FEBS Letters 443 (1999) 326–330 FEBS 21454

UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells

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Received 3 December 1998; received in revised form 10 December 1998

Abstract Uncoupling proteins (UCPs) are a family of mitochondrial transporter proteins that have been implicated in thermoregulatory heat production and maintenance of the basal metabolic rate. We have identified and partially characterized a novel member of the human uncoupling protein family, termed uncoupling protein-4 (UCP4). Protein sequence analyses showed that UCP4 is most related to UCP3 and possesses features characteristic of mitochondrial transporter proteins. Unlike other known UCPs, UCP4 transcripts are exclusively expressed in both fetal and adult brain tissues. UCP4 maps to human chromosome 6p11.2–q12. Consistent with its potential role as an uncoupling protein, UCP4 is localized to the mitochondria and its ectopic expression in mammalian cells reduces mitochondrial membrane potential. These findings suggest that UCP4 may be involved in thermoregulatory heat production and metabolism in the brain.

Key words: Uncoupling protein; Thermoregulation; Heat production; Metabolic rate; Brain temperature

1. Introduction

Food intake and energy expenditure are highly regulated biological processes that are central to the control of body weight [1]. Identification of leptin and its receptors has greatly facilitated our understanding of these processes [2]. Recently, much effort has been directed toward understanding the underlying molecular mechanisms of energy expenditure and its regulation. A family of mitochondrial proteins, termed uncoupling proteins (UCPs), have been identified and implicated as potential modulators of thermoregulation, body composition, metabolism, and obesity [3–9].

UCPs are mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis. As a result, energy is dissipated in the form of heat [3]. Mitochondrial proton leak has been observed in the major oxygen-consuming tissues, including liver, kidney, brain and muscle, contributing substantially to the standard metabolic rate [10–12]. The first characterized uncoupling protein, UCP1, is normally present only in brown adipocytes, where it is highly inducible by norepinephrine, thyroid hormones, retinoic acid, or cold exposure [3]. Mice lacking UCP1 are cold-intolerant, suggesting that UCP1 is involved in adaptational thermoregulatory heat production [13,14]. Conversely, UCP2 is widely expressed in human tissues, highest in skeletal muscle and white adipose tissue and lowest in brain and testis [4,6]. In situ hybridization analyses revealed UCP2 transcripts in mouse brain. A high level of expression was detected in the hypothalamus, but was not affected by cold acclimation [15]. Transcripts of the recently identified UCP3 are abundant in rodent skeletal muscle and brown fat [8,9]. Its expression is regulated by dietary and hormonal manipulations including thyroid hormone, β3-adrenergic agonists and leptin [7–9,16], suggesting a role in the regulation of energy expenditure. Herein, we report the identification and characterization of a novel uncoupling protein, termed UCP4. Like other UCPs, UCP4 was localized to the mitochondria and its ectopic expression in mammalian cells led to reduced mitochondrial membrane potential. Unlike other known UCPs, UCP4 transcripts were found to be exclusively present in the brain.

2. Materials and methods

2.1. Database searches

Both the LIFESEQ database (Incyte Pharmaceuticals, Palo Alto, CA) and the public EST (expressed sequence tag) database were searched for novel sequences that had significant homology to the known uncoupling proteins. The resultant human EST sequences were assembled using DNASTAR software (Oxford Molecular Group, Inc.). Subsequent 'searching and extending' cycles led to the identification of a cDNA that encoded a full length open reading frame. A full length cDNA was obtained by polymerase chain reaction (PCR) from a human adult brain cDNA library and confirmed by DNA sequencing.

