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**The characterization of human adenylate kinases 7 and 8 demonstrates differences in kinetic parameters and structural organization among the family of adenylate kinase isoenzymes**

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Short title: Characterization of human adenylate kinases 7 and 8

Differences in expression profiles, substrate specificities, kinetic properties and subcellular localization among the adenylate kinase (AK) isoenzymes, have been shown to be important for maintaining a proper adenine nucleotide composition for many different cell functions. In the present study human AK7 was characterized and its substrate specificity, kinetic properties and subcellular localization determined. In addition, a novel member of the human adenylate kinase family, with two functional domains, was identified and characterized and assigned the name AK8. AK8 is the second known human AK with two complete and active AK domains within its polypeptide chain, a feature that previously was shown also for AK5. The full length AK8, as well as its two domains AK8p1 and AK8p2, all showed similar AK enzyme activity. AK7, full length AK8, AK8p1 and AK8p2 phosphorylated AMP, CMP, dAMP and dCMP with ATP as phosphate donor and also AMP, CMP and dCMP with GTP as phosphate donor. Both AK7 and the full length AK8 showed highest affinity for AMP with ATP as phosphate donor and proved to be more efficient in AMP phosphorylation as compared to the major cytosolic isoform AK1. Expression of the proteins fused with the green fluorescent protein demonstrated a cytosolic localization for both AK7 and AK8.

Keywords: adenylate kinase, nucleotide metabolism, nucleotide phosphorylation

Abbreviations used: AK, adenylate kinase; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; GFP, green fluorescent protein

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## INTRODUCTION

Adenylate kinases (AK) [EC 2.7.4.3] are phosphotransferases that catalyze the interconversion of adenine nucleotides. The adenylate kinases are important in cellular energy homeostasis and the maintenance of high levels of ATP in a combined action of adenylate kinase activity and mitochondrial oxidative phosphorylation [1]. The presence of several isoforms of adenylate kinases in mammalian tissues is already well established [2]. The presence of multiple isoforms is a specific feature of adenylate kinases that is only shared with the guanylate kinases among the nucleotide and nucleoside kinases. Seven different adenylate kinase isoenzymes have been identified in human tissues and six of them have been thoroughly characterized. The major cytosolic isoform AK1 is present at high levels in skeletal muscle, brain and erythrocytes, whereas the major mitochondrial isoform AK2 is expressed in the mitochondrial intermembrane space of tissues rich in mitochondria like liver, heart and skeletal muscle. AK3 and AK4 are located in the mitochondrial matrix, with AK3 having its highest expression levels in the liver, heart and skeletal muscle. AKs 1 – 3 are expressed at higher levels compared to the other AK isoenzymes, AKs 4 – 7, which seem to have more specialized functions in certain cell types. AK4 is expressed at low levels in brain, kidney, liver and heart tissues, and it is reported to contain an N-terminal mitochondrial import sequence that remains uncleaved after import into the mitochondria [3]. AK5 is cytosolic, or both cytosolic and nuclear depending on the transcript variants, with two separate functional domains and, in contrast to the multi-tissue expression profiles of most other AKs, it is expressed almost exclusively in brain [4, 5]. As far as AK6 is concerned, fluorescence microscopy revealed a nuclear localization, but there is no confirmed expression profile available [6]. AK7 seems to have a tissue-restricted expression and its activity has been associated with cilia function [7].

The existence of several AKs with different subcellular localizations, expression profiles, substrate specificities and kinetic properties provides evidence of specialized functions in specific cellular processes. Our studies aim at getting a complete picture of all AKs expressed in mammalian cells and their role in the homeostasis and synthesis of adenine nucleotides that are required for a variety of cellular metabolic processes. The AKs are also important for DNA and RNA synthesis and may contribute to the activity of pharmacologically active nucleoside and nucleotide analogs.

In this study we have expressed and characterized AK7 and determined its substrate specificity, kinetic properties and subcellular localization. In addition, a novel member of the AK family with two functional domains has been identified and characterized and it was assigned the name AK8. By the identification and characterization of AK7 and AK8 we believe that the identification of members of the AK family of isoenzymes is close to being completed. Our results demonstrate that in addition to differences in tissue expression, subcellular localization and structural organization, the AKs show differences in kinetic parameters with both high and low substrate affinity enzymes.

## EXPERIMENTAL

### Cloning and sequencing

The IMAGE clone 4828427 (GenBank ID: BC035256) for AK7 was purchased from RZPD and the IMAGE clone 5744517 (GenBank ID: BC050576) for AK8 was purchased from the American Type Culture Collection.

