Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression

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Abstract Brown adipose tissue (BAT) and skeletal muscle are important sites of nonshivering thermogenesis. The uncoupling protein-1 (UCP1) is the main effector of nonshivering thermogenesis in BAT and the recently described ubiquitous UCP2 [1] has been implicated in energy balance. In an attempt to better understand the biochemical events underlying nonshivering thermogenesis in muscle, we screened a human skeletal muscle cDNA library and isolated three clones: UCP2, $UCP3_L$ and $UCP3_S$. The novel UCP3 was 57% and 73% identical to human UCP1 and UCP2, respectively, highly skeletal muscle-specific and its expression was unaffected by cold acclimation. This new member of the UCP family is a candidate protein for the modulation of the respiratory control in skeletal muscle.

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1. Introduction

The uncoupling protein-1 (*UCP1*) gene encodes a unique mammalian mitochondrial proton carrier that induces heat production in brown adipose tissue (BAT) by uncoupling respiration from ATP synthesis [for review, see [2]]. Despite its tissue specificity, UCP1 is a member of the mitochondrial carrier protein family, which includes the ATP/ADP translocator, the phosphate, the 2-oxoglutarate/malate and the citrate (tricarboxylate) carriers [for review, see [3]]. Human *UCP1* gene, located in 4q31, is made up of six exons and encodes a 307-amino-acid sequence with no targeting presequence [4]. Like other mitochondrial carriers, UCP1 is inserted into the inner mitochondrial membrane by means of 6 α-helical hydrophobic domains, each encoded by one of the six exons [5]. Its polypeptide chains consist of three tandemly related sequences of about 100 amino acids, each encoded by two exons and corresponding to two transmembrane domains [6]. Topological studies suggest that both the C- and N-terminal ends of UCP1 are oriented towards the cytosolic side of the inner mitochondrial membrane [7].

Structure–function studies have been performed on UCP1 and a putative purine nucleotide binding domain, analogous to that observed in the ATP/ADP carrier, has been described, which is located between amino acids 273 and 295 [8] in rat UCP1 (SWISS-PROT P04633). It has been suggested that cysteine-302 participates in the modulation of UCP1 activity by fatty acids [9] and that amino acids 13–105 are necessary for the targeting and insertion of UCP1 into the mitochondrial inner membrane [10]. Conserved residues, characteristic of the mitochondrial carrier proteins, have been described: their presence at the borders of transmembrane domains 1, 3 and 5 is characteristic of all members of this family and they are referred to as mitochondrial energy-transfer-protein signatures [11].

Contrary to the ATP/ADP translocator, which is a more elementary carrier, UCP1 is highly regulated [12]. Its activity is decreased by purine nucleotides di- or triphosphates and increased by fatty acids [2].

BAT is an important effector of nonshivering thermogenesis in rodents [2]. In humans, BAT expressing UCP1 is found in the newborn; it decreases during infancy but is still detected in the adult [13]. Under physiological conditions, BAT does not play a significant role in nonshivering thermogenesis in adult humans.

Very recently a novel member of the UCP family (UCP2) has been described [1]. This protein, which is widely expressed in human tissues, might play a role in energy balance.

Skeletal muscle contributes for up to 40% of whole-body epinephrine-induced thermogenesis in humans [14]. In the rat, in vivo administration of the β_3-adrenoceptor agonist BRL 28410 was found to stimulate skeletal muscle heat production [15] and recently, Nagase et al. [16] reported that a chronic treatment of obese yellow KK mice with the β_3-adrenoceptor agonist CL 316,243 induced ectopic expression of UCP1 in the skeletal muscle. Therefore, epinephrine-induced thermogenesis in human skeletal muscle [14] might be mediated by a skeletal muscle UCP.

In search for UCP homologues in human skeletal muscle, we screened a skeletal muscle cDNA library and isolated three cDNA clones: UCP2, $UCP3_L$ and $UCP3_S$. Whereas UCP2 mRNA was found in all tissues studied, as shown by Fleury et al. [1], UCP3 was highly skeletal muscle-specific. These new members of the UCP family are candidate proteins for the modulation of tissue respiratory control, with UCP3 being highly specific for skeletal muscle.

