (**A**) shows a field containing a COS-1 cell expressing L-CPT$_1$-EGFP and (**B**) shows the same cell (and surrounding cells) in which mitochondria were visualized by Mitotracker. (**C**) is an overlay of (**A**) and (**B**). (**D**) shows a field containing a COS-1 cell expressing L-CPT$_1$-EGFP, and in (**E**) the same cell is shown with the ER visualized using an anti-calnexin antibody in conjunction with a rhodamine-labelled second antibody. (**F**) is an overlay of (**D**) and (**E**).

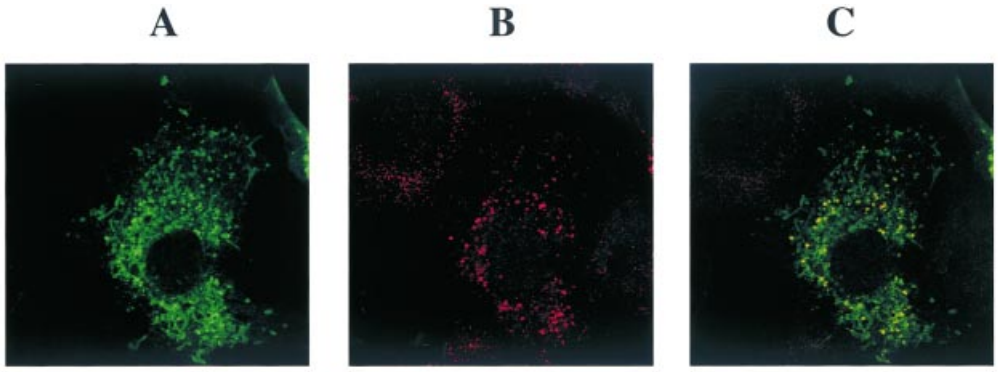

Figure 3 Confocal microscopy of peroxisomes in COS-1 cells expressing the L-CPT$_1$-EGFP fusion protein

Cells were fixed with paraformaldehyde. (**A**) shows a field containing a COS-1 cell expressing L-CPT$_1$-EGFP and (**B**) shows the same cell (and surrounding cells) in which peroxisomes were visualized using an anti-catalase antibody (**B**). (**C**) is an overlay of (**A**) and (**B**).

chondrial targeting and insertion into the mitochondrial outer membrane [29]. A small-sized construct was used in order to facilitate detection on SDS/PAGE of any increase in molecular mass of the translation product due to glycosylation at engineered N-glycosylation sites (see below). Translation of pL-CPT$_1$-T gave a product of approx. 36 kDa, in agreement with the predicted molecular mass of 37.4 kDa. A significant proportion (typically 50–80 %, see Figures 4 and 7) of L-CPT$_1$-T became associated with pancreatic microsomes in a manner that could not be abolished by extraction with alkaline carbonate (Figure 4). Resistance of an *in vitro* translation product to extraction by alkaline carbonate (0.1 M Na_2CO_3, pH 11.5, for 60 min on ice) has often been taken as evidence for its integration into membranes [29–32]. Membrane-association of the L-CPT$_1$-T translation product was also implied following sucrose-density-gradient centrifugation of the pancreatic microsomes (Fig-

Table 2 DNA constructs used for *in vitro* translations with pancreatic microsomes

Name of construct	Description
pL-CPT_1-FL	Full-length liver isoform of CPT_1 (L-CPT_1)
pL-CPT_1/ΔTMD_1/ΔTMD_2-FL	pL-CPT_1-FL with amino acids 53–75 and 103–122 deleted
pL-CPT_1-T	Amino acids 1–328 of L-CPT_1
pM-CPT_1-T	Amino acids 1–329 of muscle isoform of CPT_1 (M-CPT_1)
pL-CPT_1/ΔNCS-T	pL-CPT_1-T with amino acids 4–28 deleted
pL-CPT_1/ΔTMD_2-T	pL-CPT_1-T with amino acids 103–122 deleted
pL-CPT_1/ΔTMD_1/ΔTMD_2-T	pL-CPT_1-T with amino acids 53–75 and 103–122 deleted
pL-CPT_1/ΔTMD_2/glyc1/glyc2-T	pL-CPT_1/ΔTMD_2-T with N-glycosylation sites at residues 30 and 182

