Cytological evidence that the C-terminus of carnitine palmitoyltransferase I is on the cytosolic face of the mitochondrial outer membrane

Feike R. VAN DER LEIJ^{*1}, Anita M. KRAM[†], Beatrijs BARTELD^{S*}, Han ROELOFSEN^{*‡}, Gioia B. SMID^{*}, Janny TAKENS^{*}, Victor A. ZAMMIT[§] and Jaap R. G. KUIPERS^{*}

^{*}Department of Pediatrics, Research Laboratory CMCV 2nd floor, Groningen University Hospital, Groningen University Institute for Drug Exploration, University of Groningen, P.O. Box 30001, NL-9700 RB Groningen, The Netherlands, [†]Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands, [‡]Department of Internal Medicine, Groningen University Institute for Drug Exploration, University of Groningen, P.O. Box 30001 NL-9700 RB Groningen, The Netherlands, and [§]Department of Cellular Biochemistry, Hannah Research Institute, Mauchline Road, Ayr, KA6 5HL Scotland, U.K.

Carnitine palmitoyltransferase I (CPT I) is a key enzyme in the regulation of β -oxidation. The topology of this enzyme has been difficult to elucidate by biochemical methods. We studied the topology of a fusion protein of muscle-type CPT I (M-CPT I) and green fluorescent protein (GFP) by microscopical means. To validate the use of the fusion protein, designated CPT I–GFP, we checked whether the main characteristics of native CPT I were retained. CPT I–GFP was expressed in HeLa cells after stable transfection. Confocal laser scanning microscopy in living cells revealed an extranuclear punctate distribution of CPT I–GFP, which coincided with a mitochondrial fluorescent marker. Immunogold electron microscopy localized CPT I–GFP almost exclusively to the mitochondrial periphery and showed that the C-terminus of CPT I must be on the cytosolic face of the

mitochondrial outer membrane. Western analysis showed a protein that was 6 kDa smaller than predicted, which is consistent with previous results for the native M-CPT I. Mitochondria from CPT I–GFP-expressing cells showed an increased CPT activity that was inhibited by malonyl-CoA and was lost on solubilization with Triton X-100. We conclude that CPT I–GFP adopts the same topology as native CPT I and that its C-terminus is located on the cytosolic face of the mitochondrial outer membrane. The evidence supports a recently proposed model for the domain structure of CPT I based on biochemical evidence.

Key words: amino acid sequence, energy metabolism, enzymology, human, mitochondria.

INTRODUCTION

In mammals, β -oxidation of long-chain fatty acids is the major metabolic pathway for energy utilization in most organs and tissues. To permit the mitochondrial β -oxidation of long-chain fatty acids, intracellular transport of long-chain acyl moieties from the cytosol into the mitochondrial matrix is needed. The catalysed transfer of long-chain fatty acyl groups from CoA to carnitine by carnitine palmitoyltransferase I (CPT I) is a key regulatory step in this process. The exchange of acylcarnitine and carnitine over the inner mitochondrial membrane, mediated by carnitine acylcarnitine translocase, and the reverse reaction of CPT I, catalysed in the mitochondrial matrix by carnitine palmitoyltransferase II (CPT II), complete the net import of long-chain acyl-CoA (reviewed in [1]). The role of CPT I is important for the understanding of metabolic changes in diseases such as diabetes [2] and cardiac hypertrophy [3], and physiological changes such as those that occur after birth [4,5].

There are at least two isoforms of CPT I, encoded by separate genes with different expression patterns. Of the two isoforms identified, the liver-type enzyme (L-CPT I, encoded by the *CPT1A* gene) has been studied most extensively in isolated liver mitochondria, the subject of choice in many mitochondrial studies. The other isoform, the muscle-type enzyme (M-CPT I, encoded by the *CPT1B* gene) shows qualitatively similar but quantitatively distinct biochemical properties. Both isoforms are detergent-labile and catalyse the same reaction but have different

affinities to carnitine and different sensitivities to the natural inhibitor malonyl-CoA (reviewed in [1]). Complementary DNA sequences of both isoforms from several species are known. The deduced protein sequences show approx. 62% identical amino acid residues when the different CPT I isoforms are compared [6,7].

The compartmentalization of CPT I and CPT II to the mitochondrial outer and inner membranes respectively is now clearly established [1]. However, the topology of CPT I has been difficult to elucidate. After Murthy and Pande [8] showed that CPT I does not reside in the inner membrane but in the outer membrane, it was believed that the active site of CPT I is located at the intermembrane space aspect of the outer membrane, whereas the site of inhibition by malonyl-CoA is on the cytosolic aspect. However, more recent studies with several approaches suggest that CPT I is incorporated into the mitochondrial outer membrane by the insertion of two transmembrane domains, leaving the short N-terminal domain and the long C-terminal domain of the protein on the cytosolic face of the mitochondrion [9,10]. This topology results in the exposure of sites of substrate binding and malonyl-CoA inhibition on the cytosolic aspect of the outer membrane, as indicated by their accessibility to immobilized acyl-CoA substrate and malonyl-CoA [10]. This model is plausible in view of the hydropathic distribution profiles of CPT I isoforms, as can be deduced from their cDNA sequences ([1,11], and the present study). Although the view on CPT I topology as outlined by Fraser et al. [10] has been recognized by

Abbreviations used: CPT, carnitine palmitoyltransferase; CLSM, confocal laser scanning microscopy; EM, electron microscopy; GFP, green fluorescent protein; L-CPT I, liver isoform of carnitine palmitoyltransferase I; M-CPT I, muscle isoform of carnitine palmitoyltransferase I.

