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Biochemical and Biophysical Research Communications 349 (2006) 383–390
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Functional analysis of skunk cabbage SfUCPB, a unique uncoupling protein lacking the fifth transmembrane domain, in yeast cells ☆

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Received 8 August 2006
Available online 22 August 2006

Abstract

Skunk cabbage, *Symplocarpus foetidus*, expresses two uncoupling proteins (UCPs), termed SfUCPA and SfUCPB, in the thermogenic organ spadix. SfUCPB exhibits unique structural features characterized by the absence of the putative fifth transmembrane domain (TM5) observed in SfUCPA, which is structurally similar to UCP1, and is abundantly expressed in the thermogenic spadix. Here, we conducted a series of comparative analyses of UCPs with six transmembrane domains, SfUCPA and rat UCP1, and TM5-deficient SfUCPB, using a heterologous yeast expression system. All UCPs were successfully expressed and targeted to the mitochondria, although the expression level of SfUCPB protein was approximately 10% of rat UCP1. The growth rate, mitochondrial membrane potential, and ATP content were significantly lower in cells expressing SfUCPB than in those expressing rat UCP1 and SfUCPA. These results suggest that SfUCPB, a novel TM5-deficient UCP, acts as an uncoupling protein in yeast cells.

Keywords: Skunk cabbage; Uncoupling protein; SfUCPB; Mitochondrial membrane potential; Yeast

Uncoupling proteins (UCPs) belong to the family of mitochondrial carriers, which consist of six transmembrane domains. They are present in the inner membrane and catalyze dissipation of the mitochondrial proton gradient without ATP synthesis. A well-known UCP, UCP1, mediates adaptive non-shivering thermogenesis in mammalian brown adipose tissues [1,2]. Recently, UCP1 homologues have been found in mammals and plants [3–6]. In our previous paper, we also reported two UCP-like genes, encoding SfUCPA and SfUCPB, from the thermogenic spadix of skunk cabbage, *Symplocarpus foetidus* [7]. Intriguingly, their deduced amino acid sequences suggested that SfUCPB is a novel UCP lacking the fifth transmembrane domain (TM5) found in SfUCPA, which exhibits six transmembrane domains like UCP1. Moreover, SfUCPB gene expression was abundant in the spadix compared to that of SfUCPA [7]. However, its physiological function is still unknown, and the existence of SfUCPB-like UCPs lacking the fifth transmembrane domain has not yet been reported in other organisms.

UCPs as well as mitochondrial carriers have a tripartite structure composed of three repeated domains of about 100 amino acid residues. These residues consist of two transmembrane regions and one extended matrix loop [1,3–6,8,9]. Each UCP1 domain plays an important role in mitochondrial targeting and regulation of its activity by fatty acids and purine nucleotides. Specifically, UCPs and other members of the mitochondrial carrier protein family, including the ADP/ATP carrier protein (AAC) and the phosphate carrier (PiC), do not contain any targeting signals at their N terminus [10,11]. In the case of rat UCP1

☆ *Abbreviations:* BSA, bovine serum albumin; $DiOC_6$, 3′3-dihexyloxacarbocyanine; $DiSC_2$, 3′3-diethylthiadicarbocyanide iodide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, *p*-(trifluoromethoxy) phenylhydrazone; PGK, 3-phosphoglycerate kinase; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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doi:10.1016/j.bbrc.2006.08.058

(rUCP1), the central matrix loop (CML) contains the complete mitochondrial targeting signal for directing UCP1 to the inner membrane [12] (Fig. 1). Although it remains unclear how fatty acids interact with UCPs at a structural level, purine nucleotides bind to UCP1 via three crucial Arg residues (Arg 84, 183, and 277 in rat UCP1 (rUCP1)) and purine nucleotide binding domains (PNBD) that are conserved in the known UCP homologues [1–6]. Interestingly, SfUCPB, lacking the TM5, possesses the CML, crucial Arg residues, and PNBD as found in rUCP1 [7]. From these observations, we raised the question of whether SfUCPB, an abundantly expressed TM5-deficient UCP (UCPtm5s) in the thermogenic spadix of skunk cabbage [7], is targeted to mitochondria and exhibits uncoupling properties in contrast to rUCP1 and skunk cabbage SfUCPA, both of which have six transmembrane domains (UCPtm6s).