AK7 cDNA was cloned into NdeI/BamHI sites of the pET16b plasmid vector (Novagen) using the primers: AK7 forward, 5'-

GAAGGTCGTCATATGGCTGAAGAAGAGGAAACTGCTGCTCTCA-3' with Nde1 site and AK7 reverse, 5'-CGCGGATCCTCACTGTGCTTCAGGATTGTTCTTGAAGAGATATT-3' with BamH1 site and into Nhe1/BamH1 sites of pEGFP-N1 plasmid vector (Clontech) using the primers: AK7GFP forward, 5'-TCGAGCTAGCATGGCTGAAGAAGAGGAAACTGCTGCTCTCACG-3' with Nhe1 site and AK7GFP reverse, 5'-GTGAGGATCCCGTCCAGCGAGCTATCTTC-3' with BamH1 site.

AK8, AK8p1 and AK8p2 cDNAs were cloned into Nde1 site of pET16b using the primers: AK8p1 forward, 5'-GAAGGTCGTCATATGGACGCCACTATCGCCCCGCACCGTATC-3', AK8p1 reverse, 5'-GAAGGTCGTCATATGTCACGGGGTGAACGGGGCATTAGTACCGATGGTT-3', where a stop codon was introduced, AK8p2 forward, 5'-GAAGGTCGTCATATGGCCCCGTTACCCCCGAGGGTGCTGCTGCTC-3', where a start codon was introduced, and AK8p2 reverse, 5'-GAAGGTCGTCATATGTCAGGGGATTTTCTTGGGCAGGGGATTAAT-3'. AK8p1 forward and AK8p2 reverse primers were used for the cloning of the full length AK8. For cloning of full length AK8 into Xho1/EcoR1 sites of pEGFP-N1 the oligonucleotides AK8GFP forward, 5'-AGATCTCGAGCTGGAAGTTCTGTTCCAG-3' with Xho1 site and AK8GFP reverse, 5'-GTGAGAATTCGGGGGATTTTCTTGG-3' with EcoR1 site were used. The plasmids were sequenced by MWG-Biotech (Ebersberg Germany) to verify the DNA sequences.

### Expression and purification

The plasmids were transformed into the *Escherichia coli* strain BL21 (DE3) pLysS (Stratagene) and single colonies were inoculated into LB-medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. Bacteria were grown at 37 °C and protein expression was induced at OD<sub>600</sub> 0.9 with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 12 h at 27 °C. The expressed proteins were purified using TALON Metal Affinity Resin (Clontech) as described by the manufacturer. The size and purity of the recombinant proteins were verified by SDS-PAGE and the protein concentration was determined with Bradford Protein Assay (BIO-RAD) using BSA as standard. The proteins were aliquoted and stored at -80 °C.

### In vitro translation

AK8, AK8p1 and AK8p2 were synthesized with PURExpress® *In Vitro* Protein Synthesis Kit (NEB) in the presence of [<sup>35</sup>S] methionine (Perkin Elmer), following the manufacturer's instructions. 2 µl of each reaction and 5 µl of Rainbow [<sup>14</sup>C] methylated protein molecular weight marker (Amersham) were loaded on a NuPAGE 4-12% Bis-Tris gel (Invitrogen). The gel was washed with Gel-Dry drying solution, dried with Dry Ease Mini Cellophane (Invitrogen) and used for autoradiographic detection.

### Enzyme assays

The substrate specificity of the recombinant AK7, the full length AK8 and its two domains were assayed by thin layer chromatography (TLC) as described before [8]. The nucleoside monophosphates and triphosphates were purchased from Sigma. [<sup>32</sup>P] ATP/GTP and [<sup>32</sup>P] UTP/CTP/TTP were purchased from Perkin Elmer. All assays were performed in 10 µl-volume reactions containing 50 mM Tris-HCl pH 7.6, 5 mM MgCl<sub>2</sub>, 1 mM unlabeled NTPs, 0.1 µCi/µl radiolabeled NTPs (3000 Ci/mmol), 1 mM NMPs or dNMPs and 0.5 µg of recombinant enzyme. After incubation for 1 h at 37 °C, 2 µl of the reaction products were

separated on poly (ethyleneimine)-cellulose F chromatography sheets (Merck); soaked in methanol prior to use. The nucleotides were separated in 0.5 M ammonium formate pH 3.5 for 2 h. TLC sheets were then autoradiographed by phosphorimaging plates (BAS 1000, Fuji Photo Film).

To determine the kinetic properties of AK1, AK7 and AK8, the non-radiolabeled products were separated and quantified by reversed-phase high-performance liquid chromatography (HPLC) using a Chromolith™ column (RP-18e, 100-4.6 mm) (Merck) as described before [9]. All assays were performed in 25 µl-volume reactions containing 5 mM of phosphate donor, 50 mM Tris-HCl pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM DTT and 0.5 mg/ml BSA. 25 ng of AK7, 20 ng of AK8 and 10 ng of AK1 and different concentrations of substrate (from 1 µM to 10 mM) were used to determine the  $K_m$  and  $V_{max}$  values and thereafter the  $V_{max} / K_m$  ratios were calculated. Human AK1 was purchased from Abcam.

