Comparison of 2 expression systems using COS7 cells and yeast cells for expression of heart/muscle-type carnitine palmitoyltransferase 1 (CPT1b)

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Footnotes:
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Abbreviations used: CPT1, carnitine palmitoyltransferase 1

Abstract

Carnitine palmitoyltransferase 1 (CPT1), catalyzing the transfer of the acyl group from acyl-CoA to carnitine to form acylcarnitine, is located at the outer mitochondrial membrane. Because it is easily inactivated by solubilization, expression systems using living cells are essential for its functional characterization. COS7 cells or yeast cells are often utilized for this purpose; however, the advantages/disadvantages of the use of these cells or the question as to how the CPT1 enzyme expressed by these cells differs are still uncertain. In this study, we characterized the heart/muscle-type isozyme of rat CPT1 (CPT1b) expressed by these 2 cellular expression systems. The mitochondrial fraction prepared from yeast cells expressing CPT1b showed 25% higher CPT1 activity than that obtained from COS7 cells. However, the expression level of CPT1b in the former was 3.8 times lower than that in the latter; and thus, under the present experimental conditions, the specific activity of CPT1b expressed in yeast cells was estimated to be approximately 5 times higher than that expressed in COS7 cells. Possible reasons for this difference are discussed.

Introduction

The catabolic reaction of long-chain fatty acids, i.e., β-oxidation, takes place in the mitochondrial matrix space. Because fatty acids cannot permeate into this space, they are transported across the mitochondrial inner membrane in the form of acylcarnitines. For this, fatty acids are first converted into acyl-CoA by acyl-CoA synthase. Then, the acyl-CoA is converted into acylcarnitine by carnitine palmitoyltransferase 1 (CPT1, EC 2.3.1.21) located on the outer mitochondrial membrane. The acylcarnitine thus formed is transported across the mitochondrial inner membrane by carnitine/acylcarnitine carriers; and carnitine palmitoyltransferase 2 (CPT2), located on the inner mitochondrial membrane, carries out the reverse reaction of CPT1 and regenerates acyl-CoAs for the process of β-oxidation. Of these processes, the process of the conversion of acyl-CoA into acylcarnitine is known as the rate-limiting step; and CPT1 is regarded as the rate-limiting enzyme of β-oxidation (for review, see refs. 1-4).

Three isozymes of CPT1, CPT1a, 1b, and 1c, are known to be expressed in mammals [5-7]. CPT1a is mainly expressed in the liver and kidney; CPT1b is expressed in heart, skeletal muscle, and brown adipose tissue; and CPT1c is mainly expressed in the brain and testis. Functional characterization of these CPT1 isozymes is very important to understand how the catabolism of fatty acids is regulated in various tissues. However, the enzymatic characterization of CPT1 is difficult, because this enzyme is easily inactivated upon solubilization with detergents [8,9]. For this reason, functional analysis of this enzyme is mainly carried out by using expression systems such as mammalian cells or yeast cells [10-13]. However, the question as to how these 2 expression systems differ from each other is still uncertain; and even discrepant results have been obtained with these 2 experimental systems [14,15]. Thus, to conduct effective studies on the functional properties of CPT1, characterization of these 2 expression systems is an utmost important issue to be clarified.

In the present study, we expressed rat CPT1b isozyme in COS7 cells and yeast cells,

and characterized the properties of the obtained mitochondrial fractions from these expression systems.

Materials and Methods

Materials

COS7 cells were obtained from the Health Science Research Resources Bank (Osaka). The haploid strain of *Saccharomyces cerevisiae* W303-1B (MATα *ade2-1 leu2-3, 112 his3-22, 15 trp1-1 ura3-1 can1-100*) was used as yeast cells. The Origami B(DE3)pLysS strain was purchased from Merck KGaA (Darmstadt, Germany). The mammalian expression plasmid vector pCXN2 was a gift from Dr. Miyazaki [16]. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were procured from Nissui (Tokyo) and Equitech-Bio (Kerrville, TX), respectively. Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA); and L-[*methyl*-^{3}H] carnitine hydrochloride (specific radioactivity: 3.11 TBq/mmol), from GE Healthcare UK, Ltd. The BCA protein assay kit was purchased from TaKaRa Bio Inc. (Otsu Japan).