Since there has been no report of genes encoding UCP-like proteins in budding yeast [13,14], yeast heterologous expression systems appear appropriate for initial functional analyses of skunk cabbage UCPs, although UCP overexpression in yeasts causes an artificial uncoupling in mitochondria [13–17]. Thus far, mammalian UCPs [18–22] and potato UCP (StUCP) [23,24] have been successfully analyzed using yeast heterologous expression systems, showing the direct effects of their activities on mitochondria, such as change in membrane potential and sensitivity to purine nucleotides.

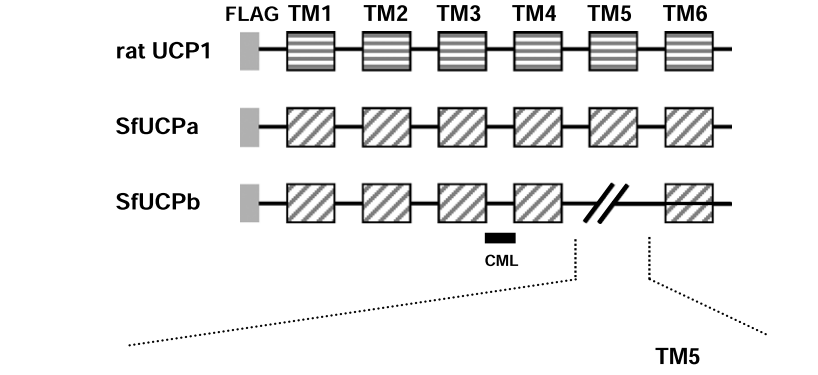
In this paper, we report functional analyses of skunk cabbage SfUCPB in mitochondria using a yeast expression system and compare them with UCPtm6s, rUCP1, and SfUCPA. SfUCPB expressed in yeast is targeted to mitochondria, lowers the mitochondrial membrane potential, and increases the basal oxygen consumption, which suggests that SfUCPB acts as a novel uncoupler. The possible mechanisms of uncoupling function of SfUCPB are also discussed.

Materials and methods

Construction of expression vectors. Each DNA sequence, encoding rUCP1 (Accession No. M11814), SfUCPA (Accession No. AB024733), and SfUCPB (Accession No. AB024734), was amplified by PCR using KOD-plus DNA polymerase (TOYOBO, Tokyo, Japan). The PCR forward (fwd) and reverse (rev) primers were synthesized as follows:

rUCP1-fwd: 5′-ATGGTGAGTTCGACAACTTCC-3′
rUCP1-rev: 5′-GGCTTAATTAAAGTCGCCTATGTGGTGCAGT-3′
SfUCPa/b-fwd: 5′-ATGGGCGATCACGGCCCGAGGA3′
SfUCPa-rev: 5′-GGCTTAATTAACACACCGGTAGAGAAAATTC CACAA-3′
SfUCPb-rev: 5′-GGCTTAATTAACCAGGATCCACACCGGT AGA-3′

Each reverse primer contained an additional Pac I site. The rUCP1 cDNA was obtained from Dr. Daniel Ricquier (Centre National de la Recherche Scientifique, France). Construction of the UCP expression vectors was performed using the pESC-URA vector (Stratagene, CA, USA). pESC-URA was digested with Bgl II and then blunt-ended using a DNA blunt-

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RatUCP1 173 :LWKGTTPNLMRNVIINCTELVTYDLMKGALVNHHILADDVPCHLLSALVAGFCTTLLASP 232
SfUCPa  176 :LWTGLGPNIARNAIINAAELASYDQVKQTILKLPGFSDNIFTHILAGLGAGFFAVCIGSP 235
SfUCPb  176 :LWTGLGPNIARNAIINAAELASYDQVKQ------------------------------- 203
             **.*..**..**.***..**..**..*.

RatUCP1 233 :VDVVKTRFINSLPGQYPSVPSCAMTMYTKEGPAAFFKGFAPSFLRLGSWNVIMFVCFEQL 292
SfUCPa  236 :VDVMKSRMMGDS--AYKSTFDCFIKTLKNDGLLAFYKGFIPNFGRLGSWNVIMFLTLEQV 293
SfUCPb  204 :---MKSRMMGDS--AYKSTFDCFIKTLKNDGPLAFYKGFIPNFGRLGSWNVIMFLTLEQV 258
               .*.*.....  .*.*...*.........*..**.***.*.*.**********...**.

