The N-terminal Domain of Rat Liver Carnitine Palmitoyltransferase 1 Contains an Internal Mitochondrial Import Signal and Residues Essential for Folding of Its C-terminal Catalytic Domain*

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We have previously shown that the first 147 N-terminal residues of the rat liver carnitine palmitoyltransferase 1 (CPT1), encompassing its two transmembrane (TM) segments, specify both mitochondrial targeting and anchorage at the outer mitochondrial membrane (OMM). In the present study, we have identified the precise import sequence in this polytopic OMM protein. In vitro import studies with fusion and deletion CPT1 proteins demonstrated that none of its TM segments behave as a signal anchor sequence. Analysis of the regions flanking the TM segments revealed that residues 123–147, located immediately downstream of TM2, function as a noncleavable, matrix-targeting signal. They specify mitochondrial targeting, whereas the hydrophobic TM segment(s) acts as a stop-transfer sequence that stops and anchors the translocating CPT1 into the OMM. Heterologous expression in Saccharomyces cerevisiae of several deleted CPT1 proteins not only confirms the validity of the “stop-transfer” import model but also indicates that residues 1–82 of CPT1 contain a putative microsomal targeting signal whose cellular significance awaits further investigation. Finally, we identified a highly folded core within the C-terminal domain of CPT1 that is hidden in the entire protein by its cytosolic N-terminal residues. Functional analysis of the deleted CPT1 proteins indicates that this folded C-terminal core, which may belong to the catalytic domain of CPT1, requires TM2 for its correct folding achievement and is in close proximity to residues 1–47.

In mammals, the mitochondrial carnitine palmitoyltransferase (CPT, EC 2.3.1.21) 1 is the key regulatory enzyme of long chain fatty acid oxidation (1). This enzyme catalyzes the conversion of long chain acyl-CoA to acylcarnitines, which permits, in cooperation with the carnitine/acylcarnitine translocase and the CPT2, their transport from the cytoplasm into the mitochondrial matrix to undergo β-oxidation. CPT1 is tightly regulated by its physiological inhibitor, malonyl-CoA, the first committed intermediate of fatty acid biosynthesis (1). The liver mitochondrial CPT1 isoform is anchored into the outer mitochondrial membrane (OMM) in an Ncyto-Ccyto orientation via two α-helical hydrophobic transmembrane (TM) segments (TM1, residues 48–75; TM2, residues 103–122). Its N terminus (residues 1–47) and its large C-terminal domain (residues 123–773) are facing the cytosol, whereas the loop connecting TM1 and TM2 is exposed in the intermembrane space (2, 3). Apart from mitochondria, microsomes and peroxisomes also contain membrane-bound malonyl-CoA-sensitive CPTs (4, 5), which share similar functional properties with the mitochondrial CPT1, have an identical molecular mass of about 88 kDa, and were immunoreactive with antibodies raised against distinct linear epitopes of the mitochondrial CPT1 (6). Whether these enzymes are identical or similar is still a matter of debate. This raises the crucial question of how the mitochondrial CPT1 is specifically imported into the OMM and whether multiple or hierarchical targeting sequences could exist within a single polypeptide allowing distinct subcellular locations.

Nuclear-encoded mitochondrial proteins are synthesized as precursors in the cytosol and harbor signals that mediate primarily, via a specific interaction with the outer mitochondrial receptors (Tom complex), their mitochondrial targeting and specify their intramitochondrial sorting (7). The targeting signals in matrix-destined preproteins are cleavable N-terminal presequences, positively charged and that have the potential to adopt amphipathic α-helices (8). By contrast, integral OMM proteins are synthesized as noncleavable proteins (9) and therefore are targeted to mitochondria by means of internal signals. How this is accomplished is still not clear, although clues have begun to emerge from studies of bitopic proteins, such as the Saccharomyces cerevisiae Tom70 and Tom6 and the mammalian Bcl-2 protein. Their targeting and insertion into the OMM have been shown to be mediated by their unique hydrophobic TM segment that functions as a “signal anchor sequence” selective for the OMM (10–12). An alternative to the signal anchor sequence model is the combination of a matrix-targeting signal with a hydrophobic stop-transfer sequence. Primarily based on the import studies of artificial bitopic chimeric proteins (13), this model has been shown to be valid for the Neurospora crassa bitopic Tom22 protein (14). Very few investigations have been performed on the nature of the targeting and/or topogenic signals of integral polytopic OMM proteins over the past 15 years. These proteins fall into two classes, namely those that contain transmembrane β-sheets, such as porin and the yeast Tom40, and those with α-helical hydrophobic TM segments. In the case of porin and Tom40, limited information regarding structural determinants of these β-barrel proteins is available (15–19), but the precise nature of their targeting signals remains unclear. Bearing in mind the structural difference between the two classes of polytopic OMM proteins, it would appear unlikely that the targeting and/or...
topogenic signals operate in a uniform manner. Thus, the rat liver CPT1 could be a useful model to study the mechanisms involved in mitochondrial targeting and membrane insertion of OMM proteins containing more than one a-helical hydrophobic TM segment.

We have previously shown that the N-terminal domain (residues 1–147) of CPT1 contains all of the information for mitochondrial targeting, OMM insertion, and membrane orientation (20). In the present study, we have identified the precise internal import signal of CPT1 by using two complementary approaches. In vitro import assay of fusion and/or deletion CPT1 proteins shows that its import into the OMM does not occur by a “signal anchor” but by a “stop-transfer” mechanism with the involvement of an internal matrix-targeting signal located immediately downstream of TM2. Heterologous expression in S. cerevisiae of several deleted CPT1 proteins not only confirms the validity of this model but also allows new insights into the folding of the C-terminal catalytic domain of CPT1. The functional importance of certain residues within the N-terminal domain for maintenance of a putative catalytic core is discussed.

