Structure and expression of human mitochondrial adenylate kinase targeted to the mitochondrial matrix

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The previously isolated cDNA encoding human adenylate kinase (AK) isozyme 3 was recently renamed AK4. Consequently, human AK3 cDNA remains to be identified and we have little information about the functional relationship between human AK3 and AK4. In pursuit of the physiological roles of both the AK3 and AK4 proteins, we first isolated an authentic human AK3 cDNA and compared their expression. Nucleotide sequencing revealed that the cDNA encoded a 227-amino-acid protein, with a deduced molecular mass of 25.6 kDa, that shares greater homology with the AK3 cDNAs isolated from bovine and rat than that from human. We named the isolated cDNA AK3. Northern-blot analysis revealed that AK3 mRNA was present in all tissues examined, and was highly expressed in heart, skeletal muscle and liver, moderately expressed in pancreas and kidney, and weakly expressed in placenta, brain and lung. On the other hand, we found that human AK4 mRNA was highly expressed in kidney, moderately expressed in heart and liver and weakly expressed in brain. Western-blot analysis demonstrated expression profiles of AK3 and AK4 that were similar to their mRNA expression patterns in each tissue. Over expression of AK3, but not AK4, in both *Escherichia coli* CV2, a temperature-sensitive AK mutant, and a human embryonic kidney-derived cell line, HEK-293, not only produced significant GTP:AMP phosphotransferase (AK3) activity, but also complemented the CV2 cells at 42 °C. Subcellular and submitochondrial fractionation analysis demonstrated that both AK3 and AK4 are localized in the mitochondrial matrix.

Key words: cDNA cloning, Northern-blot analysis, subcellular localization, Western-blot analysis.

INTRODUCTION

Homoeostasis of adenine nucleotides in cells is important for performing and maintaining multiple cellular functions that depend on cellular energy metabolism [1]. The adenylate kinases (AKs) are a family of enzymes that catalyse the interconversion of three adenine nucleotides in the cell: $Mg^{2+}ATP$ (or GTP) + AMP ↔ $Mg^{2+}ADP$ (or GDP) + ADP [2,3]. In vertebrates, three isozymes, AK1 and AK2 (ATP:AMP phosphotransferases, EC 2.7.4.3) and AK3 (GTP:AMP phosphotransferase, EC 2.7.4.10), were originally classified [2–6]. AK1 is present in the cytosol of skeletal muscle, brain and erythrocytes, whereas AK2 exists in both the cytosol and the mitochondrial intermembrane space of liver, kidney, spleen and heart. AK3, GTP:AMP phosphotransferase, is localized exclusively in the mitochondrial matrix of various tissues. Recently, two additional AK isozymes, AK4 and AK5, were identified [7,8], and the previously reported human AK3 [9] was recently renamed AK4 [7]. AK4 cDNA, which is highly homologous to human AK3, was isolated from rat and mouse by the differential-display procedure and its mRNA was strongly detected in brain and liver [7]. However, the activity and localization of AK4 protein were not characterized [7]. AK5 was found in the cytosol of brain tissue and both ATP:AMP phosphotransferase and GTP:AMP phosphotransferase activities were detected [8]. As a result, there are presently five known types of AK isozyme, but an authentic human AK3 has not so far been identified.

AK is indispensable for the growth of *Escherichia coli* [10] and *Schizosaccharomyces pombe* [11], indicating that it is an essential enzyme for life in a single cell. However, it was reported that no phenotypic change except for haematological abnormality was present in a patient with AK1 deficiency due to homozygous base substitution in exon 6 of the *AK1* gene [12]. In addition, the recent gene disruption analysis of *AK1* also demonstrated that *AK1*-knockout mice show no phenotypical changes under normal conditions, but under metabolic stress compromised energetics were detected in the heart and skeletal muscle, suggesting physiological significance for AK-catalysed phosphoryl transfer between intracellular compartments in cellular energetic homoeostasis [13,14]. Thus the physiological role of each AK isozyme and the functional co-operation of AK isozymes in multicellular organisms requires further study. In this report, to investigate further the physiological role of the AK isozyme system in the cellular compartment and to identify an authentic human AK3, we performed PCR-mediated cloning of human AK3 cDNA and determined the enzyme activity, tissue distribution and subcellular localization of the AK3 protein in comparison with the uncharacterized human AK4 protein.

MATERIALS AND METHODS

Materials

Phosphocellulose and Affi-Gel Blue columns were obtained from Whatman and Bio-Rad Laboratories, respectively. Human

Abbreviations used: AK, adenylate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RACE, rapid amplification of cDNA ends; IPTG, isopropyl β-D-thiogalactoside; CBB, Coomassie Brilliant Blue.

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The nucleotide sequence data for human AK3 cDNA reported in this paper have been assigned the GenBank Nucleotide Sequence Database under accession number AB021870.

AK4cDNA [9] was provided kindly by Dr Gangfeng Xu (University of Utah, Salt Lake City, UT, U.S.A.).

