Assessment of uncoupling activity of the human uncoupling protein 3 short form and three mutants of the uncoupling protein gene using a yeast heterologous expression system

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Abstract The human uncoupling protein 3 gene generates two mRNA transcripts, uncoupling protein 3L and uncoupling protein 3S, which are predicted to encode long and short forms of the uncoupling protein 3 protein, respectively. While uncoupling protein 3L is similar in length to the other known uncoupling proteins, uncoupling protein 3S lacks the last 37 C-terminal residues. A splice site mutation in the human uncoupling protein 3 gene, resulting in the exclusive expression of uncoupling protein 3S, and a number of point mutations in the uncoupling protein 3 gene have been described. This study compares the biochemical activity of uncoupling protein 3S as well as three mutants of the uncoupling protein 3 gene (V9M, V102I, R282C) with that of uncoupling protein 3L utilizing a yeast expression system. All proteins were expressed at similar levels and had qualitatively similar effects on parameters related to the uncoupling function. Both uncoupling protein 3S and uncoupling protein 3L decreased the yeast growth rate by 35 and 52%, increased the whole yeast basal O_2 consumption by 26 and 48%, respectively, and decreased the mitochondrial membrane potential as measured in whole yeast by uptake of the fluorescent potential-sensitive dye 3′3-dihexyloxacarbocyanine iodide. In isolated mitochondria, uncoupling protein 3S and uncoupling protein 3L caused a similar (33 and 35%, respectively) increase in state 4 respiration, which was relatively small compared to uncoupling protein 1 (102% increase). A truncated version of uncoupling protein 3S, lacking the last three C-terminal residues, Tyr, Lys and Gly, that are part of a carrier motif that is highly conserved among all mitochondrial carriers, had a greatly reduced uncoupling activity. The two naturally occurring uncoupling protein 3 mutants, V9M and V102I, were similar to uncoupling protein 3L with respect to effects on the yeast growth and whole yeast O_2 consumption. The R282C mutant had a reduced effect compared to uncoupling protein 3L. In summary, uncoupling protein 3S and the three mutants of uncoupling protein 3 appear to be functional proteins with biochemical activities similar to uncoupling protein 3L, although uncoupling protein 3S and the R282C mutant have a modestly reduced function.

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Abbreviations: UCP, uncoupling protein; $DiOC_6$, 3′3-dihexyloxacarbocyanine iodide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone

Key words: Uncoupling protein 3; Long and short form; Mutation; Yeast expression system; Uncoupling activity

1. Introduction

Uncoupling proteins (UCPs) are members of the mitochondrial carrier family. Based on a high homology and available functional data, UCPs are believed to represent a subfamily among the larger mitochondrial carrier superfamily. UCP1 was the first UCP to be identified [1–5] and biochemically characterized. UCP1 uncouples mitochondrial respiration from ATP synthesis by decreasing the transmembrane proton gradient [6–8]. The activity of UCP1 is inhibited by purine nucleotides, such as GDP [6]. UCP1 is expressed exclusively in brown adipose tissue where it is believed to play an important role in thermogenesis.

Recently, a number of proteins with high homology to UCP1 were identified, including UCP2 [9,10], which is widely expressed, and UCP3 [11–13], which is expressed primarily in skeletal muscle. Functional studies of the various UCPs, using yeast or mammalian cell expression systems, have been published [9,10,13–16], indicating that UCP2 and UCP3 both have uncoupling activity. In a recent study of UCP3 using a yeast expression system, we found that the uncoupling activity of UCP3 differs from UCP1 [17]. In whole yeast, UCP3 was found to reduce yeast growth and increase the whole yeast basal O_2 consumption to a greater extent than UCP1. In contrast to the strong effects of UCP3 in whole yeast, the increase in state 4 respiration in isolated mitochondria of yeast bearing UCP3 was small compared to that observed with UCP1. This discordance between the stronger effect of UCP3 in whole yeast and the weak effect in isolated mitochondria compared to UCP1, as well as the finding that UCP3 in contrast to UCP1 is not subject to tight GDP inhibition, suggests a different regulation of the two UCPs.

