Uncoupling protein 1 in fish uncovers an ancient evolutionary history of mammalian nonshivering thermogenesis

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Jastroch, Martin, Sven Wuertz, Werner Kloas, and Martin Klingenspor. Uncoupling protein 1 in fish uncovers an ancient evolutionary history of mammalian nonshivering thermogenesis. Physiol Genomics 22: 150–156, 2005. First published May 10, 2005; 10.1152/physiolgenomics.00070.2005.—Uncoupling proteins (UCPs) increase proton leakage across the inner mitochondrial membrane. Thereby, UCP1 in brown adipose tissue dissipates proton motive force as heat. This mechanism of nonshivering thermogenesis is considered as a monophyletic trait of endothermic placental mammals that emerged about 140 million years ago and provided a crucial advantage for life in the cold. The paralogues UCP2 and UCP3 are probably not thermogenic proteins but convey mild uncoupling, which may serve to reduce the rate of mitochondrial reactive oxygen species production. Both are present in endotherms (mammals and birds), but so far only UCP2 has been identified in ectothermic vertebrates (fish and amphibia). The evolution of UCPs is of general interest in the search for the origin of mammalian UCP1-mediated nonshivering thermogenesis. We here show the presence of UCP1 and UCP3 in ectothermic teleost fish species using comparative genomics, phylogenetic inference, and gene expression analysis. In the common carp (Cyprinus carpio), UCP1 is predominantly expressed in the liver and strongly diminished in response to cold exposure, thus contrasting the cold-induced expression of mammalian UCP1 in brown adipose tissue. UCP3 mRNA is only found in carp skeletal muscle with expression levels increased fivefold in response to fasting. Our findings disprove the monophyletic nature of UCP1 in placental mammals and demonstrate that all three members of the core UCP family were already present before the divergence of ray-finned and lobe-finned vertebrate lineages about 420 million years ago.

proton leak; brown adipose tissue; common carp; uncoupling protein 2; uncoupling protein 3

IN BROWN ADIPOCYTES, thermogenic uncoupling protein (UCP)1 increases proton leakage of the mitochondrial inner membrane and thereby dissipates proton motive force as heat (23). UCP1-mediated proton leakage is activated by fatty acids and inhibited by purine nucleotides. This molecular mechanism catalyzes nonshivering thermogenesis in newborns, small mammals, and hibernators. The biological role of UCP2 and UCP3 is controversial. There is speculation on the involvement of UCP3 in skeletal muscle thermogenesis of mammals and birds (21, 36), whereas others address the function of UCP3 to the export of redundant fatty acids from the mitochondrial matrix (15, 33). Several physiological functions have been proposed for UCP2 including the regulation of ATP-to-ADP ratios in pancreatic β-cells (42) or the modulation of reactive oxygen species production in the defence system of macrophages (19, 22). In fact, all members of the core UCP family are activated by superoxides and/or carbon-centered radicals conveying mild uncoupling, which through prevention of reverse electron transfer may function to diminish endogenous superoxide production in the mitochondrial matrix (11, 12, 36). It has been suggested recently that this mild uncoupling could be the original ancient function of UCPs serving to protect cells against oxidative damage (7). Verification of this hypothesis requires the identification and comparative functional analysis of UCPs in phylogenetically ancient vertebrates.

The evolution of UCPs and their contribution to proton leak-dependant adaptive nonshivering thermogenesis are of general interest as this invention may have facilitated the development of endothermy. Evidence for nonshivering thermogenesis in vertebrates other than placental mammals, e.g., birds (10) and marsupials (30), as well as in brain heater cells of the billfish and butterfly mackerel (3), has been reported. In the billfish and butterfly mackerel, nonshivering thermogenesis in brain heater cells is responsible for cranial endothermy, but the involvement of UCPs could not be proven by immunodetection with heterologous antibodies (2). In nonplacental mammals, physiological evidence for and against nonshivering thermogenesis was found (24), but neither morphological nor molecular proof for the existence of brown adipose tissue and UCP1 could be found (14, 17, 18). Therefore, it was concluded that brown adipose tissue and UCP1 evolved about 140 million years ago in placental mammals, providing them with the crucial advantage to maintain high body temperature in the cold (4, 8). Pertaining to UCP3, of which the physiological function remains to be resolved, orthologues have so far been identified in endotherms, including marsupials (17), placentals (5), and birds (28, 39), but not in ectothermic vertebrates.

Here, we demonstrate that all three UCPs are already present at the evolutionary stage of ectothermic teleost fish. As exemplified in the common carp (Cyprinus carpio), gene expression of UCP1 and UCP3 is regulated in response to cold and fasting in a tissue-specific manner.