Preparation of antiserum against rat CPT1b

Polyclonal antibody against rat CPT1b was raised in an adult New Zealand White rabbit as described in our previous report [17]. A peptide with the amino acid sequence of TGSHKKQDLQDLFRKASEC (amino acid 621–638, reported by Yamazaki et al. [18], plus one C-terminal Cys residue to confer reactivity with maleimide-activated keyhole limpet hemocyanin), was synthesized by means of a Shimadzu peptide synthesizer, model PSSM-8, and was used as the immunogen.

cDNA encoding rat CPT1b

The DNA fragment corresponding to the open reading frame of the cDNA encoding CPT1b was prepared by PCR by using DS112-36 as a template [6]. Primers used for amplification of the cDNA were GE2321

(5’-ACTAAACCCCCATATGGCGGAAGCACAC) and GE2324 (5’-TGTGGATCCTGGTCTCAGCTGTCAGTC). By using these 2 primers, restriction sites of *Nde*I and *Bam*HI were created at the 5’ and 3’ end of the cDNA fragment, and these 2 restriction sites were used for preparation of the expression vectors.

Bacterial expression of rat CPT1b

Bacterial expression of rat CPT1b was achieved in Origami B(DE3)pLysS strain, by using pColdIII/CPT1b as an expression vector. Briefly, a culture of Origami strain harboring the pColdIII/CPT1b vector (250 ml) was incubated until its OD_{600} had reached 0.4. Then, the protein expression inducer IPTG was added to a final concentration of 0.4 mM, and the cell suspension was further incubated at 15° C for 12 hrs. Cells were harvested by centrifugation, and re-suspended with 4 ml of TE. The resulting cell suspension was subjected to 3 cycles of freeze/thaw/sonication, and the expressed CPT1b protein was recovered as the TE-insoluble precipitate, which was resuspended in 0.3 ml of TE and used as the standard protein of rat CPT1b.

Expression of rat CPT1b in COS7 cells

Expression vector of rat CPT1b in mammalian cells was constructed by using pCXN2 [16]. For transient expression of rat CPT1b, expression vector pCXN2/CPT1b was introduced into COS7 cells by using Lipofectamine 2000, as described previously [18].

Preparation of mitochondrial fraction from cultured COS7 cells

Two days after transfection, the cultured cells were harvested; and then they were suspended in ice-cold KCl medium (150 mM KCl and 5 mM Tris–Cl buffer, pH 7.2). Next, the cells were disrupted by homogenization with a Potter-type homogenizer. The obtained homogenate was centrifuged at 800×*g*, 4º C for 10 min, and unbroken

cells obtained as the pellet were subjected to further homogenization; and this procedure was repeated 3 times. The cell lysates thus obtained were pooled and then centrifuged at 10,000×*g*, 4º C for 10 min. The resulting pellet was resuspended in ice-cold KCl medium, and used as the mitochondrial fraction.

Expression of rat CPT1b in yeast cells

The expression vector of rat CPT1b in yeast cells was constructed by using pYO326-YA2P [19]. For the expression of CPT1b, W303-1B cells were transformed with the expression vector pYO326-YA2P/CPT1b.

Preparation of mitochondrial fraction from yeast cells

Preparation of the mitochondrial fraction from cultured yeast cells was performed essentially as described previously [20]. Briefly, yeast cells were twice washed with cold distilled water and then incubated in medium containing 10 mM dithiothreitol (DTT) and 0.1 M Tris–SO_4, pH 8.0, at 30º C, after which they were centrifuged at 100 rpm for 15 min. The DTT-treated cells were resuspended in sorbitol medium (1.2 M sorbitol and 20 mM KPi, pH 7.4) and then incubated (30º C, 100 rpm, 15 min) with 11.39 mg of zymolyase 20T in sorbitol medium per g of yeast cells to form spheroplasts. The spheroplasts were twice washed with sorbitol medium, and subsequently ground in mannitol medium (10 mM Tris–HCl, pH 7.4, containing 0.6 M mannitol, 0.1 mM EDTA, and 10 μM p-APMSF) at low speed in a chilled Potter–Elvehfem homogenizer. Next, the homogenate was centrifuged for 5 min at 800×*g*. The pellet was discarded, and the supernatant was centrifuged for 10 min at 6800×*g*. The resulting mitochondrial pellet was resuspended in mannitol medium.

