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UCP3: An Uncoupling Protein Homologue Expressed Preferentially and Abundantly in Skeletal Muscle and Brown Adipose Tissue

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Uncoupling proteins (UCPs) are inner mitochondrial membrane transporters which dissipate the proton gradient, releasing stored energy as heat. UCP1 is expressed exclusively in brown adipocytes while UCP2 is expressed widely. We now report the molecular cloning of a third uncoupling protein homologue, designated UCP3. At the amino acid level, hUCP3 is 71% identical to hUCP2 and 57% identical to hUCP1. UCP3 is distinguished from UCP1 and UCP2 by its abundant and preferential expression in skeletal muscle in humans, and brown adipose tissue and skeletal muscle in rodents. Since skeletal muscle and brown adipose tissue are believed to be important sites for regulated energy expenditure in humans and rodents, respectively, UCP3 may be an important mediator of adaptive thermogenesis. Since UCP3 is minimally expressed in human heart and other critical organs, it is a promising target for anti-obesity drug development aimed at increasing thermogenesis.

Calories are expended within mitochondria in a highly regulated fashion. Oxidation of fuels generates a proton electrochemical gradient across the inner mitochondrial membrane and re-entry of these protons via ATP synthase drives conversion of ADP to ATP. Uncoupling proteins (UCPs) are inner mitochondrial membrane transporters which dissipate the proton gradient, releasing stored energy as heat (1, 2), and are therefore potentially important determinants of metabolic efficiency. UCP1, the first uncoupling protein to be identified (3-5), is expressed exclusively in brown adipose tissue, an important site of energy expenditure in rodents (6-8). However, UCP1 may be of lesser importance in humans in whom the mass of brown adipose tissue is limited. A second uncoupling protein, termed UCP2 (9) or UCPH (10) was recently identified. In contrast to UCP1, UCP2 is expressed in many tissues, including sites not thought to mediate adaptive thermogenesis. We now report the molecular cloning of a third uncoupling protein homologue, designated UCP3, which is distinguished from UCP1 and UCP2 by its preferential expression in skeletal muscle and brown adipose tissue, two important sites for regulated energy expenditure in humans (11-15) and rodents (8).

MATERIALS AND METHODS

RACE (rapid amplification of cDNA ends) cloning of UCP3. Full-length UCP3 cDNA sequences were generated using the Marathon cDNA Amplification Kit, human skeletal muscle Marathon-Ready cDNA (both from Clontech Laboratories, Palo Alto, CA) and an antisense primer (5′ TTC ACC ACG TCC ACC GGG GAT GCC ACC 3′). PCR was carried out using ExTaq Polymerase (TaKaRa), Taq Start Antibody (Clontech Laboratories) and the following conditions: 1.5 min. at 94°C, 20 sec. at 98°C and 4 min. at 68°C for 30 cycles.

Northern blot assays. Human Multiple Tissue Northern Blots (#7760-1, #7759-1 and #7767-1) containing approximately 2 μg of polyA RNA per lane were purchased from Clontech Laboratories (Palo Alto, CA). All hybridizations, membrane washes and membrane strippings were performed according to manufacturers specifications. The blots were first hybridized to a hUCP3 probe, washed and exposed to film for 1-18 hours, then stripped, rehybridized to a hUCP2 probe and exposed to film for 18 hours. The hUCP3 probe was a 293 bp fragment corresponding to residues #211-308. The hUCP2 probe was a 1125 bp fragment spanning the entire open reading frame. The specific activities of both hybridization probes were similar. Mouse Northern blots were generated using total RNA isolated from a number of tissues and equal loading of lanes was established using ethidium bromide florescence. The mouse Northern blots were hybridized using the hUCP3 probe described above or a 1207 bp mUCP2 probe which spans the entire open reading frame.

RNase protection assays. Partial human UCP-3 and UCP-2 probes were generated by reverse transcriptase-PCR using total RNA from human muscle as follows: two primers (5′ GCA GTC TTG AAG AAC GGG ACA CC 3′ and 5′ TGG CAG TAG GGG GCA CAT CT