2.2. Expression constructs

cDNA encoding UCP4 was cloned into pcDNA3 (Invitrogen) with or without a NH_2-terminal Flag tag. DNA primers for pcDNA3UCP4: 5′-CGCGGATCCCGTTATCGTCTTGCGCTACTGC (U401) and 5′-GCGGAATTCTTAAAATGGACTGACTCCACTCATC (U406); for pcDNA3Flag-UCP4: 5′-CGCGGATCCGAAATGGACTACAAGGACGACGATGACAAGTCCGTCCCGGAGGAGGAGG (U410) and U406. cDNA encoding human UCP3 was obtained from a melanoma cDNA library by PCR and subsequently cloned into pcDNA3 with or without a NH_2 Flag tag and confirmed by DNA sequencing.

2.3. Northern blot analysis

Human multiple tissue Northern blots (Clontech) were probed with the full length human UCP4 cDNA or UCP2 cDNA according to the manufacturer's instructions. The blots were subsequently hybridized with β-actin cDNA.

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PII: S0014-5793(98)01713-X

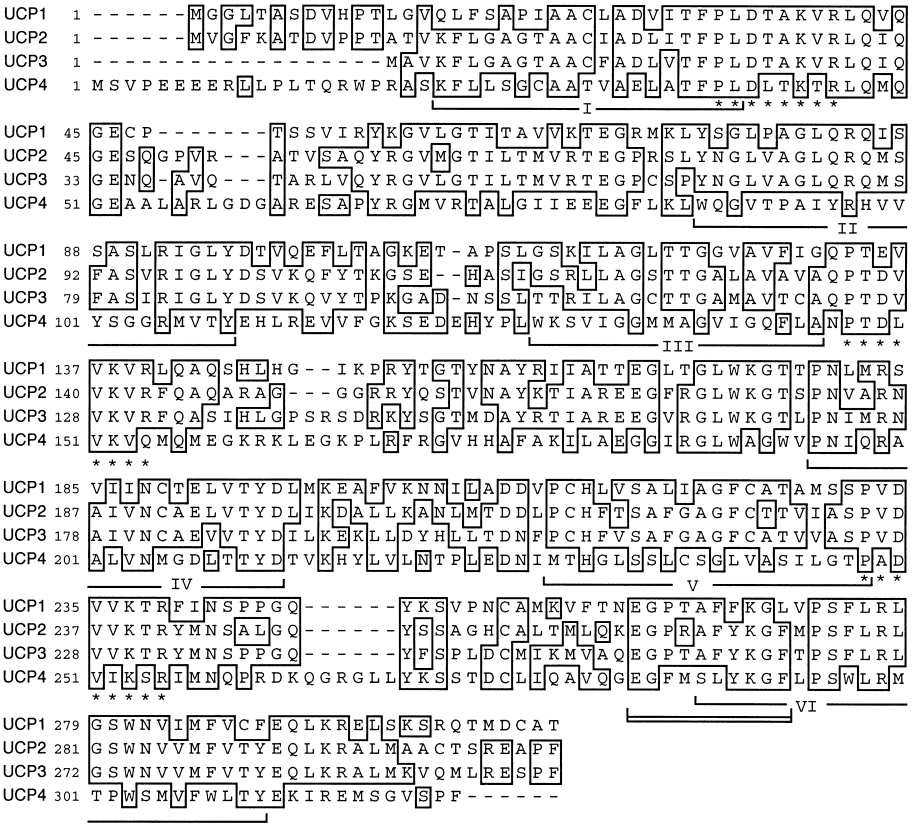

Fig. 1. Deduced protein sequence of UCP4 and sequence alignments of uncoupling proteins. The open reading frame for UCP4 defines a mitochondrial membrane protein of 323 amino acids. The GenBank accession number for the sequence: AF110532. The six putative transmembrane domains are single underlined and labeled I–VI. The three mitochondrial transporter protein signature motifs are indicated by asterisks. The putative purine nucleotide binding site is double underlined. Alignment was done with Align software (Genentech, Inc.).