RatUCP1 293 :KKELMKSRQTVDCTT                                              307
SfUCPa  294 :KKFFIK-----EVPN                                              303
SfUCPb  259 :KKFFIK-----EVPN                                              268
             **...*      ....
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Fig. 1. Schematic representation of UCP constructs. Rat UCP1 and SfUCPA have six transmembrane regions (TM1-6), which is a typical structure for mitochondrial carrier proteins. SfUCPB is devoid of the fifth transmembrane region (TM5). A FLAG tag was added to the N terminus of all constructs (upper panel). A sequence alignment of the UCP constructs around the TM5 region of rat UCP1 and SfUCPA is also depicted (lower panel). The TM5 is boxed, and asterisks indicate perfect matches and dots represent conservative changes among the three UCP constructs. Gaps introduced to optimize the sequence alignment are represented by dashes. The alignment was performed with the GENETYX program.

ing kit (Takara Bio, Shiga, Japan) before digestion with Pac I. Each PCR product, from rUCP1, SfUCPA, and SfUCPB cDNA, was also digested with Pac I. These PCR products were introduced into pESC-URA, in which UCP gene expression is under the control of the *gal10-adh1* promoter. A FLAG epitope tag was added at the N terminus of the UCP sequences (Fig. 1) as described by Zhang et al. [22].

S. cerevisiae haploid YPH499 (Mat a) strain (Stratagene) was transformed with each UCP expression vector using the Fast yeast transformation kit (Genotech, CA, USA). The same strain was transformed with the empty vector as a negative control.

Induction of UCP expression in yeast cells. Yeast cells with the expression vectors were selected on SL-ura medium (0.67% Difco yeast nitrogen base without amino acids (BD, MD, USA), 0.19% SC-URA (Qbiogene, CA, USA), 2% lactate, and 0.1% glucose, pH 6.0) with 2% Bacto agar (BD). Single colonies were inoculated in SL-ura liquid medium and grown to an OD600 of 2–3. The yeast cells were diluted to a final OD600 of 0.04–0.08 in the same medium with the lactate concentration increased to 3% and glucose removed. Incubation of the cultures was performed using baffled flasks with silicone caps on a shaking incubator (TAL-RS310, THOMAS, Tokyo, Japan) at 155 rpm. After approximately 15 h, 1% galactose was added to the medium. All incubation was conducted at 30 °C. Growth rates of the yeast cells from 2 to 10 h after addition of 1% galactose were determined using a published approach [25].

Isolation of yeast mitochondria. Mitochondria were prepared as described by Glick and Pon [26]. The yeast cells were harvested 4 h after addition of 1% galactose. Spheroplasts were obtained by digestion of the cells with Zymolyase 20 T (5 mg/g yeast cells) in 40 ml of digestion buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4) at 30 °C for 30 min. Zymolyase 20 T was removed by two washes in digestion buffer and then the final pellet was resuspended in 20 ml of homogenization buffer (0.6 M sorbitol, 20 mM MES-KOH, pH 6.0, and 0.1% BSA) with 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The spheroplasts were homogenized using a Dounce homogenizer and the supernatant was saved after centrifugation. The pellet was resuspended in 7 ml of homogenization buffer with PMSF and homogenized using a 15-ml Potter-Elvehjem tissue grinder (Alcan Packaging, NJ, USA) at 1000–1200 rpm for 5 min. After centrifugation, all supernatants were saved and used for mitochondrial purification. Mitochondria were purified by differential centrifugation and washed with a mitochondrial stabilization buffer (0.6 M sorbitol, 20 mM HEPES–KOH, pH 7.4). All preparation except for the spheroplast preparation was performed at 4 °C. The amount of mitochondrial and cytosolic proteins was measured by the Bradford method using a protein assay kit (Bio-Rad Laboratories, CA, USA).

Western blot analysis. For Western blot analysis, yeast cells harvested 4 h after addition of 1% galactose were resuspended in a sample buffer (60 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol) and heated at 95 °C for 5 min. Extracts from $2–6 \times 10^6$ yeast cells were separated by SDS–PAGE [27]. SDS–PAGE was also conducted using 5 μg each of mitochondrial and cytosolic proteins. After these proteins were electrotransferred onto PVDF membrane, the FLAG-coupled UCPs were detected with anti-FLAG monoclonal antibody (Sigma-Aldrich, MO, USA). Detection of mitochondrial porin and cytosolic 3-phosphoglycerate kinase (PGK) was performed using the corresponding anti-yeast monoclonal antibody (anti-porin, 16G9; anti-PGK, 22C5) (Molecular Probes, OR, USA). These signals were detected by SuperSignal west Femto maximum sensitivity substrate (Pierce Chemical, IL, USA).