Measurements of protein concentration

The protein concentration of mitochondrial fractions was measured by use of a BCA protein assay kit with bovine serum albumin as the standard.

Measurements of CPT1 activity

Activity of CPT1b was measured according to the procedure described previously [18]. Briefly, the reaction was initiated by the addition of the mitochondrial fraction (50 µl containing about 30 µg mitochondrial protein) to a prewarmed reaction solution (450 µl) consisting of 150 mM KCl, 1 mM EDTA, 0.25 mM reduced glutathione, 1.3 mg/ml fatty acid-free BSA, 0.5 mM L-carnitine, 50 µM palmitoyl-CoA, 2 mM KCN, 37 kBq L-[*methyl*-^{3}H] carnitine, and 50 mM Hepes buffer, pH 7.4. After incubation at 30º C for 20 min, the reaction was terminated by the addition of 500 µl of 1.2 M HC1. The palmitoyl L-[*methyl*-^{3}H]carnitine formed was extracted into 500 µl of *n*-butanol, and the butanol phase was back-extracted with 200 µl of water. Then, 200 µl of the final organic phase was taken for liquid scintillation counting. As described in our previous report, the formation of palmitoyl L-[*methyl*-^{3}H] carnitine occurred in an incubation time-dependent manner at least up to 20 min. Thus, the amount of palmitoyl L-[*methyl*-^{3}H] carnitine formed by this time was used to calculate the activity of CPT1b.

Western blotting

Expression levels of CPT1b in individual expression systems were evaluated by Western blotting according to the procedure described previously [14]. Specific antibody against CPT1b was prepared as stated above, and that against the β-subunit of F_1-ATPase was prepared as described previously [14].

Results and discussion

Comparison of yields of mitochondrial fractions and activities of expressed CPT1b between COS7 cells and yeast expression systems

As CPT1 is easily inactivated by solubilization with detergents, enzymatic activities of CPT1 are usually studied by using the mitochondrial fraction. To discuss the usefulness of these 2 expression systems for studies on the properties of CPT1b, we considered it to be important to characterize these systems with respect to the following points: i) yield of mitochondrial fraction, ii) observable CPT1 activity of the mitochondrial fraction, and iii) properties of the expressed enzyme. Thus, we first examined the yield of the mitochondrial fraction and observable CPT1 activity in it for the individual expression systems.

As starting materials, transfected cells in 2 culture dishes (100-mm diameter) and 500 ml of YPgal culture having reached an OD_{600} of 0.8 – 1.2 were used for expression systems of COS7 cells and yeast cells, respectively. We repeated preparation of the mitochondrial fraction from the individual expression systems more than 5 times, and the mean values ± SD (n = 3) of the yield of the mitochondrial fraction and observed CPT1 activity in it for each system are shown in **Table I**.

The amount of total protein in the cellular lysate obtained from yeast cells as a starting material was approximately 7-fold greater than that from the COS7 cells. Regardless of the use of relatively distinct amounts of total cellular proteins as starting materials, we recovered relatively similar amounts of proteins (i.e., 0.86 and 0.57 mg proteins) as mitochondrial fractions from these 2 expression systems. Possibly these results indicate the higher purity of the mitochondrial fraction of yeast cells than that of COS7 cells. It is quite reasonable that the use of larger numbers of cells as starting materials would be desirable for the preparation of mitochondrial fractions of higher purity. We could easily increase the amount of total cellular proteins of COS7 cells by

using larger numbers of culture dishes; but in this case, the experimental cost would become significant.