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3′) were designed to amplify 210 bp of hUCP-2 and another two primers (5′ GGA CTA CCA CCT GCT CAC TG 3′ and 5′ CCC GTA ACA TCT GGA CTT T 3′) were designed to amplify 302 bp of hUCP3. Both PCR products were subcloned into PGMT easy TA cloning vector (Promega). Identity and orientation was confirmed by sequencing. Linearized templates for synthesis of antisense UCP-3 and UCP-2 transcripts were prepared using *Spe* I. Linearized templates for synthesis of reference RNA (i.e. sense transcripts) were prepared using *Nco* I. A 103 bp cDNA corresponding to human cyclophilin was used as an internal control (Ambion) to correct for variations in RNA amount. Antisense probes and reference RNAs were *in vitro* transcribed from linearized template DNA using T7 and Sp6 (Stratagene) RNA polymerase and labeled (antisense transcripts only) with [^{32}P]-UTP at high specific activity (1500 cpm/pg). Transcription reactions were treated with RNase free DNase I, ethanol precipitated in the presence of 2.5M ammonium acetate. Purified reference RNA was used for preparing standard curves to determine absolute abundance of UCP-3 and UCP-2 mRNA transcripts. Protected bands were visualized by autoradiography and quantitated by phosphoimager analysis using image Quant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

The expressed sequence tag (EST) database (http://www.ncbi.nlm.gov) was screened for sequences homologous to UCP1. One human EST, deposited by the Washington University, St. Louis - Merck & Co. EST project, was identified which was similar but not identical to hUCP1 and hUCP2 (accession no. AA192136, IMAGE clone no. 628529). This clone originated from a human skeletal muscle cDNA library (#937209, Stratagene, La Jolla, CA). The bacterial stock for clone 628529 was obtained from Genome Systems (St. Louis, MI) and was found to contain an insert of ~ 1.3 kb, which included the C-terminal third of the open reading frame. The coding region within clone 628529 was fully resequenced. 5′ RACE (Rapid Amplification of cDNA Ends) and human skeletal muscle Marathon-Ready cDNA were used to clone sequences corresponding to the coding region presumed to represent hUCP3.

UCP3 cDNA sequence contains a 5′ untranslated region of at least 183 bases, an open reading frame of 936 bases, a 3′ untranslated region of approximately 1.1 kb, a polyadenylation signal and a polyA tail. The UCP3 mRNA transcript is predicted to be equal to or greater than 2.2 kb. UCP3 protein, as deduced from the open reading frame, is composed of 312 amino acids and is estimated to have a molecular weight of ~34 kD (Fig. 1). At the amino acid level, hUCP3 is 71% identical to hUCP2 and 57% identical to hUCP1. Hydrophilicity plots also demonstrate that these three proteins are similar. Many of the nonidentical residues in hUCP3 are conservative substitutions which in most cases correspond to residues found in either mUCP2 (9, 10) or UCP1 from various species (16). Based upon the high degree of homology between UCP1, UCP2 and UCP3, it is likely that UCP3 uncouples mitochondrial respiration.

In order to establish the tissue distribution of UCP3 in humans, Northern blot analyses were performed (Fig. 2). UCP3 was abundantly expressed in skeletal muscle, generating a dominant mRNA transcript of ~ 2.4 kb (1 hour exposure). With longer exposure (18 hours), a much weaker UCP3 signal (2.4 kb) was detected in heart, thyroid and bone marrow. No UCP3 signal was detected in a large number of other tissues and organs (Fig 2). The longer exposures (18 hours) of the human UCP3 Northern blots also revealed the presence of a smaller mRNA transcript (~ 1.6 kb). Of note, the 294 bp hUCP3 probe employed was 75% identical to hUCP2. Rehybridization of the same blots with hUCP2 confirmed that this smaller 1.6 kb signal was UCP2 (Fig 2). The UCP2 signal, as previously reported (9, 10), was widely expressed being most abundant in spleen, thymus, bone marrow, trachea, and lymph node, and somewhat less abundant in skeletal muscle

