The N-terminal Domain of Rat Liver Carnitine Palmitoyltransferase 1 Mediates Import into the Outer Mitochondrial Membrane and Is Essential for Activity and Malonyl-CoA Sensitivity*

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The rat liver carnitine palmitoyltransferase 1 (L-CPT1), an integral outer mitochondrial membrane (OMM) protein, is the key regulatory enzyme of fatty acid oxidation and is inhibited by malonyl-CoA. In vitro import of L-CPT1 into the OMM requires the presence of mitochondrial receptors and is stimulated by ATP but is membrane potential-independent. Its N-terminal domain (residues 1–150), which contains two transmembrane segments, possesses all of the information for mitochondrial targeting and OMM insertion. Deletion of this domain abrogates protein targeting, whereas its fusion to non-OMM-related proteins results in mitochondrial targeting and OMM insertion in a manner similar to L-CPT1. Functional analysis of chimeric CPTs expressed in Saccharomyces cerevisiae shows that this domain also mediates in vivo protein insertion into the OMM. When the malonyl-CoA-insensitive CPT2 was anchored at the OMM either by a specific OMM signal anchor sequence (pOM29) or by the N-terminal domain of L-CPT1, its activity remains insensitive to malonyl-CoA inhibition. This indicates that malonyl-CoA sensitivity is an intrinsic property of L-CPT1 and that its N-terminal domain cannot confer malonyl-CoA sensitivity to CPT2. Replacement of the N-terminal domain by pOM29 results in a less folded and less active protein, which is also malonyl-CoA-insensitive. Thus, in addition to its role in mitochondrial targeting and OMM insertion, the N-terminal domain of L-CPT1 is essential to maintain an optimal conformation for both catalytic function and malonyl-CoA sensitivity.

The mitochondrial carnitine palmitoyltransferase (CPT1; EC 2.3.1.21) system in cooperation with the carnitine/acylcarnitine translocase permits long-chain acyl-CoA to be transferred from the cytosol to the mitochondrial matrix to undergo β-oxidation (1). The CPT system consists of two membrane-bound enzymes, CPT1 and CPT2. CPT1 is an integral protein of the outer mitochondrial membrane (OMM), loses activity upon solubilization of mitochondria by strong detergents and exists under two isoforms, the liver (L-CPT1; 88 kDa) and the muscle (82 kDa) (2). By contrast, CPT2 is loosely associated with the inner face of the inner mitochondrial membrane (IMM), is released in active soluble form by detergents, and only one ubiquitous isoform exists (2). A unique feature of CPT1 is its potent inhibition by malonyl-CoA, the first committed intermediate of fatty acid biosynthesis (3). This provides a mechanism for physiological regulation of β-oxidation in liver and other tissues (1, 4). Apart from mitochondria, rat liver microsomes and peroxisomes contain also both membrane-bound/malonyl-CoA-sensitive and soluble/malonyl-CoA-insensitive (luminal) CPT-like enzymes (5–8), which share similar functional properties with their mitochondrial counterparts. Thus, a similar fatty acid transport operates in mitochondria, microsomes, and peroxisomes, but it seems that the components involved in these systems are all different (6). This raised the crucial question of how L-CPT1 is specifically targeted to mitochondria and inserted into the OMM.

Most mitochondrial proteins are encoded by nuclear genes, synthesized as precursors in the cytosol, targeted to mitochondria, and finally imported into their respective mitochondrial subcompartments. Mitochondrial targeting and transport is due to the interaction of targeting signals within the precursors with an import receptor complex located in the OMM and named the Tom complex (9). In the case of most matrix proteins, and especially for CPT2 (10), their N-terminal presequences function as cleavable matrix-targeting signals that initiate translocation across both mitochondrial membranes in a membrane potential-dependent fashion. By contrast, OMM proteins do not contain cleavable signal presequences and therefore must be 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 yeast Tom70p and the human Bcl2 (11). Their targeting and insertion into the OMM have been shown to be mediated by a unique hydrophobic transmembrane region that functions as a "signal anchor sequence" (12, 13). Integral polypeptide (multispanning) OMM proteins fall into two classes, namely those that contain transmembrane β-sheets (porin) and those with α-helical hydrophobic transmembrane segments. The targeting signals for polypeptic OMM proteins still have not been defined, and although limited information regarding structural determinants of the β-barrel porin is available (14–16), the precise nature of its targeting components remains unclear, as does the issue of whether porin is inserted loop by loop or in a single concerted step.

Rat mitochondrial L-CPT1 and CPT2 share 50% of homology in the major part of their sequences with the exception of their N termini (17). The extended N-terminal domain (about 150 amino acids) of L-CPT1 bears no significant similarity to CPT2
and contains two hydrophobic transmembrane segments (H1, residues 48–75; H2, residues 103–122) (17, 18). Both the N and C termini of L-CPT1 are exposed to the cytosol, while the short loop connecting H1 and H2 is exposed to the intermembrane space (18). Thus, L-CPT1 could be a useful model to study the mechanisms involved in targeting and membrane insertion of OMM proteins containing more than one α-helical transmembrane segment.