PCR cloning of human AK3 cDNA

The gene-specific oligonucleotide primers 5′-GGN GSI CCI GGI GCN GGI AAR GG-3′ (where I indicates 2′-deoxyinosine), corresponding to amino acid sequence GAPGSGK, as the 5′ sense primer, and 5′-GAA TGT TTC CAA CAC CCC TTT TT-3′, corresponding to amino acid sequence KGVLETF, as the 3′ antisense primer, were designed on the basis of the amino acid sequences corresponding to the conserved region of mitochondrial AK3 cDNA from bovine, rat and mouse (accession numbers M25757, D13062 and AB020203). The primers were used to amplify human liver Marathon-Ready cDNA (Clontech) as described previously [15]. The approx. 600 bp fragments thus amplified were gel-purified and subcloned into pGEM-T Easy vector (Promega).The nucleotide sequences of purified plasmid DNAs were determined by the dideoxy chain-termination method [16]. To isolate the remainder of the 5′ and 3′ portions of the cDNA by the 5′- and 3′-rapid amplification of cDNA ends (RACE) method [17], we prepared the following gene-specific primers: 5′-CCA GAT TAT GTC ACG ACT CGG CTG-3′ and 5′-AAG CCA GTC CTG GAA TAT TAC CAG-3′ as the sense primers, and 5′-TCA GCT CGA AGT GTG TAG TGA TGC-3′ and 5′-TGG AAG TGT CCT TGG AAA ACC ATC-3′ as the antisense primers. 5′- and 3′-RACE were performed using the same Marathon-Ready cDNA.

Northern-blot analysis

A multiple-tissue Northern-blot filter (Clontech) was probed with ^{32}P-labelled human AK3 or AK4 cDNA fragments as described previously [18]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [19] was also used to monitor the quantity and integrity of mRNA after detaching the above probes.

Construction of expression plasmids for human AK3 and AK4

To express recombinant AK3 and AK4 proteins in *E. coli*, the cDNA fragments corresponding to each coding region were generated by PCR using human liver Marathon-Ready cDNA and human AK4 cDNA as DNA templates. We chose to express both the full-length proteins (hAK3 and hAK4) and the fusion protein with a hexahistidine tag at their C-termini (AK3His and AK4His). Primers were designed to introduce *Nco*I and *Kpn*I sites at the 5′ and 3′ ends of the coding sequence, respectively, allowing for subcloning of each coding region at the corresponding sites into pSE380 bacterial expression vector (Invitrogen). The fragments corresponding to hAK3 and hAK4 were obtained with the following primers: a sense primer (5′-AGC CAT GGG GGC GTC CGC GCG GCT GCT GCG-3′) and an antisense primer (5′-TGG TAC CTC ATG GAG TAA CTG AAG CTT TCT GGC TTC TTT G-3′) for hAK3, and a sense primer (5′-AGC CAT GGC TTC CAA ACT CCT GCG CGC GGT-3′) and an antisense primer (5′-TGG TAC CTC AAT ATG CTT CTT TGG ACT GAA TAG GTG TGA-3′) for hAK4 (the *Nco*I and *Kpn*I restriction sequences are underlined). For construction of AK3His and AK4His expression vectors, a sense primer (5′-AGC CAT GGG GGC GTC CGC GCG GCT GCT GCG-3 ′) and an antisense primer (5′-TGG TAC CTC AAT GAT GAT GAT GAT GAT GTG GAG TAA CTG AAG CTT TCT GGC TTC TTT G-3′) for AK3His and a sense primer (5′-AGC CAT GGC TTC CAA ACT CCT GCG CGC GGT-3′) and an antisense primer (5′-TGG TAC CTC AAT GAT GAT GAT GAT GAT ATG CTT CTT TGG ACT GAA TAG GTG TGA T-3′) for AK4His were prepared. Amplification was performed at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min for 30 cycles. The amplified DNA fragments were gel-purified and then subcloned between the *Nco*I and *Kpn*I sites of pSE380. The plasmids were named pSE/AK3, pSE/AK4, pSE/AK3His and pSE/AK4His, respectively. The sequences of each construct were verified by complete sequencing in both directions.

To express both human AK3 and AK4 in eukaryotic cells, the same DNA fragments in the pGEM-T Easy vector were cleaved with *Not*I and recloned into the pCIneo mammalian expression vector (Promega), which were named pCI/AK3, pCI/AK3His, pCI/AK4 and pCI/AK4His, respectively.

Expression of recombinant AK3 and AK4

Cells of *E. coli* strain CV2, a temperature-sensitive AK mutant [10], were transformed with either pSE/AK3 or pSE/AK4, and both AK3 and AK4 proteins were overproduced by treating the cells with 1 mM isopropyl β-D-thiogalactoside (IPTG) at 30 °C for 6 h. The bacterial cells were harvested by centrifugation, washed once with PBS and then resuspended in 40 ml of 50 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 1 mM PMSF. The cell suspensions were disrupted by sonication with ten 20 s treatments at half-maximum power of a sonicator (Branson model 450) on ice. The soluble lysates were obtained by centrifugation at 9000 ***g*** for 15 min. All procedures for the preparation of soluble lysates were conducted at 4 °C.

The mammalian expression plasmids were introduced into HEK-293 cells by the method of lipofection, and the cellular extracts were prepared for further biochemical analysis as described previously [15].