We next assessed the CPT1b activity in the mitochondrial fraction from each cellular system. As mentioned in our previous reports, the mitochondrial fraction prepared from COS7 cells transfected with a sham vector (specifying no cDNA of CPT1b) showed almost negligible catalytic activity, indicating the expression level of the endogenous CPT1 to have been very low. Likewise, in the case of the yeast cells, there was almost negligible catalytic activity (data not shown). On the contrary, when we assessed the enzymatic activity of CPT1b in the mitochondrial fractions prepared from COS7 cells and yeast cells, the values were similar, as shown in **Table I**. In an exact sense, the activity observed for the mitochondrial fraction from the yeast cells was 25% higher than that observed for the COS7 cells. If we assume that the expression levels of CPT1b and specific activities of expressed CPT1b were identical between the 2 mitochondrial fractions obtained from COS7 cells and yeast cells, the above results are quite reasonable. This higher CPT1b activity observed for yeast mitochondria could be attributable to their higher purity than those of COS7 mitochondria.

We also examined the sensitivity of the expressed CPT1b from each expression system to its intrinsic metabolic inhibitor, malonyl-CoA. As shown in **Fig. 1**, the enzyme from either system was inhibited more than 80% with 10 μM malonyl-CoA. However, at the lower concentration of 1 μM malonyl-CoA, the expressed CPT1b showed distinct sensitivities to the inhibitor; i.e., the activity of the enzyme from COS7 cells was suppressed about 80%, whereas that from yeast cells was inhibited only 50%. The reason for this difference is uncertain.

Preparation of standard CPT1b protein by bacterial expression system and its quantification by Coomassie staining

For further characterization of the mitochondrial fraction prepared from the

individual expression systems, evaluation of the amount of CPT1b in each seemed important. To enable quantitative evaluation of the amounts of CPT1b in the 2 mitochondrial preparations, we next prepared "standard" CPT1b by using a bacterial expression system. We first tried to express CPT1b in *E. coli* strain BL21(DE3), but remarkable expression, i.e., that easily detectable by SDS-PAGE and subsequent Coomassie staining, was not achieved. Thus, we further tested its expression in other strains, i.e., Rosetta 2(DE3)pLysS and Origami B(DE3)pLysS. Although a satisfactory expression level of CPT1b was not obtained even by using these strains, CPT1b was slightly expressed in the Origami B(DE3)pLysS strain as inclusion bodies. Thus, we collected the insoluble proteins from lysates of this bacterium. When a sample of CPT1b protein thus prepared was subjected to SDS-PAGE and subsequent Coomassie staining, a protein band showing an estimated molecular size of CPT1b was observed as one of the major protein bands, as shown in **Fig 2**. Ideally, a standard protein should have an amino acid sequence completely identical to that of the native protein, but the standard protein thus prepared had 6 additional amino acid residues (MetAsnHisLysValHis) encoded by the TEE (translation enhancing element) sequence in the pColdIII vector. For this reason, in an exact sense, this standard protein (88.97 kDa) would be expected to show a slightly slower migration than the native protein (88.22 kDa). As this protein band was not observed with the bacterial lysate prepared from the *E. coli* strain in which the sham vector had been introduced, this protein band was suggested to be the expressed CPT1b. The amount of CPT1b standard protein was quantified in terms of its staining intensity in polyacrylamide gel stained with Coomassie dye, with bovine serum albumin used as a standard.

Use of specific antibody against CPT1b and standard CPT1b protein for quantification of CPT1b present in the mitochondrial fraction from each cellular system

We next assessed the amounts of CPT1b in the individual mitochondrial fractions

prepared from COS7 cells and yeast cells. For this assessment, we first prepared a specific antibody against CPT1b by using a synthetic peptide as the immunogen. Specific reactivity of the raised antibody was confirmed by using bacterially expressed CPT1b as an authentic CPT1b (data not shown). When the mitochondrial fraction prepared from either expression system was subjected to Western analysis using the specific antibody against CPT1b, a clear protein band was observed (**Fig. 3**). As these protein bands were not observed with the mitochondrial fractions prepared from COS7 cells and yeast cells in which sham expression vectors had been introduced, they were concluded to be the expressed CPT1b. It should be emphasized that no immunostained band was observed with the mitochondrial fraction prepared from COS7 cells transfected with the sham expression vector, thus indicating that the expression level of the endogenous CPT1b in this cell line was negligible. As COS7 cells were derived from kidney tissue of an African green monkey, even if heart/muscle-type isozyme (CPT1b) was not detectable, the expression of the liver-type isozyme (CPT1a) could be considered. However, even if liver-type isozyme (CPT1a) was expressed, its expression level would not have been high, because the mitochondrial fraction prepared from COS7 cells transfected with the sham vector showed almost negligible catalytic activity, as mentioned above.