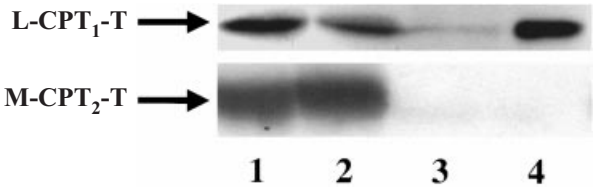

Figure 4 *In vitro* transcription/translation of truncated forms of L-CPT_1 and M-CPT_1

In vitro transcriptions and translations in the presence of canine pancreatic microsomes were performed. Lane 1, total translation products; lane 2, sucrose wash supernatants; lane 3, alkaline carbonate extract supernatants; lane 4, alkaline carbonate-extracted pellets. In control experiments, where carrier rat liver microsomes were added after translations had been performed in the absence of canine pancreatic microsomes, no L-CPT_1-T polypeptide was detected in the alkaline carbonate-extracted pellet (results not shown).

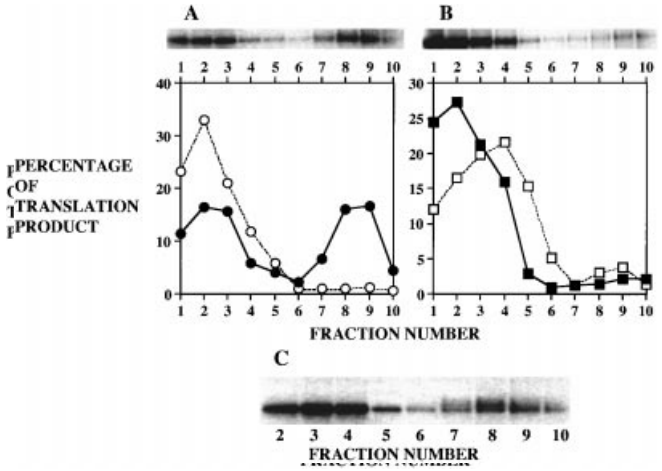

Figure 5 Sucrose-density-gradient centrifugation of *in vitro* translation products

In vitro transcriptions and translations were performed followed by dialysis and sucrose-density-gradient centrifugation of the products (without carbonate extraction) where sucrose concentration was increased from 0.25 M (fraction 1) to 1.4 M (fraction 10). (**A**) L-CPT_1-T, translated without (open symbols) or with (closed symbols) canine pancreatic microsomes. (**B**) L-CPT_1-L, translated without (open symbols) or with (closed symbols) canine pancreatic microsomes. (**C**) Equivalent translations with and without canine pancreatic microsomes were carried out and then pooled before sucrose-density-gradient centrifugation, which showed that glycosylation of AADA (as shown by the appearance of products of greater molecular mass) was essentially confined to fractions 7–9. Glycosylation of AADA was inhibited by *N*-acetyl Asn-Tyr-Thr-carboxamide (results not shown). Preliminary experiments (results not shown) established that, in the absence of microsomes, most of the translation product was in fractions 2–4, whereas in the presence of microsomes it was mostly in fractions 7–10.

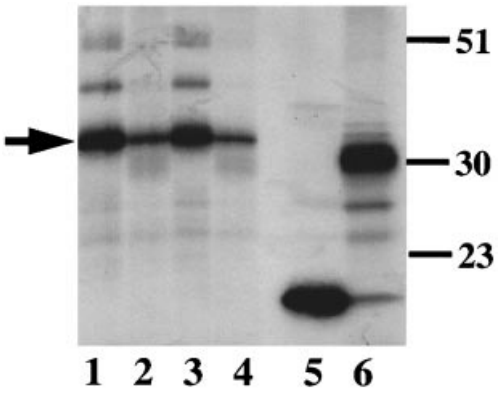

Figure 6 *In vitro* transcription/translation of the mutant L-CPT_1/ΔTMD_2/glyc1/glyc2-T and of yeast α-mating factor