¹ To whom correspondence should be addressed (e-mail f.r.van.der.leij@med.rug.nl).

others [12,13], the significance of the model should be tested by the use of techniques complementary to those used before. Therefore we wished to study CPT I topology by microscopical means.

So far, the available antibodies against CPT I have been shown to be unsuitable for localization studies by immunogold electron microscopy (EM) (F. Fraser and V. A. Zammit, unpublished work). We therefore decided to use the jellyfish 'enhanced' green fluorescent protein (GFP) reporter system, which has been used successfully to study protein trafficking to the mitochondrial matrix [14] and the mitochondrial outer membrane [15]. Here we report the use of the GFP domain of the fusion protein for its fluorescent and immunogenic properties, and provide evidence that the C-terminus of CPT I is on the cytosolic face of the mitochondrial outer membrane.

EXPERIMENTAL

Materials

The human cervix epithelial carcinoma cell line HeLa S3 (ATCC-CCL2.2) was from the laboratory collection of the Department of Radiobiology (University of Groningen, Groningen, The Netherlands). Dulbecco's modified Eagle's medium, fetal calf serum and geneticin (G418) were from Life Technologies (Paisley, Scotland, U.K.). The vectors pEGFP-N2 and pEGFP-C1 were from Clontech (Palo Alto, CA, U.S.A.), pGEM7 was from Promega (Madison, WI, U.S.A.) (GenBank accession numbers U55762, U57608 and X65310 respectively). Unless stated otherwise, solvents were from Merck (Darmstadt, Germany), and other chemicals were from Sigma (Milwaukee, WI, U.S.A.).

Gene fusion

For the expression of recombinant CPT I, the coding sequence of the *CPT1B* cDNA [7] (Genbank accession number Y08682), which codes for human M-CPT I, was cloned behind the cytomegalovirus immediate early promoter, and the 3' end encoding the C-terminus was fused to *GFP* in pEGFP-N2. A *Hind*III site was introduced slightly upstream of the *CPT1B* stop codon by the use of primer 2H [7] in PCR-mediated site-directed mutagenesis. The resulting fragment was cloned as an *Eco*RI-*Hind*III fragment in pGEM7, then recut with *Sph*I and *Bam*HI. This 3' part of the gene was joined to a 5' *Xho*I-*Sph*I fragment by ligation into a *Xho*I-*Bam*HI digest of pEGFP-N2. The construct was designated pCE200; its sequence integrity was confirmed by dideoxy sequencing. The fused coding sequences were linked with a spacer region as outlined in Figure 1. The

plasmid pEGFP-C1 was used as a control. Enhanced GFP is encoded by a synthetic form of the *gfp* gene from the jellyfish *Aequorea victoria*, modified for the high efficiency of translation in human cells and for enhanced fluorescent properties [16,17].

Transfection and cell culture

Transfection of HeLa cells was performed by using the calcium precipitation method [18], with slight adaptations [19]. Stable transfectants were selected by fluorescence microscopy after a first selection on 1000 µg/ml G418. Transfected colonies were rescued by treatment with trypsin for 1 min in small glass cylinders, each surrounding one colony. After treatment with trypsin, colonies were subcultured in medium with 400 µg/ml G418 (2.5 ml per well); 12–20 clones were picked for each construct. Four lines were used for further study: two CPT I-GFP expressing lines and two GFP control lines. Green fluorescent cultures were grown on coverslips for confocal laser scanning microscopy (CLSM) and EM. Large-scale cultures (80 ml in 225 cm² flasks) were grown to obtain cells for the isolation of mitochondria and for the partial disruption of cells for analysis by EM. Of each representative cell line, 10⁵ cells were subjected to FACS® analysis in a Coulter Elite flow cytometer (Beckman Coulter, Miami, FL, U.S.A.).

CLSM

MitoTracker CMXRos-H₂ (Molecular Probes Europe BV, Leiden, The Netherlands) was used as mitochondrial marker. Coverslips with cells grown to approx. 75% confluence were incubated with 100–200 nM Mitotracker in Dulbecco's modified Eagle's medium without fetal calf serum for 30 min at 37 °C, then washed with PBS and put on ice. From each glass coverslip with attached cells, a small chamber was constructed by putting a clean coverslip on top, separated by spacers and kept together with silicon grease. A Leitz DM IRB inverted microscope (Leica, Heidelberg, Germany) with a True Confocal Scanner 4D (Leica) equipped with an argon-krypton laser was used. Double-label images were taken sequentially at 568 and 590 nm (excitation and emission) and at 488 and 515 nm. The 590 nm filter was of long-pass type; the 515 nm filter was a band-pass filter.