In the present study, we have characterized the requirements for in vitro import of the rat L-CPT1 into isolated rat liver mitochondria, and by using deletion and fusion protein approaches, we have investigated the role of the N-terminal domain (residues 1–150) of L-CPT1 in mitochondrial targeting and anchoring at the OMM. In addition, through heterologous expression of several chimeric CPTs in Saccharomyces cerevisiae, we have confirmed the validity of the in vitro import results and explored the functional importance of the N-terminal domain of the rat L-CPT1.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, DNA Manipulations, and Cloning Strategies**

*Escherichia coli* DH5α strain was used to propagate various plasmids and their derivatives. The transcription plasmid pGEM4 (Promega) was used for cloning DNA fragments, for making constructs, and for in vitro transcription/translation. All of the pGEM4 constructs were under the control of the SP6 promoter. All DNA manipulations (restriction, ligation) were performed according to the instructions provided by the manufacturers’ protocols of the respective enzymes. The full-length rat L-CPT1 and CPT2 cDNA inserts were retrieved from pYes2.0-CPT1 and subcloned into pGEM4. These plasmids were designated as pL-CPT1 and CPT2 cDNA inserts were retrieved from pYes2.0-CPT1 and generated a 377-nucleotide stretch of the mature coding sequence of rat CPT2 beginning at amino acid 26. The PCR was performed by using the manufacturers’ protocols of the respective enzymes. The full-length rat L-CPT1 was amplified for 30 cycles with a 3′-primer (5′-CGG GTG ACC AGT GCT GTC GGG-3′) introducing an *Eco*RI site and the 3′-primer (5′-CCG CAT GAG GTG CCT GCG ATC-3′) containing a *Bam*HI site and ligated into pGEM4-DFHR as an *Eco*RI–*Bam*HI fragment. The final construct encodes a fusion protein between the first 147 amino acids of L-CPT1 and the reporter protein DHFR. Four amino acids (Gly-Ser-Gly-Ile) were introduced into the joining region. The fidelity of all PCR reactions and the quality of DNA subcloning were confirmed by DNA sequence analysis.

**In Vitro Coupled Transcription-Translation**

All constructs were transcribed and translated using the TNT® SP6 coupled reticulocyte lysate system according to the manufacturer’s protocols (Promega) in the presence of [35S]methionine (Amersham Pharmacia Biotech, France). Lysates containing the labeled synthesized proteins were stored at −80 °C until used for the in vitro import assay.

**Isolation of Rat Liver Mitochondria**

Male Wistar rats (200–300 g) bred in our laboratory were fed ad libitum on a standard laboratory chow diet (62% carbohydrate, 12% fat, and 26% protein in terms of energy) with continuous access to water. Rat liver mitochondria were isolated in an isolation buffer (0.3 M sucrose, 5 mM Tris-Cl, 1 mM EGTA, pH 7.4) using differential centrifugation and then further purified on self-forming Percoll gradients (31% (v/v) Percoll) (22). Protein concentration was determined by the method of Lowry et al. (23) with bovine serum albumin as a standard. Purified mitochondria were resuspended in isolation buffer at a protein concentration of 20 mg/ml.

**In Vitro Import**

Freshly isolated rat liver mitochondria (0.5 mg of protein/ml) were resuspended in import buffer (250 mM sucrose, 2 mM bovine serum albumin, 20 mM Hepes-KOH, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM diithiothreitol, 5 mM phosphocreatine, 100 μM phosphocreatine kinase, 0.4 mM GTP, 1 mM NADH, 0.6 mM spermidine, pH 7.6). After preincubation for 3 min, either at 4 or 30 °C, radiolabeled proteins (2.5%, v/v) were added, and import was performed either at 4 or 30 °C for the indicated time periods. The import reaction was stopped by dilution in 4 volumes of ice-cold KCl buffer (250 mM sucrose, 10 mM Hepes, 80 mM KCl, pH 7.6). Samples were split into two aliquots, one corresponding to the standard import reaction, centrifuged at 12,000 × g for 5 min at 4 °C, and washed in EDTA buffer (250 mM sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.6). One of the aliquots (representing total protein binding to mitochondria) was directly analyzed on SDS-PAGE (24). The other aliquot was subjected to protease treatment (in the case of CPT2 and Su9-DHFR) or carbonate extraction (in the case of OMM proteins). For protease treatment, mitochondria were resuspended in KCl buffer and treated for 15 min on ice with either 500 μg/ml (for CPT2) or 200 μg/ml (for Su9-DHFR) of trypsin (Sigma). Protease treatment was stopped by adding 5 mg/ml of soybean trypsin inhibitor (STI; Sigma). After a 10-min incubation on ice, mitochondria were washed in EDTA buffer containing STI (1 mg/ml). For carbonate extraction, mitochondria were resuspended in 0.1 M Na2CO3 (pH 11.5) at a final protein concentration of 0.2 mg/ml and incubated on ice for 30 min. After centrifugation at 100,000 × g for 30 min at 4 °C, integral membrane proteins were recovered in the pellet, while soluble and peripheral proteins present in the supernatant were trichloroacetic acid-precipitated. Samples were then subjected to SDS-PAGE and exposed to Hyperfilms-MP (Amersham Pharmacia Biotech). Import rates were quantified by scanning the fluorographs.

**Pretreatment of Rat Liver Mitochondria with Trypsin**

Mitochondria (5 mg/ml) were incubated on ice with 25 μg/ml of trypsin for 20 min. Then STI was added at a final concentration of 2.5 mg/ml, and mitochondria were further incubated on ice for 10 min. After centrifugation in a microcentrifuge, mitochondria were washed in EDTA buffer containing 1 mg/ml of STI, resuspended in isolation buffer at a protein concentration of 20 mg/ml, and finally used for import reactions.
Manipulation of ATP Levels and Abolition of Mitochondrial Membrane Potential (ΔΨ)

After a 3-min preincubation of mitochondria at 30 °C in import buffer without any energy-regenerating system, mitochondrial ATP levels were depleted by a further 10-min incubation at 30 °C in the presence of 20 μM oligomycin (Sigma). Then apyrase (40 units/ml; Sigma) was added, and mitochondria were incubated for 10 min at 30 °C before the addition of lysate. Prior to its addition to the import reaction, reticulocyte lysate was ATP-depleted by apyrase treatment for 10 min at 30 °C. For examination of the ΔΨ-dependence of import, mitochondria were incubated on ice for 5 min with 1 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma) before import.