2.4. Subcellular localization

MCF7 cells were transfected with a DNA construct expressing either Flag-UCP3 or Flag-UCP4 using FuGENE transfection reagent (Boehringer Mannheim). The transfected cells were fixed in 3% formaldehyde at room temperature for 15 min and permeabilized with 1% Triton X-100 for 15 min. The cells were incubated with both anti-Flag monoclonal antibody (10 μg/ml, Kodak) and anti-cytochrome *c* oxidase polyclonal antibody (3 ng/ml) for 20 min, washed and subsequently incubated with Cy3-conjugated and FITC-conjugated (Jackson Laboratories) secondary antibodies before examination by confocal microscopy.

2.5. Analysis of mitochondrial membrane potential by spectrofluorometry

293T cells were transfected with either pcDNA3 vector alone, or constructs expressing UCP3 or UCP4 using FuGENE transfection reagent. Mitochondrial membrane potential was analyzed as previously described [17,18]. Briefly, a mitochondrial membrane potential-sensitive dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, Eugene, OR) was prepared as a stock solution (5 mg/ml) in dimethyl sulfoxide (DMSO, Sigma). To prepare the staining medium, the stock solution was diluted to 1 mg/ml with DMSO, and then further diluted to 10 μg/ml with prewarmed (37°C) culture medium and filtered through a 0.2 μm filter to exclude aggregated JC-1. Transfected cells were trypsinized and resuspended in culture medium. 1.5×10^6 cells were pelleted and resuspended in 0.5 ml of the staining medium and incubated in the presence or absence of 50 μM CCCP (carbonylcyanide *m*-chlorophenylhydrazone, Sigma) at 37°C in the dark for 30 min. The stained cells were washed, resuspended in 1 ml culture medium, and examined by spectrofluorometry (RF5000U spectrofluorophotometer, Shimadzu, Japan). A subset of cells were analyzed by flow cytometry (Coulter EPICS Elite ESP, Hialeah, FL). For spectrofluorometric analysis, excitation was at 488 nm and fluorescence intensity was measured over the spectrum 500–615 nm. Flow cytometry analysis was performed with an argon laser of single 488 nm as excitation, a filter transmitting 525 ± 20 nm in FL1 channel, and a filter transmitting above 590 nm in FL2 channel. A minimum of 10,000 cells per sample were analyzed.

2.6. Statistical analysis

The mean ratios of red (593 nm) versus green (532 nm) fluorescence intensity peaks from spectrofluorometry were compared across treatments (nine independent transfections per treatment). Differences were analyzed using Fisher's protected least significant difference.

3. Results and discussion

3.1. Identification and molecular cloning of a new member of the uncoupling protein family

To identify novel uncoupling proteins, we searched ESTs using the protein sequence of human UCP3 [16]. Several overlapping human EST sequences were initially identified that showed significant homology to the known UCPs. A cDNA clone containing a full length open reading frame (ORF) was obtained by PCR as described in Section 2. This ORF encoded a protein of 323 amino acids with a predicted molecular weight of 36 061 Da and a p*I* of 9.28. Database searches, protein sequence alignment and comparative analyses revealed that this molecule was a novel protein and most related to the established UCPs, possessing 34%, 33% and 29% amino acid identities to human UCP3, UCP2 and UCP1, respectively. Interestingly, this molecule was also related to plant uncoupling proteins and a putative nematode uncoupling protein

with 38–46% amino acid identities [19–21]. Based on its protein sequence and uncoupling activity (see below), this novel molecule was termed uncoupling protein-4, or UCP4.

Like other UCPs, UCP4 possessed six putative transmembrane domains, three mitochondrial transporter protein signature motifs and a putative nucleotide binding site (Fig. 1). The uncoupling activity of UCP1 is modulated by nucleotides and free fatty acids (FFA) [3]. UCP1 lacking its putative nucleotide binding site is resistant to inhibition by nucleotides and substitution of Phe-267 by Tyr leads to enhanced uncoupling activity [22–24]. However, like UCP2 and UCP3, UCP4 had a Tyr residue at this position (Fig. 1). Moreover, the carboxy-terminus of UCP1 has been implicated in the activation of its uncoupling activity by FFA. Substitution of Cys-305 by Ala or Ser results in either decreased or increased activation by FFA, respectively [25]. Curiously, UCP2 has an Ala (Ala-307), whereas UCP4 (Ser-321) and UCP3 (Ser-298) have a Ser at this position, suggesting that the uncoupling activities of UCPs may be regulated differently by nucleotides and FFA.