**EXPERIMENTAL PROCEDURES**

Construction of Fusion and Deletion CPT1 Proteins

*Escherichia coli* DH5α strain was used to propagate various plasmids and their derivatives. The transcription plasmid pGEM4 (Promega) was used for cloning DNA fragments, making constructs, and *in vitro* transcription/translation. All of the pGEM4 constructs were under the control of the SP6 promoter. All DNA manipulations (restriction and ligation) were performed according to the instructions provided by the manufacturers’ protocols for the respective enzymes. The same strategy was used to generate all dihydrofolate reductase (DHFR) fusion constructs in which different parts of the N-terminal end of CPT1 were fused to DHFR. cDNAs coding for these regions were amplified by polymerase chain reaction (PCR) using different primers and pGEM4-CPT1Δ3’ (21) as template. All 5’-primers introduced an EcoRI restriction site (in front of a start codon), whereas the 3’-primers contain a BamHI restriction site, except for pCPT1-(33–47)-DHFR. DNA fragments encoding CPT1-(1–32), CPT1-(1–47), and CPT1-(1–82) were amplified by PCR using the same 5’-primer as previously described for pCPT1-(1–147)-DHFR (20) and the respective 3’-primers 5’-GCC GCA GAT CTG TTT GAG-3’ as template. The PCR product was ligated into pGEM4-DHFR to give pCPT1-(97–147)-DHFR (20) and the respective 3’-primer 5’-AAA ATG AGC CCC TCG TGG TGG TTC TTT-3’ (including the SacI restriction site of DHFR). The PCR product was digested by SacI and ligated into pCPT1-(122–147)-DHFR cut by the same enzymes to generate pCPT1-(1–47)-OM-DHFR and pCPT1-(122–147)-OM-DHFR.

**pCPT1Δ2**—Deletion of the first 82 amino acids of CPT1, CPT1Δ2, was achieved by amply DNA coding for CPT1-(1–82) by PCR using the same 5’-primer previously used to generate pCPT1-(1–147)-DHFR and the 3’-primer 5’-GGG CCT AGC GAG GGG-3’ as template and the same 5’-primer as previously described for pCPT1-(1–147)-DHFR. The PCR product was cloned into pGEM4-CPT1Δ3’ cut by the same enzymes to generate pCPT1Δ3’. DNA encoding CPT1-(1–82) was amplified by PCR using the same 5’-primer previously used to generate pCPT1-(1–147)-DHFR and the 3’-primer 5’-GGG CCT AGC GAG GGG-3’ as template. The PCR product was cloned into pGEM4-CPT1Δ3’ deleted from its large EcoRI-BglII fragment which codes for the first 147 amino acids of CPT1. The resulting pCPT1Δ3’–148 encodes a protein in which amino acid 82 is fused to residue 149 and possesses one extra amino acid (Arg) in the joining region.

**pCPT1Δ31–148**—This construct was obtained by excising the BglII-BamHI fragment from pGEM4-CPT1Δ3’ and re-ligating the plasmid. This results in a CPT1 protein in which amino acid 30 is fused to amino acid 149.

**pCPT1Δ31**—DNA encoding CPT1-(122–147) was amplified by PCR using pGEM4-CPT1Δ3’ as template and the same 5’-primer as previously described for pCPT1-(1–147)-DHFR and the 3’-primer 5’-GGG CCT AGC GAG GGG-3’ as template. This EcoRI-BglII PCR product was ligated into pGEM4-CPT1Δ3’ digested by EcoRI and BglII to obtain pCPT1Δ31–148. One amino acid (Arg) was introduced between residues 122 and 149.

cDNAs encoding the deleted CPT1 proteins were retrieved from pGEM4 as EcoRI-SalI insert and subcloned into the yeast expression vector pYeDP1–8 containing the full-length CPT1 (pYeDP-CPT1) (21) cut by the same enzymes to obtain pYe-CPT1Δ31–148, pYe-CPT1Δ31–148, and pYe-CPT1Δ31–148. Each cDNA was placed under the control of the inducible GAL10 promoter present in the vector. The fidelity of all PCRIs and the quality of DNA subcloning were confirmed by DNA sequencing.

In Vitro Synthesis of Precursor Proteins and Import into Mitochondria

Radiolabeled precursor proteins were synthesized by *in vitro* transcription-translation using the TNT® SP6-coupled reticulocyte lysate system (Promega) in the presence of [35S]methionine (Amersham Pharmacia Biotech) according to the manufacturer’s protocols. Isolation of purified rat liver mitochondria was performed as described previously (20). In *in vitro* import of radiolabeled proteins into mitochondria was performed as previously indicated (20). Dissipation of the membrane potential (∆Ψ) by 1 μM of carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma), mitochondrial pretreatment with trypsin (Sigma), postmitochondrial treatment with trypsin, alkanol extraction with 0.1 M NaClO4, as well as analysis of the import reactions by SDS-PAGE and fluorography were performed as described previously (20).

Submitochondrial Localization of Imported Proteins

After import, samples were split into 5 eq aliquots (70 μg of mitochondrial protein). Mitochondria were centrifuged at 12,000 × g for 5 min at 4°C, washed in KC1 buffer (250 mM sucrose, 10 mM Hepes, 80 mM KC1, pH 7.6), and resuspended (0.5 mg of protein/ml). Mitochondria were then diluted 10-fold either in KC1 buffer (nonswollen mitochondria) or in a swelling buffer (20 mM Hepes-KOH, pH 7.4) and subjected to trypsin (100 μg/ml) when indicated. After a 20-min incubation on ice, 2.8 mg/ml soybean trypsin inhibitor (STI, Sigma) was added, and sam-
samples were further kept on ice for 10 min. Mitochondria/mitoplasts were resolubilized by centrifugation and washed in EDTA buffer (250 mM sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.6). Mitoplasts were then solubilized by 0.5% (v/v) Triton X-100 (Sigma) in the absence or presence of trypsin (400 µg/ml). After centrifugation, both the pellet and the supernatant were incubated 5 min at 65 °C to inhibit trypsin, and the solubilized proteins present in the supernatant were trichloroacetic acid-precipitated. Samples were submitted to SDS-PAGE, blotted onto nitrocellulose, and analyzed by fluorography. The efficiency of swelling and solubilization of the mitoplasts was assessed by immunostaining.

