A Quest for the Origin of Mammalian Uncoupling Proteins

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Abstract. Nonshivering thermogenesis is dependent on the presence of UCP1 in brown adipose tissue. The function of UCP2 and UCP3 is most likely related to mitochondrial superoxide metabolism and/or fatty acid oxidation. These three members of the core UCP family are known in eutherians but had not been found in marsupials and monotremes so far. The objective of our search is to determine the origin of UCP1 and classical nonshivering thermogenesis. Furthermore, our approach to characterize UCP2/UCP3 in distantly related animal species will assist in the functional annotation of these proteins.

We recently reported on the molecular identification, tissue-distribution, and physiological regulation of UCP2 and UCP3 mRNA in the marsupial *Antechinus flavipes* (yellow-footed Antechinus). Despite separate evolution of the marsupial lineage since 130 million years, our data suggest a conserved physiological role of these UCPs. Here, we present the immunological detection of marsupial UCP3 in skeletal muscle using antibodies raised against mouse/rat UCP3. A comprehensive phylogenetic analysis led us to hypothesize that all uncoupling proteins were already present at the evolutionary stage of modern teleost fishes and questions the unique presence of UCP1 in placental mammals. However, the search for UCP1 in nonplacental mammals has been unsuccessful so far and is most likely hampered by the more rapid evolution of UCP1 as compared to other UCPs.

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The Quest for UCP1 in Nonplacental Mammals

In placental mammals, uncoupling protein 1 (UCP1) is responsible for nonshivering thermogenesis in brown adipose tissue mitochondria. The protein is inserted with six ???-helical transmembrane domains into the inner mitochondrial membrane and catalyzes proton flux into the matrix. Protonmotive force normally driving ATP synthesis is thereby dissipated as heat. In mice acutely exposed to the cold this unique mechanism of heat production is essential for survival (Enerbäck et al., 1997), and in small placental hibernators, brown adipose tissue mass is increased in relation to body mass as compared to nonhibernating mammals (Heldmaier, 1971). In the past, physiologists, morphologists, and biochemists have repeatedly questioned the common view that brown adipose tissue and UCP1 are monophyletic traits of placental mammals. In some marsupial species, treatment with sympathomimetics increased resting metabolic rate, resembling the adrenergic stimulation of nonshivering thermogenesis in brown adipose tissue of placental mammals (Loudon et al., 1985), but this finding was not confirmed in other studies. The interscapular fat deposit of Bennett's wallaby consists of multilocular adipocytes (Loudon et al., 1985), but so do white adipocytes in cold-stressed placental mammals (Loncar et al., 1988). The brownish appearance of the interscapular fat depot in the small marsupial carnivores *Sminthopsis macroura* and *Antechinus flavipes* (unpublished observation) indicates a high tissue content of mitochondria, but only the identification of UCP1 in marsupial adipose tissue and the demonstration of uncoupled mitochondrial respiration would provide hard evidence for the existence of brown adipose tissue in marsupials.

It is well established that purine nucleotides bind to UCP1 and inhibit proton transport activity in the absence of free fatty acids (Klingenspor, 2003). Indeed, increased GDP-binding to interscapular brown adipose tissue mitochondria of the Benett's wallaby suggested the presence of UCP1 (Loudon et al., 1985). Furthermore, UCP1-like immunoreactivity was reported in the interscapular fat deposit of *Sminthopsis crassicaudata* using an antibody raised against squirrel UCP1 (Hope et al., 1997). However, by using rodent probes or primers deduced from rodent UCP1 sequences, UCP1 mRNA could not be detected in the Tasmanian bettong (Rose et al., 1999) and the Tasmanian devil (Kabat et al., 2003). In our laboratory the attempts to identify UCP1 mRNA in marsupial adipose tissue with heterologous probes were futile as well. Previously, the use of a labelled oligomer from a conserved region of UCP1 had been successful to detect the mRNA in a variety of placentals (Brander et al., 1993). However, in our

hands several attempts to amplify marsupial UCP1 by RT-PCR using different primers deduced from conserved regions of the UCP1 coding sequence failed. Taken together, the monophyletic nature of the UCP1 gene in placental mammals has not been seriously challenged, as sequence data providing unambiguous proof for the presence of UCP1 in nonplacental mammals have not been reported.