Purification of recombinant AK3 and AK4

Purification of AK3 and AK4 proteins in the bacterial extracts was performed by sequential affinity chromatography. The cleared lysates were loaded on to a 1 ml Affi-Gel Blue column equilibrated with 50 mM Tris/HCl, pH 8.0. The column was washed with 20 ml of 50 mM Tris/HCl, pH 8.0. Proteins were eluted with 5 ml of 2 M NaCl in 50 mM Tris/HCl, pH 8.0. The aliquots containing AK3 or AK4 were combined and concentrated with a Centricon-10 concentrator (Amicon). The fractions from the Affi-Gel Blue column containing AK3 or AK4 protein were diluted 100-fold and loaded on to a 1.0 ml P11 phosphocellulose column equilibrated with 30 mM Tris/HCl/1 mM EDTA/0.1 mM dithiothreitol, pH 7.5. After loading the sample, the column was washed successively with 20 ml portions of 30 mM Tris/HCl/1 mM EDTA/0.1 mM dithiothreitol, pH 7.5, until the effluent had an A_{280} of less than 0.1. The AK3 or AK4 protein was eluted with 5 ml of 0.2 M NaCl/30 mM Tris/HCl/1 mM EDTA/0.1 mM dithiothreitol, pH 7.5, and 0.5 ml fractions were collected. The proteins were recovered from these steps in a total of 0.5 ml. Protein concentration was determined by the Lowry method [20] or the Bradford method [21] with BSA as a standard. Protein samples from each purification step were resolved by 0.1 % SDS/PAGE (10 % gel) and stained with Coomassie Brilliant Blue (CBB) R-250 to check the purity.

Enzyme assays

ATP:AMP phosphotransferase (AK1 and AK2) and GTP:AMP phosphotransferase (AK3) activities were measured by a coupled pyruvate kinase/lactate dehydrogenase assay as described previously [22]. Nucleotides such as UTP, TTP, ITP, GMP, CMP, TMP, UMP and IMP were also tested for the assay as substrates.

Antiserum preparation using recombinant His-tagged AK4 as an immunogen

A bacterial culture of CV2 cells containing pSE380/AK4His was lysed by sonication, and the recombinant proteins were purified by affinity chromatography on a TALON CellThru Affinity Resin column (Clontech) following the manufacturer's instructions. The fractions containing AK4His were determined by SDS/PAGE analysis and subsequent Western-blot analysis with anti-His antibody (Invitrogen). The peak fractions were collected and the eluted proteins were purified further by preparative electrophoresis on 0.1% SDS/PAGE (10% gel). The portion containing the AK4 protein in the gel was excised and the AK4 proteins were electroeluted from the gel. The mass (29 kDa) of the proteins was found to be maintained after electrophoresis and electroelution. Polyclonal anti-AK4 antibodies (αAK4Ab) against the AK4His fusion protein were prepared as described previously [23].

Western-blot analysis

Human tissue extracts (brain, heart, kidney, liver, skeletal muscle; Protein Medlays) were purchased from Clontech. Mouse tissue extracts were prepared and aliquots corresponding 100 μg of each tissue extract, bacterial lysates and cellular extracts of HEK-293 cells containing recombinant protein or purified AK protein were subjected to 0.1% SDS/PAGE (10% gel) and electroblotted on to a nitrocellulose membrane as described previously [15]. Antibodies were used with a 1:2000 dilution of αAK1Ab [24], αAK2Ab [23], αAK3Ab [23] and αAK4Ab.

Subcellular and submitochondrial fractions from mouse liver and kidney

Total cell lysates were prepared from liver and kidney of a 6-week old Balb/c mouse. Cytosol and mitochondrial fractions were obtained as described previously [25,26]. To test the intactness of the isolated mitochondria, the respiratory control ratio (RCR) of each mitochondrial preparation was analysed as described previously [27]. Samples with an RCR greater than 3.0 were used for further analysis.

Submitochondrial fractionation was performed as described previously [26,28]. For the preparation of mitoplasts, mitochondria were suspended in 20 mM Hepes/KOH, pH 7.4, and then placed in a test tube on ice for 30 min. The mitoplasts were recovered by centrifugation at 4000 ***g***, and then resuspended in 50 μl of 10 mM Hepes/KOH, pH 7.4, containing 220 mM mannnitol and 70 mM sucrose. The mitoplasts were treated further with 250 μg/ml proteinase K with or without 1% Triton X-100 at 4 °C for 30 min, followed by the addition of 5 μl of 4 mg/ml PMSF to terminate the protease reaction. The reaction mixture in a microcentrifuge tube was centrifuged at 12000 ***g*** for 10 min, and then the precipitates were subjected to 0.1% SDS/PAGE (10% gel). Separated proteins in the gel were transferred electrophoretically to a nitrocellulose membrane. The membrane was analysed with the antibodies (αAK2Ab [23], αAK3Ab [23] and αAK4Ab) and the positions of AK proteins were visualized by the ECL Western-blotting detection reagents (Amersham).

RESULTS

Isolation and sequence analysis of the cDNA encoding human AK3

A cDNA that was highly homologous to bovine, rat and mouse AK3 cDNA was isolated by PCR. We further isolated a 0.7 kb cDNA from 5′-RACE and a 1.0 kb cDNA from 3′-RACE. Sequencing analysis of these cDNA fragments revealed that the 1.8 kb human AK3 cDNA (accession number AB021870) covered one open reading frame that directs a 227-residue protein with a molecular mass of 25.6 kDa and which showed high sequence similarity to the bovine, rat and mouse AK3 cDNA sequences (73%, 88% and 85% at the nucleotide level, 75%, 90% and 91% at the amino acid level, respectively), but it was very different from the renamed human AK4 (60.1% at the nucleotide level, 57.4% at the amino acid level). The predicted pI value of the deduced protein was 9.66. The sequence (GCG GCC ATG G) around the presumptive start codon was in good accordance with the Kozak consensus sequence [29].