In vitro transcriptions and translations were performed without (lanes 1, 3, 5) or with (lanes 2, 4, 6) canine pancreatic microsomes without further processing (sucrose and alkaline carbonate washing) of the translation products. Lanes 1–4, L-CPT_1/ΔTMD_2/glyc1/glyc2-T (indicated by the arrow); lanes 5 and 6, yeast α-mating factor. It should be noted that the amount of CPT_1 translation product synthesized in the presence of microsomes (lanes 2 and 4) was less than in the absence of microsomes (lanes 1 and 3), a phenomenon that was reproducibly observed.

ure 5A). Provided microsomes were present during translation, a significant proportion of the L-CPT_1-T translation product was recovered in fractions 7–9. These fractions were judged to contain 'import-competent' membranes by the following criterion. When AADA (an authentic microsomal protein which is membrane-inserted and glycosylated within microsomes [25]) was translated, products of greater molecular mass, indicative of glycosylation, were apparent in fractions 7–9 (Figure 5C). As a further control, the DNA construct pM-CPT_1-T was translated (Figure 4). M-CPT_1-T has 60% amino acid sequence identity overall when aligned with L-CPT_1-T (89, 43 and 35% identity in the NCS, TMD_1 and TMD_2 regions respectively) but a fusion protein of full-length M-CPT_1 with green fluorescent protein does not target to the ER when transfected into HeLa cells [33]. Figure 4 shows that negligible M-CPT_1-T was found in the carbonate-extracted pellet. Taken all together, this series of experiments initially suggested that a specific and authentic insertion of L-CPT_1-T into microsomal membranes could have occurred.

However, further experiments in which mutants of pL-CPT_1-T were transcribed/translated provided strong evidence that this was not the case. The translation products L-CPT_1/ΔTMD_2-T, L-CPT_1/ΔTMD_1/ΔTMD_2-T and L-CPT_1/ΔNCS-T were present in similar amounts in carbonate-extracted pellets to L-CPT_1-T (results not shown). Therefore association of the truncated form of L-CPT_1 with microsomal membranes did not depend on the topographical features NCS, TMD_1 and TMD_2, which might have been expected to play a role in membrane association/anchorage. Additionally, this membrane-association was not dependent on an active ER translocation system, since comparable amounts of carbonate-resistant L-CPT_1-T were found with NEM-treated microsomes (results not shown). Finally, we transcribed/translated the mutant pCPT_1/ΔTMD_2/glyc1/glyc2-T, which contained a potential N-glycosylation site on either side of TMD_1. The rationale was that if the polypeptide underwent

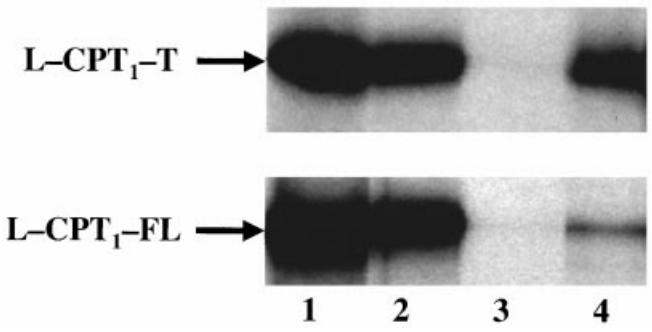

Figure 7 ***In vitro*** **transcription/translation of full-length L-CPT$_1$**

In vitro transcriptions and translations were performed in the presence of canine pancreatic microsomes. Lane 1, total translation products; lane 2, sucrose wash supernatants; lane 3, alkaline carbonate extract supernatants; lane 4, alkaline carbonate-extracted pellets.