Identification of Uncoupling Proteins in Marsupials

Even in tissues other than brown adipose tissue, a significant portion of mitochondrial respiration is due to proton leakage across the inner mitochondrial membrane but the biochemical cause for this leak has not been resolved (Rolfe and Brown, 1997). In 1997, paralogous proteins similar to UCP1 were found (Boss et al., 1997; Fleury et al., 1997). Because of their sequence and structural similarity, and their uncoupling activity in certain experimental conditions, they were named UCP2 and UCP3. In placental mammals UCP2 is ubiquitously expressed in multiple tissues, whereas UCP3 is restricted to skeletal and heart muscle. Pertaining to their phylogenetic distribution, UCP2 and UCP3 are described in placental mammals. Moreover, UCP2 is also found in fish (Stuart et al., 1999) and a UCP-like protein is present in birds (Raimbault et al., 2001). None of the core UCP family members have been identified so far in invertebrates.

A thermogenic function of UCP2 and UCP3 in brown adipose tissue is unlikely since they do not compensate for defective thermogenesis in UCP1 ablated mice, and ablation of the UCP2 or UCP3 gene does not impair energy balance, cold resistance, or nonshivering thermogenesis in rodents (Cannon and Nedergaard, 2004). However, a recent study suggests a possible thermogenic activity of UCP3 in skeletal muscle of mice in response to extasy treatment (Mills et al., 2003). Accumulating evidence suggests that UCP3 may play a role in mitochondrial lipid metabolism operating as a fatty acid anion exporter (Himms-Hagen and Harper, 2001; Schrauwen et al., 2003). Other nonthermogenic-uncoupling functions have been assigned to UCP2, including the reduction of superoxide generation in mitochondria by mild uncoupling, diminished pancreatic insulin secretion by lowering the cytosolic ATP/ADP ratio, neuroprotection and modulation of the immune system (Nedergaard and Cannon, 2003).

We cloned full-length marsupial UCP2 and UCP3 cDNAs in *Antechinus flavipes* and a UCP2 cDNA in *Sminthopsis macroura* (stripe-faced dunnart) (Jastroch et al., 2004). Initial hybridization analyses using heterologous probes from mouse and hamster revealed the presence of the UCP2 gene and transcript

but not UCP1 and UCP3. Since the marsupial UCP3 sequence was not detectable using heterologous rodent cDNA probes, we applied an alternative cloning strategy based on conserved blocks within the UCP3 coding sequence. We deduced consensus oligomers from placental UCP3, also including bird UCP. We suggest that the latter is most likely the avian orthologue of UCP3, which is supported by the physiological regulation of gene expression (Evock-Clover CM et al., 2002) and phylogenetic relation to mammalian UCP3 (Fig. 3). We amplified a 480 bp cDNA fragment from skeletal muscle cDNA of *A. flavipes,* which we used to isolate a full-length UCP3 clone (Genbank Acc. #AY519198) from a cDNA library.

In *A. flavipes* exposed to 5° C for two days, the mRNA levels of UCP2 and UCP3 in interscapular adipose tissue and in skeletal muscle were not increased (Jastroch et al., 2004), which appears to exclude a potential role in adaptive thermogenesis in marsupials. However, we recently achieved the immunological detection of UCP3 in protein extracts from skeletal muscle (Fig. 1). UCP3 protein levels in cold-exposed *A. flavipes* were elevated nearly 2-fold. In contrast to the mRNA data, this finding suggests a role of UCP3 for muscle thermogenesis.

We also studied the regulation of UCP2 and UCP3 mRNA in response to two days of food deprivation. Whereas UCP2 mRNA remained unchanged in most tissues investigated, UCP3 mRNA was upregulated 6-fold in heart and 2.5-fold in skeletal muscle. This mode of physiological regulation is consistent with the upregulation of UCP3 expression in skeletal muscle of rodents induced by fasting but not compatible with a thermogenic function of UCP3. Since muscle energy expenditure in fasted animals is lowered, the efficiency of mitochondrial ATP synthesis is unlikely to be reduced in this negative state of energy balance.

When Did the UCP1 Gene Emerge in Vertebrate Evolution?

One hundred thirty million years of evolution separated marsupials and eutherian mammals, leading to sequence variations in the UCP genes that may impose some difficulties in their identification in marsupials. In this respect, the search for UCP2 orthologues is less difficult due to the high sequence conservation in distantly related taxa, such as amphibians, fish, and rodents. In retrospect, the high identity of rodent and marsupial cDNA sequences (90%) facilitated detection of the marsupial gene and the corresponding mRNA by heterologous hybridization assays. However, for UCP3 this approach completely failed due to only 80% global identity between marsupial and rodent UCP3. The search for regions in the coding sequence conserved across phylogenetically distinct species