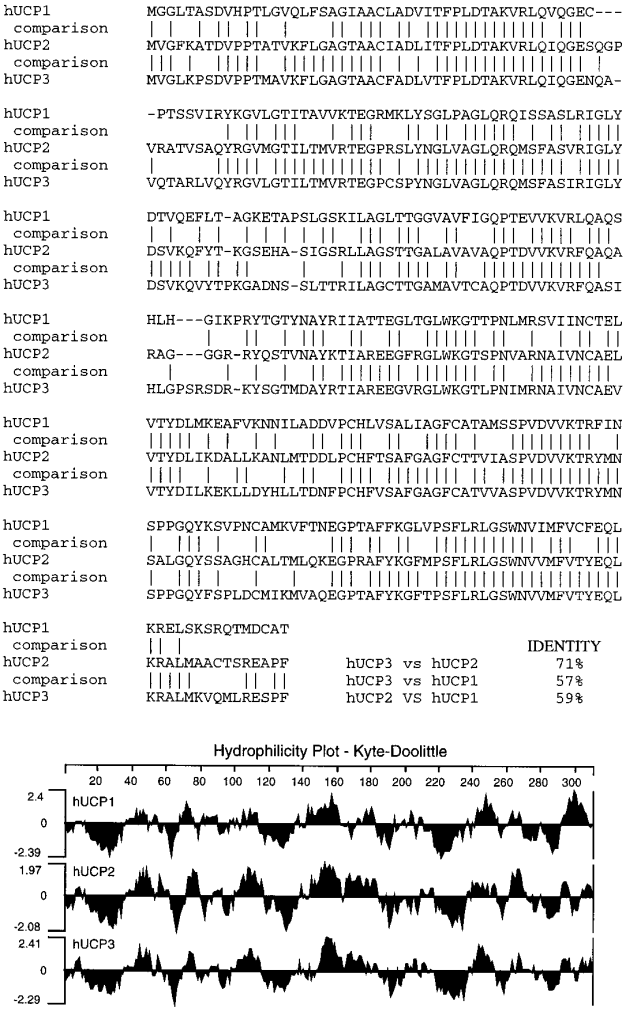

FIG. 1. Comparison of human UCP1, UCP2 and UCP3. Sequence alignments were performed using the ALIGN program (21). The hydrophilicity plots for hUCP2 and hUCP3 were generated using the method of Kyte and Doolittle (22). The Genbank accession number for hUCP3 is AF001787.

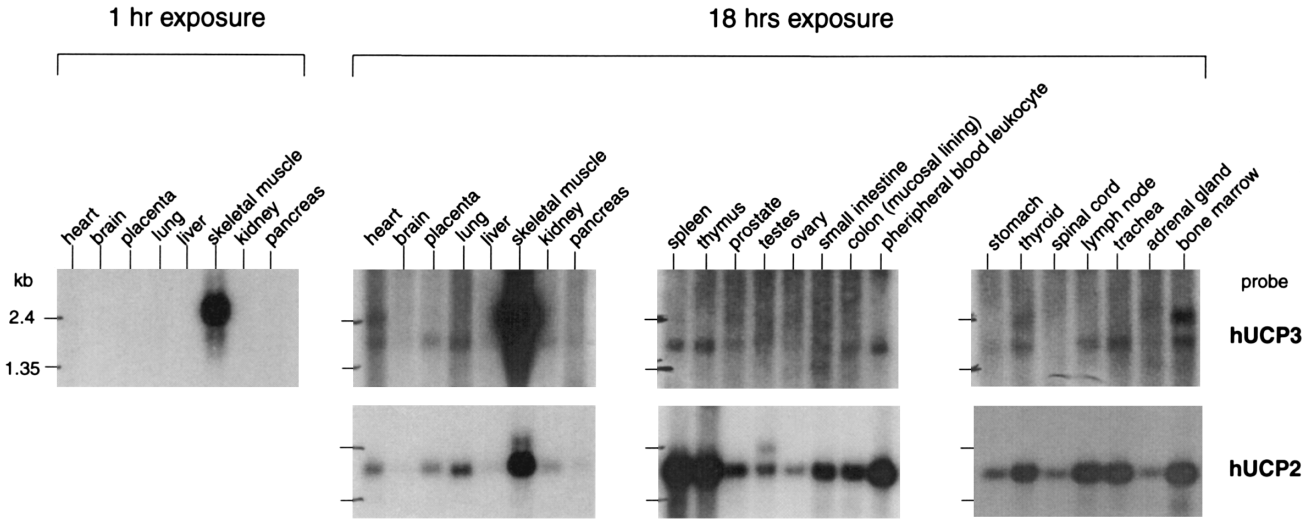

FIG. 2. Northern blot analyses of human UCP2 and UCP3 mRNA levels were performed as specified in the methods section. Filters were exposed to film for 1 or 18 hours.

and a number of other tissues. UCP2 was also abundantly expressed in white adipose tissue as previously reported (10) (data not shown). Sensitive and quantitative RNAse protection assays for hUCP2 and hUCP3, and standard curves derived from *in vitro* transcribed hUCP2 and hUCP3 RNA sense transcripts, revealed that UCP3 mRNA was approximately 2.5-5 fold more abundant than UCP2 mRNA in human skeletal muscle (data not shown). The UCP3 RNase protection assay also revealed that little or no UCP3 mRNA was expressed in human subcutaneous white adipose tissue and UCP3 levels in the heart were less than 1% of those detected in skeletal muscle.