Electron microscopy

Cells grown on coverslips were fixed in 0.1% (v/v) glutaraldehyde (Fluka Chemie AG, Buchs, Switzerland) in PBS at 4 °C overnight. Fixed cells were washed once in PBS, scraped from the coverslips, dehydrated with a graded ethanol series and embedded in Unicryl (BB International, Cardiff, South Glam., U.K.). Ultrathin sections were labelled by the method of Slot and Geuze [20] with rabbit anti-GFP antibody diluted 1:1000 (Clontech) and 15 nm gold-conjugated goat anti-(rabbit IgG) (Amersham International, Little Chalfont, Bucks., U.K.). We refer to this method as post-embedding EM. For pre-embedding labelling, (5–6) × 10⁷ cells were partly disrupted by ten mild strokes by hand in a Potter-Elvehjem homogenizer in 2 ml of 20 mM Tris/HCl, pH 7.4, containing 250 mM sucrose. A loose pellet was sedimented by gravity for 1 h and the supernatant was centrifuged over 20% (v/v) Percoll at 10000 g for 10 min. The pellet was washed in PBS containing 250 mM sucrose, 1 mM EDTA, 5 mM Mops, pH 7.2, and 1 mM PMSF, then resuspended to 0.5–1 mg/ml protein. Of the suspension, 80 µl was incubated with 10 µl of undiluted anti-GFP for 1 h at 0 °C, washed and incubated for 1 h at 0 °C with gold-conjugated goat anti-(rabbit IgG), diluted 1:10. A final wash was followed by fixation as described by

Figure 1 Outline of the construct used for expression of CPT I-GFP

An almost full-length cDNA of the human *CPT1B* gene, which encodes M-CPT I, was placed behind the human cytomegalovirus (CMV) immediate-early promoter, and fused in frame to GFP. The latter was followed by the SV40 poly(A) signal sequence for the termination of transcription. The start (ATG) and stop (TAA) codons of the translational fusion are indicated. The protein sequence of the fusion site of CPT I-GFP and that of the C-terminal protein sequence of human (wild-type) M-CPT I are depicted below.

Kiebler et al. [21]. The same pre-embedding method was used for mitochondria. Control samples received no primary antibody. All preparations were counterstained with 4% (w/v) uranyl acetate and Reynold's lead citrate and observed in a Philips EM 400 electron microscope (Philips, Eindhoven, The Netherlands).

Preparation of mitochondria

Cultures grown in parallel were harvested, then washed in PBS at 4 °C. Further handling of the samples was performed on ice. Mitochondria were isolated by the method of Maeda et al. [22]. All measurements were performed with sonicated frozen samples (stored at -20 °C for use within 2 weeks, or as aliquots at -80 °C for longer-term use) or with fresh preparations, in which mitochondria were either swollen by the addition of 2 vol. of deionized water, or sonicated. Sonication was with two 15 s pulses at 8 W. Protein measurements were by the bicinchoninic acid method [23], as supplied in a kit (Pierce, Rockford, IL, U.S.A.). Citrate synthase was measured as described [24] to monitor the enrichment of mitochondrial proteins.

Measurement of CPT activity

CPT activity was measured by using the forward exchange assay [25], with adaptations as described below, and with L-[methyl-¹⁴C]carnitine as labelled substrate (Amersham; 2.1 GBq/mmol; 10 kBq per reaction). The linear stage of fatty-acyl[¹⁴C]carnitine production was measured in 50 mM Hepes (pH 7.4)/150 mM KCl/1 mM EDTA/1 mM dithiothreitol/1.3 mg/ml BSA (fatty acid free)/500 μ M carnitine/100 μ M palmitoyl-CoA. Malonyl-CoA (100 or 200 μ M) or Triton X-100 (2%, v/v) were added 1 min before measurement of the activity. Substrate concentrations of 10 μ M palmitoyl-CoA and 500 μ M carnitine were used to measure the sensitivity to malonyl-CoA at pH 7.0. However, the use of a lower pH influences inhibition by malonyl-CoA only at concentrations lower than 10 μ M [26]. All reactions were at 30 °C, in a final volume of 0.5 ml. The reactions were stopped by the addition of an equal volume of 1.2 M HCl. The radioactive acylcarnitine formed was extracted with 0.5 ml of butan-1-ol, which was subsequently washed three times with butan-1-ol-saturated water. The radioactivity in 0.2 ml of the butan-1-ol phase was quantified with UltimaGoldXR (Packard, Meriden, CT, U.S.A.) as scintillant in a Packard Tricarb 2500TR liquid-scintillation analyser.

Western blot analysis

Proteins of mitochondria of cells expressing CPT I-GFP and of total cell homogenates of GFP-expressing cells were separated by SDS/PAGE [15% and 12.5% (w/v) gel] respectively. Aliquots (3 μ g) of protein were solubilized in buffer containing 1.5% (w/v) SDS. Gels were run with a prestained molecular mass marker (Bio-Rad, Veenendaal, The Netherlands) yielding bands of 19.8, 28.3, 33.5, 46.9, 80 and 104 kDa, and a second molecular mass marker (BDH, Poole, Dorset, U.K.) yielding bands of 43, 56, 77, 97, 116 and 200 kDa. Blotting and detection was by standard methods, with rabbit anti-GFP (Clontech) and HRP-conjugated pig anti-rabbit IgG (Dako, Glostrup, Denmark), with the use of an enhanced chemiluminescence kit (Amersham) and exposure times of 20–60 min.

