Recombinant human uncoupling protein-3 increases thermogenesis in yeast cells

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Abstract The long form of human uncoupling protein-3 (hUCP3L) is highly homologous to thermogenin (UCP1), the uncoupling protein of brown fat mitochondria, but is expressed predominantly in skeletal muscle. Its putative role is to regulate the coupling efficiency of oxidative phosphorylation and thus thermogenesis in skeletal muscle, a major thermogenic tissue in higher mammals. To study the functional relevance of hUCP3L, the protein was expressed in yeast cells under the control of the galactose promoter. Expression of hUCP3L induced a series of phenotype changes in the yeast cells. The cellular growth and the mitochondrial membrane potential were both diminished. The portion of cellular respiration coupled to oxidative phosphorylation decreased from 57% to 11% ($P < 0.001$) and the cellular heat production, as measured by direct microcalorimetry, was increased by 33.3 ± 3.2% ($P < 0.001$) after induction of UCP3L. These observations demonstrate for the first time the intrinsic thermogenic properties of hUCP3L in intact cells.

Key words: Uncoupling protein; Thermogenesis; Yeast; Respiration

1. Introduction

The formation of an electrochemical gradient of protons across the inner mitochondrial membrane is essential for coupling the oxidation of metabolic substrates with the synthesis of ATP. An increase in the proton permeability of the energy transducing mitochondrial membrane results in the dissipation of metabolic energy in the form of heat. In thermogenic tissues such as the brown fat, the release of heat from the mitochondria is a physiological process which makes an important contribution to resting energy expenditure in rodents. This process is mediated by a membraneous 33 kDa protein named thermogenin or uncoupling protein 1 (UCP1) whose expression is unique to the brown fat mitochondria [1]. The protein, discovered more than 20 years ago [2,3], has meanwhile been cloned and thoroughly characterized (for reviews see [4–7]). Several laboratories have considered the possible involvement of brown fat and UCP1 in the development of obesity [8]. In higher mammals, however, brown fat is present only in newborn animals and essentially disappears a few days after birth. Interestingly, however, proton leaks have been observed in mitochondria from many other tissues where UCP1 is not expressed [9] even though the mechanisms underlying the increase in proton permeability have not yet been clarified [10]. For instance, a large proportion (up to 50%) of oxygen consumption of skeletal muscle, a major thermogenic tissue in humans, is devoted to heat production [10]. The recent discovery of the novel UCP1 gene homologues, UCP2 [11,12] and UCP3L [13–15], which encode for putative mitochondrial proteins with similar function, has restimulated the interest in exploring a possible role of UCPs in regulating thermogenesis and thus obesity also in humans. While low amounts of UCP2 are detectable in a variety of peripheral and central tissues in humans, UCP3L is found only in skeletal muscle where it is expressed in considerable amounts [11–15]. UCP3L could therefore be an important regulator of energy expenditure in humans.

In the present work, we present data on the effects of the expression of recombinant human UCP3L (hUCP3L) in yeast cells and demonstrate that this protein is in fact a genuine uncoupler of mitochondrial respiration and thus can considerably increase thermogenesis.

2. Materials and methods

Oligonucleotides were purchased from Microsynth (Balgach, Switzerland) and human skeletal muscle poly(A)$^+$ mRNA from Clontech (Heidelberg, Germany). Restriction and modification enzymes were from Roche Diagnostics (Rotkreutz, Switzerland) or Gibco BRL (Basel, Switzerland). Basic components for growth media were from Difco Laboratories (Detroit, MI). Adenine, tryptophan, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and lactic acid were purchased from Fluka (Buchs, Switzerland) and zymolyase from ICN (Lucerne, Switzerland). $DiOC_6$ was from Molecular Probes Inc. (Eugene, OR). Triethyl tin bromide (TET) was from Aldrich Chem. Co. (Steinheim, Germany). Human UCP3L antibodies were from Alpha Diagnostic International (San Antonio, TX).