Measurement of mitochondrial membrane potential in vivo. Mitochondrial membrane potential in the yeast cells was measured with the potential-sensitive fluorescent probe 3′3-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes) as described by Zhang et al. [22]. The yeast cells were harvested 4 h after addition of galactose. The measurement was performed with 100 nM $DiOC_6$ by flow cytometry (FACS Vantage, Becton Dickinson, NJ, USA).

ATP assay in cellular extracts. Cellular ATP content was measured using yeast cell extracts as described below. $1–3 \times 10^7$ cells harvested 4 h after addition of galactose were resuspended in 0.25 ml Tris–EDTA buffer (0.1 M Tris–acetate (pH 7.8), 2 mM ethylenediaminetetraacetic acid (EDTA)). The suspension was mixed with an equal volume of ice-cold TCA/EDTA solution (10% trichloroacetic acid, 2 mM EDTA) and incubated for 10 min at 4 °C. After centrifugation, the supernatant was diluted 100-fold in Tris–EDTA buffer. ATP assay of the dilutions was performed using the ATP bioluminescent assay kit (Sigma-Aldrich) and a luminometer (Microtec, Chiba, Japan).

Whole yeast O_2consumption. O_2 consumption of whole yeast was measured according to Zhang et al. [22]. The basal O_2 consumption and maximum O_2 consumption induced with 2 μM carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were measured in a Clarke-type oxygen electrode chamber (Hansatech, King's Lynn, UK) at 25 °C with 1.2 ml cell suspension containing 1×10^8 cells in 3% lactate SL medium. For measurement, yeast cells were harvested 4 h after addition of 1% galactose.

Measurement of membrane potential of isolated mitochondria. Membrane potential of mitochondria isolated from the yeast cells was measured with the potential-sensitive fluorescent probe 3, 3′-diethylthiadicarbocyanide iodide ($DiSC_2(5)$) (Acros Organics, NJ, USA) as described by Bouillaud et al. [19]. Measurement of each 50 μg of mitochondrial protein was performed using a measurement buffer (0.65 M mannitol, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 0.5 mM ethyleneglycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mg/ml BSA, and 20 mM Tris–maleate, pH 6.8) with 0.61 μM $DiSC_2(5)$ in a spectrofluorophotometer (RF-5300PC, SHIMADZU, Tokyo, Japan) with excitation at 620 nm and emission at 670 nm (5 nm bandwidth).

Results

rUCP1, SfUCPA, and Sf UCPB expressed in yeast cells are targeted to the mitochondria

We transformed yeast with genes encoding two classes of UCPs: SfUCPB missing TM5 and UCPtm6s containing six transmembrane domains (SfUCPA and rUCP1). After transformation, to determine UCP expression in the yeast, cellular extracts were separated by SDS–PAGE and analyzed by the Western blot. In this analysis, the UCPs, which had a FLAG tag at the N terminus (Fig. 1), were detected by an anti-FLAG antibody. As shown in Fig. 2A, the expression level of SfUCPB was lower than that of UCPtm6s, although a mitochondrial marker, porin, had similar expression levels in these yeast cells. Quantitative measurements of each expressed UCP indicated that the expression of SfUCPB was about one-tenth of that of rUCP1, whereas the expression of rUCP1 and SfUCPA was similar (Fig. 2B).

Moreover, to determine mitochondrial targeting of the expressed UCPs, Western blot analysis was performed with mitochondrial and cytosolic fractions isolated from the yeast cells. As shown in Fig. 3, cytosolic PGK and mitochondrial porin were strongly detected in the cytosolic and mitochondrial fractions, respectively. As shown in Fig. 3, expression of SfUCPB and UCPtm6s was mainly detected in the mitochondrial fraction and their expression levels were similar to expression in the cellular extracts (Fig. 2). These results indicate that although SfUCPB was expressed at a lower level than UCPtm6s, it could be targeted to the mitochondria.

