A novel nuclear-localized protein with special adenylate kinase properties from *Caenorhabditis elegans*

Ruitong Zhai\textsuperscript{a,c,1}, Geng Meng\textsuperscript{a,b,1}, Yanmei Zhao\textsuperscript{a}, Bin Liu\textsuperscript{a}, Genfa Zhang\textsuperscript{c}, Xiaofeng Zheng\textsuperscript{a,b,}\textsuperscript{*}

\textsuperscript{a} National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China
\textsuperscript{b} Department of Biochemistry and Molecular Biology, College of Life Sciences, Peking University, Beijing 100871, China
\textsuperscript{c} College of Life Sciences, Beijing Normal University, Beijing 100873, China

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Abstract The adrenal gland protein AD-004 like protein (ADLP) from *Caenorhabditis elegans* was cloned and expressed in *Escherichia coli*. Enzyme assays showed that ADLP has special adenylate kinase (AK) properties, with ATP and dATP as the preferred phosphate donors. In contrast to all other AK isoforms, AMP and dAMP were the preferred substrates of ADLP; CMP, TMP and shikimate acid were also good substrates. Subcellular localization studies showed a predominant nuclear localization for this protein, which is different from AK1–AK5, but similar to that of human AK6. These results suggest that ADLP is more likely a member of the AK6 family. Furthermore, RNAi experiments targeting ADLP were conducted and showed that RNAi treatment resulted in the suppression of worm growth.

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Keywords: AD-004 like protein; Adenylate kinase activity; Nuclear localization; RNAi; *Caenorhabditis elegans*

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1. Introduction

Adenylate kinase (AK) (EC 2.7.4.3) is a nucleoside monophosphate kinase (NMPK) that catalyzes a reversible phosphotransferase between nucleoside triphosphates and monophosphates. It plays an important role in cell metabolism, including the synthesis of DNA and RNA molecules [1]. Adenylate kinases can also phosphorylate nucleoside analogs used in the treatment of cancer and viral infection [1–7]. In mammalian tissues, five isoforms of AK, AK1 to AK5, have been identified [8,9]. Recently, a new AK isoform that has a different subcellular localization, AK6, has been identified and characterized by crystal structure determination and enzymatic assays [9].

Adrenal gland protein AD-004 like protein (ADLP) from *Caenorhabditis elegans* (*C. elegans*) is a functionally unknown protein containing 182 amino acids. A Walker motif with conserved sequence Gly-X-X-Gly-X-Gly-Lys in its N-terminal region was found [10] that encodes the phosphate-binding loop and is one of the characteristics of ATPases and NMPKs (Fig. 1A). This suggested that ADLP might possess ATPase activity, adenylate kinase activity, or both. Furthermore, the sequence alignment indicated that ADLP shares about 40% homology with the newly identified human AK6 (Fig. 1B), though the sequence similarity with other AKs (AK1–AK5) is lower than 20%. This promoted us to determine whether ADLP from *C. elegans* possesses features that resemble human AK6 and belong to this novel AK isoform family. So far, in *C. elegans*, only C.AK1 was found according to the NCBI annotation, but no further information relating to C.AK1 has been reported [11].

In this paper, we report the cloning and characterization of AD-004 like protein from *C. elegans*. Enzymatic activity assays and subcellular localization of this protein demonstrate that it is a novel adenylate kinase in *C. elegans*, and is similar to human AK6. Result from RNA interference (RNAi) experiments suggest that this protein may play important role in worm growth.

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2. Materials and methods

2.1. Cloning and expression of *C. elegans* ADLP

Total RNA was extracted from *C. elegans* using TRIZOL Reagent (Invitrogen, USA), and the cDNA of the target gene was amplified by reverse transcriptase coupled PCR (RT-PCR) (AccessQuick RT-PCR System, Promega) following the manufacturer’s instructions. The primers were designed by introducing NdeI and BamHI restriction enzyme sites as follows: 5'-gaattctatgtgaacagccagaaactgc-3' (the underline indicates the NdeI site) and 5'-caggatctacaatcctggggtggtgc-3' (the underline indicates the BamHI site). The amplified cDNA fragment was inserted into a PET28a vector. The positive clone was confirmed by PCR and DNA sequencing, followed by transformation into *Escherichia coli* expression strain Rosetta (DE3). The expression, solubility test and large-scale purification of the recombinant protein were performed by following the procedure from our previous study [12].