### Cell culture and transfection

HeLa cells were seeded in µ-Slides (Ibidi) and cultured in Dulbecco's Modified Eagle's Medium with 10% (v/v) fetal calf serum (Gibco BRL, Carlsbad, CA, USA), 100 U/ml penicillin and 0.1 mg / ml streptomycin. Cells were grown at 37 °C in a humidified incubator with 5 % CO<sub>2</sub>. Plasmids were transfected into cells using FuGENE HD transfection reagent (Roche) as described in the manufacturer's protocol. The cells were stained 30 min with 100 nM MitoTracker Red (Molecular Probes) 48 h after transfection. Cell fluorescence was imaged using a Leica DMI6000B with Leica Application Suite AF software v1.8.

## RESULTS

### Expression of AK7 and the different forms of AK8

The cDNA predicted to encode AK7 is longer than previously identified sequences of single-domain AKs (figure 1A). The AK7 encoding cDNA contained an N-terminal 368 amino acid sequence with no clear homology to any known sequence. The full length AK7 cDNA was expressed and shown to produce a 656 amino acid protein with a molecular weight of ~ 74 kDa. According to the databases, the gene product was expected to be 723 amino acid residues but the presence of a stop codon at the end part of the gene product cloned in this study suggests that there may exist more than one transcript variants of AK7. The gene is localized on chromosome 14 and consists of 18 exons with a total length of 96.67 kb. The AK domain of AK7 showed high conservation with the other single domain AKs, AK1-4 and AK6 (figure 1B).

The cDNA of AK8 was identified in the same genomic library in the open reading frame 98 of chromosome 9 and its expression produced a 479 amino acid protein. Since the AK8 polypeptide contains two p-loops, we expressed its possible two domains by inserting: i) a stop codon before the second p-loop to translate only the first domain (268 amino acids) and ii) a start codon before the second p-loop to translate only the second domain (216 amino acids) (figure 2A). Our previous studies of other AKs suggest that a short N-terminal amino acid fragment before the p-loop structure is crucial for the correct folding and activity of the enzyme. A start codon was therefore introduced in a way that an N-terminal fragment, consisting of amino acid residues MAPFTP, is present before the p-loop of AK8p2. The two-domain AK protein sequence of AK8 is similar to the previously characterized two-domain sequence of AK5 (figure 2B). The full length AK8 and its two domains were synthesized with a cell-free *in vitro* translation system in order to verify their expression and their sizes that were ~53, 33 and 27 kDa for full length AK8, AK8p1 and AK8p2 respectively (figure 3). A phylogenetic tree analysis suggests that AK7 and AK8 are more distantly related to AK1 than the previously characterized AKs (figure 4).



### Substrate specificity of AK7 and the different forms of AK8

An initial screening of phosphotransferase activity was performed using TLC. AMP, CMP, GMP, UMP, dAMP, dCMP, dGMP, dTMP and dUMP were tested as phosphate acceptors at 1 mM concentrations with 1 mM of the phosphate donors ATP, CTP, GTP, TTP or UTP. Similar substrate specificity was observed for all of the investigated enzymes. With ATP as phosphate donor AK7 (data not shown), AK8, AK8p1 and AK8p2 phosphorylated AMP, CMP, dAMP and dCMP and in addition to that they phosphorylated AMP, CMP and dCMP when GTP was used as phosphate donor (figure 5). There was no phosphorylation of substrates using CTP, TTP or UTP as phosphate donors. The phosphorylation reactions were confirmed by using the *in vitro* translated enzymes to exclude the possibility of bacterial contamination of the recombinant enzyme preparations (data not shown).

### Kinetic properties of AK7 and AK8

The substrates and phosphate donors identified to be most efficiently utilized by AK7 and AK8 in the initial TLC screening were used to determine the kinetic properties of the enzymes. AK7 showed the highest affinity for AMP with ATP as phosphate donor with a  $K_m$  of 1  $\mu\text{M}$  and a  $V_{max}$  of 1130 pmol/ $\mu\text{g}/\text{min}$  (table 1). The affinity for dAMP and CMP was also high with a  $K_m$  of 28  $\mu\text{M}$  and a  $V_{max}$  of 860 pmol/ $\mu\text{g}/\text{min}$  for dAMP and a  $K_m$  of 1.2  $\mu\text{M}$  and a  $V_{max}$  of 150 pmol/ $\mu\text{g}/\text{min}$  for CMP. The affinity of dCMP for AK7 with ATP as phosphate donor was too low for kinetic determinations. The best substrate for AK8 was AMP when ATP was used as phosphate donor, followed by CMP (table 2). AK8 was less efficient in dAMP phosphorylation, as compared to AK7, with a  $K_m$  of 630  $\mu\text{M}$  and a  $V_{max}$  of 1360 pmol/ $\mu\text{g}/\text{min}$ . As judged by the TLC assays AMP was the preferred substrate for both AK7 and AK8 also when using GTP as phosphate donor but the affinity of the substrates were too low to obtain accurate values for kinetic determinations.