Computer analysis

Standard gene analysis programs were from the Lasergene package for Apple PowerPC (DNASTar, Madison, WI, U.S.A.).

The Protean module was used to plot hydropathic distributions by the method of Kyte and Doolittle [27], a method known to be reliable for the analysis of organellar proteins [28]. The neural network-based PredictProtein program (www.embl-heidelberg.de/predictprotein/ppDoPred.html) was applied to check predictions of transmembrane helices at high accuracy [29].

RESULTS

CLSM indicates predominant sorting of CPT I-GFP to mitochondria

We constructed a gene encoding a fusion protein between human M-CPT I and GFP, and localized the gene product in transfected cells by fluorescence microscopy. The transfected colonies showed easily detectable levels of green fluorescence, irrespective of the construct introduced (encoding CPT I-GFP or non-fused GFP). As judged by eye, the mean level of fluorescence was higher in cells expressing non-fused GFP than in cells expressing CPT I-GFP.

CLSM showed that the green fluorescence in CPT I-GFP cells was not homogeneously distributed within the cells but was confined to extranuclear structures (Figures 2a, 2d, 2g and 2h). These structures resembled mitochondria with regard to their punctated subcellular distribution and thread-like appearance (compare with [14]). Additional indication that the CPT I-GFP-containing structures were mitochondria was obtained by the use of MitoTracker CMXRos-H₂. This compound is sequestered in mitochondria and fluoresces red after oxidation. In our experiments, the resulting CMXRos fluoresced at the same sites as CPT I-GFP (Figures 2b, 2c, 2e and 2f). Sometimes non-overlapping red fluorescent spots were seen (see Figures 2f–2h) that probably result from the oxidation of CMXRos-H₂ in peroxisomes. The first cultures of (poly)clones might have consisted of cells derived from different gene-integration events. This chimaerism was useful because within single preparations it provided images of cells with and without green fluorescent structures (Figures 2f and 2g). The confocal laser scanning image of control cells showed green fluorescence, homogeneously distributed within the cytosol and nuclei, and lower levels of green fluorescence in the nucleoli (Figure 2i). This was in agreement with our expectations based on similar studies [16,17,30].

Evidence for mitochondrial sorting of CPT I-GFP by post-embedding immunogold EM

We applied post-embedding immunolabelling to ultrathin sections of coverslip-grown cells. Strong evidence for mitochondrial sorting of CPT I-GFP was supplied by an analysis of cells with anti-GFP antibodies and secondary antibodies labelled with 15 nm gold particles. The distribution of gold particles was in or near mitochondria. Most gold particles were restricted to the periphery of the mitochondria, suggesting the localization of CPT I-GFP to the mitochondrial outer membrane. However, it was evident that the resolution was limited by the post-embedding approach. In this, complexes of primary and gold-labelled secondary antibodies are formed at the surfaces of the preparations; these complexes therefore give rise to signals at a defined distance from the exact location of the GFP epitopes. If the sizes of antibodies and gold particles are taken into account, most of the signal was within the expected distance from the outer membrane.

Figure 2 Confocal laser scanning images of transfected HeLa cells and subcellular distribution of fluorescent signals

Images of cells expressing CPT I-GFP (**a–h**) and cells expressing non-fused GFP (**i**) were taken with the use of the mitochondrial marker MitoTracker CMXRos (**a–h**) for co-localization. The upper two rows of images of surface-attached cells (**a–c** and **d–f**) show the green fluorescence of CPT I-GFP (**a, d**), the red fluorescence of MitoTracker CMXRos (**b, e**) and combined images (**c, f**). Combined images are also shown for surface-attached cells in (**g**) and for cells in suspension in (**h**). The cells expressing non-fused GFP (**i**) show green fluorescence in the cytosol and nuclei, with lower levels of green fluorescence in the nucleoli. Scale bars, 10 μ m.

Results from GFP control cells were used for comparison. Post-embedding labelling of these cells showed gold particles at nuclear and cytosolic sites. The overall numbers of gold particles in immunolabelled preparations of GFP cells were smaller than in preparations of CPT I-GFP cells. However, because a soluble protein has one more degree of freedom than its membrane-bound counterpart for escape from the labelling procedure, the labelling densities are not directly comparable [31]. The estimated numbers of gold particles at sites other than mitochondria in CPT I-GFP cells were smaller than those in GFP cells and

similar to those in cells that had not been treated with the primary antibody.

Occasionally, several gold particles were clustered together near mitochondria in CPT I-GFP cells. However, clusters of signal can arise non-specifically and can depend on the electrostatic state of the gold conjugate. Although we did not observe many gold clusters in control experiments, the observation of gold clusters should be interpreted with care. In our opinion, the observation of gold clusters does not allow extrapolation to the membrane distribution of the original signal.

Figure 3 Pre-embedding electron microscopy of mitochondria from CPT I-GFP cells

(a) Micrograph from an experiment in which partly disrupted cells yielded intact mitochondria inside complete cell structures. (b) A preparation of isolated mitochondria. The thin sections were 100–200 nm thick. The plane and curve of the mitochondrial outer membranes relative to the plane of the cut surface determine the spreading of the 15 nm gold particles: at an angle of 90° the multilayer of gold results in overlapping electron-dense particles, whereas at different angles or at more convex parts the particles are more disperse. The primary antibody used was polyclonal anti-GFP and was applied before the thin sections of embedded material were cut. Scale bars, 0.5 μ m.