3.2. Tissue distribution of UCP4

The transcripts of the known uncoupling proteins have distinct tissue distribution patterns [3,6,9]. To examine the expression pattern of UCP4 transcripts, two human multiple tissue Northern blots were hybridized with an UCP4 cDNA probe. Interestingly, UCP4 transcripts (3.3 kb) were only detected in the brain (Fig. 2A). To examine UCP4 transcript distribution within the brain, two human brain multiple tissue Northern blots were analyzed. Abundant transcripts were detected in most of the brain tissues, with low levels found in spinal cord, medulla, corpus callosum and substantia nigra (Fig. 2B, top). To compare the expression of UCP2 and UCP4 transcripts in the brain, a human brain multiple tissue blot was probed with the human UCP2 cDNA. The UCP2 transcripts were detectable in all the brain tissues examined (Fig. 2B, bottom right). Intriguingly, the highest levels of UCP2 transcripts were found in the spinal cord and medulla where the lowest UCP4 transcripts were detected, whereas much lower levels were observed in the other brain tissues.

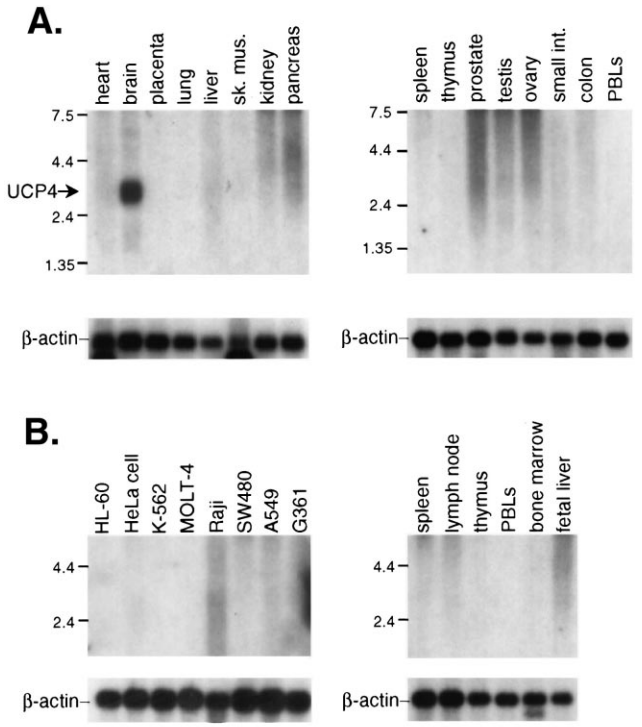

Fig. 2. Tissue distribution of UCP4. Human adult tissue, immune tissue and cancer cell line Northern (RNA) blots (A); human adult brain and fetal tissue Northern blots (B) (Clontech) were probed with UCP4 or UCP2 cDNA according to the manufacturer's instructions. The blots were subsequently probed with a β-actin cDNA. PBL, peripheral blood leukocytes; Sk. mus., skeletal muscle; Sm. int., small intestine; HL-60, promyelocytic leukemia; HeLa cell, HeLa cell line; K-562, chronic myelogenous leukemia; MOLT-4, lymphoblastic leukemia; Raji, Burkitt's lymphoma; SW480, colorectal adenocarcinoma; A549, lung carcinoma; G361, melanoma.

To determine whether UCP4 expression was developmentally regulated, a human fetal tissue blot was examined. The UCP4 transcript was only detected in the fetal brain (Fig. 2B, bottom left). Taken together, these analyses demonstrate that UCP4 expression is brain-specific.

Next, we performed radiation-hybrid analysis to determine chromosome location for UCP4. UCP4 was mapped to human chromosome 6p11.2–q12 with SHGC-34952 being the closest genomic marker.