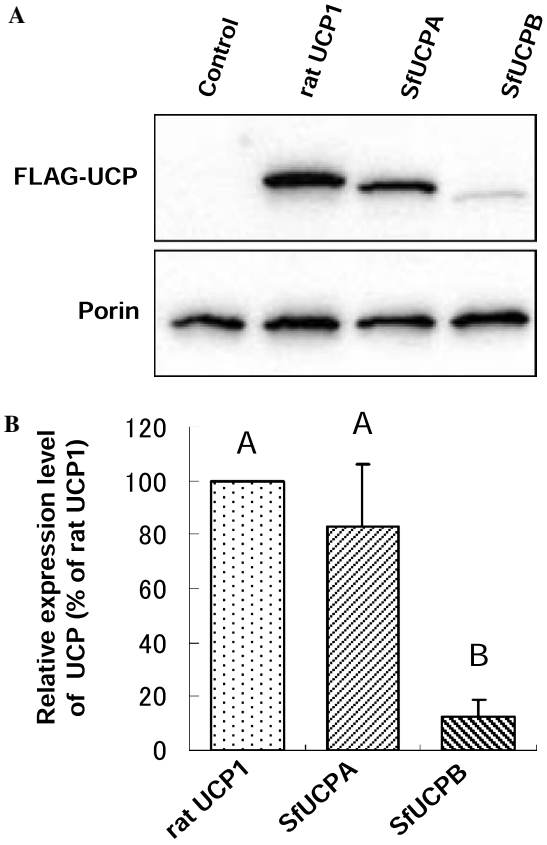

Fig. 2. Western blot analysis for identification of UCP protein expression in whole cells. (A) Yeast cells were harvested 4 h after addition of 1% galactose, and extracts from 2×10^6 cells were separated by SDS–PAGE and analyzed by Western blot with antibodies specific for FLAG and porin. (B) Relative expression level of each UCP was quantified after normalization using porin as a control. Values are means ± SD of three independent experiments. Letters above bars indicate a significant difference from values annotated with different letters ($p < 0.01$, Tukey test).

SfUCPA and SfUCPB inhibit cell growth

Since SfUCPB was detectable in mitochondria (Fig. 3), the effects of its expression on yeast cells were analyzed by measurement of growth rate. The cell growth of UCP-expressing yeast before addition of galactose was similar to that of controls with the empty vector (data not shown). However, the growth rate after addition of galactose became slower than that of the controls, showing that UCPs, except for rUCP1, caused significantly slower cell growth compared to controls (Table 1). Furthermore, SfUCPB significantly suppressed growth compared to rUCP1 ($p < 0.05$), although the effect of SfUCPB was not significantly different from SfUCPA.

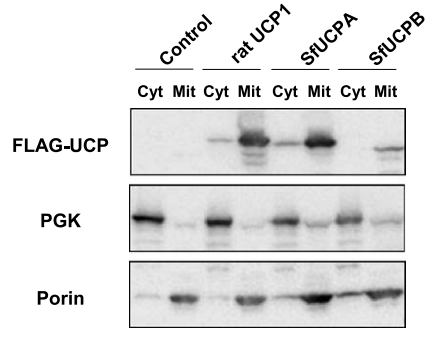

Fig. 3. Western blot analysis for targeting of UCP proteins to the mitochondria. Protein fractions (5 μg) of the mitochondria and cytosol isolated from yeast expressing UCP proteins were separated by SDS–PAGE and analyzed by Western blot with antibodies specific for FLAG, porin and PGK. Culture conditions were the same as in Fig. 2.

Table 1
Growth rates of UCP-expressing yeast cells

	Doubling time (h)
Control	5.1 ± 0.1
Rat UCP1	6.1 ± 0.7
SfUCPA	7.3 ± 0.8*
SfUCPB	8.1 ± 1.0**

Doubling time of yeast growth was determined using OD_{600} data of the yeast cells from 2 to 10 h after addition of galactose. Values are means ± SD for three independent measurements.
* $p < 0.05$.
** $p < 0.01$ indicate significant difference from control with the empty vector (Tukey test).

SfUCPA and SfUCPB decrease mitochondrial membrane potential in vivo

The mitochondrial membrane potential of UCP-expressing yeast cells was measured by flow cytometry and compared with controls. Before addition of galactose, yeast cells containing each of the UCP genes had a mitochondrial membrane potential similar to that of controls (data not shown). After addition of galactose, yeast cells expressing the UCPs, except for rUCP1, had a membrane potential lower than that of the controls (Fig. 4), which suggests that UCP mediated dissipation of the membrane potential. SfUCPB decreased the membrane potential about 2-fold more than SfUCPA (Fig. 4). These results suggest a higher uncoupling activity of SfUCPB *in vivo* than that of UCPtm6s.