Figure 4 Flow cytometry and Western blot analysis

(a) The distribution of fluorescence of cells expressing CPT I-GFP (left panel) and control cells expressing non-fused GFP (right panel) as determined by flow-cytometry. Cell counts are indicated. The FITC channel was used for measuring green fluorescence. (b) Anti-GFP immunoblot analysis of mitochondrial proteins from CPT I-GFP cells (left panel) and total cell proteins from GFP cells (right panel). The sizes of the protein bands are indicated at the right. CPT I-GFP shows an apparent band size of 109 kDa and a presumed proteolytic degradation product of 98 kDa. Non-fused GFP migrates to the distance expected for a 30 kDa protein.

Demonstration that the CPT I-GFP C-terminus is exposed on the cytosolic face of the mitochondrial outer membrane by pre-embedding immunogold EM

The resolution of post-embedding immunogold EM in the experiments described above did not allow firm conclusions to be drawn about the membrane distribution of the gene product and its orientation within the outer membrane. To address the latter question properly we used partly disrupted cells to ensure the intactness of mitochondria and incubated these preparations with antibodies before embedding and sectioning. If the orientation of the C-terminus were to be towards the inter-membrane space of the mitochondria, the antibodies directed against GFP would not have access to GFP epitopes. In contrast, exposure of the GFP domain on the cytosolic aspect of the membrane would result in specific labelling of the mitochondrial outer membrane.

The results showed clear-cut labelling on the outer surface of mitochondria (Figure 3a). The preparations sometimes contained perfectly intact cells without any gold localized in the cells, indicating that these cells had not been disrupted in the mild procedure that we used. Free mitochondria were also observed but most labelled mitochondria were inside partly disrupted cell structures.

When isolated mitochondria were used for pre-embedding labelling, the results confirmed those obtained with partly disrupted cells. The mitochondria from CPT I-GFP cells showed massive labelling at the mitochondrial surface (Figure 3b). Because the thickness of the ultrathin sections was approx. 100–150 nm, the spreading of the 15 nm gold particles in these preparations was determined by the convex shape of mitochondria and by the angle between the mitochondrial outer membrane and the cut surface of these sections. On visual inspection of the EM images, these preparations of mitochondria showed a high proportion of intact mitochondria, with occasional examples of swollen and broken mitochondria and membrane remnants. Interestingly, even when mitochondria with disrupted outer membranes were obtained, they did not show any labelling

Figure 5 CPT activity in mitochondria isolated from CPT I-GFP and GFP cells

CPT activity was measured in the forward direction with 500 μ M carnitine, and either 100 μ M (left panel) or 10 μ M (right panel) palmitoyl-CoA in the reaction mixture. Palmitoyl-CoA (100 μ M) was used for measuring total CPT activity (black bars) and CPT activity after the addition of 100 or 200 μ M malonyl-CoA (white bars) or 2% (v/v) Triton X-100 (grey bars). CPT activity is given in nmol of product/min per mg of protein. Results are means $\pm$ S.D. for four experiments (error bars), except for the measurements after Triton X-100 solubilization, in which only two experiments were performed in each case.

of the inner aspect of the outer membrane, confirming that the C-terminal domain of the protein was entirely located on the cytosolic aspect. In contrast with the results obtained by post-embedding labelling, the gold label remained on one side, namely the cytosolic side of the mitochondrial membrane, owing to fixation of the antibody complex before embedding.

Whereas the first microscopical results might have been obtained from chimaeric clones, we observed variable levels of fluorescence even after prolonged microscopically monitored propagation. FACS analyses, shown in Figure 4(a), indicated a striking difference between CPT I-GFP and GFP cells with regard to the distribution of cells with different levels of fluorescence. The level of green fluorescence ranged over three orders of magnitude in CPT I-GFP cells and over one order of magnitude in GFP cells. CPT I-GFP cells showed an unequal distribution of green fluorescence, whereas GFP cells showed a gaussian distribution (Figure 4a). With regard to the relative level of fluorescence, the FACS analyses confirmed the microscopical data: the overall level of green fluorescence was lower in CPT I-GFP cells than in GFP cells.

CPT I-GFP fusion protein retains major CPT I characteristics

To investigate whether the topology of CPT I-GFP reflected that of native CPT I and therefore to rule out the possibility that the cytosolic position of the C-terminus was an artificial consequence of a misfolded and inactive protein, the molecular mass and the capacity of the fusion protein to express CPT I activity were determined.

The molecular mass of CPT I-GFP is of interest because it is known that the electrophoretic behaviour of the M-CPT I protein deviates from that predicted from the gene sequence (82 kDa after SDS/PAGE, compared with a predicted size of 88 kDa) [1,32]. The CPT I-GFP fusion construct was predicted to yield a protein with a molecular mass of 115 125 Da. Western analysis of mitochondrial proteins from CPT I-GFP cells resulted in two bands that were recognized by the anti-GFP antibody (Figure 4b, left panel): a major band of 109 kDa and a minor band of 98 kDa. The latter is possibly a proteolytic artifact. However, attempts to eliminate the presence of this smaller band by the use of protease inhibitors during the centrifugation of mitochondria remained unsuccessful. The GFP in control GFP cells was expected to have a molecular mass of 29 373 Da. Western analysis of a total homogenate of these cells showed a 30 kDa band (Figure 4b, right panel), indicating no abnormality.