Yeast Culture and Subcellular Fractionation

The yeast expression vector pYeDP1-8/10 containing the various deleted CPT1 proteins was used to transform S. cerevisiae (haploid strain W303: MATa, his3, leu2, trpl, 1, ura3, ade2-1, can1-100) according to Ref. 22. Methods for yeast culture and subcellular fractionation were as previously described (21, 23).

Miscellaneous Methods and Chemicals

Protein concentrations were determined by the method of Ref. 24 with bovine serum albumin as standard. Swelling of yeast mitochondria and Western blotting were performed as described previously (21). The antisera used were against the rat liver CPT1 (1/3000–1/10,000), the yeast cytochrome b1 (1/1000), and the yeast mtHSP70 (1/10,000). CPT activity was assayed at 30 °C as palmitoyl-L-[1-14C]carnitine from L-[methyl-3H]carnitine formed from L-[methyl-3H]carnitine (290 µM; 10 Ci/mol) and palmitoyl-CoA (80 µM) in the presence of 1% bovine serum albumin (w/v) as described previously (21). Malonyl-CoA concentration was 150 µM. When indicated, mitochondria were solubilized by 0.5% Triton X-100 as described in Ref. 20 and centrifuged at 16,000 × g for 10 min at 4 °C to sediment the insoluble membrane residues, and the supernatants were used for CPT assay.

Statistics

Results are expressed as means ± S.E. Statistical analysis was performed using the Mann-Whitney U test.

RESULTS

The Mitochondrial Targeting Signal of CPT1 Resides within Residues 97–147—To investigate the precise location of the mitochondrial targeting signal of CPT1 within its N-terminal domain, we first asked whether each half of this domain could play an equivalent role in this process. For this purpose, CPT1-(1–82) and CPT1-(97–147) were fused to a non-OMM-related protein, the cytosolic mouse DHFR (Fig. 1A). CPT1-(1–82)-DHFR was used as a positive control protein, since CPT1-(1–147)-DHFR and CPT1-(97–147)-DHFR were imported into the OMM in a temperature- and trypsin-sensitive receptor-dependent manner. The latter being exposed on the cytosolic face of mitochondria (20).

Radiolabeled CPT1-(1–147)-DHFR, CPT1-(1–82), and CPT1-(97–147) were synthesized in vitro and used to perform import reactions to determine their specific import requirements. In all cases, efficiency of import into the OMM was assayed using the alkaline extraction method, which removes all proteins that are not integrated into the membranes (10, 20). A significant amount of alkaline-resistant CPT1-(1–82)-DHFR and CPT1-(97–147)-DHFR was recovered as alkaline-resistant forms (Fig. 2A), confirming their insertion into the OMM with the mitochondrial receptors and to be specifically inserted into the OMM. Similarly, deletion of residues 83–148 in CPT1 abrogated in vitro the ability of the protein to be imported into rat liver mitochondria in a temperature- and trypsin-sensitive receptor-dependent manner, whereas deletion of residues 1–82 did not (data not shown). These results suggest that the signal sequence of CPT1 mediating mitochondrial targeting may reside within residues 97–147.

The Transmembrane Segments of CPT1 Do Not Function as Signal Anchor Sequences—To determine whether one or both of the TM segments of CPT1 function as a mitochondrial signal anchor sequence, we fused CPT1-(48–75), CPT1-(97–122), and CPT1-(48–122) to DHFR (Fig. 2A). When import reactions were performed in the absence of added mitochondria, a small amount of CPT1-(48–75)-DHFR, CPT1-(97–122)-DHFR, and CPT1-(48–122)-DHFR was recovered as alkaline-resistant forms (Fig. 2B, lane 2), likely due to protein aggregation. Import in the presence of mitochondria led to an increase in the level of these alkaline-resistant forms (Fig. 2B, compare lanes 2 and 4) that were totally digested by trypsin (Fig. 2B, compare lanes 4 and 5). Thus, the presence of TM1 and/or TM2 allowed insertion of DHFR into the OMM, the latter facing the cytosol. However, in contrast to CPT1-(1–147)-DHFR and CPT1-(97–147)-DHFR (Fig. 1B), there was no significant changes in the amount of inserted CPT1-(48–75)-DHFR, CPT1-(97–122)-
DHFR, and CPT1-(48–122)-DHFR when import was performed with trypsin-pretreated organelles (Fig. 2C, compare lanes 4 and 6). These results show that insertion of TM1 and/or TM2 into the OMM does not require the trypsin-sensitive surface receptors in contrast to CPT1. Thus, targeting of CPT1 to mitochondria is not mediated by its TM segments, ruling out the hypothesis that they may serve as signal anchor sequences. Therefore, the mitochondrial targeting signal of CPT1 must reside in regions flanking the TM segments.