SfUCPB and rUCP1Δ5 reduce cellular ATP content

Lowering of the cellular mitochondrial membrane potential by SfUCPA and SfUCPB as shown in Fig. 4 would inhibit oxidative phosphorylation in yeasts and result in a decrease of the cellular ATP content because UCPs dissipate the mitochondrial proton gradient without ATP synthesis. For instance, UCP3-expressing yeasts are inhibited in oxidative phosphorylation [17]. Thus, the ATP content of UCP-expressing yeast cells was measured and compared with that of controls. Although the ATP levels of all yeast cells were similar before addition of galactose (data not shown), those of UCP-expressing yeast

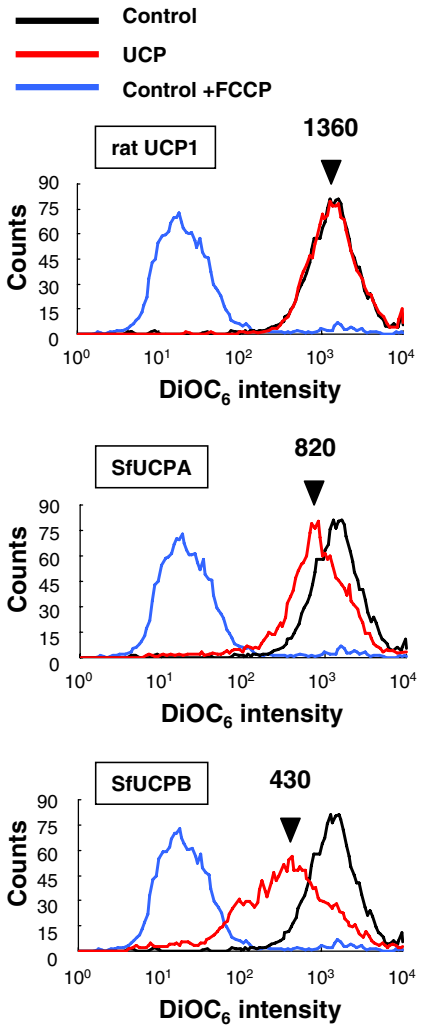

Fig. 4. Change of mitochondrial membrane potential in UCP-expressing yeast cells. Yeast cells were harvested 4 h after addition of 1% galactose. The mitochondrial membrane potential *in vivo* was measured using a membrane potential-dependent fluorescent probe, $DiOC_6$. Fluorescence histograms of a control that was transformed with the empty vector and yeast expressing UCPs are superimposed. Control + FCCP represents the addition of an uncoupler, FCCP, to the control. Values are mean $DiOC_6$ intensity of the histogram peaks in the respective cells expressing UCPs. These presented data are representative of several independent experiments.

cells after addition were decreased compared with controls. The ATP levels in yeast cells expressing SfUCPA and SfUCPB were significantly lower than in those expressing rUCP1 (Fig. 5).

Effect of UCPs on O_2 consumption in yeast cells

Oxygen consumption was measured using whole yeast cells expressing rUCP1, SfUCPA, and SfUCPB. Basal oxygen consumption was defined as the relative percentage of FCCP-induced oxygen consumption [22]. Although no significant increase was observed in rUCP1, SfUCPA, and SfUCPB increased the basal O_2 consumption of whole yeasts by 10.8% and 11.3%, respectively (Table 2).

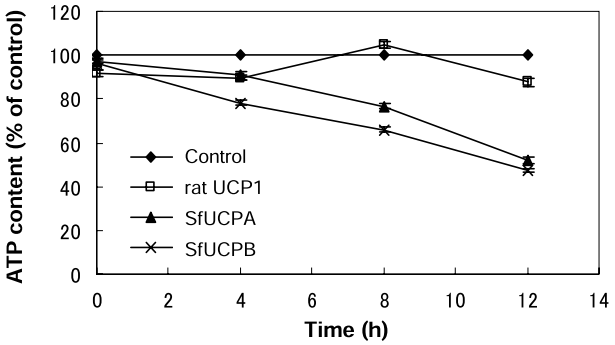

Fig. 5. ATP content in extracts from yeast cells expressing UCPs. Yeast cells were harvested at the time point indicated after addition of 1% galactose. Extracts were obtained from 1×10^7 to 3×10^7 cells and cellular ATP content was measured using a 100-fold dilution of the extracts. Values are means ± SD of three independent measurements.

Table 2
Basal O_2 consumption in yeast cells

	Basal O_2 consumption (% of rate with FCCP)
Control	55.4 ± 0.8
Rat UCP1	58.2 ± 1.1
SfUCPA	61.4 ± 0.7*
SfUCPB	62.6 ± 0.7*

Yeast cells were harvested 4 h after addition of 1% galactose. The whole yeast O_2 consumption was measured in 3% lactate containing medium. The values are means ± SD of six independent measurements.
* $p < 0.01$ indicates significant difference from the control vector (Tukey test).