2. Materials and methods

2.1. Library screening

Total RNA from rat tibialis anterior muscle and interscapular BAT were purified by the method of Chomczynski and Sacci [17], and amplified by reverse transcription-polymerase chain reaction using primers corresponding to conserved domains of rat UCP1, i.e. in positions 279–298 (UCPRF) and 1021–1044 (UCPRR) on GenBank Accession M11814. Single fragments of similar size were obtained

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Abbreviations: UCP, uncoupling protein; BAT, brown adipose tissue

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from both tissues. The BAT PCR product's sequence was identical to that of the rat UCP1 cDNA, whereas that from the tibialis anterior muscle was different, bearing ca. 60% identity to rat UCP1. This cDNA, referred to as rat atypical UCP cDNA, was used as a probe to screen a human skeletal muscle cDNA library (Stratagene, #936215, La Jolla, CA).

2.2. Clone sequencing

cDNA clones were purified by Qiagen plasmid kit according to the manufacturer's instructions (Quiagen, Santa Clarita, CA) and sequenced using standard protocols for the ABI373A automated sequencer with the M13-20 and T3 primers and gene-specific primers until the sequence was determined on both strands.

2.3. Human tissue samples

The human Multiple Tissue Northern blot (#7760-1) was from Clontech Laboratories Inc. (Palo Alto, CA). Pieces of human omental white adipose (10–40 g) or abdominal skeletal muscle (800 mg) were obtained during intra-abdominal surgery. Pieces of perirenal brown adipose tissue weighing about 1.5 g were obtained during renal surgery from infants (mean age: 3 months). The project has been approved by the Ethics Commission of the Department of Surgery, Faculty of Medicine, University of Geneva.

2.4. Rat treatment and tissue samples

Seven-week-old Sprague-Dawley male rats fed ad libitum standard laboratory chow were caged individually with a 12-h light-dark cycle. Cold adaptation was performed at 6°C for 20 days during which the rats were pair-fed to control rats kept at 23°C. They were killed by cervical dislocation. All experiments were performed in accordance with our institutional guidelines.

2.5. Northern analysis

Total RNA (12–20 μg) was electrophoresed in a 1.2% agarose gel containing formaldehyde, as described by Lehrach et al. [18] and transferred to Electran Nylon Blotting membranes (BDH Laboratory Supplies, Poole, UK) by vacuum blotting. Probes were labeled by random priming with [α-^{32}P]dCTP (Amersham, Bucks, UK) to a specific radioactivity of approximately 1×10^9 dpm/μg DNA. RNA blots were hybridized for 2 h at 65°C in QuikHyb (Stratagene, La Jolla, CA), then washed in a solution of 2×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate (SDS) at 50°C twice for 5 min and in 0.1×SSC/0.1% SDS at 50°C for 5 min. Blots were exposed to Hyperfilm ECL films (Amersham, Bucks, UK) at −80°C with intensifying screens. Size estimates for the RNA species were established by comparison with an RNA Ladder (Gibco BRL, New York, NY). The signals on the autoradiograms were quantified by scanning photodensitometry using ImageQuant Software Version 3.3 (Molecular Dynamics). Hybridization of the blots with a [γ-^{32}P]ATP-labeled synthetic oligonucleotide specific for the 18S rRNA subunit was used to correct for differences in the amounts of RNA loaded onto the gel.

3. Results and discussion

Screening of ca. 1 000 000 phages from the human skeletal muscle cDNA library with a rat atypical UCP cDNA probe (see Section 2) resulted in the isolation of 10 positive clones. Among them, three different categories were found by sequencing. The predicted polypeptides of these three clones which contain 309, 312 and 275 amino acids are illustrated in Fig. 1. The 309-amino-acid protein is UCP2 recently isolated by Fleury et al. [1]. The two other proteins have their first 275 amino acids in common, suggesting that they are isoforms. They have amino acid identities of 57% and 73% to human UCP1 and UCP2, respectively and, as such, are considered as novel proteins referred to as UCP3 long and short forms, $UCP3_L$ and $UCP3_S$, respectively (GenBank Accessions U84763 and U82818). Like other mitochondrial carriers, UCP2 and $UCP3_L$ contain six predicted transmembrane domains (Fig. 1). Potential purine nucleotide binding domains extend between amino acid residues 276–298 in UCP2 and 279–301 in $UCP3_L$. $UCP3_S$ lacks the sixth potential transmembrane region and the purine nucleotide binding domain implicated in the control of the coupling efficiency of UCP1 [8]. Binding of guanosine diphosphate (GDP) to UCP1 is