A comparison of the kinetic parameters of AMP as substrate for AK7 and AK8 with the more abundant AK1 showed that the  $K_m$  of AK1 was in the millimolar range whereas the  $K_m$  of AK7 and AK8 were in the micromolar range (table 3). Also the  $V_{max}$  of AK1 was in a different range as compared to the  $V_{max}$  of AK7 and AK8. The ratio  $V_{max}/K_m$ , that is used to define the overall efficiency of an enzyme, was higher for AK7 and AK8 as compared to AK1.

### Subcellular localization of AK7 and AK8

The subcellular prediction softwares used did not indicate any targeting signal within the amino acid sequences of the investigated enzymes. Nevertheless, the highest probability, according to the softwares, was a cytosolic localization for both enzymes. This was confirmed by *in vivo* expression of AK7 and full length AK8 as fusion proteins with the Green Fluorescent Protein (GFP) demonstrating that both proteins had a cytosolic localization (figure 6).

## DISCUSSION

With the data of the present study the human AK family of enzymes now has 8 members. These enzymes have high sequence similarity with distinguishable conserved AK domains and catalyze similar reversible phosphoryl transfer reactions between adenine nucleotides. In this study AK7 and AK8 were expressed and characterized. Both AK7 and AK8 were shown to have the highest affinity for AMP as substrate but both these enzymes also recognized dAMP, CMP and dCMP as substrates. The preferred substrate of all AKs is

AMP and their main phosphate donor is ATP, although some can phosphorylate several other substrates and use other NTPs as phosphate donors. AK1 and AK2 can utilize all NTPs as phosphate donors but they specifically recognize AMP as substrate and, as we show in the present study, AK1 also phosphorylates dAMP [10]. AMP is also the main phosphate acceptor of AK3, but the phosphate donor specificity is restricted to GTP and ITP [11]. AK4 phosphorylates AMP, dAMP, CMP and dCMP with ATP and GTP as phosphate donors, a pattern of substrate and phosphate donor specificity that AK4 shares with AK5, AK7 and AK8 with some differences in kinetics [3, 5]. AK6 can use all phosphate donors to phosphorylate AMP, dAMP, CMP and dCMP [6]. Taken together, the common feature of the different AKs is to participate in AMP conversion and ATP homeostasis. Whether the additional substrate affinities have any physiological importance has not yet been shown.

There are significant differences in the phosphorylation efficiency among the AKs demonstrated both in the present and earlier studies. When the kinetic properties of AK7 and AK8 were determined, AK1 was included as a reference enzyme since AK1 is the major AK in human cells. Both AK7 and AK8 showed a higher affinity for AMP as substrate, as compared to AK1, but a much lower maximal catalytic rate. When both substrate affinity and maximum velocity of AMP phosphorylation were combined and expressed as  $V_{max}/K_m$ , both AK7 and AK8 showed higher efficiency in AMP phosphorylation as compared to AK1. This feature could compensate the low expression levels of these enzymes compared to the expression profile of AK1. The high AMP affinity of AK7 and AK8 may relate to specific functions in certain cell types, such as kinocilia-bearing cells where AK7 was initially identified [7]. There may be a special demand on high local ATP production in kinocilia expressing cells as well as in other cells with similar high energy demanding functions. Of the previously characterized human AK4, AK5 and AK6, all of these enzymes have different kinetic parameters that may indicate that they contribute to adenosine nucleotide homeostasis in different microenvironments and have specific cellular functions [3, 5, 6].

All previously reported AKs with a single AK domain (AKs 1-4 and AK6) have a size of around 200 amino acids whereas the presently studied AK7 is substantially larger. However, the AK domain of AK7 has approximately the size of the single-domain AKs. The first N-terminal 368 amino acid domain of AK7 does not contain any of the conserved AK sequences and other indications of its potential function were not suggested by blast searches of this N-terminal sequence. Whether this domain encodes a functional polypeptide or not, there is no evidence for any contribution to the AK activity of AK7. In a previous study human AK5 was identified to have two enzymatically active AK domains and we now demonstrate the existence of a second human AK with two complete AK domains. This enzyme was named AK8 because it was the eighth human AK isoenzyme identified. Although AK8 has a similar two-domain structure as found in AK5, AK8 is different from AK5 in that it apparently has only one transcript whereas the AK5 open reading frame has two transcripts, which differ in a 27 amino acid Tag sequence [4]. However, when the two AK domains of AK8 were expressed separately they both showed similar AK activity. The AK8 protein thus may harbor two functionally active domains in the same polypeptide chain, something that is supported by the activity data presented in our study. Whether different AK isoenzymes can enhance their combined activity by formation of functional complexes with multiple sequential catalytic domains is an important issue for future investigations. As of today only monomer forms of AKs are known, but future studies may reveal if oligomerisation is of physiological significance for regulating the activity of the AK isoenzymes. According to databases and the literature there are at least two AKs in lower species with more than one possible catalytic domain; a *Drosophila melanogaster* predicted AK that consists of 562 amino acids with two domains and a sea urchin sperm flagellar AK (920 amino acids) with triplicated domains [12]. With the picture getting completed regarding