Construction of Yeast Expression Plasmids

The cDNAs encoding the various chimeric proteins were retrieved from pGEM4 and subcloned into the yeast expression vector pYeDP1/8–10 (25). First, the EcoRI–Smal fragment encoding for CPT1-(1–147)–CPT2 was inserted into the pYeDP1/8–10 cut by the same enzymes in order to obtain the pYe-CPT1-(1–147)–CPT2 plasmid. The pOM29-CPT2 cDNA was also cloned into the pYeDP1/8–10 as an EcoRI–Smal fragment to give the pYe-pOM29-CPT2 plasmid. The third construct designated as pYe-pOM29-CPT2Δ148 was obtained by excision from pGEM4 of the EcoRI-blunted HindIII fragment encoding for pOM29-CPT2Δ148 and by insertion into pYeDP1/8–10 digested by EcoRI and Smal.

Yeast Culture, Subcellular Fractionation, and Isolation of Yeast Mitochondria

Each cDNA was placed under the control of the inducible GAL10 promoter and the different constructs were used to transform S. cerevisiae (haploid strain W303: Matα, his3, leu2, trp1, ura3, ade2–1, can1–100) according to Ref. 26. Methods for yeast culture, subcellular fractionation, and isolation of yeast mitochondria were as described previously (21).

Miscellaneous Methods and Chemicals

Swelling of yeast mitochondria and Western blotting were performed as described (21). Immunoblotting was performed as in Ref. 27 using the ECL detection system (Pierce) according to the supplier’s instructions. The antisera used were against the yeast cytochrome b6 (11,000), the yeast mitochondrial HSP70 (15,000), the yeast porin (11,000), the rat liver mitochondrial CPT1 (13,000) and rat porin (13,000) (28). CPT activity was assayed at 30 °C as palmitoyl-L-[methyl-3H]carnitine formed from L-[methyl-3H]carnitine (200 μM; 2 Ci/mmol) and palmitoyl-CoA (80 μm) in the presence of 1% bovine serum albumine (w/v) as described previously (29). Malonyl-CoA concentration was 150 μM. When the CPT assay was performed using detergent-solubilized mitochondria, mitochondria were solubilized by 5% Triton X-100 as described in Ref. 30.

The insoluble membrane residue was sedimented by centrifugation at 15,000 × g for 30 min at 4 °C, and the supernatant was used for CPT assay. No CPT activity could be detected in the residual pellet fraction after solubilization. The PCR reagents and T4 DNA Polymerase were purchased from Biolabs (Ozyme, Saint-Quentin en Yvelines, France). Restriction enzymes and T4 DNA ligase were obtained from Life Technologies, Inc. Yeast culture media products were from Difco, and Zyppurchased from Biolabs (Ozyme, Saint-Quentin en Yvelines, France).

Statistics

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

RESULTS

Import of L-CPT1 into Mitochondria Leads to Its Insertion into the OMM in a Time- and Temperature-dependent Manner—An initial series of experiments was designed to determine whether L-CPT1 could be imported into rat liver mitochondria. The first criterion used was the tight membrane association of the native L-CPT1 (31). Membrane insertion of proteins can be assessed by measuring their levels of resistance to extraction with alkaline buffer (12, 32, 33). This procedure distinguishes both soluble and peripherally membrane-bound proteins from integral proteins such as porin (33) or Tom70p (12). When purified rat liver mitochondria were submitted to a sodium carbonate extraction (pH 11.5), native L-CPT1 re-mained in the pellet fraction (Fig. 1A), while CPT2 and the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (data not shown) were recovered in the supernatant fraction (Fig. 1C). When purified rat liver mitochondria were incubated at either 4 or 30 °C as described for C. The amount of imported L-CPT1 resistant to alkaline extraction was quantified by densitometry scanning of fluorographs. The percentage of imported L-CPT1 was expressed as the percentage of total L-CPT1 bound to mitochondria after an import reaction for 60 min at 30 °C (without alkaline treatment).