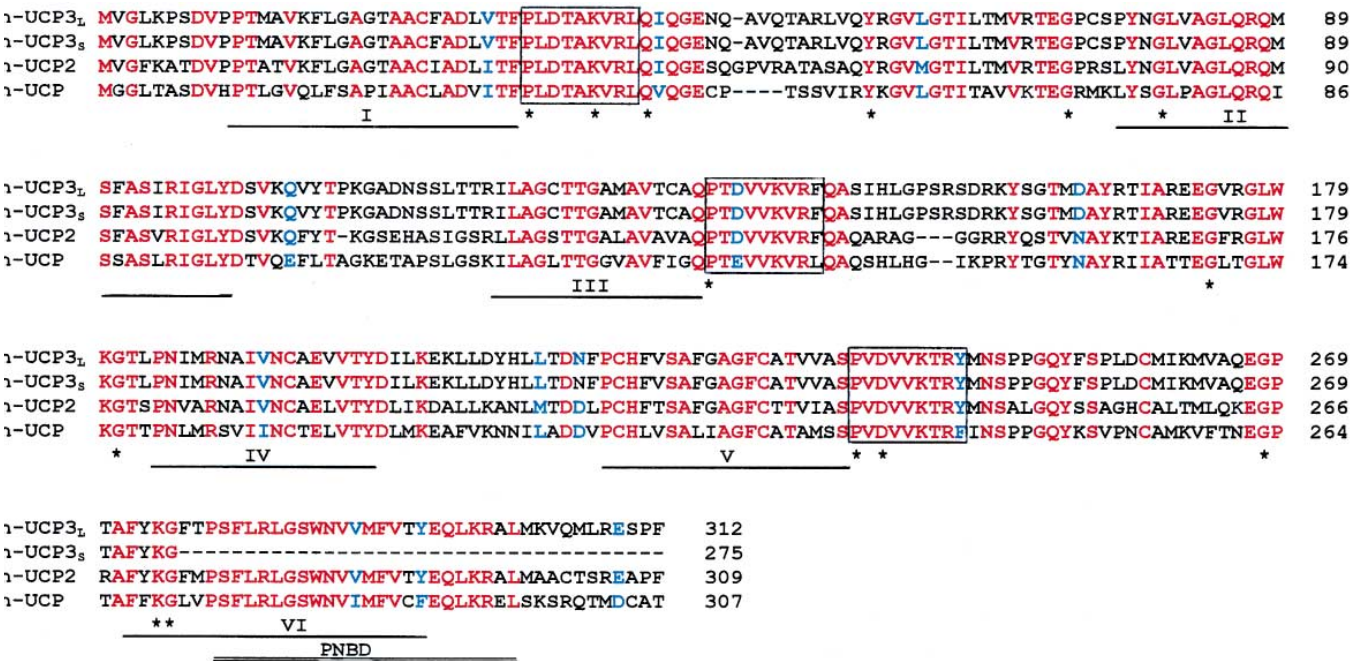

Fig. 1. Amino acid sequence alignments of human $UCP3_L$, $UCP3_S$, UCP2 (GenBank Accession U82819) and UCP1 (UCP) obtained with the ClustalW Multiple Sequence Alignment program (K.C. Worley, Human Genome Center, Baylor College of Medicine). The sequences are presented in single letter code. Gaps introduced into the sequences to optimize alignments are illustrated with a dash. Identical and similar amino acids are highlighted in red and blue, respectively. Potential transmembrane α-helices are underlined and numbered in roman numerals (I–VI). The potential purine-nucleotide binding domain (PNBD) is underlined twice. The three mitochondrial energy-transfer-protein signature domains are boxed. The 14-amino-acid residues which are totally conserved in mitochondrial carrier proteins are shown with asterisks.