the members of the human AK family, the question addressed was how many AKs exist in other organisms. *Escherichia coli* is known to encode for one single AK and interestingly when searching the databases the number of AK isoenzymes appeared to increase with more complex organisms. According to the databases *Saccharomyces cerevisiae* encodes 3 AKs, *Caenorhabditis elegans* encodes 4 AKs and *Drosophila melanogaster* encodes 6 different AK isoenzymes.

There are correlations found between AK expression and certain human disorders although conclusive confirmation of genetic AK alterations as the cause of the disease may still be lacking. In humans, AK1 deficiency caused by a nonsense homozygous mutation, has been associated with mild chronic haemolytic anaemia and psychomotor impairment [13]. Reticular dysgenesis, the most severe form of human inborn immunodeficiencies, is characterized by complete absence of granulocytes and lymphocytes and has been linked to mutations of the AK2 gene [14]. The mitochondrial localization of AK2 implies a central role in providing the energy required for the proliferation of hematopoietic precursors and in controlling cell apoptosis [15, 16]. Increased AK4 protein levels have been detected in cultured cells exposed to hypoxia and in an animal model of amyotrophic lateral sclerosis, a neurodegenerative disease in which oxidative stress is implicated [17]. A recent study showed that pancreatic  $\beta$ -cells express AK1 and AK5 that play an important role in regulating K-ATP channel activity and thereby control insulin secretion [18]. Autoantibodies targeted against AK5 have been traced in two cases of non-viral immune-mediated limbic encephalitis [19]. The identification of the nuclear-localized AK6 gave evidence on nucleotide metabolism in the nucleus and the energy supply routes between the mitochondria and the nucleus [20]. The involvement of AK7 in human disease has been reported in recent studies where it was related to the expression of AK7 in tissues rich in epithelium with cilia [7]. The AK8 gene (C9ORF98) was identified to be one of the genes that have a negative regulatory role in epithelial cell migration [21]. Both AK1 and AK7 have been deleted in mice models and found to have specific phenotypic alterations. In a study of AK1-deficient mice they were shown to have low AMP-activated protein kinase (AMPK) phosphorylation in skeletal muscle, consistent with limited AMP production [22]. In addition, deletion of the AK1 gene accelerated the loss of cardiac contraction on ischemic challenge [23]. An AK7-deficient mouse model presented pathological signs of microtubular defects, decreased ciliary beat frequency, hydrocephalus, abnormal spermatogenesis, mucus accumulation and acute respiratory responses upon allergen challenge [7]. In summary, the vast literature on AK associated alterations and disorders supports the importance of AK activity for many vital processes in humans and in other organisms.

The number of AK isoenzymes present in different subcellular localizations and with different tissue and cell expression illustrates the importance of maintaining a sufficient AK activity for proper cell and organism functions. All of the 8 human AKs that now have been characterized catalyze the reversible reaction  $ATP + AMP \leftrightarrow 2ADP$  and their contribution to energy transfer and metabolic signaling has been demonstrated to be central for cell viability and functions. The present study contributes to complete the picture of the family of human AK isoenzymes and should be an important contribution to clarify their role in human health and disease.

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**Table 1.** HPLC-based kinetic properties of the recombinant human adenylate kinase 7 with ATP as phosphate donor. Values are presented as mean  $\pm$  standard deviation from three independent experiments. NM, not measurable.

Substrate	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ (pmol/ $\mu\text{g}/\text{min}$ )	$V_{max}/K_m$
AMP	1 $\pm$ 0.2	1130 $\pm$ 17	1130
dAMP	28 $\pm$ 12	860 $\pm$ 19	31
CMP	1.2 $\pm$ 0.8	150 $\pm$ 59	125
dCMP	NM	NM	-

**Table 2.** HPLC-based kinetic properties of the recombinant human adenylate kinase 8 with ATP as phosphate donor. Values are presented as mean  $\pm$  standard deviation from three independent experiments. NM, not measurable.