Tissue distribution of AK3 and AK4 mRNAs

To examine the tissue distribution of AK3 mRNA, we performed Northern-blot analysis (Figure 1). AK3 transcripts were detected in all tissues examined, and were strongly expressed in heart, liver and skeletal muscle, moderately expressed in kidney and pancreas, and weakly expressed in brain, placenta, and lung (Figure 1, left-hand panel). The 3.2 kb transcripts were expressed predominantly in all tissues. Transcripts with larger and smaller sizes were also observed at 5, 1.8 and 1.35 kb, especially in heart, liver and pancreas. On the other hand, multiple AK4 transcripts were detected (Figure 1, central panel). The longest transcripts were about 8.0 kb in size and strongly expressed in kidney and heart, moderately expressed in liver, and weakly expressed in brain. In placenta and lung, AK4 mRNA was hardly detectable. Multiple transcripts of either AK3 or AK4 mRNAs may be derived from alternative splicing and/or differential selection of a poly(A)$^+$ addition site in a similar way to the bovine *AK2* and *AK3* genes [30–32]. Although Yoneda et al. [7] reported that AK4 mRNA was predominantly expressed only in brain and liver, our analysis demonstrated the highest expression of AK4 mRNA in kidney, high expression in liver and heart, and lower expression in brain. Densitometric analysis of each signal of AK4 transcripts relative to that of GAPDH (Figure 1, right-hand panel) revealed that the relative levels of the major 8.0 kb AK4 transcripts were 2.0 (kidney), 0.71 (brain), 0.51 (liver) and 0.39 (heart). Recently we also found the same expression patterns of AK4 mRNA in mouse tissues (Y. Yamashiro, K. Fujisawa and T. Noma, unpublished work). The results indicated that the expression of AK3 mRNA appeared to be regulated in a housekeeping-type manner and that expression of AK4 mRNA appeared to be regulated in a tissue-specific manner.

Tissue distribution of AK3 and AK4 proteins

We next performed Western-blot analysis to examine the relative level of protein contents of each AK isozyme in human and mouse tissues (Figure 2). AK3 was present in all five tissues examined in human (Figure 2A, upper panel), and abundantly in liver and kidney, moderately in heart, skeletal muscle, brain and testis, and less in lung and pancreas of mouse (Figure 2B, upper panel). In contrast, AK4 was detected abundantly in kidney, moderately in liver and weakly in brain and heart, and was not detectable in skeletal muscle in human tissues (Figure 2A, lower panel). Similar abundant expression was also observed in mouse kidney and liver (Figure 2B, lower panel), but AK4 was hardly detectable in mouse heart. The electrophoretic analysis demonstrated that the molecular mass of AK4 (29 kDa) was slightly greater than that of AK3 (28 kDa). The tissue-specific expression of the AK4 protein was consistent with the profile of mRNA expression, as shown in Figure 1.

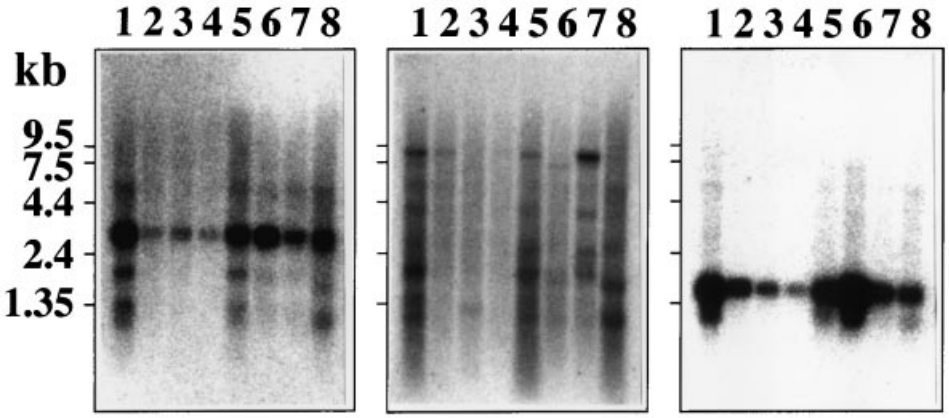

Figure 1 Northern-blot analysis of poly(A)$^+$ RNA from various human tissues

A blot filter containing 2 μg of poly(A)$^+$ RNA from each of various tissues (Clontech) was hybridized to a 0.5 kb fragment of AK3 cDNA (left-hand panel), a 1.7 kb fragment of human AK4 cDNA [9] (central panel) and a 0.7 kb fragment of human GAPDH cDNA [19] (right-hand panel). Probing the same filter with GAPDH cDNA indicated that the loaded amounts of each mRNA varied, but the integrity was confirmed. RNA sources were as follows: lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. Positions of the size markers (kb) are shown on the left.