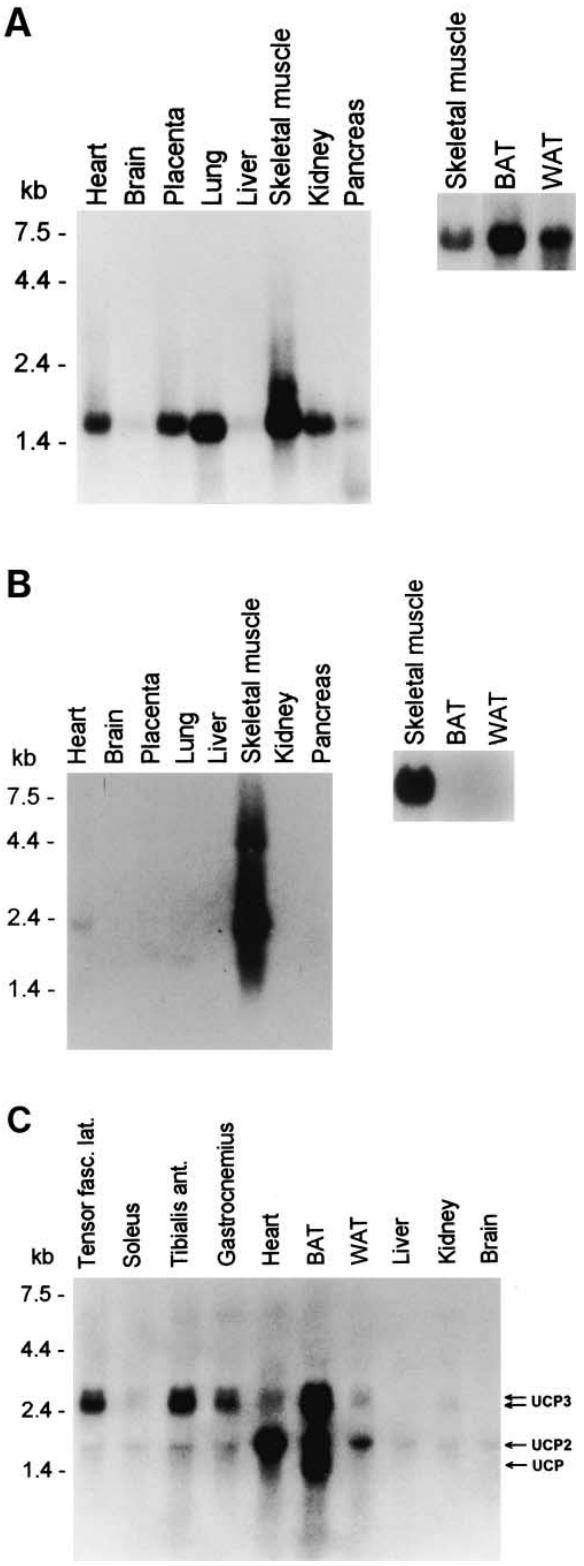

Fig. 2. Expression of UCP2 and UCP3 in human and rat tissues. A: Autoradiogram of a Northern blot of various human tissue poly(A) RNA hybridized with a ^{32}P-labeled human UCP2 probe consisting of the full-length UCP2 cDNA. The molecular size markers are indicated in kb. Inset: UCP2 expression in total RNA. Brown adipose tissue (BAT); white adipose tissue (WAT). B: Autoradiogram of a Northern blot of various human tissue poly(A) RNA hybridized with a ^{32}P-labeled human UCP3 probe consisting of the full-length UCP3$_S$ cDNA. Inset: UCP3 expression in total RNA. C: Autoradiogram of a Northern blot of various rat tissue total RNA. The ^{32}P-labeled rat atypical UCP cDNA probe used (see Section 2) hybridized to the rat 3 UCP species, i.e. UCP1 (1.4 kb), UCP2 (1.7 kb) and UCP3 (2.5 and 2.8 kb).
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known to induce a conformational change leading to inhibition of H^+ and Cl^- permeability [2]. Therefore, UCP3$_S$ would exert its biological effect without any control by GDP. As also illustrated in Fig. 1, other structural features of mitochondrial carriers are observed in UCP2 as well as in UCP3$_L$ and UCP3$_S$: the three mitochondrial energy-transfer-protein signatures [11], which can be identified at the border and downstream of the first, third and fifth potential transmembrane domains, and the 14 conserved mitochondrial carrier residues [3].