3.3. Subcellular localization of UCP4 in mitochondria

To examine the subcellular location of UCP4, human breast carcinoma MCF7 cells were transfected with either pcDNA3-Flag-UCP3 or pcDNA3Flag-UCP4. The cells were stained with anti-Flag and anti-cytochrome *c* oxidase (a mitochondrial marker). The cell staining patterns for either cytochrome *c* oxidase (green image; Fig. 3A,D) or UCP3 or UCP4 (red image; Fig. 3C,F) showed a typical mitochondrial punctate staining throughout the cytoplasm with no nuclear or cell surface staining. As expected, UCP3 or UCP4 was co-localized with cytochrome *c* oxidase, as indicated by a yellow image for the double staining (Fig. 3B,E), consistent with the notion that UCP3 and UCP4 are localized in the mitochondria.

3.4. Ectopic expression of UCP4 in mammalian cells resulted in decreased mitochondrial membrane potential

The biochemical activities of UCPs have been studied in both mammalian and yeast cells in vitro [3,4,6,16]. Expression of the known UCPs in these cells leads to a decline in mitochondrial membrane potential [3,4,6,8,9,16]. JC-1 is a fluorescent dye that has been shown to be a reliable indicator of the mitochondrial membrane potential changes in intact cells [17,18,26]. To test whether UCP4 has an uncoupling activity, we transiently transfected human embryonic kidney 293T with vector or DNA construct expressing either UCP3 or UCP4 (see inset, Fig. 4A) and measured the mitochondrial membrane potential. Relative to controls, expression of UCP3 in 293T cells significantly reduced the fluorescent peak value ratio (593 nm/532 nm) by 15% ($P < 0.01$; Fig. 4A), indicative of a decline in mitochondrial membrane potential. Similar results were obtained when cells were transfected with the UCP4-expressing construct, where the ratio dropped by 19% vs. control ($P < 0.01$; Fig. 4A,B). The results obtained by spectrofluorometry were further confirmed by flow cytometry analysis where the integrated red-to-green intensity ratios fell by 18% and 24% in UCP3- and UCP4-transfected cells, respectively ($P < 0.01$ vs. control; $n = 3$/treatment). Cells treated