2.1. Construction of expression vectors and their introduction into yeast

hUCP3L cDNA was generated by RT-PCR from human skeletal muscle poly(A)$^+$ mRNA using the two oligonucleotides hUCP3LF-100 (5′-TCTCCTTGGACCTCCTCTCG-3′) and hUCP3LR-1124 (5′-CACGTTAGCTACCAGTGGCC-3′). The amplified sequence, which contained the complete coding region, was introduced into the vector pCR2.1 (Invitrogen, Groningen, The Netherlands). 5′ and 3′ untranslated regions were removed by enzymatic amplification using Pwo polymerase and the forward primer hUCP3LF-*Bgl*II (5′-GGAGATCTATGGTTGGACTGAAGCCTTCAGACGT-3′) and the reverse primer hUCP3LR-*Eco*RI (5′-GGAATTCTCAAAACGGTGATTCCCGTAACATCT-3′). The resulting fragment was double digested with *Bgl*II and *Eco*RI and then introduced into the yeast expression vector pYeDP60 [16] previously digested with *Bam*HI and *Eco*RI. By this means, the hUCP3L cDNA could be cloned as close as possible to the Gal-Cyc promoter in the vector pYeDP60. Sequence analysis was performed confirming the coding sequence for hUCP3L. The haploid yeast strain W303-B1 (*Saccharomyces cerevisiae*: *Mat*a; *ade*2-1; *his*3-11, -15; *leu*2-3, -112; *ura*3-1; *can*^{R}; *cyr*$^+$) was transformed by the Li-acetate method [17] with the hUCP3L cDNA containing and the empty pYeDP60 expression vectors. The yeast clones

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; UCP, uncoupling protein; TET, triethyl tin bromide; BAT, brown adipose tissue; TAM, thermal activity monitor; RCI, respiratory control index

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containing the vectors were selected for uracil auxotrophy on synthetic minimal glucose medium SGAT (0.1% casamino acids, 0.7% YNB, 2% glucose, 25 mg/l tryptophan and 40 mg/l adenine).

2.2. Expression of hUCP3L in yeast

Yeast cells from glycerol stocks were grown at 28°C with moderate agitation in SMAT medium (0.1% casamino acids, 0.7% yeast nitrogen base, 2% lactic acid, 50 mg/l tryptophan and 40 mg/l adenine, adjusted to pH 4.5 with KOH). Expression of hUCP3L was induced by the addition of 1/10 vol. of 10×YPL (20% D-galactose, 10% yeast extract, 20% bactopeptone) to the SMAT medium when the cultures reached an OD_{600} of 0.2–0.5.

2.3. Growth comparisons on solid medium

Agar plates containing SGAT or SLAT (identical to SGAT except that 2% D-galactose was used as a carbon source instead of glucose) were used. Yeasts were grown on a SMAT medium and diluted with 0.9% NaCl solution to 30 000 cells/ml. 40 μl of the diluted culture were then pipetted on the agar plates. The plates were incubated for 48 h at 28°C before evaluation of growth.

2.4. Mitochondrial membrane potential measurements in whole cells

Yeast clones were grown on a SMAT medium and then hUCP3L expression was induced for 8 h as described above. Yeast cells were then washed twice with 0.9% NaCl and finally resuspended in 10 mM Tris-MES (pH 6.3) supplemented with 20 mM glucose to an OD_{600} of 0.2. The yeast cells were then incubated for 30 min with the membrane potential sensitive dye, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), at a final concentration of 175 nM. The fluorescence was measured in triplicate in Dynatech microtiter plates using a Cytofluor 2350 fluorimeter (Millipore Corporation, Bedford, MA) with wavelength settings at 485 nm for excitation and 530 nm for emission. When necessary, the mitochondrial uncoupler CCCP or the mitochondrial electron transport blocker NaN_3 were added at various concentrations to the cells 10 min before the dye.

2.5. Oxygen consumption of whole cells

About 6×10^7 cells (corresponding to 0.36 mg total protein assuming that 1 cell contains 6 pg protein) were added to 3 ml of 10 mM Tris-MES (pH 6.3) supplemented with 20 mM glucose at 30°C and respiration was followed using an oxygen electrode of the Clark type (Yellow Springs Instruments, Yellow Springs, OH). TET and CCCP (final concentrations of 100 μM and 10 μM, respectively) were added sequentially after oxygen consumption reached a steady state value.