MATERIALS AND METHODS

Phylogenetic inference and comparative genomics. We conducted a comprehensive search for Ucp genes by blasting zebrafish (Danio rerio) and pufferfish (Fugu rubripes) genomes with full-length coding se-

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quences of mammalian UCPs (Ensembl Genome Browser, http://www.ensembl.org). To verify the membership of identified candidates to the core UCP family, the predicted protein sequences of putative fish UCPs were aligned [ClustalX, ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/ (37)] together with all known UCP protein sequences available in public databases and subjected to phylogenetic inference using the Neighbour joining method (Felsenstein J. PHYLP Phylogeny Inference Package version 3.6. Distributed by the author, Seattle, WA: Univ. of Washington, 2004. http://evolution.genetics.washington.edu/phylip.html). Bootstrapping involved 1,000 replicates, and the consensus tree was illustrated using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Physical gene maps of verified Ucp loci were scaled based on assemblies of the Ensembl Genome Browser (http://www.ensembl.org). Genes located up- and downstream of Ucp genes in these loci were blasted against mammalian genomes for the highest score.

Cold acclimation and fasting of the common carp. Six-month-old common carp (C. carpio) provided by the Department of Inland Fisheries (Berlin, Germany) were kept under a natural photoperiod limited gene conservation (16). Therefore, the nearly com-

In the search for UCP orthologues in the animal mammalian genomes for the highest score.

volved 1,000 replicates, and the consensus tree was illustrated using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Physical gene maps of verified Ucp loci were scaled based on assemblies of the Ensembl Genome Browser (http://www.ensembl.org). Genes located up- and downstream of Ucp genes in these loci were blasted against mammalian genomes for the highest score.

RESULTS

Genomic identification of UCP1, UCP2, and UCP3 in te-

leost fish. In the search for UCP orthologues in the animal kingdom, heterologous hybridizations are not applicable due to limited gene conservation (16). Therefore, the nearly com-

pleted genome projects of the zebrafish (D. rerio) and puffer-

fish (F. rubripes) were searched for the presence of UCP genes. In addition to Ucp2, which has been previously identified in fish expressed sequence tag (EST) libraries (35), we discovered two novel Ucp-like genes in the databases of both teleost genomes. On the amino acid level, their intraspecific identities with zebrafish UCP2 were 68% and 74%, respectively.

To elucidate the relationship of these novel Ucp-like genes to the known mammalian orthologues, we compared their genomic location in mammals and fish. Pertaining to mammalian Ucp1, an identical order of neighboring genes is seen, thereby demonstrating a region of conserved synteny within the mammalian lineage (Fig. 1). Surprisingly, this region of conserved synteny is also found in the genomes of the zebrafish and pufferfish and encloses one of the novel fish Ucp genes. On zebrafish chromosome 1, a region spanning ~50 kb contains this novel Ucp gene flanked by gene A (ENSDART0000026397) and gene B (ENSDART0000034884), of which orthologous counterparts also neighbor Ucp1 in mammals (Fig. 1). Therefore, this Ucp gene is unequivocally identified as the Ucp1 orthologue in fish.

The second novel Ucp-like gene is located in direct vicinity of the zebrafish Ucp2 gene resembling the juxtaposition of Ucp2 and Ucp3 in the human and mouse (26), rat (www.ensembl.org), and bovine (34). Furthermore, homologous genes neighboring the Ucp2–Ucp3 cluster were also conserved in both mammalian and fish genomes, forming a region of conserved synteny (Fig. 1). Therefore, the second novel Ucp is annotated as the Ucp3 orthologue in fish.

Phylogenetic inference. UCP1 and UCP3 protein sequences were deduced from the respective genes identified in the zebrafish and pufferfish (www.ensembl.org). The intraspecific identity of UCP1 to UCP2 and UCP3 is 68% and 71%, respectively, whereas the identity of UCP2 to UCP3 amounts to 74%. In cross-species alignments of UCPs, we highlighted regions of particular interest previously identified by the study of structure-function relationships (Fig. 2). Phylogenetic inference definitely grouped all three proteins into the core UCP family and positioned UCP2 within the respective vertebrate orthologues (Fig. 3). However, despite the unambiguous annotation of fish UCP1 and UCP3 based on conserved synteny, the phylogenetic relationship to their respective orthologues within the UCP core family could not be resolved (Fig. 3).

Regulation of gene expression. To investigate tissue-specific expression as well as the regulation in response to cold exposure and fasting, we cloned cDNAs of all three UCPs in the common carp (C. carpio). RT-PCR amplified a 300-bp UCP1 fragment from liver cDNA, a 490-bp UCP3 fragment from skeletal muscle cDNA, and a 1,141-bp UCP2 fragment from spleen cDNA. These PCR products were cloned into pGEM-T-Easy (Promega) and sequenced. The UCP1 cDNA fragment served to screen the carp liver cDNA library. UCP2 and UCP3 cDNAs were directly used to generate probes for Northern blot analysis.