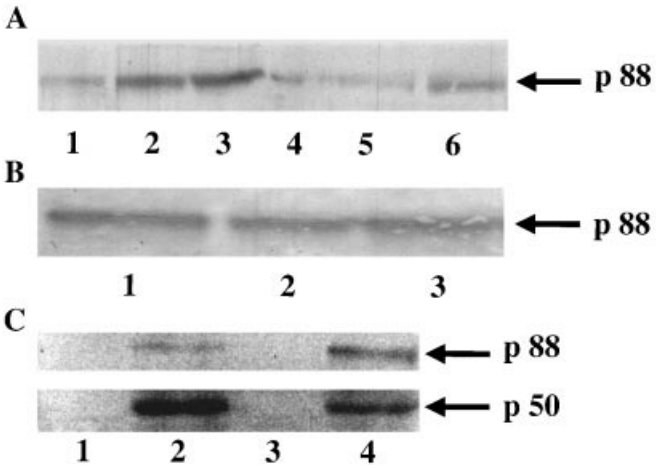

Figure 8 Detection of L-CPT$_1$ in rat liver microsomal membranes

(**A**) Western blots of microsomal membrane proteins (lanes 1–3) and proteins from purified mitochondrial outer membrane fraction (lanes 4–6) from fed rats were probed with L-CPT$_1$ anti-peptide antibody. The following amounts of CAT activity were loaded: 1.2 m-units (lanes 1 and 4), 2.4 m-units (lanes 2 and 5) and 4.8 m-units (lanes 3 and 6). (**B**) Western blots of solubilized, partially purified microsomal membrane CAT probed with L-CPT$_1$ anti-peptide antibody. Lane 1, 0.6 m-unit of the Superdex 200 Peak I (as described in [13]); lane 2, microsomal membranes (0.6 m-unit of CAT activity); lane 3, microsomal membranes (1.2 m-units of CAT activity). (**C**) Western blots of liver microsomal membrane proteins which had been labelled with DNP-etomoxiryl-CoA (lanes 2 and 4) or control membrane proteins which had not been treated with DNP-etomoxiryl-CoA (lanes 1 and 3) were probed with an anti-DNP antibody. Microsomes were isolated from fed (lanes 1 and 2) or starved (lanes 3 and 4) rats. Protein (160 μg) was loaded in each lane, which corresponded to 3.1 m-units and 9.1 m-units of CAT activity for membranes isolated from fed and starved animals respectively. Preliminary experiments established that DNP-etomoxiryl-CoA inhibited microsomal CAT activity by $> 95\%$.

transmembrane insertion, regardless of its orientation, one of the N-glycosylation sites should be luminal and hence glycosylated to give a slower-migrating species on SDS/PAGE. However, we obtained no evidence for glycosylation of this polypeptide by microsomal membranes which readily glycosylated yeast α-mating factor (Figure 6). This strongly suggested that the truncated L-CPT$_1$ did not adopt an authentic transmembrane configuration.

Some full-length translation product (L-CPT$_1$-FL) of molecular mass approx. 88 kDa was also recovered in the carbonate-extracted pellet. However, routinely, recovery of this was small in terms of the total amount of full-length translation product and compared with the proportion of L-CPT$_1$-T, which was resistant to carbonate extraction (Figure 7). After sucrose-density-gradient centrifugation of the pancreatic microsomes there was no association of the L-CPT$_1$-FL translation product with the 'import competent' fractions 7–9 over and above that seen in the absence of microsomes (Figure 5B). On this basis, the small proportion of L-CPT$_1$-FL observed in the carbonate-extracted pellet (Figure 7) was not due to insertion of the protein into microsomal membranes.

Investigations with rat liver microsomal fractions

Malonyl-CoA-inhibitable CAT activity in purified rough microsomes was 10.4 ± 1.1 nmol/min per mg of protein ($n = 4$, mean $\pm$S.E.M.), which is within the range of activities reported previously [11,13,17]. In accord with previous work by Fraser et al. [11], these rough microsomes contained an approx. 88 kDa polypeptide (p88) which cross-reacted with an antibody against residues 428–441 of L-CPT$_1$ (the anti-C antibody of [19]).

Figure 8(A) compares Western blots of microsomes and mitochondrial outer membranes adjusted for equal units of CAT activity in gel lanes. The p88 signal was generally greater in the lanes containing microsomal membrane proteins compared with those containing mitochondrial outer membrane proteins. This may simply reflect difficulty in solubilizing CPT$_1$ from mitochondrial outer membranes [13]. While this information may indicate that there is sufficient p88 protein in microsomal fractions to account for the observed CAT activity, an additional experiment was required to verify this conclusion. We therefore performed Western-blotting of solubilized Superdex 200 gel-filtered microsomal CAT (Peak I, as described in [13]) and found that, standardized against CAT activity, the p88 signal was comparable with that in 'intact' microsomes (Figure 8B), strongly suggesting that p88 is the major and possibly only malonyl-CoA-inhibitable CAT in microsomal fractions.