Sensitivity of yeast mitochondria to palmitate and GDP

UCP1 activity is stimulated by fatty acids and inhibited by purine nucleotides [1–6,16–19]. Thus, to investigate the response to these regulators in cells expressing SfUCPA and SfUCPB, the membrane potential of UCP-expressing mitochondria was measured with a fatty acid, palmitate, and a purine nucleotide, GDP, using a fluorescent dye, $DiSC_2(5)$. As shown in Fig. 6, in the rUCP1-expressing mitochondria, palmitate reduced the membrane potential and GDP allowed it to recover, while their addition to mitochondria isolated from controls resulted in no detectable changes in membrane potential. SfUCPA-expressing mitochondria were also sensitive to palmitate, but insensitive to GDP. On the other hand, mitochondria expressing SfUCPB responded slightly to palmitate but lost sensitivity to GDP (Fig. 6).

Discussion

UCP1 plays a crucial role in adaptive thermogenesis by uncoupling mitochondrial respiration from ATP synthesis

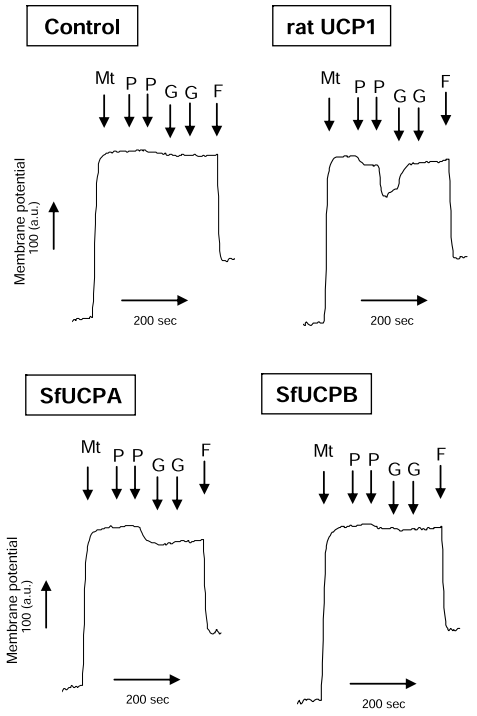

Fig. 6. Response of membrane potential to palmitate and GDP in mitochondria isolated from yeast cells. Mitochondria were isolated from yeast cells harvested 4 h after addition of 1% galactose. Mitochondrial membrane potential was measured using the membrane potential-dependent fluorescent probe $DiSC_2(5)$. After mixing of 50 μg mitochondrial protein with 3 mM NADH (Mt), 64 μM palmitate (P), 1 mM GDP (G), and 10 μM FCCP (F) were subsequently added. These presented data are representative of several independent experiments.

and increasing energy expenditure [1–6]. Based on sequence homology among skunk cabbage UCPs (SfUCPA and SfUCPB) and mammalian UCP1, it was predicted that SfUCPA and SfUCPB would have a similar function to UCP1 in the thermogenic spadix of skunk cabbage [7]. Thus far, however, the biological function of skunk cabbage UCPs, especially SfUCPB, an abundantly expressed UCP in the thermogenic spadix, has not yet been studied.

The expression level of SfUCPB in yeast was approximately one-tenth that of rUCP1, whereas the expression of SfUCPA was similar to that of rUCP1 (Fig. 2). Notably, overexpression of UCPs in yeast cells leads to artificial uncoupling [15,16,28–30]. For instance, Harper et al. suggest that the GDP-insensitive uncoupling due to UCP2 and UCP3 is artificially caused by their overexpression [28]. In the present study, however, the membrane potential of rUCP1-expressing mitochondria was markedly decreased by addition of palmitate and recovered fully with GDP (Fig. 6), which are native characteristics of UCP1 [1,2]. Because rUCP1 showed the highest expression level (Fig. 2), our yeast expression system appears to represent physiological properties of the native UCPs rather than uncoupling artifacts due to overexpression.