The human UCP3 gene generates two mRNA transcripts, UCP3L, encoding a protein similar in length to UCP1 and UCP2, and UCP3S, encoding a protein which lacks the last 37 C-terminal residues [11,18]. These 37 residues are encoded by exon 7 which is missing from UCP3S due to a cleavage and polyadenylation signal in intron 6. Termination of message elongation at this polyadenylation signal occurs approximately 50% of the time. If the polyadenylation signal in intron

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6 is bypassed, message elongation proceeds until a second polyadenylation signal, located downstream of exon 7, is reached and thus, UCP3L is generated [18]. Mice do not express a transcript which is equivalent to human UCP3S [19]. In mouse, a UCP3 variant mRNA, murine UCP3S, was found [19]. The predicted protein lacks the last 94 C-terminal residues. Murine UCP3S mRNA was much less abundant than murine UCP3L.

All known members of the mitochondrial carrier superfamily are believed to have six transmembrane domains [20,21]. In contrast to UCP3L, UCP1 and UCP2, the putative human UCP3S lacks the sixth C-terminal transmembrane domain. Thus, it is possible that UCP3S is unstable and/or inactive. Import of UCP3S into the inner mitochondrial membrane or the function of UCP3S may be impaired.

In a recent study by Argyropoulos et al. [22], a heterozygous polymorphism in the splice donor junction of exon 6 was found in 20% of Gullah-speaking African Americans and 16% of members of the Mende tribe in Sierra Leone, but not in Caucasians. No homozygous subjects for the polymorphism were detected among the Gullah-speaking African Americans. 1% homozygotes among the members of the Mende tribe. This polymorphism results in loss of the splice function and is therefore predicted to give rise to a protein that is identical to UCP3S. Argyropoulos et al. [22] also showed reduced basal fat oxidation rates and increased respiratory quotients in the group of heterozygotes for the polymorphism, suggesting that UCP3S has a reduced or no function.

A different study by Chung et al. [23] identified heterozygous as well as homozygous individuals for the splice site mutation, predicted to give rise to the UCP3S protein, among an African American population. In addition, three DNA sequence variants in the UCP3 gene were found which result in the alteration of three amino acids (V9M, V102I, R282C) that are highly conserved among the UCPs. No consistent and significant metabolic phenotype was associated with the splice site mutation or any of the three point mutations except for a small elevation of the plasma glucose concentration in individuals with the V9M mutation.

A study by Millet et al. [24] found no difference in the absolute amount and ratio of UCP3L and UCP3S mRNA in lean versus obese subjects, indicating that obesity in these patients is not caused by altered transcription or alternative splicing of the UCP3 gene. Caloric restriction resulted in a 3-fold parallel increase in both UCP3L and UCP3S mRNA, which suggests that there is no regulation of UCP3 gene expression at the level of alternative splicing.

In this study, we utilized a yeast heterologous expression system to compare the biochemical activity of the long and short form of UCP3 and the three UCP3 sequence variants. We have previously used the yeast growth rate, whole yeast O_2 consumption and state 4 respiration in isolated mitochondria to assess the biochemical activity of UCP3L in comparison to UCP1 and demonstrated uncoupling activity for both proteins that appears to be regulated differently [17]. Expression of the oxoglutarate carrier, a closely related mitochondrial carrier that is thought to have no uncoupling activity, had no effect on any of these parameters [17]. Therefore, the yeast expression system appears to be a suitable way of assessing the activity of UCP3S compared to UCP3L. In this study, a truncated UCP3S, lacking the last three C-terminal residues (Tyr, Lys, Gly), that are part of a mitochondrial carrier motif that is highly conserved among all mitochondrial carriers of known sequence [20,21], was studied as an additional negative control.