The CPT1 Protein Is Targeted to Mitochondria by an Internal Matrix-targeting Signal—Hydrophobic cluster analysis is an efficient method for predicting secondary protein structure and segmentation (25). This method predicts two putative amphiphilic β-strands within residues 1–32 (β1, residues 8–14; β2, residues 19–23) and three amphipathic α-helices (residues 33–47, 76–102, and 123–147) flanking the TM segments (26). To test whether these regions have the capacity to target a reporter protein to mitochondria, we constructed a series of proteins in which CPT1-(1–47), CPT1-(1–32), CPT1-(33–47), CPT1-(76–102), and CPT1-(122–147) were fused to DHFR (Fig. 3A). We used the Su9-DHFR protein, which consists of the presequence of N. crassa Fg-ATPase subunit 9 preceding DHFR (27), as a positive control for protein import into the mitochondrial matrix. In all cases, the efficiency of the import of these radiolabeled fusion proteins into mitochondria was estimated by determining their protection toward trypsin proteolysis. Su9-DHFR was imported into the mitochondrial matrix, where it became processed to its mature-form size, which was inaccessible to exogenously added trypsin (Fig. 3B, compare lanes 4 and 8). Following import at 30 °C in the presence of mitochondrial CPT1-(1–47)-DHFR, CPT1-(1–32)-DHFR, and CPT1-(76–102)-DHFR were recovered in association with mitochondria but were almost totally digested by exogenous trypsin (Fig. 3B, compare lanes 4 and 8). This indicated that these CPT1 residues were unable to drive DHFR into the mitochondria. By contrast, CPT1-(33–47)-DHFR and CPT1-(122–147)-DHFR became largely insensitive to added trypsin (Fig. 3B, compare lanes 4 and 8), demonstrating that they were at least translocated across the OMM. Acquisition of the protease protection occurred in a temperature- and receptor-dependent manner (Fig. 3B, compare lane 3–5) and needed the presence of a membrane potential (ΔΨ) (Fig. 3C, compare lanes 3 and 4). To ascertain the location of CPT1-(33–47)-DHFR and CPT1-(122–147)-DHFR to the matrix, mitochondria were subfractionated by a swelling procedure in the presence of trypsin. As expected from previous studies (21), the endogenous CPT1 protein was resistant to trypsin digestion in intact mitochondria (due to a folded state of its large cytosolic C-terminal domain) (Fig. 3D, lane 3) and was partially degraded into an 83-kDa fragment when the OMM was disrupted upon swelling (Fig. 3D, lane 8), confirming their localization into the matrix. These results indicate that residues 33–47 and 122–147 behave as matrix-targeting signals. Surprisingly, when residues 1–32 were added to CPT1-(33–47)-DHFR, import of DHFR into the mitochondrial matrix was totally inhibited (Fig. 3B). Thus, the matrix-targeting function of residues 33–47 was abrogated by the first 32 amino acids of CPT1. This explained why residues 33–47 did not support import of CPT1-(1–32)-DHFR (Fig. 1B) or CPT1Δ33–147. To study further the role of residues 122–147 in mitochondrial targeting, import of CPT1Δ121 was analyzed (Fig. 3A). In contrast to CPT1Δ31–148 (data not shown) or CPT1Δ150 (20), CPT1Δ121 was efficiently imported into mitochondria in a process that was dependent upon the temperature, the presence of trypsin-sensitive surface receptors, and a membrane potential (Fig. 3, B and C). Swelling experiments confirmed that the imported CPT1Δ121 was located into the matrix compartment (Fig. 3D). These results demonstrate that residues 122–147 function as a matrix-targeting signal, driving the import of a reporter protein into the matrix in the absence of any of the TM segments of CPT1. As soon as one TM segment was present, such as in CPT1-(97–147)-DHFR, protein translocation across the mitochondrial membranes was arrested, leading to the insertion of the corresponding protein into the OMM (see Fig. 1B). To confirm the essential role of residues 122–147 in mitochondrial targeting, we compared the receptor dependence of the import of CPT1 and CPT1Δ123–148 (Fig. 4). In contrast to the full-length CPT1, deletion of residues 123–148 within CPT1 abrogated the ability of the protein to be imported in a trypsin-sensitive receptor-dependent manner (Fig. 4B, compare lanes 3 and 4). Thus, residues 123–147 specify in vitro the mitochondrial targeting of CPT1, whereas its hydrophobic TM segment(s) likely acts as a stop-transfer sequence that stops and anchors the translocating protein into the OMM.

Residues 122–147 Exert a Retention Force on the OMM Surface—We have previously reported that the N-terminal domain of CPT1 participated in the determination of the N\textsubscript{cya}, C\textsubscript{cyto} membrane topology (20) (Fig. 5B). As shown in Fig. 2B, residues 48–122 alone, encompassing the two TM segments and the connecting loop, seemed to be sufficient for attainment of the N\textsubscript{cya}, C\textsubscript{cyto} topology of CPT1. In the case of S. cerevisiae Tom70, the N\textsubscript{cya}, C\textsubscript{cyto} orientation of the protein was reversed when its first 10 residues were replaced by a strong matrix-targeting signal (28). Our aim was to test whether amphiplicity of the regions flanking the TM segments of CPT1 could, in addition to the TM1-TM2 pairing, be an important determinant for conferring protein topology. It has been shown that the
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Fig. 3. The N-terminal domain of CPT1 contains two matrix-targeting signals. A, fusion and deletion protein constructs: Su9-DHFR corresponds to the matrix-targeting signal of N. crassa F0-ATPase subunit 9 fused to DHFR (27), CPT1-(1–47), CPT1-(1–32), CPT1-(33–47), CPT1-(76–102), and CPT1-(122–147) were fused to DHFR, whereas residues 1–121 were deleted within CPT1 (CPT1Δ121). Zigzag lines were used as in Fig. 1A. Numbers denote residues of CPT1 after which the fusion to DHFR occurs. B, import of CPT1-(33–47)-DHFR, CPT1-(122–147)-DHFR, and CPT1Δ121 is temperature- and mitochondrial receptor-dependent. Import of radiolabeled proteins was carried out as described in Fig. 1. Following import, mitochondria were washed, centrifuged, and split into 2 eq aliquots, and trypsin (200 µg/ml) treatment was performed when indicated. After inactivation of the protease with STI (4 mg/ml), mitochondria were reisolated, submitted to SDS-PAGE, and analyzed by fluorography. p, precursor; m, mature form of Su9-DHFR. 10%, percentage of input lysate of each radiolabeled protein (lane 1). C, Δp dependence of import of CPT1-(33–47)-DHFR, CPT1-(122–147)-DHFR, and CPT1Δ121. Import of the radiolabeled proteins was performed in the presence (-CCCP; lanes 2 and 3) or in the absence (+CCCP; lane 4) of a membrane potential (+Δp). Following import at 30 °C, untreated mitochondria were split into 2 eq aliquots. The first one was washed and directly submitted to SDS-PAGE (lane 2). The second one as well as CCCP-pretreated mitochondria were subjected to trypsin treatment (+Trypsin; lanes 3 and 4) as described in B. Samples were then analyzed by SDS-PAGE and fluorography. 10%, percentage of input lysate of each radiolabeled protein (lane 1), D, the imported CPT1-(33–47)-DHFR, CPT1-(122–147)-DHFR, and CPT1Δ121 are located in the matrix. After import of the radiolabeled proteins, their submitochondrial localization was determined as described under “Experimental Procedures.” Mitochondria (lanes 2 and 3) and mitoplasts (lanes 4, 5, and 7) were incubated in the absence (-Trypsin; lane 1; lane 2) or presence of 100 µg/ml trypsin (+Trypsin; lane 1; lanes 3–5 and 7). All samples were reisolated by centrifugation and washed in EDTA buffer supplemented with STI, except for lane 7. The first 3 aliquots (lanes 2–4; T, total) were directly analyzed by SDS-PAGE, and the last two were solubilized by 0.5% Triton X-100 (+TX-100; lanes 5 and 7) at 4 °C for 10 min. Trypsin (400 µg/ml) was added when indicated (+Trypsin; lane 2; lane 7), and samples were kept on ice for another 10 min. Samples were centrifuged to recover the pellet (P, lanes 5 and 7) and the supernatant (S; lanes 6 and 8) that was trichloroacetic acid-precipitated. Samples were submitted to SDS-PAGE, blotted onto nitrocellulose, and analyzed by fluorography. Immunostaining with the endogenous CPT1 and the mHsp70 was then performed. 10%, percentage of input lysate of each radiolabeled protein (lane 1). f0 denotes fragment of Hsp70 generated by trypsin treatment of solubilized mitoplasts. f1 and f2 are CPT1-processed species.