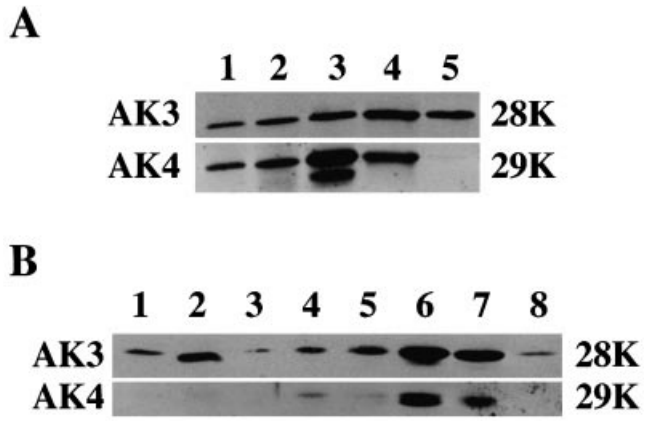

Figure 2 Tissue distributions of AK3 and AK4

Human (**A**) and mouse (**B**) tissue lysates were resolved by 0.1% SDS/PAGE (10% gel) and electrotransferred on to a nitrocellulose membrane. AK3 and AK4 expression in human and mouse tissues was analysed by Western-blot analysis as described in the Materials and methods section. Each lane contained 100 μg of tissue lysate. (**A**) Lane 1, brain; lane 2, heart; lane 3, kidney; lane 4, liver; lane 5, skeletal muscle. (**B**) Lane 1, heart; lane 2, skeletal muscle; lane 3, lung; lane 4, brain; lane 5, testis; lane 6, kidney; lane 7, liver; lane 8, pancreas. Molecular masses are shown on the right (kDa).

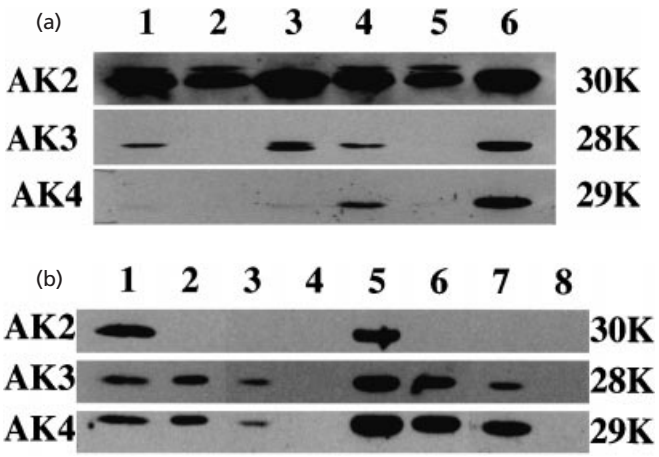

Figure 3 Subcellular localization of AK3 and AK4 in the cells

(**a**) Subcellular localization of AK3 (central panel) and AK4 (lower panel). Subcellular fractionation of liver and kidney extracts was performed as described previously [26]. Localization of AK2 in the cell was also analysed as a control, as shown previously [26]. Western-blot analysis was performed using αAK2Ab (upper panel), αAK3Ab (central panel) or αAK4Ab (lower panel) after electrophoresis with 0.1% SDS/PAGE (10% gel). Lanes 1–3, liver samples; lanes 4–6, kidney samples; lanes 1 and 4, total cellular extracts; lanes 2 and 5, cytosolic fractions; lanes 3 and 6, mitochondrial fractions. (**b**) Submitochondrial localization of AK3 (central panel) and AK4 (lower panel). Submitochondrial fractions were prepared as described in the Materials and methods section. Each fraction was separated by SDS/PAGE and transferred on to a nitrocellulose membrane. Separated proteins were analysed with αAK2Ab (upper panel), αAK3Ab (central panel) or αAK4Ab (lower panel), and visualized with enhanced chemiluminescence Western-blotting detection reagents. Localization of AK2 in the mitochondrial intermembrane space was also demonstrated as a control (upper panel). Lanes 1–4, liver samples; lanes 5–8, kidney samples; lanes 1 and 5, mitochondria; lanes 2 and 6, mitoplasts; lanes 3 and 7, proteinase K-treated mitoplasts; lanes 4 and 8, proteinase K + Triton X-100-treated mitoplasts.

Subcellular localization of AK3 and AK4

Subcellular fractionation analysis of liver and kidney and subsequent Western-blot analysis revealed that both AK3 and AK4 were localized exclusively in the mitochondrial fractions (Figure 3a, central and lower panels, lanes 3 and 6), whereas AK2 as a control was detected in both cytosolic and mitochondrial fractions (Figure 3a, upper panel, lanes 2 and 3). Further submitochondrial fractionation from the same tissue samples demonstrated that AK3 and AK4 were detected in the mitoplast and proteinase K-treated mitoplast fraction in almost the same amounts as in the whole mitochondrial fraction (Figure 3b, central and lower panels, lanes 1–3 and 5–7), whereas they were not detected in the proteinase K + Triton X-100-treated mitoplast. On the other hand, AK2, which is localized in the intermembrane space of the mitochondria, as we reported previously [26], was detected in the whole mitochondrial fraction but not in the mitoplast fraction, in which the outer membrane