To test whether CPT I-GFP also retained other important characteristics of CPT I, CPT activity was determined *in vitro* by the forward exchange assay, i.e. in the direction of catalysis by CPT I *in vivo*. When 100 μ M palmitoyl-CoA and 500 μ M carnitine were used, an increased CPT activity of approx. 20 nmol product/min per mg of protein was observed in mitochondria from CPT I-GFP cells. This increased activity was sensitive to inactivation by solubilization in Triton X-100, and to inhibition by malonyl-CoA (Figure 5, left panel).

The use of the detergent Triton X-100 is suitable for inactivating both endogenous and transgenic CPT I activity; the remaining CPT activity was presumed to represent CPT II, because we used disrupted mitochondria. With regard to the minor relative contribution of CPT II in mitochondria from CPT I-GFP cells, the use of disrupted mitochondria did not interfere with our purpose to show that CPT I-GFP expresses CPT I activity. Measurement of CPT activity under these assay conditions is a standard method for diagnostic purposes [33]. However, inhibition by malonyl-CoA is far from complete under these assay conditions. Approx. 67% inhibition was found in mitochondria from CPT I-GFP cells, and only 14% inhibition in control mitochondria (Figure 5, left panel). Because the inhibition of CPT I by malonyl-CoA is known to be partly competitive with the substrate palmitoyl-CoA [34], we also measured sensitivity to malonyl-CoA by using palmitoyl-CoA at 10 μ M instead of at 100 μ M; under these conditions the sensitivity to malonyl-CoA was indeed higher (Figure 5, right panel). The remaining activity in these mitochondria was assumed to represent CPT II and probably some remaining activity of CPT I. Because the use of lower concentrations of palmitoyl-CoA resulted in lower total activities (note the difference in scale between the left and right panels in Figure 5), the use of 10 μ M palmitoyl-CoA did not give an accurate measure of the maximum CPT capacity but was useful in assessing sensitivity to malonyl-CoA. In summary, the results shown in Figure 5 unambiguously demonstrate a greater CPT activity in mitochondria from CPT I-GFP cells than in control mitochondria. This activity is sensitive to Triton X-100 and, most importantly, malonyl-CoA.

DISCUSSION

In the present study the expression of a CPT I-GFP fusion protein in HeLa cells was used for microscopical and functional analyses to investigate CPT I topology. The human HeLa cell

line was chosen to express a tagged human M-CPT I efficiently in a species-specific background with respect to mitochondrial membrane properties. To obtain a fusion protein with the highest probability of unimpaired CPT I or GFP function, GFP was fused to the C-terminus of CPT I, not to the N-terminus. The CPT I N-terminal protein sequence is highly conserved throughout all CPT I enzymes; the domain C-terminal to the transmembrane segments is expected to be much larger (645 amino acid residues for human M-CPT I) than the N-terminal domain (55 residues). Indeed, the CPT I-GFP fusion protein retains both functions of the fusion counterparts.

At least three general characteristics of CPT I are preserved in CPT I-GFP (Figure 5). These characteristics are the ability to transfer palmitoyl chains from coenzyme A to carnitine, the inhibition of this activity by malonyl-CoA, and the loss of this activity on solubilization with Triton X-100. Therefore the addition of an almost 30 kDa GFP domain to the C-terminus of CPT I does not abolish CPT I function. This finding allows the extrapolation of the cell biological data obtained *in vitro* by immunogold EM and *in vivo* by CLSM to the topology of native CPT I. However, CPT I-GFP cells showed variable degrees of fluorescence within the cell population (Figure 4a), which might indicate a specific effect of the CPT I domain in CPT I-GFP on the stability of gene expression.

The sorting of CPT I-GFP was studied *in vivo* by CLSM (Figure 2) and *in vitro* by post-embedding immunogold EM. The predominant sorting of CPT I-GFP to the periphery of mitochondria indicates that the protein signals responsible for this are preserved. It is not known exactly which part of the protein is involved in the targeting of CPT I. From our experiments we conclude that if a C-terminal signal is involved in mitochondrial targeting, it is not obscured by the addition of GFP. Therefore a protruding C-terminal signal is not involved in the mitochondrial targeting of CPT I.