2.2. Enzyme assays

Using the purified recombinant protein, enzymatic activity of ADLP was measured following the method described previously [9]. The nucleoside monophosphates and triphosphates were obtained from Sigma. [γ-\textsuperscript{32}P]-ATP (5000 Ci/mmol) and [α-\textsuperscript{32}P]-AMP (3000 Ci/mmol) were obtained from Furui Company, China. A phosphoryltransfer assay was performed with [γ-\textsuperscript{32}P]-ATP as the phosphate donor and non-radioactive nucleoside monophosphates (Sigma, USA) as substrates. Each standard reaction mixture contains 50 mM Tris–HCl (pH 7.5), 5 mM DTT, 5 mM MgCl₂, 2 mM NMP, 1 mM [γ-\textsuperscript{32}P]-ATP and 50 ng of tested protein in a total volume of 50 μl. The reaction solution was incubated at 37 °C for 20 min, stopped by heating at 70 °C for 2 min, and the sample was centrifuged at 3000 rpm for 10 s. The reaction products were separated by thin-layer chromatography on polyethyleneimine-cellulose F chromatography sheets (Merck, USA), which were autoradiographed using a storage phosphor screen (Amer sham Biosciences, USA). A reaction mixture without ADLP was used
as negative control to subtract the background; while a commercially available AK1 (Sigma) was used as positive control. Screening of phosphate donor was performed with $[^{32}P]$-AMP as substrate and non-radioactive nucleoside triphosphate as phosphate donor following the procedure described above.

### 2.3. Green fluorescent protein fusion protein

Both N-terminal and C-terminal green fluorescent protein (GFP) fusion ADLP proteins were constructed by using the following primers: 5' - cgggaattc atgttgcttccgaacatcc - 3' (the underline indicates the EcoRI site) and 5' - cgggatcc agagttatgatctttgatcc - 3' (the underline indicates the BamHI site).

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**Fig. 1.** Alignment of the amino acid sequences of ADLP (C.AK6) with NMPKs. The alignment was performed using the program Clustal X [31]. The sequences, identified using EMBL-EBIFASTA [32], are C. elegans AK1 (C.AK1), C. elegans AK6 (C.AK6), human AK1–6, human CMPK, GMPK and TMPK in (A), and eukaryotic AK6 homologs from human (1RKBA), C. elegans (NP_496065), Drosophila (NP_610797) and Arabidopsis (BAC42255) in (B). The asterisks and black boxes indicate the strictly conserved residues. Secondary structure elements are depicted based on the human AK6 structure [9].

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the BamHI site. HeLa cells were used to carry out transfection assays as described previously [9], and visualized with a fluorescent microscope 24 hours after transfection. The nuclear dye 4,6-diamidino-2-phenyl-indole-dihydrochloride (DAPI) (1 μg/ml in mowiol) was used to stain DNA and visualize the nucleus.

2.4. RNA interference of ADLP by feeding in C. elegans

An RNA interference (RNAi) assay was performed as described by Kamath [13] with the following minor modification: the cDNA that encodes ADLP was cloned into the L4440 feeding vector (pPD129.36) [14] using PstI and BglII sites. The resulting plasmid was transformed into E. coli strain HT115 (DE3), and the L4440 vector was used as negative control. Expression of the dsRNA was induced overnight at room temperature. Two L4-stage worms were placed onto each NGM plate, followed by incubation at 20 °C for 2 days. The two L4-stage worms were re-picked, transferred onto new sets of plates, and incubated at 20 °C for 3 or 4 days for egg laying.

2.5. Real-time quantitative RT-PCR

The loss of the ADLP transcripts following RNAi was examined by real-time quantitative RT-PCR. Total RNA samples, extracted from RNAi-treated or control C. elegans, were reverse-transcribed as described above. Real-time RT-PCR was performed with the SYBR Green qPCR kit (Finnzymes) in a DNA Engine Opticon continuous fluorescence detection system (MJ Research) according to the manufacturer's instructions. The expression level of the target gene in RNAi-treated worms was compared with that of the non-treated worms. To minimize mRNA quantification errors and genomic DNA contamination biases, and to correct for inter-sample variations, we used the isocitrate dehydrogenase (IDH) gene (F35G12.2), a housekeeping gene of C. elegans, as an internal control, and the relative expression ratio was based on the expression of a target gene relative to that of IDH. Primers used for detection of ADLP (Accession No. NM_063664) transcripts were 5′-ctggtgtacacaggtg-3′ and 5′-tacctgcagcttctacgtc-3′, and those for IDH (Accession No. NM_065526) were 5′-gatggtaagggacatctggattcg-3′ and 5′-gatggtaacacaggttct-3′. Significance was determined with a T test (P < 0.05).