unique TM segment of Tom70 (residues 11–29, here termed as OM) allowed specific N\textsubscript{in}-C\textsubscript{cyto} insertion of a reporter protein into the OMM (20, 28). Our strategy was to determine whether residues 1–47 or 122–147 of CPT1 could reverse the membrane orientation of this OM segment. For this purpose, CPT1-(1–47) and CPT1-(122–147) were fused to the OM domain preceding the DHFR moiety (Fig. 5A). As expected, the radiolabeled fusion proteins CPT1-(1–47)-OM-DHFR and CPT1-(122–147)-OM-DHFR were efficiently membrane inserted following an import reaction (Fig. 5B, compare lanes 2 and 3). Like CPT1-(1–47)-DHFR, the inserted CPT1-(1–47)-OM-DHFR was totally digested by exogenously added trypsin (Fig. 5B, lane 4). This implied that DHFR was located on the cytosolic face of mitochondria and indicated that residues 1–47 did not cause retention of the N terminus of the protein on the cytosolic face of the OMM. These results were in agreement with the observed N\textsubscript{in}-C\textsubscript{cyto} membrane topology of CPT1-(1–82)-DHFR (Fig. 1B). Surprisingly, the membrane-inserted CPT1-(122–147)-OM-DHFR was resistant to trypsin treatment of intact mitochondria (Fig. 5B, lane 4), even in the absence of a membrane potential (data not shown). Upon swelling of mitochondria in the presence of trypsin, CPT1-(122–147)-OM-DHFR became totally digested by the protease (Fig. 5B, lane 5), whereas mHsp70 remained protease-protected (data not shown). These results suggested that DHFR was located in the intermembrane space compartment and that CPT1-(122–147) was able to reverse the N\textsubscript{in}-C\textsubscript{cyto} orientation of OM-DHFR. These experiments emphasize that CPT1-(122–147) but not CPT1-(1–47) may participate together with residues 48–122 in the determination of the membrane topology of CPT1.

Heterologous Expression of Various Deleted CPT1 Proteins in S. cerevisiae—Yeast cells, a system devoid of endogenous CPT activity, represent a suitable model to study the structure-function relationships of the rat liver CPT1 (21, 29, 30). To validate the importance of residues 123–147 of CPT1 for mitochondrial targeting in an in vivo setting, CPT1Δ82, CPT1Δ83–148, CPT1Δ121, CPT1Δ123–148, CPT1Δ31–148, and the full-length CPT1 were expressed in S. cerevisiae. Immunodetection of the yeast-expressed proteins in crude homogenates showed that proteins of the predicted sizes were expressed, except for CPT1Δ31–148 (Fig. 6A, lane 7). Thus, in agreement with our in vitro import experiments, deletion of residues 31–148 led to a protein that was unable to be targeted correctly to mitochondria and hence might be rapidly degraded within the cells.
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Analysis of the subcellular distribution of the yeast-expressed full-length CPT1 shows that, by contrast to our previous reports (20, 21), the amount of CPT1 recovered in the microsomal fraction represents about 27% of the total expressed protein (Fig. 6, B and C). One possible explanation for this discrepancy was that immunoblotting was performed more stringently in the present experiment, allowing increased sensitivity in the detection of the expressed protein. Recovery of CPT1 in microsomes was not due to a mitochondrial contamination of the microsomal fraction, as shown by the subcellular distribution of the mtHSP70 that was representative of all the constructs (Fig. 6B). Moreover, microsomal recovered CPT1 exhibited CPT activity (2.49 ± 0.27 nmol/min/mg of protein) that was almost totally (93%) inhibited by 150 μM malonyl-CoA (0.17 ± 0.02 nmol/min/mg of protein), ruling out that CPT1 was recovered as aggregated protein. When analyzing the subcellular distribution of the various deleted CPT1, we kept in mind that CPT1 harbors two TM segments, and hence partial deletions within the N-terminal domain might affect targeting and membrane anchorage differentially. Deletion of residues 83–148 led to a complete reverse subcellular distribution when compared with the full-length CPT1 since about 93% of CPT1Δ83–148 was recovered in the microsomal fraction (Fig. 6, B and C). Upon deletion of 123–148, both mitochondrial and microsomal fractions contained equivalent amounts of the expressed protein, underlying the absence of privileged mitochondrial targeting (Fig. 6, B and C). Conversely, deletion of residues 1–82 or 1–121 did not alter mitochondrial targeting, whereas their recovery in the microsomal fraction was almost totally abolished (Fig. 6, B and C). These results confirmed our in vitro experiments (Fig. 1 and Fig. 3) and supported the conclusion that residues 123–148 were essential for mitochondrial targeting of CPT1. The finding that deletion of residues 1–82 abrogated microsomal location of CPT1 whereas CPT1Δ83–148 was most exclusively recovered in this fraction was puzzling, since both proteins contain a TM segment that could allow their nonspecific anchorage at the microsomal membranes. The present observation suggests the presence of a putative microsomal targeting signal within residues 1–82 of CPT1.