In mice, UCP3 expression was most abundant in skeletal muscle and brown fat (Fig. 3). Significantly less expression was detected in other mouse tissues such as white adipose tissue, brain, kidney, liver and colon. As in the human mRNA studies, a smaller transcript was also detected in mouse samples. This smaller transcript most likely represents mUCP2, since it was most abundant in white adipose tissue, a site of high-level UCP2 expression (9, 10). Of note, the hUCP3 probe is 73% identical to mUCP2. Hybridization of mouse RNA samples with a mUCP2 probe confirmed that this smaller band was UCP2.

A number of studies have demonstrated that brown adipose tissue plays an important role in regulating energy balance in rodents (6-8). The tissue is highly specialized for stimulated energy expenditure with a rich vascular supply, dense sympathetic innervation, and numerous mitochondria. Brown adipocytes are further distinguished from other cell types by their expression of all three uncoupling proteins: UCP1 which is expressed exclusively in brown adipocytes (4, 5), UCP2 which is expressed widely (9, 10) and UCP3 which is expressed selectively and abundantly in brown adipocytes and skeletal muscle. Thus, brown fat is ideally suited for regulated thermogenesis.

In contrast to rodents, the abundance of brown adipose tissue in large mammals is limited and therefore brown fat may not be a significant regulator of human energy expenditure. Skeletal muscle has been implicated as an important mediator of adaptive thermogenesis in humans (11-15). Approximately 80% of the variance in resting energy expenditure between individuals can be accounted for by differences in fat-free mass

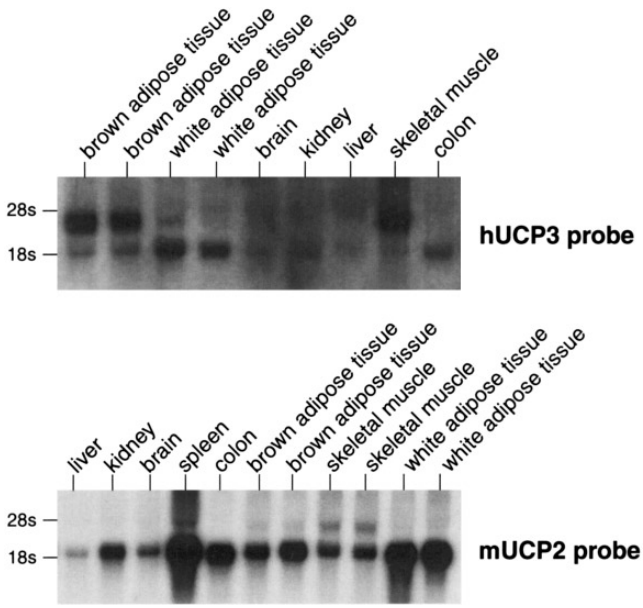

FIG. 3. Northern blot analyses of mouse UCP2 and UCP3 mRNA levels were performed as specified in the methods section.

(17), much of which is skeletal muscle. Similarly, a perfused forearm study has demonstrated that differences in skeletal muscle energy expenditure account for much of the variation in metabolic rate between individuals (13). Regulated energy expenditure in skeletal muscle is controlled, in large part, by adrenergic stimulation (11, 12, 14, 15). It is interesting to note that brown fat and skeletal muscle have in common a rich blood supply, sympathetic innervation, and abundant mitochondria. In addition, both tissues express high levels of UCP3 mRNA.

The heart continuously expends energy to maintain blood circulation and it is likely that uncoupled respiration in the heart would be detrimental. In view of this, it is notable that despite the general tendency for non-contractile, muscle-specific genes expressed in skeletal muscle to also be expressed in the heart (18), UCP3 is minimally expressed in cardiac tissue. Abundant expression of UCP3 in two thermogenic tissues, skeletal muscle and brown fat, and the relative lack of expression in other sites such as the heart, are consistent with a role for UCP3 as a molecular mediator of adaptive thermogenesis. Further studies, such as the creation of transgenic and gene knockout mice which overexpress or lack UCP3, and/or the identification of humans with UCP3 mutations will be required to establish the true physiologic role of UCP3.

One approach to anti-obesity drug development is to induce uncoupling by creating activators of one or more UCPs. β3-adrenergic receptor agonists, which increase UCP1 expression and activity in brown fat, as well as the amount of brown fat are presently under development (19, 20). However, given the relative lack of brown fat in humans, the effectiveness of these compounds is uncertain. UCP2 is another potential target, but its expression in critical organs where increased thermogenesis might produce unwanted side effects is a matter of concern. Specific activators of UCP3 expression or function, on the other hand, should selectively increase energy expenditure in skeletal muscle and brown fat, two tissues that normally have the capacity for adaptive energy expenditure. Thus, UCP3 is a potential target for anti-obesity drug development.

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