2.6. Western blotting

Antiserum against the recombinant protein was prepared by immunizing a rabbit following a procedure described previously [9]. Western blot analysis for ADLP was conducted on the protein extracts from wild-type adult worms and RNAi-treated worms by using the anti-ADLP, β-Actin was used as an internal control for normalizing the loading materials. The proteins were extracted from worms with the lysis buffer [50 mM Tris·HCl (pH 8.0), 300 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] and electrophoresed on a 15% SDS–PAGE apparatus, followed by transferal onto a nitrocellulose membrane using a wet blotting device (Bio-Rad, USA). The membrane was incubated with anti-ADLP (1:1000) or anti-β-Actin (1:500, Santa Cruz, CA, USA) primary antibodies, and subsequently incubated with anti-rabbit IgG-HPR (1:300, Huamei Company, China) secondary antibody. The membrane was developed in ECL chemiluminescence reagent (Pierce, USA) and exposed to X-ray film (Kodak, USA).

3. Results

3.1. Cloning and expression of ADLP in E. coli

The cDNA of ADLP was successfully obtained by RT-PCR, which was consistent with its predicted length, 546 bp (Fig. 2A), followed by insertion into vector pET28a, and verification by PCR analysis and DNA sequencing. An N-terminal (His)6-tag was utilized, which is convenient for protein purification.

Optimization of the expression conditions showed that ADLP was expressed well in Rosetta (DE3) at 30 °C for obtaining the maximum amount of soluble proteins. Purification of recombinant protein was performed in two steps using a Ni-affinity column and a Superdex-75 column. SDS–PAGE analysis of the purified protein showed only one band at the position corresponding to the predicted molecular weight (Fig. 2B), which was also verified by Western blot with monoclonal His-tag antibody (data not shown). The concentration of the purified protein was estimated with the Bio-Rad Protein Assay Kit, and the results showed that about 10–15 mg recombinant protein can be obtained from 1 L culture with at least 95% purity.

An antiserum against ADLP was obtained by immunizing a rabbit with the purified recombinant ADLP, and the internal expression of ADLP in C. elegans was verified by Western blot as shown in Fig. 2C.

3.2. ADLP shows the enzymatic activity of an adenylate kinase

First, a phosphoryltransfer assay with [γ-32P]ATP as a phosphate donor was performed to evaluate the catalytic activity of C. elegans ADLP, to see whether it possesses the activity of an adenylate kinase. As shown in Fig. 3A, ADLP showed adenylate kinase activity.

Therefore, we performed the following experiments to further characterize the detailed features of ADLP. Different substrates AMP, CMP, TMP, UMP, GMP, IMP, dAMP, dGMP, dCMP, and diMP were screened with the phosphate donor ATP. A commercial AK1 from chicken (Sigma) was used as a positive control. According to the results obtained (Fig. 3B), AMP and dAMP were the best phosphate acceptors of all tested NMPs. CMP and TMP showed significant activity, dCMP can also be phosphorylated to some extent, while IMP and UMP showed weak activity. Since there is structural similarity between the AK family and bacterial shikimate kinase [15], non-nucleotide substrate such as shikimate acid was examined here with the result showing that shikimate acid

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Fig. 2. Cloning, expression and purification of ADLP in E. coli Rosetta (DE3) strain, and examination of internal ADLP in C. elegans. (A) The cDNA of ADLP obtained by RT-PCR examined on a 1.0% agarose gel. (B) Purified ADLP checked by SDS–PAGE. (C) Internal expression of ADLP in C. elegans examined by Western blot analysis by using the antiserum of ADLP generated from rabbit (lane 1), and the recombinant ADLP was used as a positive control (lane 2).
could be phosphorylated in addition to the monophosphates described above (Fig. 3C).