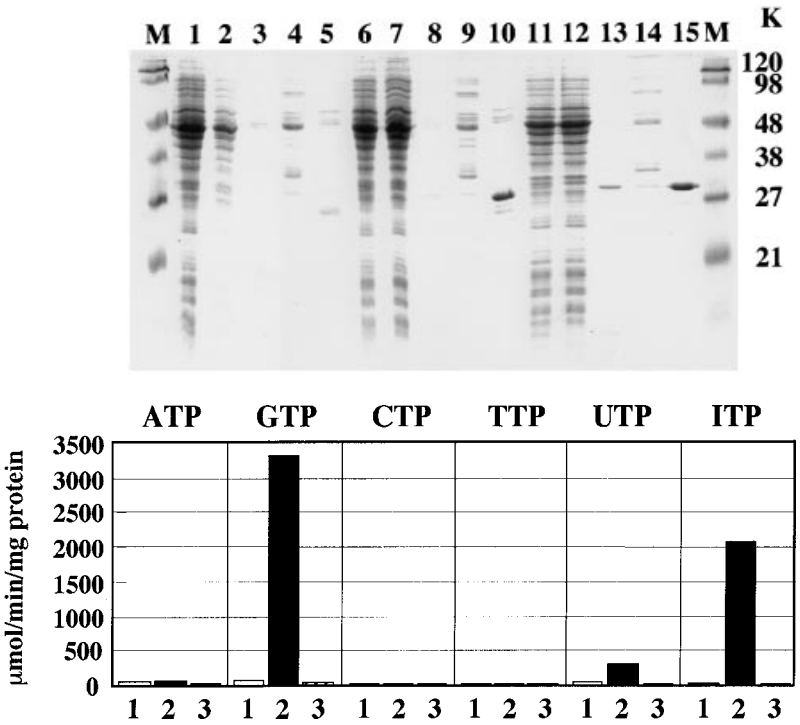

Figure 4 Expression and enzyme activity of human AK3 and AK4 in *E. coli*

(**A**) Expression of human AK3 and AK4 in *E. coli*. The pSE/AK3 and pSE/AK4 plasmids as well as pSE380 (control) were transduced into *E. coli* strain CV2 and selected. The lysates of each transformant containing pSE/AK3, pSE/AK4 or pSE380 were prepared as described in the Materials and methods section. Each sample was electrophoresed by 0.1% SDS/PAGE (10% gel) and stained with CBB R250. Lanes 1–5, samples from *E. coli* strain CV2 containing pSE380 (control); lanes 6–10, samples from CV2 containing pSE/AK3; lanes 11–15, samples from CV2 containing pSE/AK4; lanes 1, 6 and 11, crude extracts; lanes 2, 7 and 12, pass fractions from phosphocellulose column; lanes 3, 8 and 13, eluates from phosphocellulose column; lanes 4, 9 and 14, pass fractions from Affi-Gel Blue column; lanes 5, 10 and 15, eluates from Affi-Gel Blue column. Molecular masses are shown on the right (kDa). M indicates the marker lane. (**B**) Phosphotransferase activities of human AK3 and AK4. The activities were examined with various combinations of nucleotides. The phosphoryl donors are indicated at the top, and purified recombinant materials are indicated at the bottom. Columns 1, 2 and 3 indicate each purified sample from the transformants with pSE380, pSE/AK3 and pSE/AK4, respectively.

of the mitochondrion was destroyed and the proteins in the intermembrane space were lost (Figure 3b, upper panel, lanes 1, 2, 5 and 6). AK3 and AK4 in mitoplasts were resistant to simple proteinase K digestion (Figure 3b, central and lower panels, lanes 3 and 7), whereas they were completely destroyed after rupturing of the mitoplasts with a detergent, Triton X-100 (Figure 3b, central and lower panels, lanes 4 and 8). These results indicate that both AK3 and AK4 are located in the mitochondrial matrix.

Enzyme activity of AK3 and AK4

Next, to examine whether the proteins encoded by AK3 and AK4 cDNA have any AK activities, we produced both AK3 and AK4 by using bacterial expression plasmids (pSE/AK3 and pSE/AK4). CV2 cells transformed with the pSE/AK3 and pSE/AK4 plasmids and a control plasmid (pSE380) were cultured separately in the presence of IPTG for 6 h. IPTG-induced GTP:AMP phosphotransferase activity in crude extracts derived from the bacteria containing pSE/AK3, but not pSE/AK4, was specifically detected with a specific activity of about 5.0 μmol/min per mg of protein. The results were repeated with other expression plasmids that were prepared with the pKK223-3 bacterial expression vector (Pharmacia). Expression of AK3 and AK4 proteins was confirmed by SDS/PAGE analysis (Figure 4, top panel, lanes 10 and 15) and Western-blot analysis (results not shown), showing a protein of approx. 28 kDa as AK3 and a slightly larger protein (29 kDa) as AK4. The production of AK3 was about 3-fold more abundant than that of AK4. The difference in productivity may be due to the differential codon usage between human and *E. coli* or to differential protein stability, since the nucleotide sequences other than the protein-coding region were the same in AK3 and AK4.

We then purified each protein by sequential Affi-Gel Blue and phosphocellulose column chromatography and measured the AK activities. The specific activity of GTP:AMP phosphotransferase in the AK3 protein was 3200 μmol/min per mg of protein when GTP was used as a phosphoryl donor, and that of ITP:AMP phosphotransferase activity in the AK3 protein was 2100 μmol/min per mg of protein (Figure 4, lower panel). Various combinations of nucleotides were also tested for their ability to act as substrates of the purified enzyme, and only GTP, ITP and AMP gave a significant activity under our experimental conditions although UTP gave a very weak but distinct activity (Figure 4, lower panel). On the other hand, in the case of AK4 protein neither ATP:AMP phosphotransferase nor GTP:AMP phosphotransferase activities were detected at all.