Recently, *in vitro* import assays of mitochondrial phosphate carriers using yeast mitochondria have shown that a mitochondrial polytopic protein with four, but not five, spanning transmembrane segments is not translocated to the inner membrane of mitochondria [11]. The authors reported that a molecular chaperone complex (Tim9–Tim10) interacts with the transmembrane segments of polytopic proteins in the mitochondrion and protects them from aggregating. Conversely, the segments of polytopic proteins with four transmembrane segments are inefficiently associated with the Tim9–Tim10 complex and this leads to a transport incompetence of the protein [11]. However, in the present study, SfUCPB lacking the fifth transmembrane domain was targeted to the mitochondria as well as UCPtm6s, SfUCPA and rUCP1 (Fig. 3). These data clearly indicate that the TM5, which is missing in SfUCPB, is not primarily involved in targeting to mitochondria in yeast cells. These findings are also consistent with a previous report demonstrating that the CML, which is conserved in both SfUCPA and SfUCPB (Fig. 1), contains the signals for both targeting and insertion of UCP into the mitochondria [12].

It was, therefore, of great interest whether SfUCPA and SfUCPB, when successfully expressed and targeted to the mitochondria, cause any physiological changes in yeast cells. It appears that SfUCPA and SfUCPB cause slower cell growth (Table 1) and lower mitochondrial membrane potential (Fig. 4) and ATP content *in vivo* (Fig. 5), whereas rUCP1-expressing yeast cells do not show such altered phenotypic characteristics. These results seem to be partially attributable to the different responsiveness to purine nucleotides of each UCP in the mitochondria. Namely, the mitochondrial membrane potential of rUCP1 was significantly decreased by palmitate, an activator of UCP1, and this effect was completely overcome by sequential addition of GDP (Fig. 6). In contrast, SfUCPA showed an apparent response to palmitate while GDP had less of an inhibitory effect on activity. These results are consistent with the previous observation that StUCP, a potato UCP with six transmembrane domains and similar to SfUCPA, also showed susceptibility only to the fatty acid linoleate but not to purine nucleotides in isolated mitochondria [24]. Interestingly, in the case of SfUCPB, palmitate slightly affected the membrane potential, but no response was observed by addition of GDP to isolated mitochondria (Fig. 5). Because SfUCPB-expressing yeast showed the highest basal rate of oxygen consumption (Table 2), it seems likely that SfUCPB acts as an uncoupler whose activity is not significantly affected by endogenous GDP in yeast cells.

So far, genes coding for UCPs lacking a fifth transmembrane domain, except for SfUCPB, have not been found in any complete genome databases including those of *Arabidopsis* and rice. Therefore, it is of great interest to know how the absence of the fifth transmembrane region enables skunk cabbage SfUCPB to have a higher uncoupling activity than those of widely distributed UCPtm6s-like UCP1. The effects of SfUCPB on the yeast mitochondrial membrane potential were similar to that of UCP1Δ9 [19]. Such

uncoupling due to UCP1Δ9 is caused by deletion of amino acids 261–269, which contain a region related to purine nucleotide binding [19]. However, since SfUCPB has the C-terminal region that potentially binds purine nucleotides, it might have mechanisms distinct from UCP1Δ9 for such regulation. One possible explanation would be an alteration of the topological features of SfUCPB due to defects in the TM5. As mentioned above, purine nucleotides at the cytosolic site of the mitochondrial inner membrane play a significant role in the negative modulation of UCP1 activity [1,2]. Therefore, SfUCPB may lose its ability to be negatively controlled by purine nucleotides due to topological modification of its C-terminal end facing the matrix side of the inner membrane of the mitochondria. Alternatively, its deletion of TM5 may induce structural changes in SfUCPB that lead to purine nucleotides-insensitive uncoupling within the mitochondria by an unknown mechanism.

In conclusion, our data showed that the level of SfUCPB in a yeast expression system was approximately one-tenth that of UCP6tm, rUCP1, and SfUCPA, whereas SfUCPB-expressing yeast showed significant physiological alterations such as repression of cell growth and lowering of the mitochondrial membrane potential and cellular ATP content. These observations would be explained by assuming that SfUCPB functions as a novel uncoupler with five transmembrane domains. Namely, SfUCPB, successfully targeted into yeast mitochondria, acts as a GDP-insensitive uncoupler and decreases the rate of oxidative phosphorylation within the mitochondria, which leads to an increased basal oxygen consumption and lowers the level of ATP. We further suggest that the uncoupling due to SfUCPB results from the inhibition of its interaction with purine nucleotides in the mitochondrial intermembrane space, presumably because the absence of TM5 results in a topological or structural change.

Acknowledgments

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) and a Grant-in-Aid for the 21st Century COE program from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors thank Dr. Daniel Ricquier for his generous gift of the rat UCP1 cDNA cloned in the plasmid. The authors also thank Minoru Otsuka, Yoshihiko Onda, and Yasuko Ito for helpful suggestions.

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