2.6. Direct microcalorimetry of whole yeast cells

Direct heat production was measured at 30°C using a TAM (Thermal Activity Monitor, Thermometric AB, Sweden). Yeast cells were resuspended in 10 mM Tris-MES (pH 6.3) supplemented with 20 mM glucose at an $OD_{600} = 0.5$. Sealed glass ampoules (volume = 3 ml) containing 100 μl of the cell suspension were introduced in the calorimeter and read against a similar volume of cell free buffer. The power/time curves were recorded until steady state was reached (approximately 20 min).

2.7. Isolation of yeast mitochondria

A fraction enriched in mitochondria was prepared according to Guérin et al. [18]. Briefly, yeast spheroplasts were prepared by enzymatic digestion for about 20 min at 30°C with zymolyase (2 mg/g wet weight yeast). After washing and homogenization of the spheroplasts with a Dounce homogenizer, mitochondria were fractionated by differential centrifugation. The final mitochondrial pellets were resuspended in 650 mM mannitol, 20 mM Tris-maleate, 0.5 mM EGTA, 10 mM K_2HPO_4, pH 6.8.

2.8. Immunological detection of hUCP3L

After electrophoretic separation on 12% SDS-polyacrylamide gels (SDS-PAGE), proteins were electrotransferred overnight at 27 mA on a nitrocellulose membrane (blotting buffer: 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol). Detection of hUCP3L was performed with affinity purified anti-hUCP3L antibodies diluted 1000-fold and horseradish peroxidase-linked anti-goat IgGs as secondary antibodies according to the recommendations described in the Enhanced Chemiluminescence (ECL) detection system (Amersham, Switzerland).

2.9. Statistical analysis

Student's unpaired *t*-test was used to determine statistical significance, which was accepted at the $P < 0.05$ level.

3. Results

3.1. Induction of hUCP3L in yeast cells and its influence on growth

Haploid yeast (strain w303) cells were transformed with empty expression vector (control clones) or with a vector containing a hUCP3L construct (hUCP3L clones). The expression of UCP was under control of the Gal-Cyc promoter, which is strongly repressed by glucose and induced by galac-