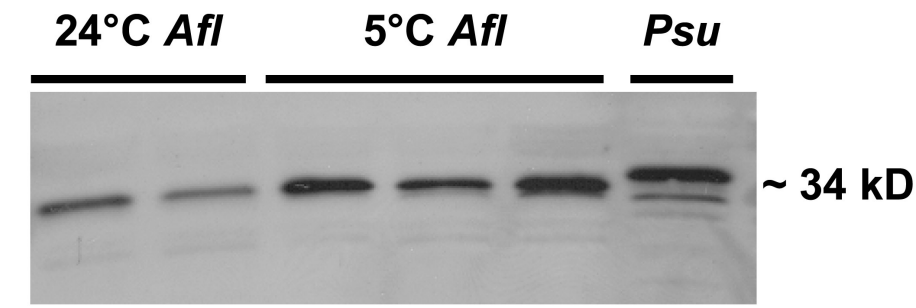

*Fig. 1: Immunological detection of UCP3 in cold and warm acclimated yellow-footed antechinus (*Antechinus flavipes, *Afl). Forty micrograms of protein from skeletal muscle per lane were subjected to Western blot analysis using an antibody against mouse/rat UCP3. An equal amount of skeletal muscle protein from the Djungarian hamster (*Phodopus sungorus, *Psu) served as a control.*

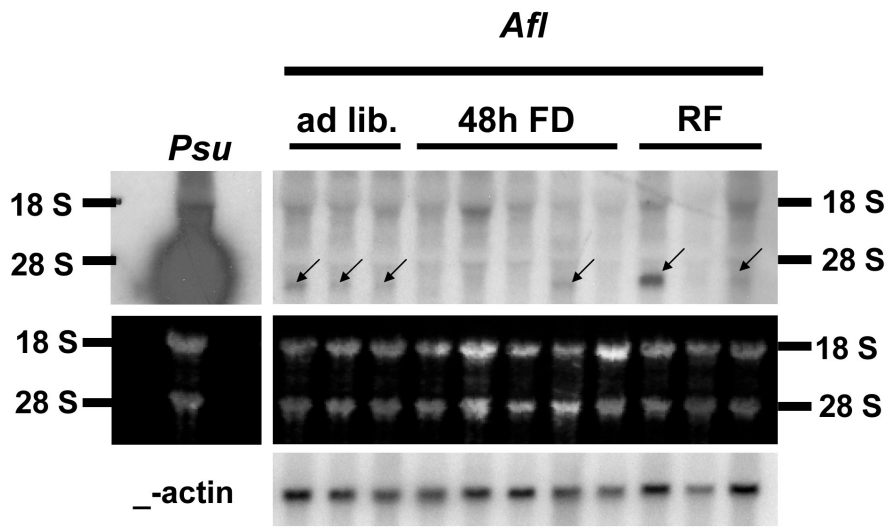

Fig. 2: Northern blot of skeletal muscle from fed, fasted, and refed A. flavipes. *Twenty micrograms of total RNA were analysed by Northern blotting with a rat UCP1 cDNA probe, as well as a mouse UCP2 and* β*-actin probe. Arrows indicate the apparent detection of marsupial UCP1. Afl:* Antechinus flavipes, *Psu:* Phodopus sungorus, *ad lib.:* ad libitum, *FD: food-deprived for 48 hours, RF: refed for 24 hours.*

led to successful PCR amplification of a marsupial UCP3 cDNA fragment. Notably, the full-length marsupial UCP3 cDNA probe did not hybridize with hamster UCP3 mRNA on Northern blots of total skeletal muscle RNA, whereas a clear albeit weaker hybridization signal was observed probing hamster spleen RNA with the marsupial UCP2 cDNA (Jastroch et al., 2004). This illustrates the limitations of heterologous hybridization assays in the search for orthologues in distantly related species.