The preferred phosphate donors were examined by testing ATP, GTP, CTP, UTP, TTP, ITP, dATP, and dGTP, using [α-32P] AMP as substrate. The results showed that ATP was the best phosphate donor, and dATP was a good phosphoryl donor, while the others were poor donors in comparison to ATP and dATP (Fig. 3D).

3.3. ADLP localized to the nucleus of HeLa cells

A potential nuclear localization motif rich in basic amino acids at the N-terminus of ADLP, RRRPN, was identified according to the predictive result of PSORT II [16] (Fig. 1). To detect the subcellular localization of ADLP, we fused it to GFP. Fig. 4 showed the fluorescence microscopy of N-terminal GFP fused ADLP, which localized to the nucleus, as demonstrated by the staining of the nucleus with DAPI. Similar results were observed from a C-terminal GFP fusion protein (data not shown).

3.4. RNAi in C. elegans

To investigate the effects of RNAi on growth, worm size and life span in detail, we have closely observed the worms’ development at 20 °C. Visible RNAi phenotype in RNAi-treated plates was observed compared to control. Following exposure to the corresponding dsRNA, compared to the control worms, a slow growth (Gro) defect was induced in the F1 generation. The size of worms in the plates treated with RNAi appears smaller than those in control plates, although there is no difference in configuration and movement (Fig. 5A).

To confirm that the observed slow growth of worms was due to silencing of ADLP transcription following RNAi treatment, we quantitatively determined the level of ADLP expression through real-time RT-PCR and Western blot analysis in RNAi-treated and wild-type worms. Fig. 5B showed the mRNA level in the absence of ADLP cDNA from RNAi-treated worms was only about 38% of that of the control without RNAi treatment, which indicated that the expression levels in the RNAi treated worms were significantly lower than those in the controls (P < 0.05). The results of the Western blot analysis showed that the expression level of the ADLP was significantly decreased in the RNAi-treated worms (Fig. 5C), in agreement with the real-time RT-PCR result. These results indicated that after RNAi treatment, the expression of ADLP was decreased at both the mRNA and protein levels.

4. Discussion

According to bioinformatics analysis by BLAST, ADLP contains a conserved domain, the P-loop, indicating that this protein has a functional correlation with ATP. In this study, we utilized a phosphoryltransfer assay to confirm that ADLP does have NMPK enzyme activity. The substrate specificity of ADLP is different from all other AKs.
According to the previous reports, AK1 and AK3 share the same phosphate acceptors, AMP and to a lesser extent dAMP, although their phosphate donors are largely different: AK1 uses only ATP, but AK3 prefers GTP and ITP [8,17]. Additionally, AK2 uses ATP and AMP as its substrates [8,18]. AK4 has not been detected to possess any activity in vitro or in vivo [17], and AK5 phosphorylates AMP and dAMP with ATP as the phosphate donor, and AMP, CMP and dCMP with GTP as the phosphate donor [4]. CTP and UTP are the preferred phosphate donors for human AK6 and AMP, dAMP, CMP, dCMP have demonstrated significant activities as substrates [9]. TMPK phosphorylates dTMP and dUMP to their respective diphosphate forms; its best phosphate donors are ATP, dATP, GTP, and dGTP, and it can also use several other phosphate donors [19]. CMPK phosphorylates CMP, dCMP, and UMP with highest efficiency using ATP and dATP as the preferred donors, although it has several other donors [20]. Guanylate kinase (GK) catalyzes the reactions from GMP and dGMP to GDP and dGTP, respectively, whose best donors are ATP, dATP, GTP, CTP, and UTP [21].

In the case of ADLP, it has three unique features compared with other characterized adenylate kinases: (a) it only uses ATP and dATP as phosphate donors, different from other AKs; (b) it can use a wide-range of nucleoside monophosphates as phosphate acceptors: the best phosphate acceptors for ADLP are AMP and dAMP, CMP and TMP are also good substrates, and dCMP is a substrate to a lesser extent; (c) besides the nucleoside monophosphates described above, non-nucleotide substrates such as shikimate acid can also be phosphorylated as the phosphate acceptor and show significant activity (Fig. 3C), different from human AK6, in which no activity was detected when using shikimate acid as substrate [9]. Sequence analysis suggested that ADLP has more similarities with shikimate kinase in shikimate-binding site than that of human AK6 (Supplementary data). This is consistent with the observation of shikimate kinase activity in ADLP but not human AK6.