Etomoxiryl-CoA and its DNP derivative are covalent inhibitors of mitochondrial CPT$_1$ [34] and microsomal CAT [13]. In accord with previous studies [11], DNP-etomoxiryl-CoA labelled p88 in microsomes (Figure 8C). Starvation increased the p88 signal by 2.7-fold and the CAT specific activity by 2.9-fold, changes which match parallel changes in CAT activity and the p88 Western-blotting signal with microsomal membranes from fed, starved and suckling rats [18]. DNP-etomoxiryl-CoA also labelled p50, an approx. 50 kDa polypeptide (Figure 8C). Other studies [12,35], on the basis of labelling with [^{3}H]etomoxir, suggested that the microsomal malonyl-CoA-inhibitable CAT was a protein of 47–50 kDa. However, this seems unlikely because starvation decreased the p50 signal by 2.0-fold.

Taken together with the findings of Fraser et al. [11], it is very difficult not to conclude that the malonyl-CoA-inhibitable CAT in rat liver microsomes is an 88 kDa polypeptide which is either identical with, or remarkably similar to, the CPT$_1$ of the mitochondrial outer membrane. We performed a computer search of the IMAGE (Integrated Molecular Analysis of Genomes and their Expression) database of ESTs for evidence of variant sequences related to L-CPT$_1$, deriving either from a separate gene or from alternative splicing of the known gene. However, we have been unable to identify any variant ESTs (results not shown) and the publication of the draft of the complete human genome sequence makes it unlikely that there is a second, undetected, copy of the gene.

Malonyl-CoA-inhibitable CAT activity has been found in liver microsomes obtained directly by centrifugation of post-mito-

chondrial supernatants [11,36,37] or after further purification by sucrose-density-gradient centrifugation ([12,13,17] and the present study). In instances where monoamine oxidase was measured, very little contamination of microsomes by this marker was observed [12,13,36]. However, L-CPT_1 activity is enriched in contact sites which are sub-domains where the mitochondrial outer and inner membranes come into close proximity [2–5]. These contact sites are also continuous with ER-related membranes [38]. On this basis, in fragmented membrane preparations, 'classical' mitochondrial outer membrane markers, such as monoamine oxidase or rotenone-insensitive NADH-cytochrome *c* reductase, are not reliable markers for some mitochondria-derived CPT_1 [2,3]. By contrast, Fraser and Zammit [2] have shown a close co-localization of CPT_1 with porin, a component of mitochondrial contact sites [39,40]. We found that the relative abundance of porin in sucrose-density-purified microsomes (17.3 ± 0.5 arbitrary units/mg of protein) was comparable with (or even greater than) that in the crude mitochondrial outer membrane fraction (12.1 ± 0.9 arbitrary units/mg of protein). Porin is absent from highly purified liver ER and Golgi fractions [41] and, although capable of translocation in cell-free systems into mitochondrial membranes, will not translocate into microsomal membranes [36,42,43]. We therefore are confident in concluding that porin in microsomes was derived from mitochondria or closely-associated structures but not from membrane structures derived from the ER. If microsomal CAT had been derived from the ER, we would have expected the ratio malonyl-CoA-inhibitable CAT activity (nmol/min per mg of protein)/porin abundance (arbitrary units/mg of protein) in microsomes to be markedly higher than in mitochondrial outer membranes. However, this was not found to be the case (0.61 in microsomes and 1.28 in crude mitochondrial outer membranes).

Concluding remarks

The findings of previous work [11,18] and of this study offer strong evidence for identity of the microsomal enzyme with L-CPT_1. In summary, liver microsomes contain a polypeptide of identical size (88 kDa) with L-CPT1, which has at least three linear epitopes in common with L-CPT_1. This 88 kDa polypeptide can be solubilized by deoxycholate under conditions which parallel the solubilization of the microsomal CAT activity. Changes in abundance of this 88 kDa polypeptide in microsomal fractions, quantified immunologically, or by binding of a covalent inhibitor, correlate with physiological changes in the activity of microsomal malonyl-CoA-inhibitable CAT. Furthermore, considerations of EST databases and of the organization of the L-CPT_1 gene lend no support to the possible existence of a close homologue of L-CPT_1.