FIG. 1. Efficient import of rat L-CPT1 and CPT2 into isolated rat liver mitochondria. A, endogenous rat L-CPT1 but not CPT2 is an integral membrane protein. Rat liver mitochondria (80 μg of protein) were incubated 30 min on ice either in the isolation buffer (Alkali) or in 0.1 M Na2CO3, pH 11.5 (+ Alkali). The mock-treated mitochondria and the membranes of carbonate-treated mitochondria (P) were reisolated by centrifugation for 30 min at 100,000 × g at 4 °C. Proteins in the supernatant (S) were precipitated by trichloroacetic acid and collected by centrifugation. Samples were analyzed by SDS-PAGE, blotting onto nitrocellulose, and immunostaining for endogenous rat L-CPT1 and CPT2. B, alkaline extraction of imported rat CPT2. The radiolabeled precursor was imported at 30 °C for 30 min into freshly isolated rat liver mitochondria. After import, mitochondria were reisolated by centrifugation and washed, and, when indicated, nonimported precursor was digested by trypsin (500 μg/ml; + Post-Trypsin) for 15 min on ice. After inactivation of the protease by soybean trypsin inhibitor (5 mg/ml), mitochondria were reisolated, washed, and subjected to SDS-PAGE either directly (+ Alkali) or after a carbonate extraction (+ Alkali) in order to recover a membrane pellet (P) and the proteins in the supernatant (S) that were precipitated by trichloroacetic acid. Import of radiolabeled CPT2 was then analyzed by fluorography and densitometry. 5%, percentage of input lysate; p, precursor; m, mature form. C, resistance of imported L-CPT1 to alkaline extraction. Radiolabeled L-CPT1 was incubated either at either 4 or 30 °C for 60 min in the import mixture in the absence (Mito.) or presence (+ Mito.) of freshly isolated rat liver mitochondria. Samples were then centrifuged, washed, and subjected to SDS-PAGE either directly (– Alkali) or after an alkaline extraction (+ Alkali). Import of radiolabeled L-CPT1 was analyzed as in B. 10%, percentage of input lysate. D, kinetics of L-CPT1 import. Import of L-CPT1 was carried out for 5–60 min at either 4 or 30 °C as described for C. The amount of imported L-CPT1 resistant to alkaline extraction was quantified by densitometry scanning of fluorographs. The percentage of imported L-CPT1 was expressed as the percentage of total L-CPT1 bound to mitochondria after an import reaction for 60 min at 30 °C (without alkaline treatment).
1A). This clearly demonstrates that L-CPT1, in contrast to CPT2, is an integral mitochondrial protein and explains the different behavior of the two proteins toward detergents (31).

We then asked whether once imported into freshly isolated rat liver mitochondria, only L-CPT1 could be inserted into mitochondrial membranes. Both L-CPT1 and CPT2 were synthesized in a TNT-coupled reticulocyte lysate system in the presence of \[^{35}S\]methionine. After incubation with rat liver mitochondria in the import mixture, the full-length precursor CPT2 was processed into its mature form, since a protein of slightly smaller size appeared (Fig. 1B). 90% of the mature CPT2 recovered in association with mitochondria was resistant to proteolysis even at high trypsin concentration (Fig. 1B), whereas the CPT2 precursor present in the reticulocyte lysate was entirely digested by trypsin (data not shown). This shows that the mature CPT2 was efficiently imported into the mitochondrial matrix. Moreover, as for the native CPT2 (Fig. 1A), the newly imported CPT2 was not inserted into the mitochondrial membranes following import, since it was completely extracted by alkaline treatment (Fig. 1B). These results show that the assay conditions used were suitable for successful in vitro mitochondrial import of CPT proteins and that alkaline treatment of mitochondria is a useful criterion for characterization of the process. In vitro transcription-translation of L-CPT1 cDNA leads to the synthesis of an 88-kDa \[^{35}S\]-labeled protein that comigrates on SDS-PAGE with the native rat L-CPT1 (data not shown). This radiolabeled L-CPT1 was then incubated in the import mixture with isolated rat liver mitochondria. The recovery of L-CPT1 bound to mitochondria was incubated in the import mixture with isolated rat liver mitochondria. The recovery of L-CPT1 bound to mitochondria was decreased by 75% of the total bound L-CPT1 was in an alkaline-resistant form after an import of 60 min. At 4 °C, the efficiency of insertion was 7-fold lower (Fig. 1D). These results show that upon import into rat liver mitochondria, L-CPT1 was inserted into the mitochondrial matrix, where it became processed to its mature size.

Import of L-CPT1 Is Dependent upon Mitochondrial Receptors, Is Stimulated by ATP, and Is \(\Delta V_m\)-independent—We next investigated whether the import of L-CPT1 needed the presence of the surface mitochondrial receptors (Tom complex). As a positive control for receptor dependence, we used the Su9-DHFR fusion protein, which consists of the presequence of Neurospora crassa F0-ATPase subunit 9 preceding the cytosolic mouse DHFR (34). Su9-DHFR was imported into the mitochondrial matrix, where it became processed to its mature size form, which was inaccessible to exogenous added trypsin (Fig. 2A, lane 3). When mitochondria were pretreated with low concentration of trypsin to remove the mitochondrial surface receptors (12, 33), import of CPT2 and Su9-DHFR was decreased by 75 and 70%, respectively (Fig. 2A, compare lanes 3 and 4). Import of L-CPT1 into trypsin-pretreated mitochondria was reduced by 60% (Fig. 2B, compare lanes 3 and 4). Although slightly lower when compared with CPT2 and Su9-DHFR, the decrease in import efficiency of L-CPT1 due to the absence of mitochondrial receptors was similar to what was observed for other OMM proteins (12, 35). The effect of trypsin pretreatment of mitochondria on the import efficiency of both L-CPT1 and CPT2 was dose-dependent and was specific for trypsin, since proteinase K pretreatment does not alter the import efficiency (data not shown). These results clearly show that import of L-CPT1 is dependent upon trypsin-sensitive mitochondrial receptors.

ATP-dependent cytosolic and mitochondrial chaperones mediate import of precursors into mitochondria by preventing their misfolding and irreversible aggregation, by driving protein translocation, and by assisting protein folding and/or assembly into complexes (9). ATP depletion of both mitochondria...
and reticulocyte lysate abolished the import of Su9-DHFR into mitochondria (Fig. 2A, compare lanes 3 and 5). Under these conditions, import of CPT2 and L-CPT1 was reduced by 80% (Fig. 2A, compare lanes 3 and 5) and 60% (Fig. 2B, compare lanes 3 and 5), respectively. Thus, insertion of L-CPT1 into the OMM is stimulated by ATP.