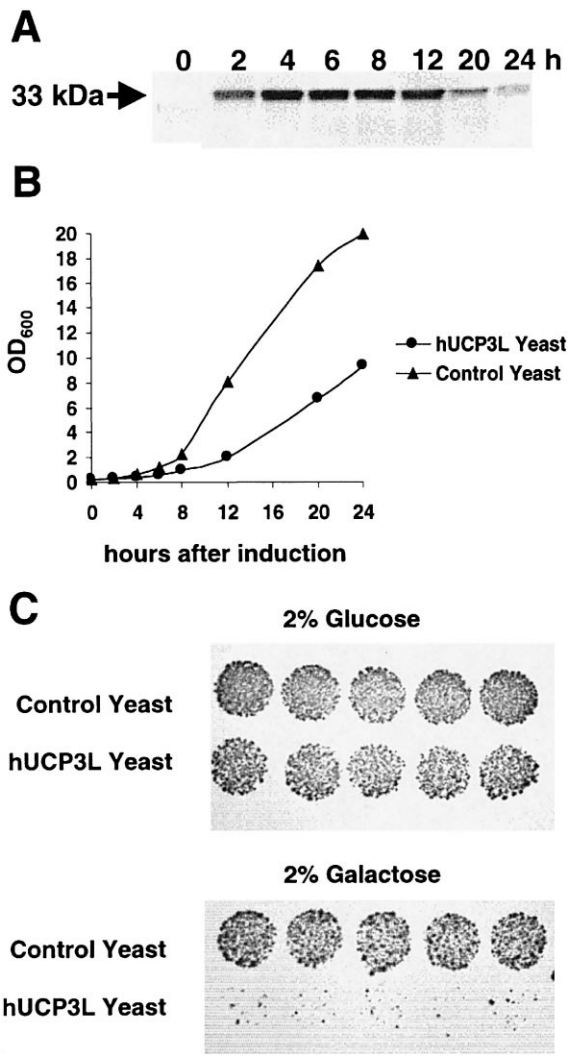

Fig. 1. Effect of hUCP3L expression on yeast growth. A: Kinetics of hUCP3L expression in yeast. Yeast was diluted ($OD_{600} = 0.01$) in SMAT medium supplemented with 2% galactose to start the induction and further incubated at 28°C. At various time points after induction, 6×10^6 cells were separated on a 12.5% SDS-PAGE and hUCP3L was immunodetected. B: Effect of UCP3L expression on yeast growth (liquid medium). Control yeast and UCP3L yeast were induced as described in A and growth was monitored. C: Effect of hUCP3L expression on yeast growth (solid medium). 1200 cells were dotted on agar plates containing glucose or galactose minimal medium. The plates were incubated for 48 h at 28°C. Five independent clones each of control yeast (empty vector) and UCP3L expressing yeast were analyzed.

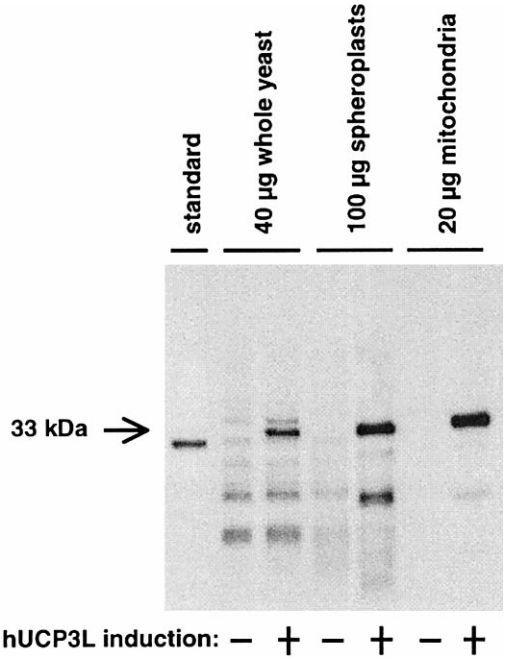

Fig. 2. Co-localization of recombinant hUCP3L with yeast mitochondria. Control yeast and hUCP3L expressing cells were spheroplasted and further subfractionated. The indicated amounts of total protein were separated on a 12.5% SDS-PAGE, transferred on a nitrocellulose membrane and UCP3L was then detected immunologically. As a positive control, a truncated form of hUCP3L expressed in *E. coli* (molecular weight of about 30 kDa) was used.

tose in the growing media. The clones were first amplified in the presence of lactate, as a non-fermentable carbon source, under vigorous shaking to favor the formation of mitochondria [18]. The hUCP3L expression was then induced by addition of galactose. Fig. 1A shows a Western blot analysis of the time dependence of the hUCP3L expression. The molecular weight (MW) of the most prominent band recognized by the anti-hUCP3L antibodies was approximately 33 kDa, corresponding to the expected size of the hUCP3L protein [13]. Recombinant hUCP3L was already detectable a couple of hours after induction and maximal levels (normalized per cell number) were obtained after 6–10 h. The intensity of the hUCP3L band then gradually decreased and was almost gone after 24 h. Since induction was studied under non-selective conditions, some cells lost the expression vector and invaded the culture. This led to a progressive dilution of the hUCP3L expressing cells, thereby explaining the low level of hUCP3L/cell found after 24 h.

Before induction, the growth characteristics of control and UCP3L yeast clones were identical in lactate or in glucose media (not shown). After induction with galactose, however, large differences in growth rates were observed, as shown in Fig. 1B. While control yeast cells resumed a rapid growth after a short lag phase of 4–6 h, the growth of yeast expressing the recombinant protein remained strongly inhibited until the expression levels of hUCP3L/cell started to decline.