2. Materials and methods

2.1. Expression vectors and mutagenesis

Constructs of human UCP3L and UCP3S using the pYES2 expression vector (Invitrogen, Carlsbad, CA, USA) were prepared as previously described [17]. The PCR primers used were designed to introduce *Hin*dIII and *Xba*I sites close to the start and stop codons, respectively. To generate mutant UCP3S (mUCP3S), lacking the last three C-terminal amino acids of UCP3S, tyrosine, lysine and glycine, the codon for tyrosine (TAC) was converted into a stop codon (TAA). The three point mutations in the UCP3 gene were generated using site-directed mutagenesis (Quickchange site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA). The codon for V9 (GTG) was changed to ATG (codon for methionine), the codon for V102 (GTC) to ATC (codon for isoleucine) and the codon for R282 (CGT) to TGT (codon for cysteine). All sequences were verified by DNA sequencing. Carrier gene expression is under the control of the *gal10-cyc1* promoter.

The *Saccharomyces cerevisiae* strain INVSC1 (Invitrogen) was transformed with the pYES2 vectors containing these constructs. Empty pYES2 vector was transformed as a negative control.

2.2. Expression of the UCPs

Yeast transformants were selected onto SC-ura plates. Single colonies were inoculated into a pre-culture grown in SC-ura medium to an optical density at 600 nm (OD_{600}) of approximately three. The yeasts were diluted to a final OD_{600} of 0.04 in 500 ml SC-ura medium with 3% lactate and no glucose and grown at 30°C with vigorous shaking to ensure good oxygenation. After approximately 36 h, 1% galactose was added and the cells were harvested after 8–12 h.

2.3. Analysis of expression of UCP3L, UCP3S and mUCP3S by Western blotting

The expression of the UCPs was detected by Western blotting with antibody to human UCP3, that was prepared against a peptide representing residues 147–166 in exon 4, as described in [17].

2.4. Measurement of the mitochondrial membrane potential in yeast cells

The mitochondrial membrane potential was measured with the potential-sensitive dye 3'3-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, Eugene, OR, USA) as previously described [17].

2.5. Whole yeast O_2 consumption

O_2 consumption of whole yeast was measured as previously described [17]. Briefly, the basal O_2 consumption and maximum O_2 consumption induced with 2 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were measured in a Clarke type oxygen electrode chamber at 25°C with 1 ml cell suspension at a final concentration of 2.5×10^8 cells/ml in 3% lactate SC medium.

2.6. Isolation of yeast mitochondria and polarography

Mitochondria were prepared as previously described [17]. Spheroplasts were prepared by enzymatic digestion with zymolyase and mitochondria were isolated by differential centrifugation after homogenization of the spheroplasts. The buffer for mitochondrial isolation contained 0.1% bovine serum albumin that was omitted during the last washing step. Mitochondrial respiration was measured in a Clarke type oxygen electrode at 30°C under the following standard incubation conditions. 0.6 M mannitol, 10 mM Tris-maleate pH 6.8, 5 mM KH_2PO_4/K_2HPO_4, 0.5 mM EDTA, 3 mM NADH, 2.5 μg/ml oligomycin and approximately 0.25 mg mitochondrial protein/ml.

2.7. Statistical analysis

Statistical analysis was performed using StatView 4.0 (Abacus Concept, Berkeley, CA, USA). All results are presented as the mean ± S.E.M. The Student's *t* test was used to evaluate the statistical significance of differences, as indicated in the figure and table legends.

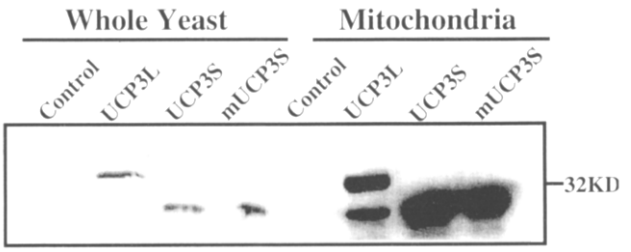

Fig. 1. Immunoblot analysis of the expression of UCP3L, UCP3S and mUCP3S in whole yeast and isolated mitochondria. 40 μg protein was used for analysis in each lane.