Because we used a known amount (30 ng) of bacterially expressed CPT1b as a standard, we could determine the expression level of CPT1b in the individual mitochondrial fractions prepared from COS7 cells and yeast cells. The amounts of CPT1b protein expressed in these fractions prepared from COS7 cells and yeast cells were determined to be 9.84 ± 0.94 ng/μg mitochondrial protein and 2.70 ± 0.66 ng/μg mitochondrial protein, respectively. The CPT1b content in the mitochondrial fraction would be affected by various factors such as i) fraction purity, ii) promoter activity, or iii) stability of the CPT1b in the mitochondrial membrane. The exact reason for the lower content of CPT1b in the mitochondrial fraction from the yeast cells is uncertain.

Of these factors, a difference in fraction purity between COS7 cells and yeast cells would be excluded as a probable answer, because the purity of the mitochondrial fraction from COS7 cells would be expected to be lower than that from yeast cells, as stated above. The interpretation that mitochondria from yeast cells have a limited content of CPT1b would be reasonable, because a possible lower level of CPT1a in yeast mitochondria than in COS7 ones was also discussed by Swanson *et al*. [11], although they did not show detailed data on the quantification of its expression levels.

As we had determined the content of CPT1b in the mitochondrial fraction from each expression system, we could roughly estimate the specific activities of CPT1b protein obtained from the 2 systems. The specific activities of CPT1b expressed in COS7 cells and yeast cells under the present experimental conditions were determined to be 0.62 and 3.00 μmol/mg protein/min, respectively. We were surprised to find that CPT1b expressed by COS7 cells and yeast cells showed remarkably different specific activities: the enzyme expressed in yeast cells showed about 5 times higher specific activity than that expressed in COS7 cells. The exact reason for this difference is uncertain, but we can suggest 2 probable answers. First, in mammalian cells such as COS7 cells, the other members of the carnitine system, i.e., carnitine/acylcarnitine carrier and carnitine palmitorytransferase 2 (CPT2), are present in the mitochondrial membrane. Although their expression levels seem not to be high, these members may have further processed the acylcarnitine formed by CPT1b. However, these members are not present in the yeast mitochondrial membrane. This difference may have caused the distinct specific activities of the expressed CPT1b. A second possible answer is the difference in phospholipid content between mammalian and yeast mitochondria, which may have affected the activity of the membrane proteins. To obtain an accurate explanation for the observed difference in the specific activity of the expressed CPT1b's, further careful studies must be carried out.

Concluding remarks

For functional characterization of membrane proteins, expression systems using living cells are effective. However, the answer to the question as to which one of the various expression systems using living cells is suitable for the target protein would be largely dependent upon the individual protein species. In the present study, we characterized 2 cellular systems, one using COS7 cells and the other, yeast cells, for expression of CPT1b. The yield of the mitochondrial fraction from yeast cells was higher than that from COS7 cells, but the expression level of CPT1b was higher in the mitochondrial fraction from COS7 cells than in that from yeast cells. More importantly, the roughly estimated specific activity of CPT1b was higher for the yeast mitochondrial fraction than for the COS7 one. Although the exact reason for the latter difference is still uncertain, the results obtained in this present study are important for further characterization of the CPT1 enzyme produced by different cellular expression systems.

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Table I Yield of mitochondrial fraction and observed CPT1 activity in it for each expression system [1)]

		expression system	
		COS7 cells	yeast cells
obtained proteins (mg)	in total cell lysate	3.40 ± 0.54	23.4 ± 4.7
	in mitochondrial fraction	0.57 ± 0.02	0.86 ± 0.12
CPT1 activity observed in the mitochondrial fraction (nmol/mg mitochondrial protein/min)		6.92 ± 2.03	8.70 ± 0.80

1) The cells in 2 culture dishes (100-mm diameter) and in 500 ml of YPgal culture reaching OD_{600} of 0.8 – 1.2 were used as starting materials for COS7 and yeast cell expression systems, respectively. Mean values ± SD of independent runs are shown.