We demonstrated differential tissue-specific mRNA expression patterns of the three UCPs in the carp (Fig. 4A). Multiple-tissue Northern blot analysis revealed preferential expression of carp UCP1 mRNA in metabolically active tissues, with highest levels in the liver, moderate levels in the brain and functional kidneys (meso- and metanephros), and lowest levels in the intestine. Carp UCP3 mRNA is exclusively expressed in skeletal muscle, with higher levels observed in red (oxidative) compared with white (glycolytic) muscle. UCP2 mRNA was found in all tissues included in the analysis, with the highest expression levels found in blood cells, the intestine, and gills.

For further physiological characterization of carp UCP1 and UCP3, we investigated the regulation of gene expression. In mammals, UCP1 gene expression in brown adipose tissue (BAT)
is induced in the cold, whereas UCP3 mRNA levels in skeletal muscle and the heart are elevated in the fasted state. Therefore, cold exposure and fasting were chosen as the physiological stimuli most likely to affect UCP1 and UCP3 gene expression in the carp. In the liver, UCP1 mRNA levels were almost diminished in carp transferred from 20 to 8°C for either 2 days or 4 wk (Fig. 4B). In contrast, UCP1 mRNA levels in the kidneys were not affected by cold exposure (ANOVA on ranks, \( P / H11022 \leq 0.3 \); data not shown).

UCP3 gene expression in skeletal muscle was not affected by cold. However, in response to 4 wk of fasting, UCP3 mRNA levels in glycolytic white skeletal muscle increased fivefold (Fig. 4B). No systematic effect of fasting on UCP1 gene expression in the liver and kidneys was observed (data not shown).

**DISCUSSION**

In the present study, we localized all three members of the core Ucp gene family in zebrafish and pufferfish genomes by comparative genomics and demonstrate tissue-specific regulation of their gene expression in the common carp (C. carpio).

Our comprehensive search for Ucp genes initially corroborated the presence of the Ucp2 gene in fish, as expected from the previous identification of UCP2 cDNA in EST libraries from carp (C. carpio) and zebrafish (D. rerio) (35). However, our search also revealed two novel Ucp genes, which were identified as new members of the core UCP family by phylogenetic inference. On the basis of conserved synteny, these novel Ucp genes were unequivocally annotated as Ucp1 and Ucp3 (Fig. 1). This comparative analysis of Ucp loci in different species illustrates the utility of syntenic relationships (1, 27, 41).

UCP1 plays a key role in nonshivering thermogenesis and was previously regarded as a monophyletic trait of placental mammals only expressed in brown adipose tissue (8). Moreover, UCP3, of which the physiological function remains to be resolved, has so far only been identified in marsupials (17) and placental mammals (5) as well as in birds (28, 39). The presence of all three Ucp genes in ectothermic fish, including Ucp1, is therefore unexpected and indicates an ancient evolutionary divergence of the core UCP family.
To further characterize the fish Ucp genes, we cloned the respective cDNAs from the common carp (C. carpio) and investigated tissue-specific expression as well as regulation in response to cold and fasting. We demonstrated that tissue distribution of UCP1 mRNA in fish is broader than in placental mammals occurring in metabolically active tissues like the liver, kidney, brain, and intestine but notably not in adipose tissue (Fig. 4A). In cold-exposed carp, UCP1 mRNA was

Fig. 2. Amino acid sequence alignments of UCP1, UCP2, and UCP3. Sequences from H. sapiens (Hsa), M. musculus (Mmu), F. rubripes (Frau), D. rerio (Dre), and Cyprinus carpio (Cca) were aligned using ClustalX 1.81 (ftp://ftp-igbmx.u-strasbg.fr/pub/ClustalX) and illustrated with Genedoc 2.601 (http://www.psc.edu/biomed/genedoc/). Most conserved amino acids are highlighted in black, with less conserved amino acids shown with a brighter background. Arrows indicate amino acids involved in pH sensing of the nucleotide binding (20). Positions of two histidines, putatively involved in the proton transport of mammalian UCP1, are boxed. Solid bars above the alignment indicate the energy transfer protein signature; the solid bar below the alignment indicates the position of a potential nucleotide binding domain (*) found in UCP1 (6).
almost diminished in the liver, whereas expression levels remained unaffected in the kidney. The cold-induced down-regulation in the liver was a rapid event as minimal expression levels were attained after 2 days and maintained in carp cold acclimated for 4 wk. This may serve to increase coupled respiration and efficiency of ATP synthesis and could be one mechanism by which the carp reduces metabolic rate and saves chemical energy at low water temperatures.