3. Results

3.1. Expression of UCP3S and UCP3L

Expression of UCP3S and UCP3L was confirmed by Western blot analysis of whole yeast protein and isolated mitochondria obtained 8 h after induction with 1% galactose (Fig. 1). Equal amounts of protein were loaded in all lanes. Fig. 1 also illustrates that both proteins are targeted to the mitochondria. UCP3S expression was on average 2–3 times greater than UCP3L expression.

3.2. The effect of UCP3S on growth

Both UCP3S and UCP3L inhibited growth of aerobically grown yeast. The average inhibition of growth by UCP3S ($35 \pm 7\%$), in comparison to control yeast, was lower than that observed with UCP3L ($52 \pm 3\%$) after growth for 14 h ($n = 11$).

3.3. The effect of UCPs on whole yeast O_2 consumption

UCP3S and UCP3L increased the basal O_2 consumption of whole yeast by 26 and 48%, respectively (Fig. 2). Addition of the uncoupler FCCP increased O_2 consumption of all yeast strains to a similar absolute rate. The observed increase in basal, but not maximally uncoupled respiration, is consistent with partial uncoupling activity induced by the presence of UCP3S and UCP3L.

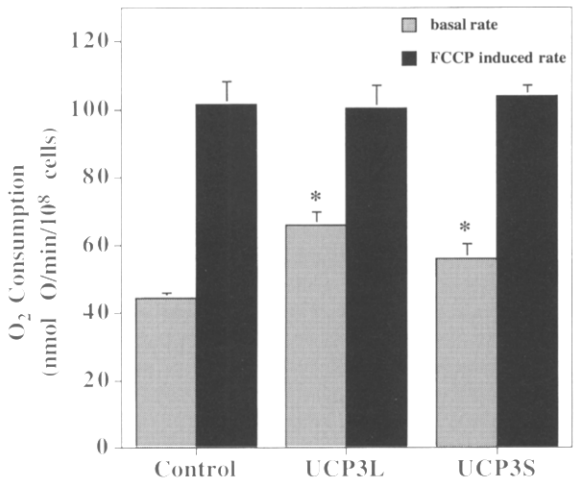

Fig. 2. Whole yeast O_2 consumption. Whole yeast O_2 consumption was measured as described in Section 2. The maximal respiratory capacity was measured by adding 2 μM FCCP. $^*P < 0.01$ versus the empty vector control.

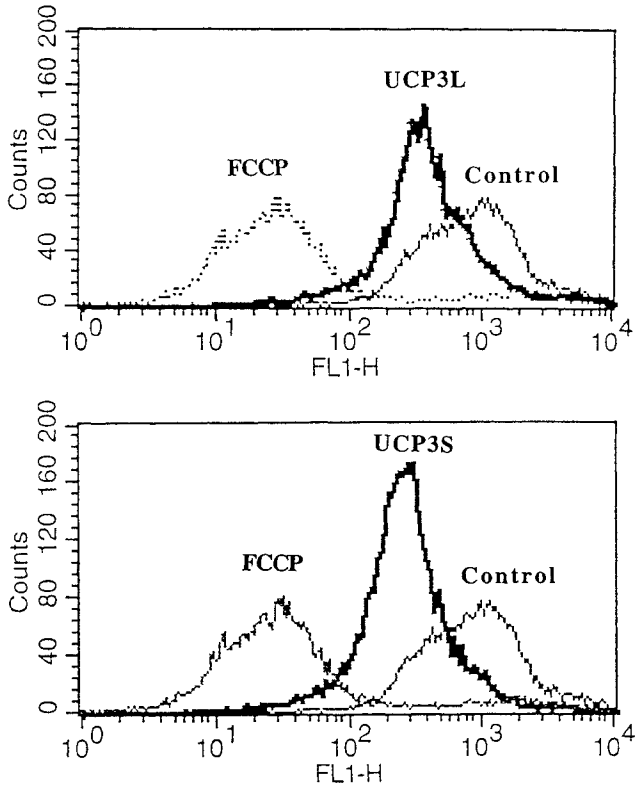

Fig. 3. Mitochondrial membrane potential of yeast expressing UCP3L and UCP3S. Fluorescence histograms of $DiOC_6$-treated cells expressing UCP3L, UCP3S and empty vector are shown. In each panel, the right histogram represents the cells with empty vector. The left histogram shows the effect of FCCP. The bold lines represent the cells expressing UCP3L and UCP3S.