Legends for Figures

Fig. 1. Inhibition of CPT1b expressed in COS7 cells and yeast cells by malonyl-CoA

For examination of the sensitivities of CPT1b expressed in COS7 cells and yeast cells toward malonyl-CoA, activities of CPT1b in these preparations were measured in the presence of various concentrations of malonyl-CoA, and their values relative to those observed in the absence of malonyl-CoA were plotted as a function of the malonyl-CoA concentration. Open circles and closed circles represent the data obtained with mitochondrial fractions from COS7 cells and yeast cells, respectively; and the perpendicular lines attached to the individual circles indicate standard deviations. Activities of CPT1b expressed in COS7 cells and yeast cells in the absence of malonyl-CoA were 6.92 and 8.70 nmol/mg mitochondrial protein/min, respectively, as shown in Table I.

Fig. 2. Preparation of standard CPT1b protein by a bacterial expression system

CPT1b protein slightly expressed as inclusion bodies in Origami B(DE3)pLysS bacteria was collected by centrifugation. It was then resuspended in 0.3 ml of TE and partially purified by repeated centrifugation. A 10-µl aliquot of the obtained suspension was subjected to SDS-PAGE using 7.5%T acrylamide gel. The major protein band highlighted with an asterisk is the expressed CPT1b.

Fig. 3. Expression-level analysis of CPT1b in the mitochondrial fractions prepared from COS7 cells and yeast cells by use of Western blotting with antibody specific for CPT1b

For evaluation of CPT1b expression levels, aliquots (5 µg protein) of the mitochondrial fraction prepared from COS7 cells or yeast cells transfected with individual expression vectors of CPT1b (designated as “cDNA +”) were subjected to

Western blotting. Mitochondrial fractions from cells transfected with sham expression vectors (designated as "cDNA –") were used as negative controls. For quantification of the expressed CPT1b, a known amount (30 ng) of bacterially expressed CPT1b was immunoblotted as a standard (designated as "STD"). CPT1b proteins in the individual samples were detected with the specific antibody and visualized by use of an ECL kit. Expression levels of CPT1b were determined from the signal intensities of the fluorograms (upper photograph). The β-subunit of F_1-ATPase (designated as "F1β") present in individual protein samples was also detected as a control. To ascertain that equal amounts of proteins had been loaded onto the gel, aliquots of each mitochondrial fraction (10 μg protein) were subjected to SDS-PAGE and stained with CBB (lower photograph).

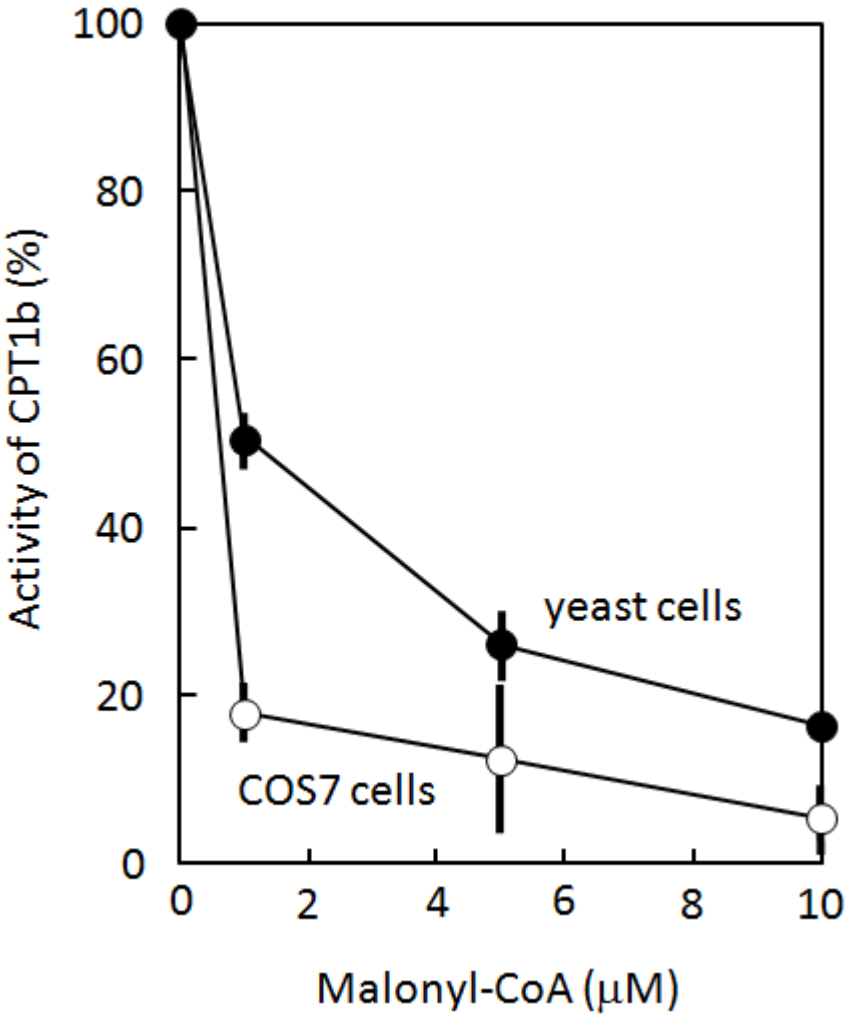
100
80
60
40
20
0
Activity of CPT1b (%)
yeast cells
COS7 cells
0
2
4
6
8
10
Malonyl-CoA (μM)