The presence of a Δψ is required for the translocation of most intramitochondrial precursors across the IMM (9), whereas the insertion of proteins into the OMM is Δψ-independent (13, 33, 36). When rat liver mitochondria were pretreated with 1 μM CCCP, a protonophore that dissipates Δψ, import of both CPT2 and Su9-DHFR was abolished (Fig. 2A, compare lanes 3 and 6), whereas insertion of L-CPT1 was unaffected (Fig. 2B, compare lanes 3 and 6). This confirms our previous results (see above) that CPT2 is imported into the mitochondrial matrix, whereas L-CPT1 is inserted into the OMM.

Specific Insertion of L-CPT1 into Mitochondria Is Mediated by Its 150 N-terminal Amino Acids—The second aim of the present study was to examine the role of the N-terminal domain of L-CPT1 (amino acids 1–150) in targeting the protein to the OMM. The various protein constructs employed are presented schematically in Fig. 3. When the first 150 amino acids of L-CPT1 were removed, the resulting protein, CPT1Δ150, shows a high propensity to aggregate when incubated in the import mixture. In the absence of added mitochondria, the protein was recovered as an alkaline-resistant form (Fig. 4A, lane 2). Moreover, by contrast to what was observed for the full-length L-CPT1, there was no significant variation in the amount of alkaline-resistant CPT1Δ150 when import was performed with intact mitochondria (regardless of temperature, ATP, and Δψ) or with trypsin-pretreated organelles (Fig. 4A, compare lane 2 with lanes 3–7). These results establish that the alkaline-resistant Δ150CPT1 moieties correspond to aggregated proteins rather than to inserted proteins. They also suggest that deletion of the 150 N-terminal amino acids of L-CPT1 abrogates the ability of the protein to be imported in vitro into rat liver mitochondria.

It is well established that the N-terminal domain of the yeast Tom70p (amino acids 1–29, pOM29) allows insertion of the protein into the OMM (36). When the mature form of CPT2 (which lacks the matrix-targeting signal and is thus incompetent for import) was fused to pOM29, the chimERIC protein pOM29-CPT2 became inserted into mitochondria in a temperature-, receptor-, and ATP-dependent manner (Fig. 4A, compare lane 4 to lanes 3, 5, and 6), as was the case for the fusion protein pOM29-DHFR (pOMD29) (12). We asked then whether the fusion of pOM29 to CPT1Δ150 could restore the ability of the truncated CPT1 to be specifically imported into mitochondria. The answer was affirmative, as shown by the fact that the recovery of pOM29-CPT1Δ150 as an alkaline-resistant form was strictly dependent upon the presence of mitochondria (Fig. 4A, compare lanes 2 and 4), was temperature-sensitive (compare lanes 3 and 4), and was strongly impaired in the absence of mitochondrial receptors (compare lanes 4 and 5) and after ATP depletion (compare lanes 4 and 6). Moreover, both pOM29-CPT1Δ148 and pOM29-CPT2 were inserted into the OMM, since their insertion was Δψ-independent (Fig. 4A, compare lanes 4 and 7). These results indicate that both L-CPT1 and pOM29-CPT1Δ148 share similar import characteristics, unlike CPT1Δ150. They strongly suggest that the first 150 N-terminal amino acids of L-CPT1 may contain the signal anchor domain of L-CPT1. In order to demonstrate this unequivocally, we tested whether the N-terminal domain of L-CPT1 confers the...
ability to anchor non-OMM-related proteins such as DHFR or the mature form of CPT2 at the OMM. Like L-CPT1 and pOM29-CPT2, but unlike CPT1Δ150, both CPT1-(1–147)-CPT2 and CPT1-(1–147)-DHFR were membrane-inserted in a process that was temperature-sensitive (Fig. 4A, compare lanes 3 and 4), receptor- (Fig. 4A, compare lanes 4 and 5), and ATP-dependent (Fig. 4A, compare lanes 4 and 6) but ΔΨ-independent (Fig. 4A, compare lanes 4 and 7). Following import, the inserted CPT1-(1–147)-DHFR was totally digested by exogenous added trypsin (Fig. 4B, compare lanes 7 and 8), confirming that the protein was inserted into the OMM with its DHFR component exposed on the cytosolic face of mitochondria. Thus, the first 147 N-terminal amino acids of L-CPT1 conferred to both DHFR and CPT2 the ability to interact with the mitochondrial receptors and to be inserted into the OMM. We conclude that in vitro the N-terminal domain of L-CPT1 mediates targeting to mitochondria and anchorage at the OMM.

Functional Analysis of Chimeric CPTs Expressed in S. cerevisiae—We have previously shown that S. cerevisiae, a system devoid of endogenous CPT activity (19), represents a valid model to study the structure-function relationships of the rat L-CPT1, since the yeast-expressed L-CPT1 was correctly targeted to and anchored at the OMM and shared the same folded conformation and sensitivity to malonyl-CoA as the native enzyme (21).