3.4. The effect of UCP3S on the mitochondrial membrane potential

UCP3S decreased $DiOC_6$ uptake as measured by flow cytometry to a similar degree as UCP3L (Fig. 3). This suggests that both proteins lower the mitochondrial membrane potential.

3.5. The effect of UCP3S on mitochondrial state 4 respiration

The capacity of UCP3S to uncouple oxidative phosphorylation was compared with that of UCP3L. The degree of uncoupling was determined by measuring the state 4 respiration of isolated mitochondria, i.e. respiration in the absence of added ADP and presence of oligomycin to inhibit ATP synthase. Under these conditions, mitochondrial O_2 consumption is due to H^+ leak through the inner mitochondrial membrane. Typical respiratory tracings for the empty vector control, UCP3S and UCP3L are shown in Fig. 4A. State 4 respiration was increased to a similar degree in mitochondria containing UCP3S and UCP3L. As previously shown [17], GDP at a concentration of 1 mM had no effect on state 4 respiration with UCP3. UCP3S and UCP3L increased state 4 respiration by 33 and 35%, when expressed as percentage of FCCP-stimulated respiration (Fig. 4B). Although this increase in state 4 respiration, expressed as percentage of the FCCP-stimulated respiration, is relatively small compared to the previously observed 102% increase with UCP1 [17], it is statistically significant compared to the empty vector control.

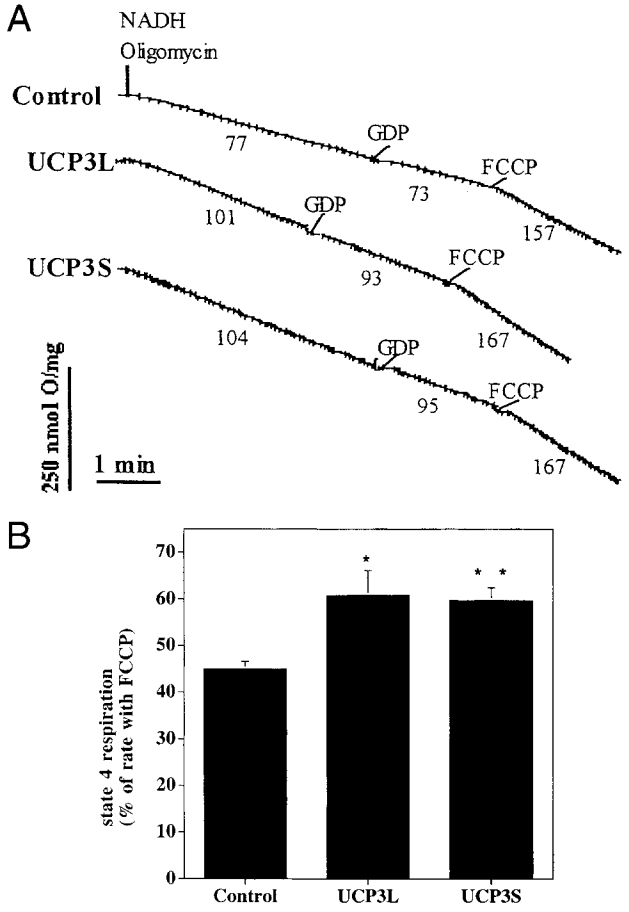

Fig. 4. Respiration in isolated mitochondria. Mitochondrial respiration was measured as described in Section 2. (A) Representative tracings for the empty vector control (Control), UCP3S and UCP3L are shown. The addition of 3 mM NADH, 2.5 μg/ml oligomycin, 1 mM GDP and 2.5 μM FCCP as well as the O_2 consumption in nmol O/min/mg mitochondrial protein are indicated. (B) State 4 respiratory rates are expressed as percentage of the maximally uncoupled respiratory rate in the presence of FCCP. The data represent the average of four experiments. $^*P < 0.05$, $^{**}P < 0.005$ versus the empty vector control.