Substrate	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ (pmol/ $\mu\text{g}/\text{min}$ )	$V_{max}/K_m$
AMP	4.1 $\pm$ 0.6	2400 $\pm$ 460	585
dAMP	630 $\pm$ 36	1360 $\pm$ 71	2.2
CMP	1.4 $\pm$ 0.9	190 $\pm$ 79	136
dCMP	NM	NM	-

**Table 3.** HPLC-based kinetic properties of the recombinant human adenylate kinase 1 with ATP as phosphate donor. Values are presented as mean  $\pm$  standard deviation from three independent experiments. NM, not measurable.

Substrate	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ (pmol/ $\mu\text{g}/\text{min}$ )	$V_{max}/K_m$
AMP	2400 $\pm$ 840	156000 $\pm$ 12000	65
dAMP	18100 $\pm$ 1500	265000 $\pm$ 11000	14.6

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## FIGURE LEGENDS

### Figure 1 Human adenylate kinase 7

(A) Map of human AK7. (B) Alignment of human AK7 with the other one-domain human AKs (black boxes indicate completely conserved amino acid residues and different shades of grey indicate different levels of conserved residues).

### Figure 2 Human adenylate kinase 8

(A) Map of the two-domain human adenylate kinases, AK8 and AK5. (B) Alignment of AK8 and AK5 (black boxes indicate completely conserved amino acid residues and different shades of grey indicate different levels of conserved residues).

### Figure 3 SDS-PAGE of the *in vitro* synthesized proteins

Lane 1; AK8p1, lane 2; AK8p2 and lane 3; full length AK8.

### Figure 4 The human adenylate kinase isoenzymes

A phylogenetic tree of the human adenylate kinases shows that AK7 and AK8 are more distantly related to AK1 as compared to AK2-AK6.

### Figure 5 Substrate specificity of human adenylate kinase 8 and its two domains

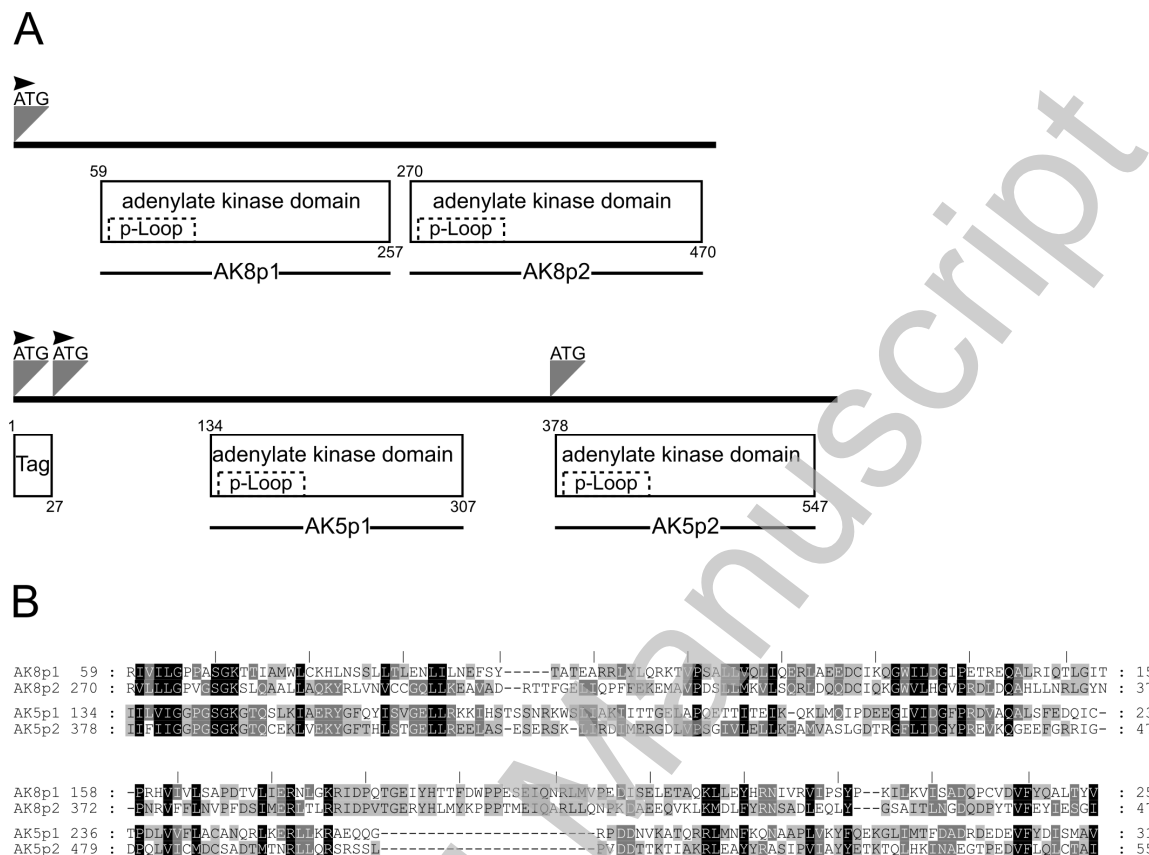
Screening of ribo- and deoxyribonucleoside monophosphate specificity of full length AK8, AK8p1 and AK8p2. 1mM of NMPs and dNMPs were used as substrates with 0.5  $\mu$ g of enzyme. (A) Unlabeled ATP and [ $\gamma$ - $^{32}$ P] ATP was used as phosphate donor. (B) Unlabeled GTP and [ $\gamma$ - $^{32}$ P] GTP was used as phosphate donor. (C<sub>1</sub>: control without enzyme or substrate; C<sub>2</sub>: control without substrate). Figures shown here are representative of at least three repetitions of each experiment.