Here we used the yeast system to establish the importance of the N-terminal domain of L-CPT1 for mitochondrial targeting of the protein in an in vivo setting. To this end, the three chimeric CPTs, pOM29-CPT1Δ148, pOM29-CPT2, and CPT1-(1–147)-CPT2, as well as the full-length L-CPT1 that served as control, were expressed in S. cerevisiae. Efficiency of yeast expression was checked by immunoblotting using crude homogenates prepared from control yeast cells transformed with the empty expression vector and from the different expressing yeast strains (Fig. 5A). As previously noted (21), antibodies against the rat L-CPT1 and CPT2 could not detect a specific signal in control yeast cells (Fig. 5A, lanes 1 and 5), whereas they readily detected the endogenous proteins in isolated rat liver mitochondria (Fig. 5A, lanes 4 and 8). As seen from lanes 2, 3, 6, and 7, there was efficient expression of L-CPT1, pOM29-CPT1Δ148, pOM29-CPT2, and CPT1-(1–147)-CPT2. Moreover, the mobility of each protein on SDS-PAGE was consistent with its deduced molecular weight. The lower molecular size protein observed in lane 6 of Fig. 5A was most likely a proteolytic fragment of pOM29-CPT2. In the case of pOM29-CPT1Δ148, the protein appeared as two closely associated bands after a long run electrophoresis (see Fig. 5B and Fig. 6). The double bands were also sometimes observed on immunoblots of the endogenous rat L-CPT1 (data not shown), and they probably arise during electrophoresis as reported for other proteins (27). Subcellular fractionation showed that the chimeric proteins expressed in yeast were localized almost exclusively to the mitochondrial fraction, as for another OMM protein, the yeast porin (Fig. 5B). Thus, as reported previously using another reporter protein (36), pOM29 was able to target CPT1Δ148 and the mature form of CPT2 to yeast mitochondria in vivo. Moreover, in agreement with the in vitro import experiments (Fig. 4A), the N-terminal domain of L-CPT1 allowed specific protein targeting to mitochondria (Fig. 5B). To examine the sub mitochondrial localization of the chimeric CPTs, intact or swollen mitochondria isolated from yeast expressing the different proteins were submitted to trypsin treatment (Fig. 6). The integrity of the OMM in intact yeast mitochondria was checked by the inaccessibility of cytochrome b$_2$ (a soluble intermembrane space protein) to trypsin proteolysis (Fig. 6). Hypotonic swelling of mitochondria was efficient, since the 10% remaining

![Fig. 5. Heterologous expression of chimeric CPTs in S. cerevisiae.](image)

Yeast cells were transformed with the empty expression vector pYeDP1/8–10 or with pYeDP1/8–10 carrying different cDNAs under the control of the inducible GAL10 promoter. Heterologous protein expression was induced by the addition of 2% galactose into the yeast culture. A, immunodetection of rat L-CPT1 and chimeric CPTs expressed in S. cerevisiae. A SDS-PAGE gel was run using either homogenate (the different expressing yeast strains (50 µg of protein; lanes 1, 2, 3, 5, 6, and 7) or mitochondria isolated from adult fed rat liver (80 µg of protein; lanes 4 and 8). Proteins were transferred onto nitrocellulose, and one half of the blot was probed with an antibody raised against rat L-CPT1 (lanes 1–4), whereas the other half was probed with an antibody against rat CPT2 (lanes 5–8). Lanes 1 and 5, control yeast cells transformed with the empty yeast expression vector; lane 2, yeast cells expressing the rat L-CPT1; lane 3, yeast cells expressing pOM29-CPT1Δ148; lanes 4 and 8, rat liver mitochondria; lane 6, yeast cells expressing pOM29-CPT2; lane 7, yeast cells expressing CPT1-(1–147)-CPT2. Results are representative of three different experiments. B, subcellular localization of chimeric CPTs expressed in S. cerevisiae. Subcellular fractions were prepared from yeast cells expressing rat L-CPT1, pOM29-CPT1Δ148, pOM29-CPT2, or CPT1-(1–147)-CPT2. Briefly, yeast mitochondria were isolated from spheroplasts by differential centrifugations as described in Ref. 21, and the postmitochondrial supernatant was further centrifuged at 150,000 × g for 1 h at 4 °C. Aliquots (50 µg of protein) of the homogenate, mitochondria, and 150,000 × g supernatant were analyzed by SDS-PAGE followed by immunoblotting with either anti-L-CPT1 or anti-CPT2 antibody as indicated. The quality of the subfractionation was assessed by immunoblotting with an anti-yeast porin antibody. Results are representative of three or four different experiments.
intactness of the IMM (Fig. 6). As shown previously (21), L-CPT1 expressed in intact yeast mitochondria adopts a folded conformation that is resistant to 10 μg/ml trypsin. A 83-kDa proteolytic fragment generated by trypsin could be observed only when the OMM was disrupted, as reported previously for the native rat L-CPT1 (18, 21). Fig. 6 shows that pOM29-CPT2, pOM29-CPT1Δ148, and CPT1-(1–147)-CPT2 present in intact mitochondria were totally degraded by 10 μg/ml of trypsin without any accompanying appearance of proteolytic fragments. This indicates that each chimeric protein was inserted into the yeast OMM with its CPT2 or CPT1Δ148 domain exposed on the cytosolic face of mitochondria. These results are in agreement with previous studies demonstrating that pOM29 anchors Tom70p into the OMM in a N-terminus orientation (36) and demonstrate that the N-terminal domain of L-CPT1 also mediates in vivo specific anchorage at the OMM. It appears that replacement of the N-terminal domain of L-CPT1 by pOM29 results in a more unfolded state of the large cytosolic C-terminal portion of the protein, which leads to exposure of newly accessible trypsin-sensitive sites that were previously masked in the folded full-length L-CPT1 (Fig. 6).