To examine the submitochondrial localization of the expressed deleted CPT1 proteins, intact or swollen yeast mitochondria were submitted to trypsin treatment (Fig. 7). The integrity of the outer and inner mitochondrial membrane was checked by the inaccessibility of endogenous cytochrome b2 (intermembrane space protein) and mtHSP70 to trypsin proteolysis, respectively. Upon trypsin treatment of intact mitochondria, CPT1Δ82, CPT1Δ83–148, and CPT1Δ123–148 were digested by the protease, and a proteolytic fragment of about 60-kDa was generated (f2 fragment) that remained membrane-anchored and detected by our CPT1 antibody raised against residues 317–430 (Fig. 7). These results indicated that (i) these deleted CPT1 proteins were anchored into the OMM with their C-terminal domain exposed to the cytosol, (ii) residues 123–148 are not essential for achievement of the correct membrane topology of CPT1, and (iii) a highly folded core exists within the cytosolic C-terminal domain of CPT1. However, the generation of the f2 fragment was less efficient in the case of CPT1Δ83–148, suggesting a partial unfolding of the C-terminal domain of this deleted protein. By contrast, CPT1Δ121 remained trypsin-protected even upon swelling and became totally digested by the protease when the inner membrane of mitoplasts was solubilized by Triton X-100 (Fig. 7). This showed that CPT1Δ121 was efficiently imported into the mitochondrial matrix but did not harbor a folded core. As shown in Fig. 3D for the native rat liver CPT1 protein, no trypsin-resistant 60-kDa fragment was generated in intact yeast mitochondria expressing CPT1 (Fig. 7). Consequently, the trypsin cleavage site at the cytosolic C terminus, previously observed for CPT1Δ82, was inaccessible in the entire protein. It became unmasked only after the cleavage by trypsin of the loop connecting TM1 and TM2 (f1 fragment), allowing the protease to generate the 60-kDa fragment (f2 fragment) (Fig. 3D and Fig. 7). These results indicate the existence of a highly folded core within the cytosolic C-terminal domain of CPT1 that is hidden by the presence of residues.

**Fig. 4.** CPT1-(123–148) mediates interaction with the mitochondrial trypsin-sensitive receptors. A, residues 123–148 were deleted within CPT1 (CPT1Δ123–148). Zigzag lines and black squares were used as in Fig. 1A. B, import of CPT1Δ123–148 is receptor-independent. Import of radiolabeled proteins was carried out for 30 min at 30 °C in the absence (-M) or presence (+M) of mitochondria either directly (lanes 2 and 3) or after a pretreatment with trypsin (Pre-Trypsin; lane 4). Following import, all the samples were submitted to alkaline extraction and analyzed by SDS-PAGE and fluorography. 10%, percentage of input lysate of each radiolabeled protein (lane 1).

**Fig. 5.** CPT1-(122–147) behaves as a membrane retention signal. A, fusion protein constructs used: CPT1-(1–47)-OM-DHFR and CPT1-(122–147)-OM-DHFR. CPT1-(1–47) and CPT1-(122–147) were fused to the signal anchor domain (OM) of S. cerevisiae Tom70 preceding DHFR. Zigzag lines were outlined as described in Fig. 3A. Black square denotes OM (residues 11–29 of Tom70p). Numbers denote residues of CPT1 after which the fusion to OM-DHFR occurs. B, CPT1-(1–47)-OM-DHFR and CPT1-(122–147)-OM-DHFR have opposite OM orientation. Following import of radiolabeled proteins at 30 °C, mitochondria were split into 4 eq aliquots (80 μg) and washed in KCl buffer. The 1st aliquot was directly analyzed by SDS-PAGE (lane 2), whereas the 2nd aliquot was submitted to alkaline extraction (+Na2CO3; lane 3) as described in Fig. 1B. The 3rd aliquot was treated with trypsin (+Trypsin; lane 4) as described in Fig. 3D, and the last aliquot was submitted to swelling in the presence of trypsin (+Swelling; lane 5). All the samples were then analyzed by SDS-PAGE and fluorography. 10%, percentage of input lysate of radiolabeled proteins (lane 1).
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FIG. 6. Heterologous expression of deleted CPT1 proteins in S. cerevisiae. A, immunodetection of rat liver CPT1 and deleted CPT1 proteins expressed in S. cerevisiae. The SDS-PAGE gel was run using either homogenate (20 μg of protein) from control yeast cells transformed with the empty vector (lane 1) or from the different yeast strains expressing the full-length CPT1 (lane 2), CPT1Δ82 (lane 3), CPT1Δ121 (lane 4), CPT1Δ123–148 (lane 5), CPT1Δ83–148 (lane 6), or CPT1Δ31–148 (lane 7). Proteins were transferred onto nitrocellulose, and the blot was probed with the rat liver CPT1 antibody. B, subcellular localization of the deleted CPT1 proteins expressed in S. cerevisiae. Yeast cells expressing the full-length CPT1, CPT1Δ82, CPT1Δ83–148, CPT1Δ121, or CPT1Δ123–148 were fractionated into homogenate (H), mitochondria (Mt), cytosol (C), and microsomes (Mc). Samples (20 μg of protein) were analyzed by SDS-PAGE and immunoblotting with the rat liver CPT1 and yeast mtHSP70 antibodies. Results are representative of three to four different experiments, and the subcellular distribution of mtHSP70 is representative for all constructs. C, bands from Western blots from three to four different experiments, as in B, were quantified by scanning densitometry. Since mitochondria, microsomes, and cytosol accounted, on average, for 9, 13, and 78% of the total homogenate protein, the signal detected in the mitochondrial and microsomal fractions has to be corrected accordingly to analyze the ratio of CPT constructs targeted to mitochondria versus microsomes. The recovery of the expressed protein in the mitochondrial (gray bars) and microsomal (open bars) fractions was expressed as percentage of total expressed protein.