### Figure 6 Cytosolic localization of human adenylate kinases 7 and 8

Fluorescent microscopy of HeLa cells expressing GFP (control), fusion protein AK7 with GFP and fusion protein full length AK8 with GFP. The mitochondria were contra-stained with Mitotracker.

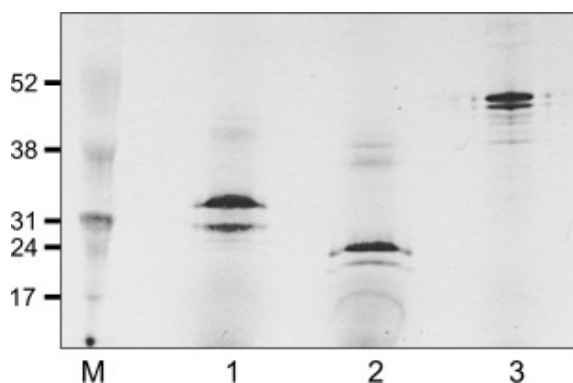


**Figure 2**



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**Figure 3**

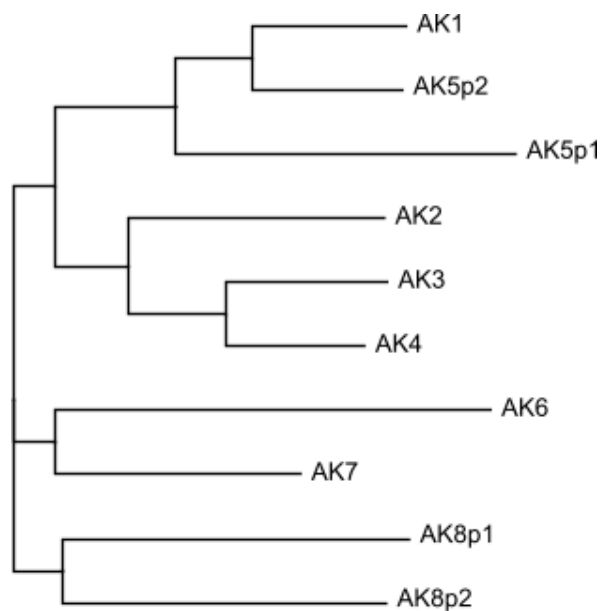
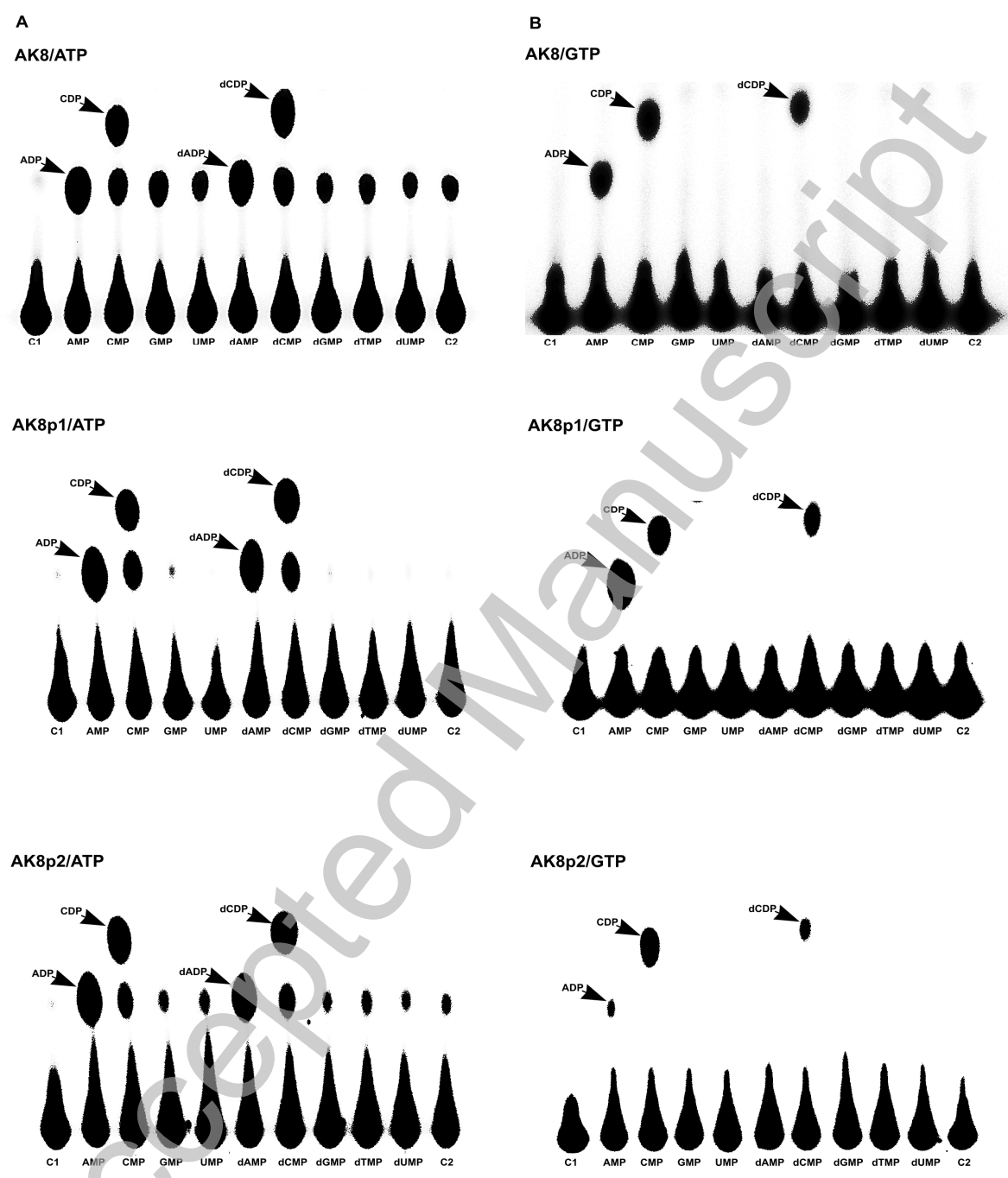
**Figure 4**