Because the use of permeabilizing agents such as digitonin has been controversial [8], we preferred the use of mild mechanical disruption of cells to leave the mitochondria intact for pre-embedding labelling. This method led to massive labelling on the cytosolic surface of these mitochondria (Figure 3a). Similar results were obtained by the labelling of isolated mitochondria (Figure 3b), whereas the analysis of membrane remnants in these preparations indicated that there was no gold labelling at the inner face of the mitochondrial membrane. Therefore new electron microscopical evidence is provided for the cytosolic compartmentalization of the relatively large C-terminal domain of the mitochondrial outer membrane enzyme CPT I. This evidence is consistent with results from studies with sequenced protein fragments [35] and deletion analysis of coding sequences [36], and with several lines of biochemical evidence provided by Fraser et al. [9,10] that indicate that both the malonyl-CoA inhibition site and the active site of CPT I are located on the cytosolic side of the mitochondrial outer membrane. Such an interpretation of CPT I topology is in agreement with predictions of CPT I topology based on its primary structure [1,35]. Given all these lines of evidence, our perception of CPT I topology is in favour of a model in which the active site of CPT I is formed by the cytosolic part of the protein. Moreover, this view of CPT I topology also explains the partly competitive nature of the inhibition of CPT I by malonyl-CoA and the fact that malonyl-CoA binding is abolished when the active site is blocked [37,38]. In CPT I, the site of regulation by malonyl-CoA is at the cytosolic side, a concept that has been beyond dispute since 1987 [1,8]. However, because inhibition by malonyl-CoA is influenced by competition between malonyl-CoA and acyl-CoA, the inhibitory action of malonyl-CoA might involve one or two

microdomains of CPT I, which must be adjacent if they are not one and the same. The location of these microdomains on the same side of the CPT I protein [37,38] is more likely to be true than the existence of separate locations on opposite sides. Hence the inhibitory site and the active site of CPT I might well be in close vicinity within the protein, on the cytosolic face of the mitochondrial outer membrane.

Human M-CPT I is likely to be representative of both L-CPT I and M-CPT I of various species with respect to membrane topology. Thus the enzymes have the same mode of action and are sensitive to the same inhibitors, and the similarity between different isoforms is fairly high, even when different isoforms between different species are compared. A computational analysis [27] of the different CPT I isoforms from rat and man verified that they show similar hydropathic distributions; high-accuracy predictions of transmembrane helices [29] indicated that only two regions (human M-CPT I positions 56–73 and 110–127) have membrane-spanning propensities (results not shown). These two regions have been referred to as H1 and H2 [1] or TMD1 and TMD2 [10] and border the part of the protein that is thought to loop into the intermembrane space.

Whereas the topologies of the enzymes within the mitochondrial outer membrane must be similar, the folding might differ within the limits of freedom given by the sequence differences. It is known that M-CPT I has a smaller apparent molecular mass, when measured by SDS/PAGE, than predicted by the primary protein sequence deduced from the gene [1,6,32]. This is an intrinsic property of the M-CPT I structure, because M-CPT I synthesized *in vitro* shows the same migration behavior as the protein expressed *in vivo* [32]. From Western analysis (Figure 4b) it can be concluded that the migration data of the CPT I-GFP recombinant protein are consistent with previous data for the native M-CPT I. The 6 kDa size difference matches the difference in the observed and predicted sizes of M-CPT I from rat [32] and man [1]. Therefore not only are general characteristics of CPT I preserved in CPT I-GFP but also a specific property of the muscle-type isoform from which the CPT I domain in the fusion protein was derived.

Our results do not exclude the possibility that a minor proportion of CPT I-GFP is functionally targeted to sites other than mitochondria, as suggested recently for a minor proportion of CPT I in rat liver preparations [39]. The proportion of CPT I-GFP localized on non-mitochondrial sites was lower than or similar to the background levels of labelling in control cells. Therefore the proportion of CPT I-GFP located on sites other than mitochondria was either too small to be detected accurately or it was absent owing to negative effects of the C-terminal fusion on non-mitochondrial targeting (as opposed to mitochondrial targeting). Because sometimes only short internal protein sequences are needed for targeting, a third possibility might be that only L-CPT I, and not M-CPT I, is functionally targeted to peroxisomes and the endoplasmic reticulum, for instance. To our knowledge, no results on M-CPT I are available on this topic.

In this study we have investigated the topology of a fusion between CPT I and GFP, and provide evidence that CPT I-GFP retains major CPT I characteristics. We conclude that CPT I-GFP topology reflects the topology of non-fused CPT I and that the C-terminus of CPT I is located on the cytosolic face of the mitochondrial outer membrane. This is the first study addressing CPT topology by CLSM and immunogold EM. The strong combination of GFP gene technology and microscopical approaches provides a promising basis for future experiments to gain further insight into this important system for energy metabolism.

We thank Mrs. W. Lemstra and Dr. E. Nollen of the Radiobiology Department of the University of Groningen for help with the transfections; Professor R. Wanders and Dr. L. IJlst of the Medical Center Amsterdam for helping us to set up CPT measurements; Mrs. N. Huijman and G. Mesander for technical assistance; B. Tebbes and H. Zaegers for the preparation of figures; and Dr. M. Veenhuis for helpful advice. This study was supported by the Netherlands Heart Foundation (NHS 94.194).