Our studies *in vivo* and *in vitro* with full-length DNA constructs contradict the notion that L-CPT_1 is targeted to the ER, despite previous detection of malonyl-CoA-inhibitable CAT activity in liver microsomal fractions [11–13,16,17,36,37]. However, our demonstration of a relatively high abundance of porin in microsomes implies that this fraction contains membrane structures derived from mitochondria. At present it is not possible to ascertain whether CPT_1 and porin in the microsomal fraction are co-localized in the same structures, but the possibility emerges that the CPT_1 in microsomes could be derived from mitochondrial contact sites. Other studies [44] have suggested that CPT_1 found in microsomal fractions may have undergone proteolytic modification of the N-terminus, a change which results in loss of sensitivity to malonyl-CoA [22]. It is therefore noteworthy that CPT_1 from contact sites has a higher IC_{50} for malonyl-CoA than that from mitochondrial outer membranes [5]. Mitochondrial outer membrane CPT_1 also has a higher K_m for fatty acyl-CoA than either contact site CPT_1 [5] or microsomal malonyl-CoA-inhibitable CAT (see Table 1 of [13]).

It also is necessary to attempt to reconcile our observations with previous studies of the expression of L-CPT_1 in *Saccharomyces cerevisiae*. In two equivalent studies, a negligible [45] or a significant (27 %) [28] proportion of full-length L-CPT_1 was found in a 100000 ***g*** pellet, the remainder being in a mitochondrial fraction. Deletion of residues 83–148 (which removes a mitochondrial targeting sequence between residues 123–147) led to almost complete recovery in the 100000 ***g*** pellet [28]. However, other studies [28,45] did not establish whether this variable proportion of 100000 ***g***-pelleted material was protein that was authentically inserted into the ER. An alternative possibility is that this pool represented protein which was expressed in excess of mitochondrial import capacity and which had collected as protein complexes in the cytosol. A particular issue that was not addressed in the latter study [45] is how deletion of mitochondrial targeting sequences might increase microsomal CPT_1 import if (as is normally the case) ER targeting is co-translational while mitochondrial targeting is post-translational. Abolition of mitochondrial targeting signals might, however, increase cytosolic complexes. Moreover, we observed no authentic ER insertion of CPT_1 based on the criterion of glycosylation, and also no decrease in microsomal membrane association of constructs lacking parts of the putative ER targeting sequence [45] relative to those in which it was fully present. It is noteworthy that in our studies we found mutagenized forms of L-CPT_1 can apparently co-sediment with membranes but that this clearly is not an index of membrane insertion.

Although our findings do not support the possibility of targeting of L-CPT_1 to the ER, our study and that of Fraser et al. [11] is not at variance with the possibility that the malonyl-CoA-inhibitable CAT of peroxisomal membranes is L-CPT_1 and it is noteworthy that the sequence of amino acids 40–55 of L-CPT_1 has similarity to sequences in some yeast proteins that are thought to be peroxisomal targeting signals [46].

Although our findings do not support the notion of the ER membrane containing a malonyl-CoA-inhibitable CAT, these are not grounds to exclude the presence of a malonyl-CoA-insensitive CAT in microsomes. Such an activity was purified [14,15], assigned a luminal localization [12,13] and shown to be immunolocically distinct from other hepatic CATs [14,15]. Although hepatic microsomes do not contain the CAC protein found in mitochondria and peroxisomes [8], microsomal vesicles are capable of importing fatty acyl-carnitine, which serves as substrate for this lumenal CAT [18,19]. This CAT in turn could provide fatty acyl-CoA for luminal forms of diacylglycerol acyltransferase [20] or acyl-CoA:cholesterol acyltransferase [47]. We suggest that acylcarnitines utilized in these ER processes would have originated from the outer face of the mitochondria.

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