Functional analysis of pOM29-CPT2 and CPT1-(1–147)-CPT2 expressed in yeast mitochondria shows that these two chimeric proteins were catalytically active (Table I). By contrast to the yeast-expressed malonyl-CoA-sensitive L-CPT1, pOM29-CPT2 and CPT1-(1–147)-CPT2 were largely insensitive to malonyl-CoA when present in intact yeast mitochondria (Table I). pOM29-CPT2 and CPT1-(1–147)-CPT2 could be solubilized in an active form by 5% Triton X-100, indicating that they are detergent-stable, whereas L-CPT1 is detergent-labile (Table I). Consequently, anchorage of CPT2 at the OMM by pOM29 or by the N-terminal domain of L-CPT1 does not appreciably modify the biochemical properties of CPT2 (malonyl-CoA-insensitive activity and detergent stability) (31). The activity of the chimeric pOM29-CPT1Δ148 protein present in intact mitochondria was 20-fold lower than that of the full-length L-CPT1 (Table I), despite similar levels of expression in yeast for these two proteins (Fig. 5A). Although in absolute terms enzyme activity of pOM29-CPT1Δ148 was low, it was highly reproducible and could be easily measured, whereas no CPT activity could be detected in mitochondria isolated from control yeast cells transformed with the empty expression vector (Table I). Moreover, the activity of pOM29-CPT1Δ148 was totally insensitive to malonyl-CoA (Table I). Thus, replacement of the N-terminal domain of L-CPT1 by pOM29 results in a chimeric protein that is anchored at the OMM but does not respond to malonyl-CoA (Table I).

**DISCUSSION**

In the present study, we have investigated how the rat mitochondrial L-CPT1 and CPT2 proteins are specifically targeted to their respective mitochondrial locations. In the case of CPT2, it has been clearly established that this protein possesses at its N terminus a typical matrix signal sequence (20) that is cleaved when the protein is fully imported into mitochondria (10). In keeping with the latter report, we show here that in vitro import of CPT2 into rat liver mitochondria exhibits the same characteristics as those reported for other intramitochondrial proteins (9), i.e. a requirement for the mitochondrial receptors, ATP, and ΔΨ across the IMM. Whereas the specific mechanisms involved in the import of L-CPT1 into the OMM were unknown, the current work establishes that this integral OMM protein can be efficiently imported post-translationally into freshly isolated rat liver mitochondria. Insertion of L-CPT1 into the OMM is a time- and temperature-dependent process that also requires the mitochondrial receptors and is stimulated by ATP but is ΔΨ-independent, in agreement with what was reported for other OMM proteins (33, 36–38). The observation that no proteolytic cleavage of L-CPT1 occurs upon its membrane insertion confirms that the N terminus is re-

**TABLE I**

Functional characterization of the chimeric CPTs expressed in *S. cerevisiae*

Mitochondria were isolated from control yeast cells transformed with the empty expression vector (None) and from yeast cells expressing the full-length L-CPT1, pOM29-CPT2, CPT1-(1–147)-CPT2, or pOM29-CPT1Δ148. 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 5% Triton X-100, as described under “Experimental Procedures.” CPT activity is expressed as nmol/min/mg of protein. Results are means ± S.E. of 3–7 separate experiments.

| Protein expressed | Detergent-solubilized mitochondria | Intact mitochondria |
|-------------------|-----------------------------------|---------------------|
|                   | Without malonyl-CoA | With malonyl-CoA | Inhibition | Without malonyl-CoA | With malonyl-CoA | Inhibition |
| None              | ND<sup>a</sup> | ND | ND | ND | ND | ND |
| L-CPT1            | 3.20 ± 0.20 | 0.20 ± 0.03<sup>b</sup> | 93.7 ± 0.8 | 4.46 ± 0.67 | 3.06 ± 0.31 | 31.9 ± 1.7 |
| pOM29-CPT2        | 3.17 ± 0.26 | 2.67 ± 0.26 | 16.0 ± 2.0 | 1.74 ± 0.33 | 1.17 ± 0.23 | 33.0 ± 2.5 |
| CPT1-(1–147)-CPT2 | 1.93 ± 0.15 | 1.75 ± 0.14 | 9.2 ± 1 | 0 | 0 | 0 |
| pOM29-CPT1Δ148    | 0.15 ± 0.05 | 0.14 ± 0.05 | 0 | ND | ND | ND |

<sup>a</sup> ND, not determined.

<sup>b</sup> p < 0.05 when compared with CPT assay performed in the absence of malonyl-CoA.
tained in the mature protein (39), as previously observed for other OMM proteins (11). Thus, mitochondrial import of L-CPT1 obeys the same general rules described for OMM proteins (11).

Also evident from the present study is that deletion of the N-terminal domain of L-CPT1 (the first 150 amino acids) results in a protein that is unable to interact \textit{in vitro} with the mitochondrial receptors and thus cannot be specifically inserted into the OMM. Conversely, fusion of the L-CPT1 N terminus to non-OMM-related reporter proteins allows them to be targeted to mitochondria both \textit{in vitro} and \textit{in vivo} and to be inserted into the OMM. We conclude, therefore, that the N-terminal domain of L-CPT1 contains all of the intrinsic information required for specific mitochondrial targeting and OMM insertion of this protein. Thus, the inability of the yeast-expressed truncated L-CPT1Δ31−148 to be recovered in association with mitochondria (19) probably resulted from the lack of this essential targeting information and not from misfolding and/or an incompetent import state of the engineered protein.