UCP2 and UCP3 have the highest predicted amino acid sequence homology to UCP1 as compared to the other members of the mitochondrial carrier protein family. Indeed, their identities to UCP1 are of 55% and of 56%, respectively, whereas those to the most closely related mitochondrial carrier protein 2-oxoglutarate/malate carrier are of 32%. UCP2 and UCP3 should therefore belong to the UCP family. Indeed, Fleury et al. [1] showed that UCP2 has uncoupling properties when expressed in yeast.

Human tissue UCP2 and UCP3 distributions were then compared by Northern blot analyses. As shown in Fig. 2A and in the inset, an UCP2 signal at a size of 1.7 kb was observed in all human tissues studied. UCP2 was expressed at the highest level in BAT > white adipose tissue > skeletal muscle. By contrast, UCP3 expression (2.3 kb signal) was restricted to skeletal muscle and heart, though it was much lower in the latter (Fig. 2B and inset). Direct comparison of the Northern blots hybridized with UCP2 and UCP3 probes showed that UCP3 was much more expressed in skeletal muscle than UCP2 in any of the tissues studied. Specific probes for the long or short form of UCP3 revealed that the two forms gave the same signal at 2.3 kb, and quantification of the signal intensity showed that both forms are expressed at similar levels in human skeletal muscle (results not shown).

The tissue distribution of UCP2 and UCP3 mRNAs was also studied in the rat using the rat atypical UCP cDNA probe, which hybridizes to the rat 3 UCP species, i.e. UCP1, UCP2 and UCP3. As shown in Fig. 2C, UCP2 was expressed in all tissues studied: heart > BAT > white adipose tissue > skeletal muscle. A major difference as compared to human UCP2 is its high degree of expression in the heart. UCP3 was most highly expressed in BAT, at a high level in tensor fascia latae (fast-twitch glycolytic), tibialis anterior (fast-twitch oxidative-glycolytic), gastrocnemius (mixed) muscles and less in soleus muscle (slow-twitch oxidative). This suggests that UCP3 is more expressed in glycolytic than in oxidative skeletal muscles. UCP3 was also detected, although at a much lower level, in rat heart and kidney, and occasionally in white adipose tissue. It has been shown in rodents but not in humans that UCP1 mRNA signal exists in two sizes [1]. It can be seen in Fig. 2C that UCP3 signal is a doublet whereas UCP2 signal is single. The sizes of the signals, as compared to an RNA ladder, were of 1.4 and 1.8 kb for UCP1, 1.7 kb for UCP2, and 2.5 and 2.8 kb for UCP3. Altogether, the results show that the tissue distributions of

UCP2 and UCP3 are very different from that of UCP1, i.e. either ubiquitous or, in humans, highly specific to skeletal muscle, respectively.

UCP2 and UCP3 expressions were studied in the tibialis anterior muscle of rats acclimated to cold, a condition known to strongly increase UCP1 in BAT [2]. It was found that 20 days of cold adaptation had no effect on UCP2 and UCP3 expressions in tibialis anterior (results not shown). This suggests that skeletal muscle UCP2 and UCP3 are differentially regulated from BAT UCP1.

The present study describes new members of the UCP family and provides evidence for candidate genes possibly involved in the uncoupling of oxidative phosphorylation specifically in skeletal muscle, an important site of nonshivering thermogenesis in humans. An understanding of the molecular and biochemical basis of skeletal muscle thermogenesis will have wide implications for both basic and clinical research in metabolic regulation in general, and in the areas of obesity and cachexia in particular.

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