Fig. 1C compares the growth on agar plates of independent yeast colonies transformed with empty or hUCP3L containing vectors. No significant difference could be detected when growth was compared in glucose containing plates. On the other hand, the growth rate of all the clones containing the hUCP3L construct was drastically reduced in plates containing galactose to allow production of UCP3L.

3.2. Co-localization of recombinant UCP3L and yeast mitochondria

To verify the translocation of recombinant UCP3L in yeast mitochondria, the expression levels of the protein were followed by Western blot analysis during subfractionation of the cells. Fig. 2 clearly shows that the recombinant protein is enriched in the mitochondrial fraction. From four independent preparations it was calculated that the specific level of UCP3L, as determined by densitometric analysis of Western blots, was 10.8 ± 2.7-fold higher in the mitochondrial fraction than in the spheroplasts fraction. A similar enrichment (12.3 ± 5.5-fold) of the mitochondrial marker cytochrome *c* oxidase was observed.

3.3. Influence of hUCP3L expression on mitochondrial function in whole cells

The fluorescence intensity of the dye $DiOC_6$ can be used as an indicator of membrane potential in biological membranes.

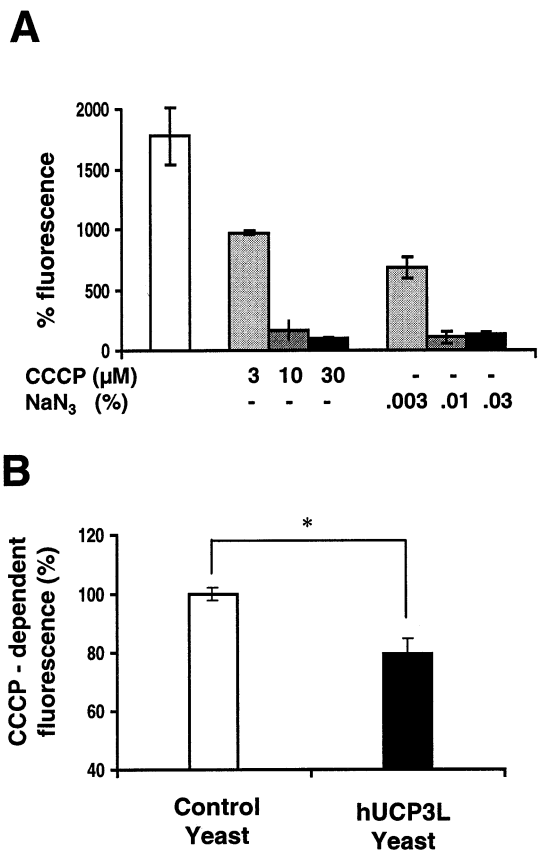

Fig. 3. Effect of hUCP3L expression on the mitochondrial membrane potential of yeast cells. A: Contribution of the mitochondrial membrane potential to $DiOC_6$ fluorescence in yeast cells. Yeast cells were incubated with the fluorescent dye $DiOC_6$ after treatment with various amounts of the mitochondrial uncoupler CCCP or the mitochondrial electron transport blocker NaN_3. B: Comparison of the mitochondrial membrane potential-related fluorescence of control and hUCP3L yeast. The mitochondrial membrane potential-related fluorescence (CCCP-dependent fluorescence) was determined after subtraction of the residual fluorescence measured in the presence of 30 µM CCCP. The value of the control yeast was set to 100% fluorescence. Values are given as mean ± S.E.M. ($n = 10$). *$P < 0.05$.