1–82. Moreover, among the deleted CPT1 proteins, only CPT1Δ82 was functionally active but showed a decreased malonyl-CoA sensitivity (Table I). The absence of CPT activity in intact yeast mitochondria expressing CPT1Δ121 could be due to its matrix location (Fig. 7). To determine whether CPT1Δ121 was still functionally active despite its unfolding, CPT activity was measured in solubilized yeast mitochondria expressing CPT1Δ121 or CPT1 as positive control. Whereas solubilization of mitochondria by 5% Triton X-100 inactivated the yeast-expressed CPT1 (20), we found that 0.5% Triton X-100, which is the concentration used for determining the sub mitochondrial localization and the trypsin resistance of CPT1Δ121 (Fig. 7), allowed CPT1 to be solubilized in an active and malonyl-CoA-sensitive form, when compared with intact mitochondria (Table I). By contrast to CPT1, the solubilized CPT1Δ121 was totally inactive (Table I), suggesting that unfolding of the protein led to its inactivation. In conclusion, these results confirm our in vitro import experiments and demonstrate that residues 123–148 function in vivo as a matrix-targeting signal specifying the mitochondrial targeting of CPT1, whereas its TM segment(s) acts as an anchoring signal allowing CPT1 insertion into the OMM.

DISCUSSION

In the present study, we identify the import signal sequence that specifies mitochondrial targeting of an OMM protein harboring two α-helical hydrophobic TM segments, the rat liver CPT1. The concordance of the two approaches used clearly shows that neither TM1 nor TM2 of CPT1 constitutes a signal for mitochondrial targeting of CPT1, whereas its TM segment(s) acts as an anchoring signal allowing CPT1 insertion into the OMM.

In the present study, we identify the import signal sequence that specifies mitochondrial targeting of an OMM protein harboring two α-helical hydrophobic TM segments, the rat liver CPT1. The concordance of the two approaches used clearly shows that neither TM1 nor TM2 of CPT1 constitutes a signal for mitochondrial targeting of CPT1, whereas its TM segment(s) acts as an anchoring signal allowing CPT1 insertion into the OMM.
Mitochondria were isolated from yeast strains expressing the full-length CPT1, CPT1Δ82, CPT1Δ83–148, CPT1Δ123–148, or CPT1Δ121. CPT activity was assayed with 80 μM palmitoyl-CoA and 200 μM carnitine in the absence or presence of 150 μM malonyl-CoA, using either intact mitochondria or mitochondria solubilized in 0.5% Triton X-100, as described under “Experimental Procedures.” Results are means ± S.E. of 3–4 separate experiments.

| Protein expressed      | Intact mitochondria | Detergent-solubilized mitochondria |
|------------------------|---------------------|-----------------------------------|
|                        | Activity            | Malonyl-CoA inhibition             | Activity                      | Malonyl-CoA inhibition               |
|                        | nmol/min/mg protein | %                                  | nmol/min/mg protein            | %                                  |
| CPT1                   | 5.75 ± 0.58         | 95.4 ± 0.4                         | 6.79 ± 0.10                    | 93.4 ± 1.5                         |
| CPT1Δ82                | 3.23 ± 0.78         | 50.6 ± 3.5                         | ND                            | ND                                 |
| CPT1Δ83–148            | Undetectable        | Undetectable                       | ND                            | ND                                 |
| CPT1Δ123–148           | Undetectable        | Undetectable                       | ND                            | ND                                 |
| CPT1Δ121               | Undetectable        | Undetectable                       | Undetectable                  | Undetectable                       |

a p < 0.05 when compared with the full-length CPT1.

ND, not determined.

Fig. 8. Model for the import pathway of CPT1. See text for details. Abbreviations used are: OMM, outer mitochondrial membrane; R, import receptors. The black area denotes the TM segments of CPT1; the internal import sequence is represented by a helix, and a states for trypsin cleavage site.

to an embedding of epitope Val14–Lys29 and should mask the matrix targeting function of residues 33–47.

The present results strongly support our working model of the import pathway for CPT1 (Fig. 8). Initially, the newly synthesized CPT1 is targeted to mitochondria by the means of its internal import sequence (residues 123–147). Subsequently, the protein interacts with the OMM import machinery, as suggested by the inhibition of its import in trypsin-pretreated mitochondria. The determination of the precise component(s) involved in this process awaits further analysis. Whereas the protein was specifically imported into the matrix in the absence of any TM segment (CPT1Δ121), the presence of TM2 proximal to residues 123–147 (CPT1Δ82) led to an OMM insertion of the protein. This clearly shows that TM2 at least acts as a stop-transfer sequence that arrests protein translocation during import across the OMM. At this stage, our working model includes two possible variations. In the “step by step model” integration of the TM segments would occur sequentially (Fig. 8a), whereas pairing of TM1 and TM2 may be a prerequisite before membrane insertion in the “single concerted step model” (Fig. 8b). The hairpin structure will exhibit a higher hydrophobic moment that would favor bilayer integration. Such a concerted partitioning of the TM segments has been described for the β-barrel Tom40 (19) and for the inner mitochondrial membrane carrier proteins (31). As reported for Tom40 (19), denaturation of the radiolabeled CPT1 precursor with urea partially decreased the efficiency of its import2 that favors the single concerted step model. Although further experimental evidences are required to discriminate between these two possibilities, the present work clearly demonstrates that the TM segments of CPT1 act as stop-transfer sequences that arrest protein translocation during import across the OMM.