The previously characterized AKs showed different subcellular locations. Both human AK6 and its homolog in yeast, Fap7, are localized to the cell nucleus [9,22], which are different from that observed in other adenylate kinase isoforms [6,23]. AK1 localizes in the cytosol, and AK2 localizes in the mitochondrial inter-membrane space [24], both AK3 and AK4 localize in the mitochondrial matrix [17,25,26], and AK5 was found in both the cytosol and nucleus [4]. Our result for ADLP from C. elegans showed that this protein was mainly localized in the nucleus (Fig. 4), which is similar to that of human AK6. The result of ADLP localization was consistent with that of bioinformatics analysis. Both LOCtree [27] and LOCKey [28] analysis suggest that ADLP indeed exists in the nucleus. Furthermore, through PSORT II [16], a useful tool for nuclear localization sequence (NLS) prediction, the N-terminal polybasic peptide RRRPN was identified as the NLS, and may explain why this protein is localized to the nucleus. Another possible explanation for this nuclear localization is that ADLP may be co-transported into the nucleus by binding to other proteins through the abundantly conserved acidic amino acids presented on the surface of the protein, as proposed for human AK6 [9]. Further experiments will be performed to investigate the mechanism of ADLP transportation.

The results of subcellular localization (Fig. 4) and enzymatic activity (Fig. 3), accompanied with the sequence alignment (Fig. 1) and an unrooted radial phylogenetic tree (Fig. 6), indicated that ADLP and human AK6 should be classified in the same family even though their substrate specificities show some differences. Therefore, we designate ADLP as C. elegans AK6 (C.AK6).

Since C.AK6 protein is identified as one of the isoforms of adenylate kinase which plays an important role in the synthesis of nucleotides and is required for a variety of cellular metabolic processes, knockdown of C.AK6 may have great effect on the growth or development of C. elegans. The result of an RNAi experiment showed a Gro phenotype (Fig. 5A), and the expression of C.AK6 was decreased at both transcriptional and translational levels, suggesting that the interference of C.AK6 may hold functions related to the growth of C. elegans. This observation is similar to that obtained from AK2 of Drosophila melanogaster and Fap7 of yeast [7,22]. Noma’s study reported that in Drosophila, lack of the AK2 gene caused significant insect growth suppression, further experimentation suggested that AK2 was essential for survival and had effects on rhythm formation [7]. In yeast, the point mutant fap7-1 was revealed to slow growth on glucose compared with the wild type [22]. In addition, Wieringa’s group found that the
loss of AK1 can induce molecular and structural adaptations to support muscle energy metabolism [29]. So the question concerning C.AK6 was raised: What is the mechanism of C.AK6 in worm growth? The result of a recent report about human AK6 (H.AK6) showed that hCINAP (H.AK6) can interact with collin, a marker protein of Cajal bodies [30]. Additionally, it is well known that Cajal bodies are involved in nuclear splicing. Since C.AK6 was found to localize to the cell nucleus, whether the slow growth of the worm resulting from RNAi knockdown of C.AK6 is due to disturbance of RNA splicing in nucleus or related events will be an interesting question to investigate.

Thus far, six AK isozymes, AK1–6 were identified that contribute to the homeostasis of cellular adenine nucleotide composition and nucleotide biosynthesis. The information obtained so far indicated the function of AK is associated with the mechanism of efficient transfer of high-energy phosphate in micro-compartments within the cell [7]. However, more effort is needed to unravel the real biological roles that AKs serve. The enzymatic characterization and phenotypic analysis of C.AK6, together with the characterization of other NMPKs, will help us to address these questions not only to illustrate the roles of adenylate kinases in energy metabolism but also link them to large-scale cellular phenomena such as signal transduction and the regulation of cell proliferation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fls.2006.05.074.

References


Fig. 6. An unrooted radial phylogenetic tree was constructed on the basis of sequence alignments of AK isofoms found in human and C. elegans, including C. elegans AK1 (AAG50236) (C.AK1), C. elegans AK6 (NP_96065) (C.AK6), human AK1 (NP_000467), human AK2 (AAH09405), human AK3 (BAA87913), human AK4 (P27144), human AK5 (Q9Y6K8), human AK6 (1RKBA), human CMPK (NP_057392), human GK (NP_000849), and human TMPK (NP_036277).