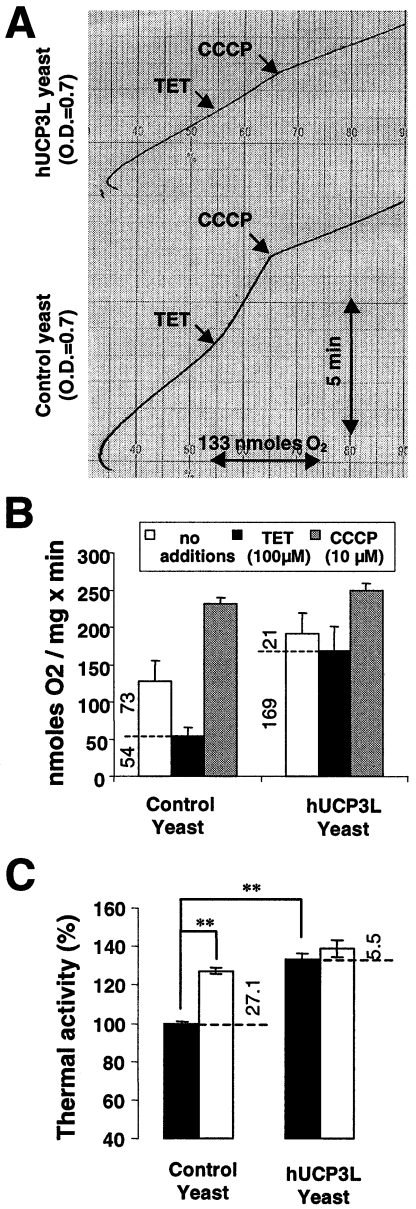

Fig. 4. Uncoupling and thermogenic effects of hUCP3 expression. A: Effect of UCP3L expression on the respiration of whole yeast cells. Representative oxygen consumption tracings of whole yeast cells as measured at 30°C with a Clark type polarographic electrode in 10 mM Tris-MES, pH 6.3 supplemented with 20 mM glucose are shown. Additions of TET (100 μM) and CCCP (10 μM) were made where indicated. B: Effect of UCP3L expression on the coupling efficiency of the respiration in yeast. Respiration rates were calculated when steady state values were reached after the sequential additions of TET and CCCP and expressed as the mean ± S.E.M. ($n = 3$). C: Thermogenic effect of UCP3L expression in yeast. Heat production of the yeast cultures used in the experiments illustrated in B was analyzed using a TAM. The values were normalized with respect to the heat power of control yeast cells (1726 μW/mg) which was set at 100%. No additions: □. CCCP addition (20 μM): ■. $^{**}P < 0.001$.

Under controlled conditions and at low dye concentrations, the signal arising from whole cells reflects mainly the membrane potential of the inner membrane of the mitochondria [19]. Fig. 3A shows that in fact, the fluorescence associated with whole yeast cells strongly decreased after the addition of CCCP, a very effective uncoupler of mitochondria. The CCCP-dependent fluorescence is normally considered to derive from the mitochondrial contribution. Similar results were obtained using NaN_3, a compound known to block electron transport by the mitochondrial cytochrome oxidase complex, thus preventing energization of the inner membrane (see Fig. 3A). According to these experiments, most of the total $DiOC_6$ fluorescence measured in whole yeast cells could be attributed to the membrane potential of mitochondria. The effect of hUCP3L expression on the mitochondrial membrane potential of intact yeast was then investigated. Fig. 3B shows that the fluorescence of hUCP3L expressing cells was significantly reduced, thus indicating a partially collapsed mitochondrial membrane potential.

Analysis of oxygen consumption of intact yeast cells in suspension was carried out using a Clark oxygen electrode. The induction of hUCP3L expression significantly increased the respiration rate of yeast cells (see Fig. 4A,B). TET, a cell permeable inhibitor of the mitochondrial ATP synthase (F_0F_1-ATPase) [20], was used to inhibit the portion of the total respiration that is coupled to oxidative phosphorylation. The residual oxygen consumption of hUCP3L cells was found to be markedly higher (about 3-fold) than that of control cells, thus showing a marked increase in uncoupling. On the other hand, the percentage of respiration coupled to ATP synthesis in hUCP3L expressing cells was significantly decreased from 57.6 ± 5.3% to only 11.9 ± 3.6% ($P < 0.001$). The low coupling efficiency of UCP3L cells was also evident by comparing the RCI_{CCCP} values (i.e. the ratio between the fully stimulated oxygen consumption in the presence of 10 μM CCCP and that measured under non-phosphorylating conditions in the presence of TET) which were found to be strongly reduced from 4.5 ± 0.9 (control cells) to 1.5 ± 0.2 (UCP3L cells) ($P < 0.01$).