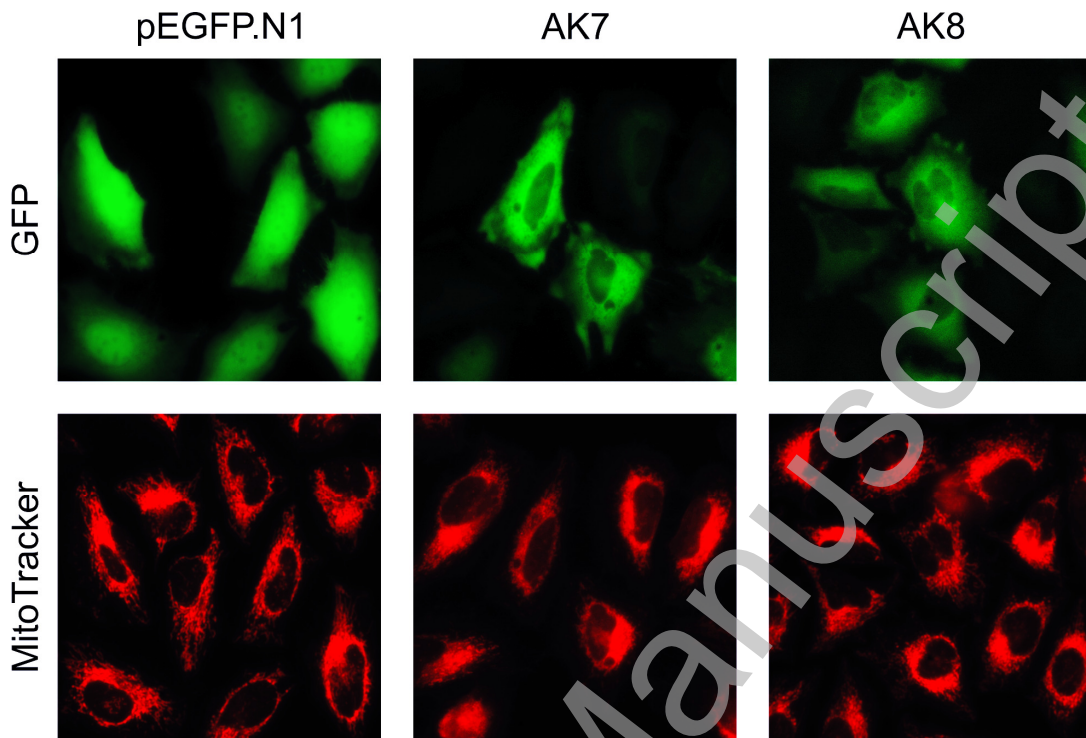
Figure 5



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**Figure 6**



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