3.6. *Characteristics of mUCP3, lacking the last three C-terminal amino acids*

mUCP3S, missing the three C-terminal amino acids tyrosine, lysine and glycine, was studied as an additional negative control. These three residues are part of the carrier motif within the third intramatrix loop and are highly conserved among all mitochondrial carriers of known sequence. The immunoblot in Fig. 1 shows that mUCP3S is expressed at a similar level compared to UCP3S in isolated mitochondria. Since the greatest effects of UCP3S and UCP3L were observed in whole yeast, we studied the effect of mUCP3S on growth and whole yeast O_2 consumption. mUCP3S inhibited yeast growth by 22.6 ± 2.6%, thus the effect was intermediate between UCP3S (35.4 ± 4.7% inhibition) and the empty vector control ($n = 5$). mUCP3S did not increase the whole yeast basal O_2 consumption. The basal O_2 consumption of yeast bearing mUCP3S was 41.9 ± 5.6 nmol O/min/10^8 cells (52.0% of the FCCP-induced rate). This rate was almost identical to the basal rate of 41.4 ± 6.1 nmol O/min/10^8 cells (51.3% of the FCCP-induced rate) with control yeast ($n = 4$). This suggests that the mutant protein has a greatly reduced activity.

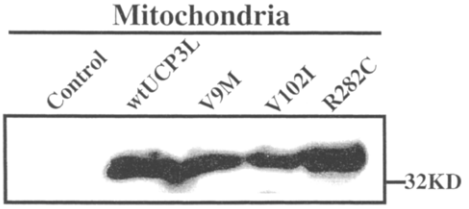

Fig. 5. Immunoblot analysis of the expression of the three UCP3L mutants in isolated mitochondria. 40 μg mitochondrial protein was used for analysis in each lane.

3.7. *Study of three mUCP3 proteins (V9M, V102I, R282C)*

The expression of the V9M and V102I mutant proteins in isolated mitochondria was similar compared to wild-type UCP3L, that of R282C slightly increased (Fig. 5). The V9M and V102I mutants had a similar inhibitory effect on growth and stimulatory effect on the whole yeast basal O_2 consumption as wild-type UCP3L (Table 1). The R282C mutant had an intermediate effect on both parameters.

4. Discussion

This study investigated the uncoupling function of the short form of UCP3. This question is important because individuals with a splice site mutation in the UCP3 gene, that results in the expression of UCP3S, have been found in different populations [22,23]. Three variants in the UCP3 sequence, resulting in the alteration of residues that are highly conserved among the UCPs, were also studied. According to Chung et

Table 1
Growth inhibition and whole yeast basal O_2 consumption in yeast expressing mUCP3L

	Growth inhibition compared to control (%) ($n = 3$)	Whole yeast basal O_2 consumption (% of FCCP-induced rate) ($n = 4$)
Control	0	47.7 ± 1.3
UCP3L	52.4 ± 5.7*	64.1 ± 4.0**
mUCP3L (V9M)	53.4 ± 2.2***	67.6 ± 3.1***
mUCP3L (V102I)	51.3 ± 9.8*	61.7 ± 2.0***
mUCP3L (R282C)	30.8 ± 3.4*	53.8 ± 1.8*

Yeast growth was measured at 30°C in 3% lactate and no glucose containing SC-ura medium as described in Section 2. At time zero, yeast was diluted to an OD_{600} of 0.20 and galactose at a final concentration of 1% was added. After 14 h, the OD_{600} was measured and growth inhibition was expressed as % inhibition compared to empty vector control yeast. Whole yeast O_2 consumption was measured at 25°C in the 3% lactate containing medium. 2 μM FCCP was added to determine the maximum respiratory capacity. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ versus the control.

al. [23], neither the splice site mutation nor the three sequence variations resulted in a significant and consistent phenotype. This may be due to the fact that UCP3 does not function primarily as an UCP or that the short form and mutants of UCP3 are functional.