3.4. Effect of UCP3L expression on cellular heat production

In the following experiments, the basal and CCCP-stimulated cellular thermogenesis was measured by direct microcalorimetry. As shown in Fig. 4C thermal power of control yeast cells was stimulated 27.1% by addition of the uncoupler CCCP ($P < 0.001$). On the other hand, UCP3L expressing yeast cells had a considerably higher unstimulated thermogenesis (133.3 ± 4.2% of control cells) ($P < 0.001$) which was only marginally stimulated (5.5%) by CCCP.

4. Discussion

UCP3L is part of a family of proteins, which includes in addition to UCP1 and UCP2 various additional mitochondrial transport proteins (such as the phosphate carrier, the adenine nucleotide translocator and the oxaloglutarate carrier). All these proteins have a similar size (about 33 kDa) and show a triplicate structure, each containing two putative transmembrane domains and a typical mitochondrial energy transfer signature [4,5]. The high degree of homology between human UCP1 and UCP3L (i.e. 57% identical) led to the suggestion that, like UCP1, UCP3L is also translocated to the inner mitochondrial membrane where it exerts the role of an uncoupler of the mitochondrial oxidative phosphorylation [13–15]. Analogously to previous strategies applied to UCP1 [21] and UCP2 [11], recombinant UCP3L has been expressed in various cell systems to probe for its putative uncoupling properties. So far, the only evidence for such a function is

based on the observation, made with cationic lipophilic dyes, of a decrease in fluorescence in whole yeast cells [15,22] or C2C12 myoblasts [23] expressing recombinant hUCP3L. Such dyes (e.g. rhodamine derivatives and $DiOC_6$) partition preferably into the inner mitochondrial membrane and their fluorescence is proportional to the level of polarization of the membranes. We could confirm that UCP3L expression induces a significant reduction of the mitochondrial potential-related fluorescence in yeast cells (Fig. 3). These results however, are fraught with potential artefacts and many interpretations are possible. Autoquenching of fluorescence could occur by high intramitochondrial concentrations of the dyes. In addition, alterations of fluorescence could reflect a change of the mitochondrial volume or of the number of mitochondria per cell rather than of the mitochondrial membrane potential. A proof of the uncoupling function of UCP3L, therefore, cannot be established solely on this basis. In this study, we provide additional conclusive evidence for the thermogenic role of UCP3L. In a series of experiments we exploited the possibility of measuring the coupling efficiency of mitochondria in situ. We observed that the overall rate of oxygen consumption was significantly increased after induction of UCP3L expression in yeast cells. In spite of this, the amount of respiration actually coupled to the synthesis of ATP decreased from 73 to 21 nmol O_2/mg/min (Fig. 4B). This could partly explain why the high energy demanding proliferative activity was so much inhibited in UCP3L expressing cells. The uncoupled respiration of UCP3L clones was considerably higher than in control clones and the difference corresponded to an extra oxygen consumption of 115 nmol O_2/min/mg protein. Since yeast cells were respiring on glucose as substrate, the theoretical energy liberated by this amount of extra oxygen for to the full combustion of glucose can be calculated to be 2.2×10^{-4} cal s^{-1} mg $protein^{-1}$ (i.e. 920 μW mg $protein^{-1}$). By using direct microcalorimetry to measure the thermogenesis of yeast cells, a measured difference in the thermal power between UCP3L clones and control cells of 625 μW mg $protein^{-1}$ was obtained (see Fig. 4C). This measured energy corresponded closely to the calculated energy liberated by the extra oxygen consumption and clearly demonstrates for the first time, that UCP3L expression causes an increase in cellular thermogenesis. One should emphasize that the expression levels of recombinant UCP3L in yeast were quite modest ($<1\%$ of the mitochondrial protein) but still sufficient to cause the marked phenotypic changes described above.

In conclusion, in this communication strong evidence has been presented demonstrating that human UCP3L, a protein expressed exclusively in skeletal muscle, has genuine intrinsic thermogenic properties. Since human skeletal muscle is considered to be a major source of heat production in the resting state, UCP3L could play a pivotal role in determining the basal metabolic rate.

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