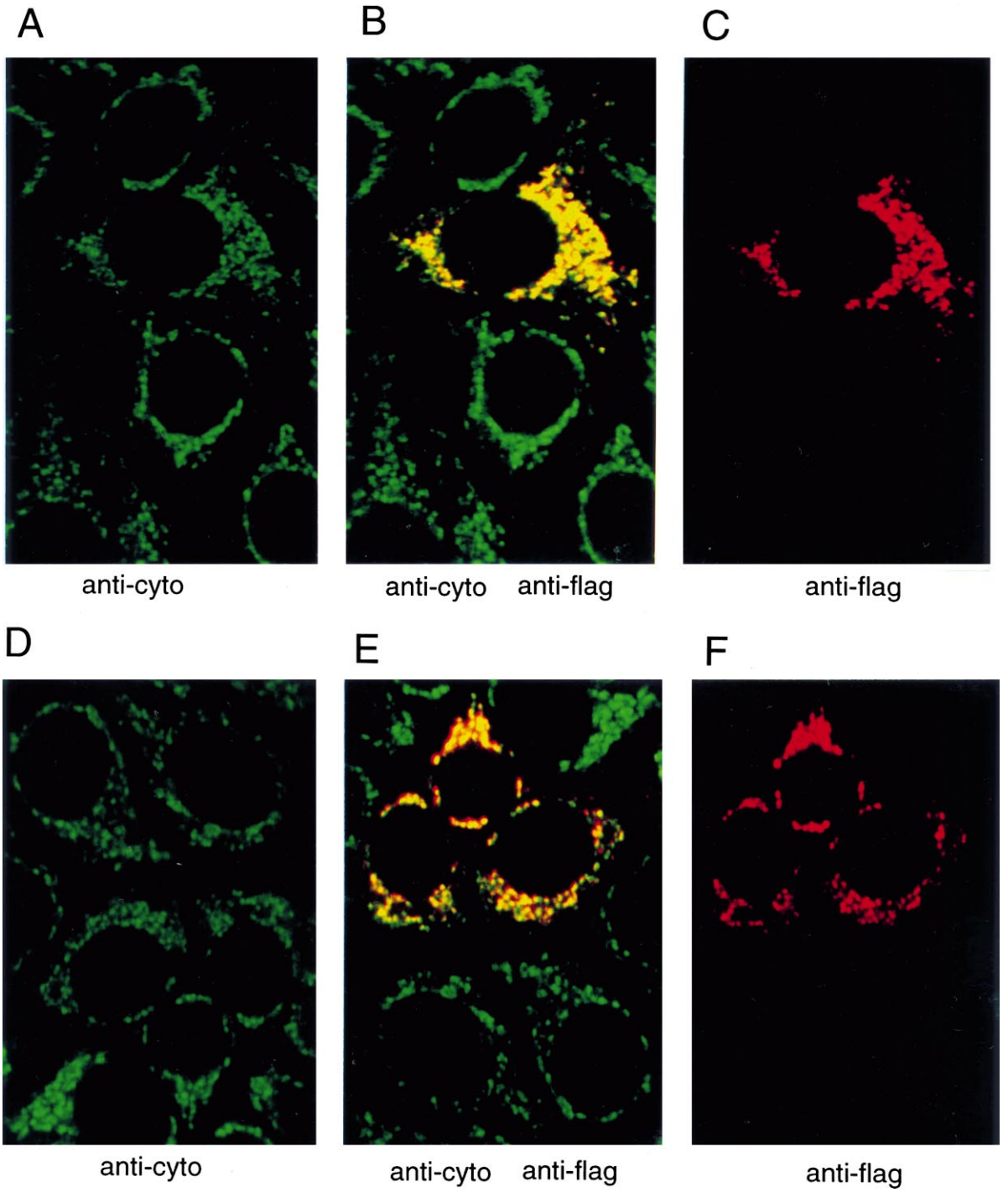

Fig. 3. Subcellular localization of UCP3 and UCP4 in the mitochondria. MCF7 cells were transfected with pcDNA3Flag-UCP3 or pcDNA3-Flag-UCP4 and stained with anti-Flag and anti-cytochrome *c* oxidase antibodies as described (see Section 2). A–C: UCP4-transfected cells. D–F: UCP3-transfected cells. Green images showing mitochondrial staining for cytochrome *c* oxidase (A, D). Fused yellow images showing double staining for cytochrome *c* oxidase and Flag-UCP4 or Flag-UCP3, respectively, indicating their co-localization in the mitochondria (B, E). Red images showing staining for Flag-UCP4 or Flag-UCP3 (C, F). Anti-Flag: anti-Flag monoclonal antibody; Anti-cyto: anti-cytochrome *c* oxidase antibody.

with a chemical uncoupler CCCP showed a dramatic reduction of the ratio (Fig. 4A,B). No difference in cell viability was observed among the cells transfected with the vector or UCP-expressing constructs when the cells were assayed for their mitochondrial membrane potential (not shown). These findings suggest that like UCP3, UCP4 is a functional uncoupling protein.

The brown fat UCP1 is involved in thermogenesis in rodents [3,13] and UCP2 and UCP3 have been implicated in the regulation of energy expenditure [4,5,8,9,16]. Although its contribution to the whole body basal metabolic rate is poorly understood, the brain has long been recognized as one of the major oxygen-consuming and metabolically active tissues where mitochondrial proton leak has been observed [11].