The evolution of UCPs is of major interest in the attempt to trace the origin of UCP1-mediated nonshivering thermogenesis in placental mammals. Physiological evidence for nonshivering thermogenesis in birds (10), marsupials (30), and billfish (3) has been reported, but either the involvement of UCPs could not be proven or experimental data suggest alternative thermogenic mechanisms. Given the presence of a UCP1 orthologue at the evolutionary level of fish, we must now conclude that the common ancestors of ray-finned and lobe-finned vertebrates already disposed of this gene about 420 million years ago. Consequently, the presence of UCP1-mediated nonshivering thermogenesis in all endothermic vertebrates has to be reconsidered. In this respect, the observed conserved synteny in the Ucp1 locus and the high sequence identity of one neighboring gene (gene B in Fig. 1, top) in fish and mammals should facilitate the identification of UCP1 orthologues in other vertebrate classes. Alternatively, the ancient Ucp1 gene may have provided the raw material to develop thermogenic UCP1 in mammals but went extinct in other vertebrate classes.

All known UCP1 orthologues catalyze a net proton translocation, and there is evidence that UCP2, UCP3, and plant UCP increase proton leakage (9, 12), but for fish UCP1 experimental data demonstrating this function are lacking at the present state. Most teleosts, like the carp, are ectotherms not able to raise and maintain their body temperature significantly above water temperature by endogenous heat production. It is therefore evident that in most fishes UCP1 does not serve as a
thermogenic protein defending elevated core body temperature. However, cranial endothermy has been reported in several species including the billfish (swordfish, sailfish, and blue marlin, Xiphidae and Istiophoridae) as well as the butterfly mackerel (Scombridae) (3). These endothermic fish possess a brain heater organ, which is derived from extraocular muscles and warms the brain and the eye up to 20°C above water temperature. Previous biochemical studies indicated that the underlying thermogenic mechanism in the modified muscle cells of the brain heater may utilize the free energy of ATP for futile calcium cycling across the sarcoplasmic reticulum membrane, thereby enabling high rates of mitochondrial respiration and energy dissipation as heat. Immunological studies have questioned the presence of UCP in heater cells, but, obviously, this issue now has to be reinvestigated (2). Notably, we detected UCP1 mRNA in the brain of the carp. The presence and thermogenic function of UCP1 in neuronal tissue or directly in brain heater cells of the billfish and butterfly mackerel would cast a new light on the evolution of nonshivering thermogenesis in vertebrates.

UCP2 gene expression in the carp is ubiquitous, reflecting the situation in marsupials (17) and placentals (5, 13, 40). Interestingly, carp UCP3 gene expression was restricted to skeletal muscle, resembling the tissue specificity of mammalian UCP3. The observed preferential expression of UCP3 in red oxidative muscle in the carp may be related to the higher mitochondrial content of this fiber type. Conversely, in ad libitum-fed rats, no significant difference in UCP3 mRNA was reported when soleus (oxidative) and gastrocnemius (glycolytic) muscle were compared (32). In the carp, we investigated the fasting response of UCP3 gene expression in white glycolytic muscle as it is known in the rat that the fasting response of UCP3 expression is most pronounced in this muscle fiber type (31). Indeed, fasting caused a fivefold upregulation of UCP3 mRNA levels. In situations of high lipid oxidation, like fasting, it has been suggested that mammalian UCP3 protects mitochondria and cells from oxidative damage either by mild uncoupling activity preventing excess mitochondrial superoxide production (7) or by fatty acid anion export impeding their accumulation and peroxidation in the mitochondrial matrix (15, 33). There is also speculation on the involvement of UCP3 in skeletal muscle thermogenesis of mammals and birds (21, 36), but the observed upregulation of UCP3 gene expression in a physiological state associated with decreased energy expenditure appears to weaken this hypothesis. Regardless of the physiological function, the high similarity in tissue distribution and fasting response of UCP3 gene expression in the rat and carp indicates that its function and the mechanisms of gene regulation are most likely conserved during evolution.

It has been suggested that teleost fish have more genes than mammals, although it is still a matter of debate whether this is due to frequent independent duplications or the result of one ancient fish-specific genome duplication at the time of modern teleost radiation ~320 million years ago (1, 25, 29, 38). However, our search for Ucp genes in zebrafish and pufferfish genomes only revealed the three orthologues known in mammals but no further sister genes. Either Ucp genes were exempt from genome duplication in ancient times or excess paralogues went extinct during the evolution of modern teleosts. Notably, the maintenance of gene redundancy in mammals supports the idea that each orthologue exhibits a distinct physiological function.

Taken together, our data disprove the monophyletic nature of UCP1 in placental mammals. All three members of the core UCP family were already present before the divergence of ray-finned (Actinopterygii) and lobe-finned (Sarcopterygii) vertebrate lineages about 420 million years ago, which suggests an ancient evolutionary history of UCPs and nonshivering thermogenesis in vertebrates.
REFERENCES


UNCOUPLING PROTEINS IN FISH

GRANTS

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