Fig. 1

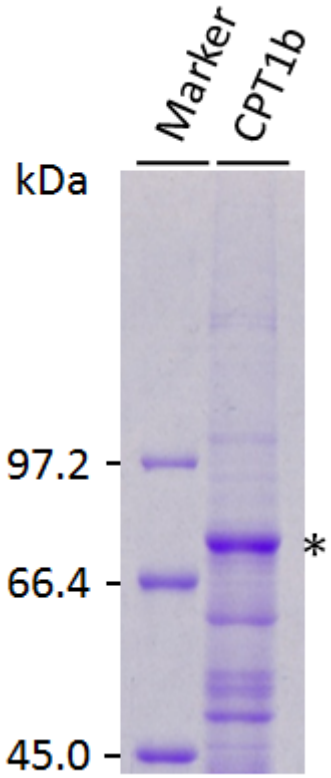

Fig. 2

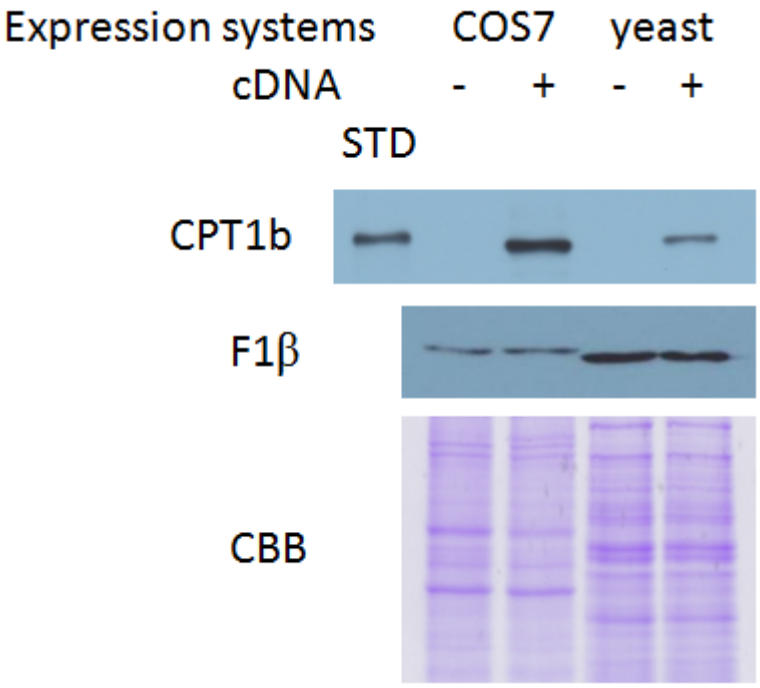

Fig. 3