To determine functional AK activity *in vivo*, complementation analysis using CV2 cells, which are temperature-sensitive mutants of endogenous AK and can grow at 30 °C but not at 42 °C, was performed as described previously [10]. Only CV2 cells containing the AK3 expression plasmid, but not the AK4 plasmid, were able to grow at 42 °C, indicating that AK3, but not AK4, has biological AK activity *in vivo*.

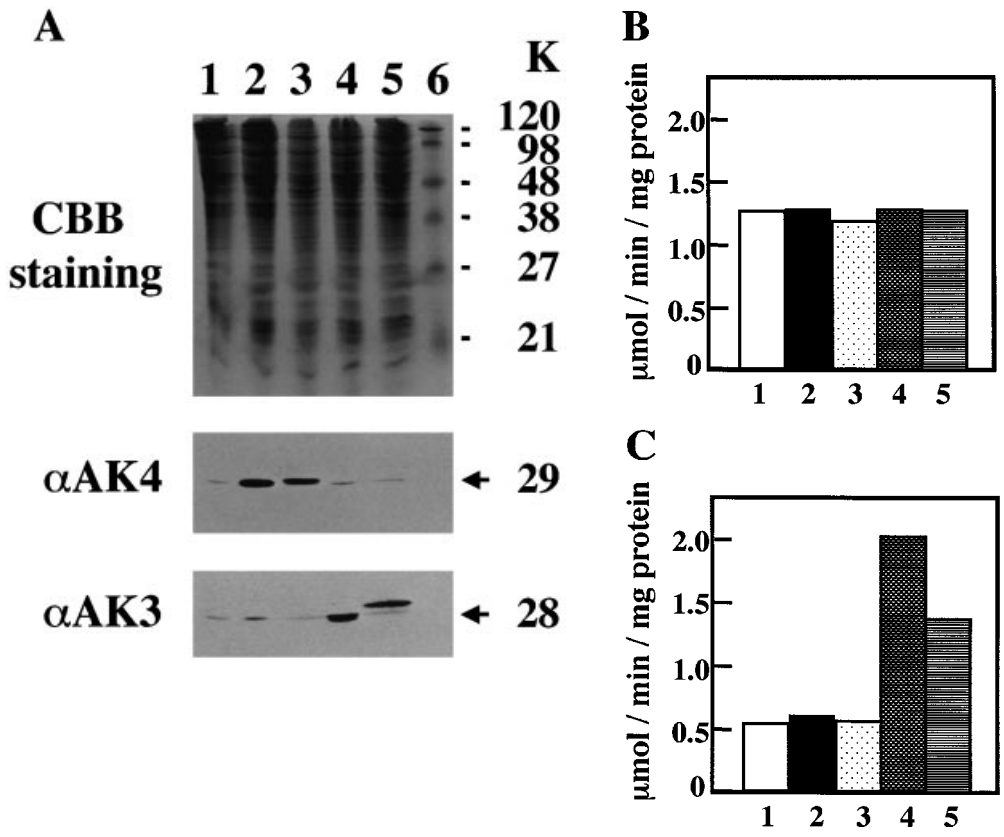

Figure 5 Expression and enzyme activity of human AK3 and AK4 in HEK-293 cells

Expression of AK3 and AK4 in HEK-293 cells. HEK-293 cells were transfected with pCI/AK3, pCI/AK3His, pCI/AK4 and pCI/AK4His plasmids, as well as the pCI plasmid as a control, and cultured for 48 h. Then the extracts of each transformant were prepared and electrophoresed on a 0.1% SDS/PAGE (10% gel) and stained with CBB R250 (upper panel). Extracts from HEK-293 cells were transfected with (lane 1) pCI, (lane 2) pCI/AK4, (lane 3) pCI/AK4His, (lane 4) pCI/AK3 and (lane 5) pCI/AK3His. Lane 6, marker lane. Molecular masses are shown on the right (kDa). The cellular proteins in other duplicated gels were transferred to nitrocellulose filters and then Western-blot analysis was performed with αAK4Ab (central panel) and αAK3Ab (lower panel). Slower migration of hexahistidine-tagged AK fusion proteins was observed in lane 3 (AK4His) and lane 5 (AK3His). (**B**) ATP:AMP phosphotransferase activities of the lysates from transfectants. (**C**) GTP:AMP phosphotransferase activities of the lysates from transfectants. (**B**, **C**) Columns 1–5 refer to the same cellular extracts from the transfectants as shown in (**A**). Almost equal levels of ATP:AMP phosphotransferase activity were detected in all samples (**B**). However, significant amounts of GTP:AMP phosphotransferase activity were only detected in samples from the transfectants with pCI/AK3 and pCI/AK3His (**C**).

To further analyse both AK3 and AK4 expression in an eukaryotic expression system, we introduced the expression plasmids pCI/AK3, pCI/AK3His, pCI/AK4 and pCI/AK4His into HEK-293 cells. Protein expression was analysed by SDS/PAGE and Western-blot analysis. CBB staining of cellular proteins extracted from each transfectant demonstrated no gross changes of the cellular constituents (Figure 5A, upper panel). Western-blot analysis with αAK4Ab and αAK3Ab revealed that each transfectant over produced the respective recombinant proteins (AK4, Figure 5A, central panel, lanes 2 and 3; AK3, Figure 5A, lower panel, lanes 4 and 5). In addition, it was also observed that both parental and transfected HEK-293 cells produced low amounts of endogenous AK3 and AK4 proteins, as shown in Figure 5(A), lower and central panels, respectively.