REFERENCES

- 1 McGarry, J. D. and Brown, N. F. (1997) *Eur. J. Biochem.* **244**, 1–14
- 2 McGarry, J. D. (1992) *Science* **258**, 766–770
- 3 Kelly, D. P. and Strauss, A. W. (1994) *New Engl. J. Med.* **330**, 913–919
- 4 Girard, J., Ferre, P., Pegorier, J. P. and Duee, P. H. (1992) *Physiol. Rev.* **72**, 507–562
- 5 Bartelds, B., Gratama, J. W. C., Knoester, H., Takens, J., Smid, G. B., Aarnoudse, J. G., Heymans, H. S. A. and Kuipers, J. R. G. (1998) *Am. J. Physiol.* **274**, H1962–H1969
- 6 Yamazaki, N., Shinohara, Y., Shima, A., Yamanaka, Y. and Terada, H. (1996) *Biochim. Biophys. Acta* **1307**, 157–161
- 7 van der Leij, F. R., Takens, J., van der Veen, A. Y., Terpstra, P. and Kuipers, J. R. G. (1997) *Biochim. Biophys. Acta* **1352**, 123–128
- 8 Murthy, M. S. and Pande, S. V. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 378–382
- 9 Fraser, F., Corstorphine, C. G. and Zammit, V. A. (1996) *Biochem. Soc. Trans.* **24**, 184S
- 10 Fraser, F., Corstorphine, C. G. and Zammit, V. A. (1997) *Biochem. J.* **323**, 711–718
- 11 Esser, V., Britton, C. H., Weis, B. C., Foster, D. W. and McGarry, J. D. (1993) *J. Biol. Chem.* **268**, 5817–5822
- 12 Cohen, I., Kohl, C., McGarry, J. D., Girard, J. and Prip-Buus, C. (1998) *J. Biol. Chem.* **273**, 29896–29904
- 13 Swanson, S. T., Foster, D. W., McGarry, J. D. and Brown, N. F. (1998) *Biochem. J.* **335**, 513–519
- 14 Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R. Y. and Pozzan, T. (1996) *Curr. Biol.* **6**, 183–188
- 15 Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G. and Youle, R. J. (1997) *J. Cell Biol.* **139**, 1281–1292
- 16 Zhang, G., Gurtu, V. and Kain, S. R. (1996) *Biochem. Biophys. Res. Commun.* **227**, 707–711
- 17 Zolotukhin, S., Potter, M., Hauswirth, W. W., Guy, J. and Muzyczka, N. (1996) *J. Virol.* **70**, 4646–4654
- 18 Graham, F. L. and van der Eb, A. J. (1973) *Virology* **52**, 456–467
- 19 Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1373–1376
- 20 Slot, J. W. and Geuze, H. J. (1984) in *Immunolabelling for Electron Microscopy* (Polak, J. M. and Varndell, I. M., eds.), pp. 129–149, Elsevier Science Publishing, Amsterdam
- 21 Kiebler, M., Keil, P., Schneider, H., van der Klei, I. J., Pfanner, N. and Neupert, W. (1993) *Cell* **74**, 483–492
- 22 Maeda, M., Doi, O. and Akamatsu, Y. (1980) *Biochim. Biophys. Acta* **597**, 552–563
- 23 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- 24 Srere, P. A. (1969) *Methods Enzymol.* **13**, 3–11
- 25 McGarry, J. D., Mills, S. E., Long, C. S. and Foster, D. W. (1983) *Biochem. J.* **214**, 21–28
- 26 Bieber, L. L. and Wagner, M. (1996) *Biochim. Biophys. Acta* **1290**, 261–266
- 27 Kyte, J. and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
- 28 van der Leij, F. R., Visser, R. G., Ponstein, A. S., Jacobsen, E. and Feenstra, W. J. (1991) *Mol. Gen. Genet.* **228**, 240–248
- 29 Rost, B., Casadio, R., Fariselli, P. and Sander, C. (1995) *Protein Sci.* **4**, 521–533
- 30 Rizzuto, R., Brini, M., Pizzo, P., Murgia, M. and Pozzan, T. (1995) *Curr. Biol.* **5**, 635–642
- 31 Slot, J. W., Posthuma, G., Chang, L. Y., Crapo, J. D. and Geuze, H. J. (1989) in *Immunogold Labelling in Cell Biology* (Verkley, A. J. and Leunissen, J. L. M., eds.), pp. 135–155, CRC Press, Boca Raton, FL
- 32 Esser, V., Brown, N. F., Cowan, A. T., Foster, D. W. and McGarry, J. D. (1996) *J. Biol. Chem.* **271**, 6972–6977
- 33 IJlst, L., Mandel, H., Oostheim, W., Ruiter, J. P. N., Gutman, A. and Wanders, R. J. A. (1998) *J. Clin. Invest.* **102**, 527–531
- 34 McMillin, J. B., Wang, D., Witters, L. A. and Buja, L. M. (1994) *Arch. Biochem. Biophys.* **312**, 375–384
- 35 Kolodziej, M. P. and Zammit, V. A. (1993) *FEBS Lett.* **327**, 294–296
- 36 Brown, N. F., Esser, V., Foster, D. W. and McGarry, J. D. (1994) *J. Biol. Chem.* **269**, 26438–26442
- 37 Declercq, P. E., Falck, J. R., Kuwajima, M., Tyminski, H., Foster, D. W. and McGarry, J. D. (1987) *J. Biol. Chem.* **262**, 9812–9821
- 38 Kolodziej, M. P. and Zammit, V. A. (1990) *Biochem. J.* **267**, 85–90
- 39 Fraser, F., Corstorphine, C. G., Price, N. T. and Zammit, V. A. (1999) *FEBS Lett.* **446**, 69–74

Received 3 March 1999/22 April 1999; accepted 24 May 1999