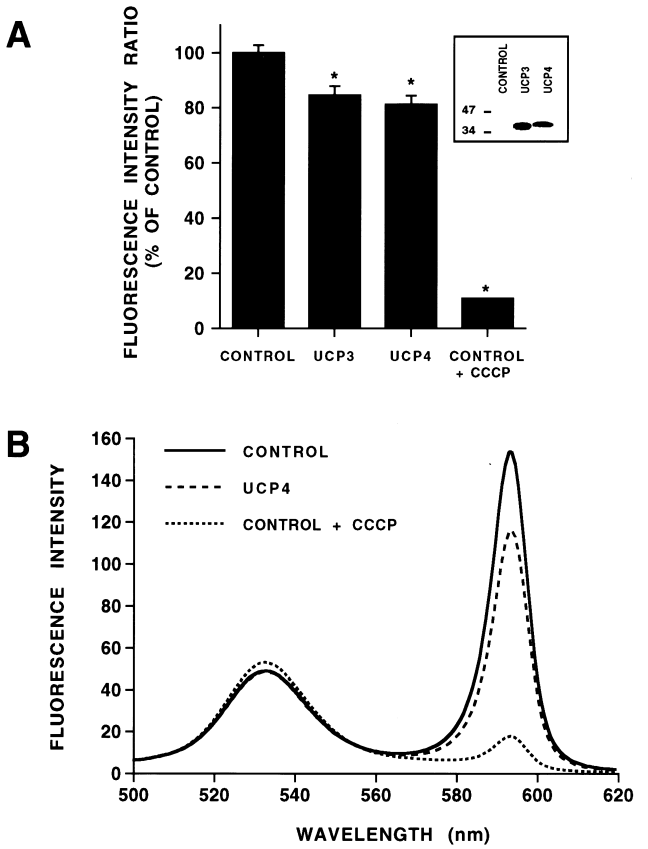

Fig. 4. Ectopic expression of UCP4 in mammalian cells reduces mitochondrial membrane potential. A: 293T cells were transfected with DNA constructs as indicated and the mitochondrial membrane potential analyzed as described in Section 2. Expression of UCP3 or UCP4 in these cells elicits a significant decline in the 593 nm/532 nm fluorescence peak ratio. The change in ratio, which tracks the mitochondrial membrane potential, was highly significant and decreased by 15–19% relative to the control ($^*P < 0.01$ vs. control, Fisher's protected LSD; $n = 9$). The NH_2-terminal Flag tag had no effect on the activity of UCP3 or UCP4 (not shown). Flag-tagged UCP3 and UCP4 expressed in 293T cells were detected by Western blot analysis using anti-Flag M2 monoclonal antibody (Kodak) and ECL detection kit (Pierce) (see inset). B: Representative fluorescence wavelength scans of transfected cells treated with JC-1 are shown. The drop in fluorescence peak intensity at 593 nm in UCP4-transfected cells relative to the control-transfected cells is indicative of a decrease in membrane potential. Cells transfected with UCP3 in parallel yielded a pattern mimicking that of UCP4 (not shown). Treatment of the control cells with CCCP strongly suppressed the peak intensity.

Thermoregulatory heat production in response to cold has also been observed in the brain [27]. Since UCP4 transcripts are exclusively expressed in the brain and it functions as an uncoupling protein in mammalian cells in vitro, it is conceivable that UCP4 may be responsible for the mitochondrial proton leak observed in brain tissue, and may potentially be involved in adaptational thermoregulation and heat production in both fetal and adult brains. In addition, since UCPs function to uncouple the cellular respiratory reaction and reactive oxygen species (ROS) have been associated with apoptosis in neurons [28], it is interesting to speculate that UCP4 may play a regulatory role in the generation of ROS, and that dysregulation of UCP4 function may be involved in development of certain pathological conditions, including neural degenerative disorders.

Acknowledgements: We thank J. Lee for the human brain cDNA library, T. Stewart and E. Filvaroff for helpful discussions, K. Luetke for reading the manuscript, D. Wood and L. Tamayo for preparing figures. Anti-cytochrome *c* oxidase was a gift from G.-A. Keller.

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