This study demonstrates that the short form of UCP3 can be expressed in yeast mitochondria and is thus not subject to premature protein degradation or failure of protein import in yeast. Using available antibodies, we have not been able to detect UCP3L or UCP3S in human skeletal muscle mitochondria, which may be due to a low sensitivity of the antibodies used and/or low expression levels of the protein in humans.

The long and short form of UCP3 have qualitatively similar biochemical activities when expressed in yeast. Both proteins reduced the yeast growth and mitochondrial membrane potential measured in whole yeast by uptake of the potential-sensitive dye $DiOC_6$ and increased the whole yeast basal O_2 consumption. The magnitude of the effects was lower with UCP3S compared to UCP3L. Although these parameters are not specific for an uncoupling function, a previous study showed that UCP1 had similar effects on these parameters [17]. In addition, the oxoglutarate carrier, a closely related mitochondrial carrier without an uncoupling function, had no effect on yeast growth and basal O_2 consumption and an effect on $DiOC_6$ uptake that was intermediate between UCPs and empty vector control. Therefore, the measurements in whole yeast suggest uncoupling activity for UCP3L and UCP3S.

Mitochondrial carrier proteins are composed of three sequence repeats, each of which contains a characteristic mitochondrial carrier sequence motif that is well-conserved among all mitochondrial carriers of known sequence [20,21]. Each of the three sequence repeats consists of two transmembrane α-helix domains and one extended matrix loop. Therefore, all mitochondrial carriers of known sequence possess six transmembrane domains. It may be argued that lack of the sixth transmembrane domain in UCP3S could prevent protein import into the inner mitochondrial membrane. However, UCP3S is truncated immediately after the third mitochondrial carrier motif. It was suggested that this carrier motif may play a role in protein importation into the inner mitochondrial membrane mediated by Tim10 and Tim12 [25]. Alternatively, the motif may facilitate correct protein folding or orientation. Therefore, as an additional negative control, we studied a mutant of UCP3S, lacking the last three C-terminal residues, tyrosine, lysine and glycine, of the carrier motif. Our results indicate a greatly reduced activity for mUCP3S compared to UCP3S. This may be due to impaired protein import of the mutant protein into the inner mitochondrial membrane. Alternatively, the three residues may be essential for the folding, orientation or function of the protein.

Our study of the three mUCP3L proteins indicates that the V9M and the V102I mutants are functional and the R282C mutation is partially functional. These findings are not surprising, since the first two mutants result in the change of one neutral residue to another. In contrast, in the R282C mutant, a basic residue is converted into the reactive cysteine residue. Therefore, an impairment in protein function is not unexpected. The residue is located within the last transmembrane domain that is missing in UCP3S. Thus, lack of arginine-282 may contribute to the quantitatively decreased activity of UCP3S. Alternatively, the additional cysteine residue in the R282C mutant may have a deleterious effect on the function of the protein.

The most specific parameter, when studying uncoupling activity, is measurement of state 4 respiration in isolated mitochondria. When UCP1 is expressed in yeast, a strong increase in state 4, that is reversed upon addition of GDP, is observed [17,26]. In contrast, expression of the oxoglutarate carrier has no effect on state 4 respiration [17]. As previously observed for UCP3L [17], UCP3S increased state 4 respiration to a relatively small degree, when compared to UCP1. This discordance between the strong effects of UCP3S and UCP3L in whole yeast and the small effect on state 4 respiration in isolated mitochondria requires further investigation. As discussed in [17], one or more unidentified activators of UCP3S and UCP3L, that are present in whole yeast, may be missing when isolated mitochondria are studied.

In summary, UCP3S could be expressed in yeast and was targeted to the mitochondria. UCP3S and UCP3L had qualitatively similar effects in whole yeast and isolated mitochondria. Given the 2–3 times higher expression level of UCP3S compared to UCP3L, the activity of UCP3S is reduced compared to UCP3L. Thus, UCP3S appears to be a functional UCP, but with a modestly reduced activity compared to UCP3L.

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