The mechanisms of recognition of the internal targeting signals present in OMM proteins and of their insertion into the membrane are poorly understood, especially in the case of multispansing OMM proteins. One model is based on the yeast bitopic protein Tom70p, which is targeted and membrane-anchored by a unique transmembrane segment (11–29 amino acids) referred to as a “signal anchor sequence” selective for the OMM (12). L-CPT1 contains within its N-terminal region two hydrophobic transmembrane segments (H1 and H2), either of which could function as a signal anchor sequence (2, 18). Deletion of the first 82 amino acids of L-CPT1, i.e., including the H1 domain, resulted in a protein that was recovered in association with mitochondrial membranes upon expression in yeast (19). However, no definitive conclusion can be drawn, because such a mitochondrial association might have resulted from an unspecified interaction of the protein with any lipid bilayer due to the hydrophobic nature of the remaining H2 domain. Thus, whether H1 and H2 play an equivalent role or only one of them acts as a specific signal anchor sequence still remains to be elucidated. An alternative to the signal anchor sequence model is the combination of a matrix-targeting signal and a stop-transfer sequence (11, 40). The former signal specifies targeting of a protein to mitochondria and initiates its translocation across the OMM, whereas the hydrophobic stop-transfer sequence arrests and anchors the translocating polypeptide into the OMM. Matrix-targeting signals are characterized by a high net positive charge and by the ability to adopt an amphiphilic helical structure upon binding to a membrane surface (41, 42). Although the two upstream regions flanking the transmembrane segments of L-CPT1 theoretically have such properties, further studies will be required to determine if one or the other could function as a matrix-targeting signal and to determine whether OMM insertion of L-CPT1 follows the signal anchor sequence or stop-transfer model. Additionally, amphipathicity of the N-terminal hydrophilic region of Tom70p (amino acids 1–10) has been identified as an important determinant in conferring protein topology (N-term-Cterm) in the lipid bilayer. Substitution of this small hydrophilic domain with a strong matrix-targeting signal causes retention of the N terminus of the protein on the cytosolic face of the OMM resulting in the insertion of the signal anchor, and hence of the entire protein, in a reverse orientation (Nterm-Cterm) (36, 43). The fact that the DHFR domain of CPT1-(1–147)-DHFR and the CPT2 domain of CPT1-(1–147)-CPT2 are located on the cytosolic face of mitochondria, as is the large cytosolic C-terminal region of rat L-CPT1 (18), suggests that the N-terminal portion of L-CPT1 could also participate in the determination of membrane topology. Additional work will be needed to establish if either of the two amphiphilic regions of the N-terminal domain of L-CPT1 functions as a retention signal involved in membrane orientation of the enzyme.

It is now well established that L-CPT1 possesses both catalytic and malonyl-CoA binding domains (19); both appear to be exposed on the cytosolic face of the OMM (18). Analysis of the primary sequence of L-CPT1 shows that its large cytosolic C-terminal region is the only part of the molecule that exhibits homology with CPT2 and other members of the acylcarnitine transferase family (17). This strongly suggests that at least the catalytic site of L-CPT1 resides within its cytosolic C-terminal domain. In the present study, we showed that anchorage of CPT2 at the OMM by pOM29 (Nterm-Cterm orientation) does not render the enzyme sensitive to malonyl-CoA inhibition. These data strengthen the view that CPT2 does not possess a malonyl-CoA binding domain and that malonyl-CoA sensitivity is an intrinsic property of L-CPT1 (2). Thus, the previous observations that the detergent-solubilized malonyl-CoA-insensitive CPT2 could become inhibitable by malonyl-CoA if reconstructed with OMM extracts (44, 45) were probably due to the reactivation of detergent-inactivated L-CPT1 (30). It is still unclear whether the malonyl-CoA binding domain of L-CPT1 is located within its N-terminal or large cytosolic C-terminal domain. The present study establishes that the N-terminal region of L-CPT1 cannot confer malonyl-CoA sensitivity to the malonyl-CoA-insensitive CPT2, allowing two hypotheses to be formulated. The first is that if malonyl-CoA binds only to the N terminus of L-CPT1, the CPT2 component of the CPT1-(1–147)-CPT2 chimera must have had a tertiary conformation that was incapable of interacting with this domain. The second is that the C-terminal region of L-CPT1 is critical for malonyl-CoA binding and that the necessary site(s) is not present in CPT2. Although we cannot distinguish between these two possibilities, the current findings clearly show that the N-terminal domain of L-CPT1 is crucial for maintaining the enzyme under a folded functional conformation. Indeed, when the large cytosolic C-terminal portion of L-CPT1 is anchored at the OMM by pOM29 and not by its own N terminus, it exhibits a less folded structure. Unfolding of the large C-terminal domain was accompanied by a huge decrease in functional activity and by a total loss of malonyl-CoA sensitivity. Although we cannot exclude the possibility that malonyl-CoA interacts to some extent with the N-terminal domain, we favor the idea that this region of L-CPT1 is essential for maintaining an optimal conformation for both catalytic function and malonyl-CoA sensitivity. Our results reinforce previous studies showing that proteolytic cleavage of the extreme N terminus of L-CPT1 by proteinase K (18) or deletion of its first 82 amino acids (19) renders the enzyme less sensitive to malonyl-CoA inhibition and/or less active. It is well established that solubilization of the OMM by detergents inactivates L-CPT1 (2). It may now be concluded that maintenance of the functional activity of L-CPT1 is not due to anchorage of the protein at the OMM \textit{per se} but needs specifically the presence of the N-terminal domain. Thus, in addition to its role in mitochondrial targeting and OMM insertion, the N-terminal domain of L-CPT1 is essential for functional activity and malonyl-CoA sensitivity of the enzyme.

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