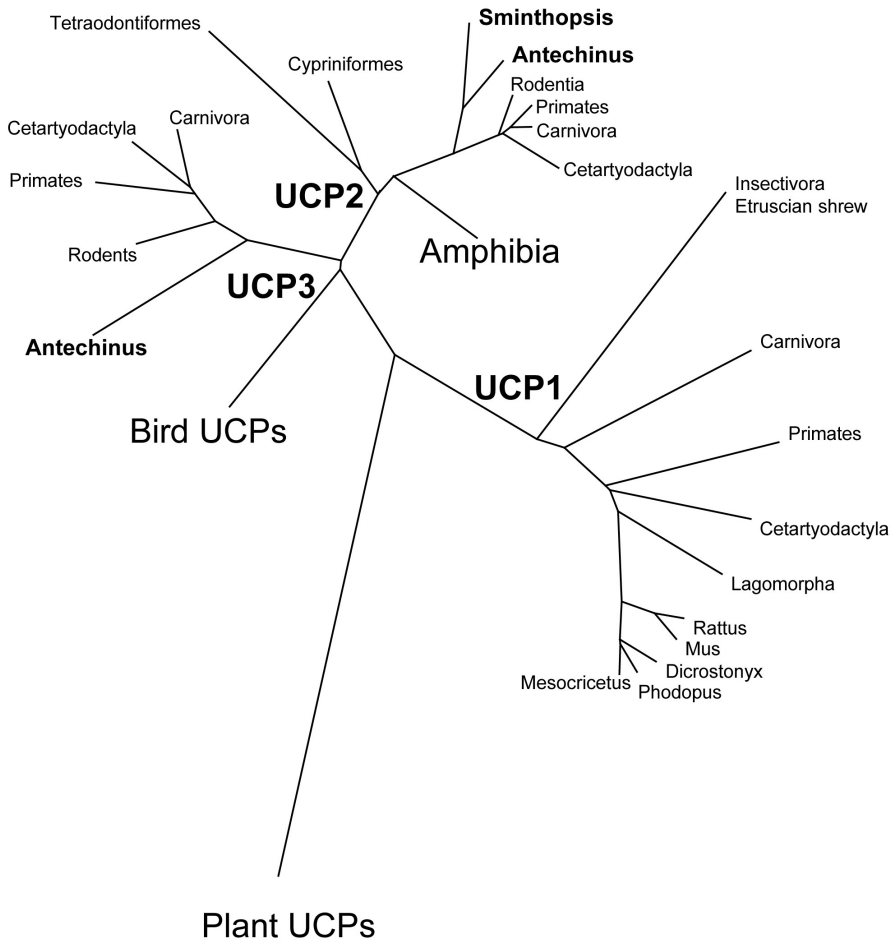

Fig. 3: Unrooted phylogenetic tree of the core UCP family using the neighbor-joining method.

Knowing this, we definitely cannot conclude that nonplacental mammals lack the UCP1 gene. We speculate that the search for marsupial UCP1 was not successful yet, since the sequence similarity to eutherian UCP1 can be expected to be rather low. Even within eutherian mammals, the comparison of UCP1 orthologues from phylogenetic distant taxa, as exemplified by comparison of Etruscan shrew UCP1 and mouse UCP1, reveals only 75% global identity (based on 245 amino acids known from shrew). Klaus and colleagues reported that the signal intensity for Etruscan shrew UCP1 mRNA is strongly diminished when using a heterologous rodent probe for hybridization analysis (Klaus et al., 1996). This indicates a rapid evolution of this protein within the eutherian infraclass and certainly complicates straightforward attempts to identify the eutherian orthologue of UCP1 in nonplacental mammals.

Despite these theoretical considerations, we screened multiple tissues sampled from fed, fasted, and refed *A. flavipes* by Northern blot analysis for the presence of UCP1 mRNA with a full-length radio-labelled rat UCP1 cDNA probe. Surprisingly, in skeletal muscle of several individuals we observed a barely detectable hybridization signal when Northern blots were washed at low stringency (indicated by arrows in Fig. 3). This apparent detection of marsupial UCP1 mRNA in skeletal muscle did not depend on feeding state and may rather be related to life history of the animals or variation in tissue sampling. Rehybridization of the same blot with UCP2 and UCP3 definitely excluded possible cross-hybridization, as judged from transcript size. Screening of skeletal muscle cDNA libraries will reveal whether the detected transcript indeed represents marsupial UCP1.

Regarding the phylogenetic tree of all known UCPs, it is well possible that UCP1 not only occurs in placental mammals. Using different outgroups, e.g., plant UCPs (Fig. 3), brain mitochondrial carrier protein (BMCP1), or the oxalacetate-malate carrier, the branching of UCP1 occurs at least at the evolutionary stage of modern teleost fish. Thus, UCP1 and UCP3 may also be found in ectothermic vertebrates unless these genes went extinct in the living species.

Orthologues are defined as proteins sharing common ancestry and function. However, if an uncoupling protein gene sharing a common ancestor with mammalian UCP1 does exist in ectothermic vertebrates, a global thermogenic function appears unlikely. The thermogenic function may rather represent a new property of UCP1 that evolved in the mammalian lineage. Despite the fact that UCP1 is regarded as a marker protein for brown adipose tissue in eutherian mammals, the expression of this protein if present might not be restricted to

adipose tissue in nonplacental mammals and ectothermic vertebrates. Notably, an involvement of UCP1 in intestinal relaxation was suggested by a study in UCP1-ablated mice (Shabalina et al., 2002), adding to the ongoing discussion as to whether the UCP1 gene is also expressed in smooth longitudinal muscle of rodents (Nibbelink et al., 2001; Rousset et al., 2003).

We therefore conclude that the search for UCP1 in nonplacental mammals has to be intensified on the molecular level.

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