The distribution of charges on either side of a membrane anchor is responsible for the orientation of proteins of both the bacterial inner membrane and the endoplasmic reticulum membrane (32, 33). However, residues 1–47, 76–102, and 123–147 of CPT1 all bear an identical net positive charge of +3. Therefore, another putative topogenic determinant could have been the amphiphilicity of the regions flanking the TM segments that may exert on the mitochondrial surface a “retention signal” functionally similar to those created and analyzed by Shore and coworkers (13, 28). The fact that residues 1–82 of CPT1 can adopt either a N\textsubscript{in}-C\textsubscript{cyto} or N\textsubscript{cyto}-C\textsubscript{in} topology in the CPT1-(1–82)-DHFR and full-length CPT1 suggests that residues 1–47 do not contain any topogenic information. Indeed, CPT1-(123–147), but not CPT1-(1–47), was able to exert in vitro such a retention signal since its fusion to the unique TM segment of the yeast Tom70 led to the inversion of its membrane topology. However, deletion of residues 123–147 within the N-terminal domain of CPT1 did not alter its membrane topology. Although we cannot exclude that CPT1-(123–147) may participate in the process of membrane insertion, our results indicate that the presence of both TM1 and TM2 is sufficient for achievement of the correct N\textsubscript{cyto}-C\textsubscript{cyto} topology of CPT1.

Besides the identification of the import signal sequence specifying the import of CPT1 into the OMM, the present study shows that a minor proportion of the yeast-expressed CPT1 was recovered as a functional enzyme in microsomes. Deletion of the first 82 N-terminal residues abolished completely this microsomal targeting, the resulting protein being recovered only into the mitochondrial fraction, whereas deletion of residues 83–148 had the opposite effect. Similar results were also obtained by Zammit and co-workers2 by using another yeast expression system (Pichia pastoris). Subcellular distribution of isoenzymes is usually achieved by the expression of two (or more) closely nuclear-related genes. However, the product of a single gene can be targeted to different locations due to the use of alternative transcription-translation initiation sites, alternative splicing, or multiple targeting signal sequences (7). Although the identity of the microsomal CPT1 remains obscure, our results suggest that residues 1–82 of the mitochondrial CPT1 may contain a putative microsomal targeting signal. Further work is required to determine whether the microsomal CPT1 corresponds to a misrouting of the mitochondrial isoen-

2 C. Prip-Buus, I. Cohen, and J. Girard, unpublished results.

3 N. T. Price and V. A. Zammit, personal communication.
zyme, and if the mitochondrial and microsomal CPT1s are encoded by a single gene. If it is not the case, the reminiscence of such a signal within the mitochondrial enzyme might result from the evolution of an ancestral CPT1 gene.

Finally, the present study allows new insights into the folding of the C-terminal catalytic domain of CPT1. Following mitochondrial targeting and OMM insertion, CPT1 must fold correctly to attain its native functional conformation that is characterized by a highly folded state resistant to trypsin proteolysis (Fig. 8c). Our current findings indicate the existence of a highly folded core in its cytosolic C-terminal domain, as emphasized by the generation of a trypsin-resistant 60-kDa fragment upon trypsin treatment of intact mitochondria expressing CPT1Δ82. Possible trypsin sites occur C-terminal to Arg-595 or -598 and Lys-631 or -634. Trypsin was able to digest the full-length CPT1 at these sites only when the loop connecting TM1 and TM2 was previously cleaved by the protease. This suggests that the highly folded core within the cytosolic C-terminal domain of CPT1 is hidden in the native protein by its cytosolic first N-terminal residues. Several lines of evidence support the idea that this folded domain may belong to the catalytic core of CPT1. First, CPT1 remained active and malonyl-CoA sensitive when solubilized by a low Triton X-100 concentration that maintained its C-terminal domain folded. Second, deletion of residues 1–82 did neither alter the folded core nor the catalytic activity but decreased the malonyl-CoA sensitivity. This is in agreement with previous studies suggesting that the catalytic domain of CPT1 resides within its cytosolic C-terminal domain, whereas its extreme N terminus is important for malonyl-CoA sensitivity (3, 20, 34). Third, deletion of residues 1–121 or 83–148 that encompassed TM2 altered both folding of the C-terminal domain and CPT activity. The fact that residues 171–186 contain the (LIVM)LVP(IVTA)P motif, which corresponds to the carnitine/choline acyltransferase family signature 1 (2), may explain why CPT1Δ123–148 was inactive despite the presence of the folded core. Indeed, deletion of residues 123–148 led to a shift of residues 171–186 to the OMM that likely alters the catalytic activity of the enzyme independently of the trypsin-resistant folded core.

The present study confirms our previous observation that the N-terminal domain of CPT1 is essential to maintain an optimal conformation for catalytic function (20). We show here that TM2 is essential to achieve the correct folding of this putative catalytic core and that residues 1–47 may be in close proximity to this domain, preventing trypsin from having access to residues at position 595, 598, 631, or 634. The extreme protease resistance that characterizes the native CPT1 is likely due to intramolecular interactions between either the cytosolic N- and C-terminal domains of the enzyme or between TM1 and TM2. Additionally, it could result from an oligomerization of the enzyme, as reported for porin (16). It is now clear that α-helical TM segments of membrane proteins can participate in highly specific interactions that drive their folding and/or oligomerization and contribute to an increasingly diverse set of functional roles (35, 36). Whether the TM segment(s) of CPT1 fulfill such functional interactions with either each other or other OMM proteins needs to be determined.

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