The ATP:AMP phosphotransferase activities were detected at a similar level in the celluar extracts of all transfectants, indicating that transfection with each expression plasmid gave no significant effects on endogenous ATP:AMP phosphotransferase activities (Figure 5B). On the other hand, the increases in GTP:AMP phosphotransferase activities over the level of control cells(Figure 5C, column 1) were detected only in transfectants with pCI/AK3 and pCI/AK3His, which produced weaker activity (Figure 5C, columns 4 and 5). Similar results were obtained with HeLa cells as another parental cell line for transfection (results not shown). Taken together, we conclude that AK4 does not produce any AK activity *in vivo*.

DISCUSSION

This study describes the isolation, expression and characterization of the cDNA encoding an authentic mitochondrial GTP:AMP phosphotransferase, which is structurally related to the previously designated AK3, which has been renamed AK4 [7,9]. We named the encoded protein human AK3 on the basis of its sequence similarity with the bovine, rat and mouse AK3 isozymes, its specific enzyme activity for GTP:AMP and ITP:AMP phosphotransferases, and its specific mitochondrial localization. Previously, we have demonstrated the presence of three AK isozymes in the invertebrate *Drosophila melanogaster*, by both enzyme activity and cross-reactivity with anti-AK isozyme antibodies [33]. In addition, we recently isolated three cDNAs encoding *Drosophila* AK1, AK2 and AK3 ([33] and K. Fujisawa and T. Noma, unpublished work). Thus the AK isozyme system appears to be generally functioning in vertebrate and invertebrate multicellular organisms. We have also isolated and characterized the mouse genomic *AK3* and *AK4* genes as

independent clones, indicating that they are encoded by distinct genes in the genome (Y. Yamashiro, K. Fujisawa and T. Noma, unpublished work).

Expression of AK3 was observed in all tissues examined at both the mRNA and protein levels, suggesting that the AK3 gene is regulated in a housekeeping manner. In addition, we also examined AK4 protein expression in human and mouse tissues, since the protein expression and enzyme activity of AK4 have not been characterized yet. Interestingly, AK4 expression was detected predominantly in kidney and weakly in liver, heart and brain, indicating that the *AK4* gene is regulated in a tissue-specific manner (Figure 1, central panel, and Figure 2). Recently, we have reported the characterization of the proximal promoter regions of both the bovine *AK3* gene and the human *AK4* gene, which was previously named *AK3* [34,35]. Their proximal promoter regions contained the four conserved sequence motifs and had the high GC content that are frequently observed in housekeeping genes, although other features in the promoter regions were different from each other. For example, multiple transcription-start points were observed in the *AK3* gene, whereas a single transcription-start point was detected in the *AK4* gene. The fact that we found tissue-specific expression of the *AK4* gene in this study suggests that regulatory regions other than the proximal promoter region are important for their differential regulation.

Subcellular fractionation analysis demonstrated that both AK3 and AK4 are localized in the mitochondrial matrix. This finding is consistent with the features of the N-terminal portions of both AK3 and AK4, which may form an amphipathic helical wheel consisting of several positively charged and hydrophobic residues, a characteristic of the mitochondrial targeting signals.

The purified AK3 protein had both GTP:AMP and ITP:AMP phosphotransferase activities, with specific activities of 3200 and 2100 μmol/min per mg of protein. Because of the high sequence similarity between AK3 and AK4 and their similar binding characteristics to phosphocellulose and Affi-Gel Blue columns, as well as their identical localizations in the mitochondrial matrix, we examined whether the AK4 protein has GTP:AMP phosphotransferase activity. Unexpectedly, we could not detect any AK activity in human AK4 protein in either bacterial or mammalian expression systems.

So far, AK isozymes are believed to have two main roles in regulating adenine nucleotide homoeostasis: maintaining the 'energy charge' in the subcellular milieu [1] and participating in high-energy phosphoryl transfer from the sites of ATP synthesis to the sites of ATP consumption via a high-energy phosphoryl transfer chain consisting of multiple AK molecules [36]. Despite numerous studies on the structure of AK proteins, little is known about the specific biochemical role of the AK proteins associated with mitochondrial metabolism or disorder. The proposed role of AK3 in the mitochondria is its function as a scavenger of GTP, which is produced by intra-mitochondrial substrate-level phosphorylation in the citric acid cycle [37]. In contrast with AK3, nothing has yet been reported about AK4 other than its cDNA sequence. This report presents basic information about the localization and AK activity of AK4 in mitochondrial nucleotide metabolism. Furthermore, our recent studies on *in situ* hybridization and immunohistochemical analysis revealed that the AK4 protein is localized in both the cortex and the outer region of the medulla in the kidney, which are mainly composed of proximal and distal tubules (T. Gondo, T. Ishihara, K. Yoshinobu and T. Noma, unpublished work). It is interesting to remember that these tubular epithelial cells have unique-specialized cellular shapes that are involved in the ion-transport process and contain abundant mitochondria in the well-developed basal infolding structure, providing the energy for active transport [38]. Therefore, elucidation of the functional role of AK4 in the kidney will provide new insights into its physiological and biochemical roles in cellular function.

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