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Identification of a Novel Nuclear-Localized Adenylate Kinase from *Drosophila melanogaster*

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Abstract—As a step to further understand the role of adenylate kinase (AK) in the energy metabolism network, we identified, purified, and characterized a previously undescribed adenylate kinase in *Drosophila melanogaster*. The cDNA encodes a 175-amino acid protein, which shows 47.85% identity in 163 amino acids to human AK6. The recombinant protein was successfully expressed in *Escherichia coli* BL21(DE3) strain. Characterization of this protein by enzyme activity assay showed adenylate kinase activity. AMP and CMP were the preferred substrates, and UMP can also be phosphorylated to some extent, with ATP as the best phosphate donor. Subcellular localization study showed a predominantly nuclear localization. Therefore, based on the substrate specificity, the specific nuclear localization in the cell, and the sequence similarity with human AK6, we named this novel adenylate kinase identified from the fly DAK6.

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Key words: cloning, protein expression, adenylate kinase activity, nuclear localization

Adenylate kinases (AK) are a family of enzymes that catalyze the reversible high-energy phosphoryl transfer reaction between adenine nucleotides ATP and AMP. These enzymes play important roles in the balance of energy metabolic processes and ATP transportation and consumption. In mammalian tissues, six isozymes of AK (AK1 to AK6) have been identified [1-3]. The subcellular localization, enzymatic activities, and tissue distributions of these AKs have been characterized [2-5]. AK1 exists in cytoplasm and is highly expressed in brain, testis, and cardiac cells. AK2 is partly localized in cytosol but mainly in mitochondrial intermembrane space. AK3 is localized in the mitochondrial matrix and is a housekeeping gene since it is expressed in all tissues except for red blood cells. AK4 is also localized in the mitochondrial matrix but expressed mainly in brain, kidney, liver, and heart. AK5 is localized in cytosol and is only identified in brain. In contrast to all other AK isoforms described above, the recently identified AK6 is mainly localized in nucleus, suggesting a novel role of AK in nuclear nucleotide metabolism [2-6].

In drosophila only three AK isozymes, DAK1-3, have been identified and investigated so far; they are related to human AK1, AK2, and AK3, respectively [3]. Among them, DAK2 is localized in both cytoplasm and mitochondria [7] and knockout of *dak2* gene resulted in significant growth suppression of the fly larvae, suggesting that DAK2 is essential for survival and has effects on rhythm formation [3].

In this work, we have identified a novel adenylate kinase by its homology to human AK6 in *Drosophila melanogaster*. This protein holds the conserved Walker motif (Gly-X-X-Gly-X-Gly-Lys), a characteristic of nucleoside monophosphate kinases (NMPKs), NMP binding domain, and a LID domain (Fig. 1a). Our experiments show that this protein can catalyze the phosphoryl transfer reaction, especially the reaction between AMP and ATP. Subcellular localization of this protein showed a dominant nuclear localization. Therefore this protein was named DAK6 due to its similar characters to HAK6. To our knowledge, this protein is the first nuclear adenylate kinase identified in *D. melanogaster*.

Abbreviations: AK) adenylate kinase; DAPI) 4,6-diamidino-2-phenylindole; GFP) green fluorescent protein; IPTG) isopropyl β-thiogalactopyranoside; NLS) nuclear localization sequence; NMPK) nucleoside monophosphate kinases.

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MATERIALS AND METHODS

Total RNA extraction and plasmid construction. Total RNA was extracted from mature *D. melanogaster* using

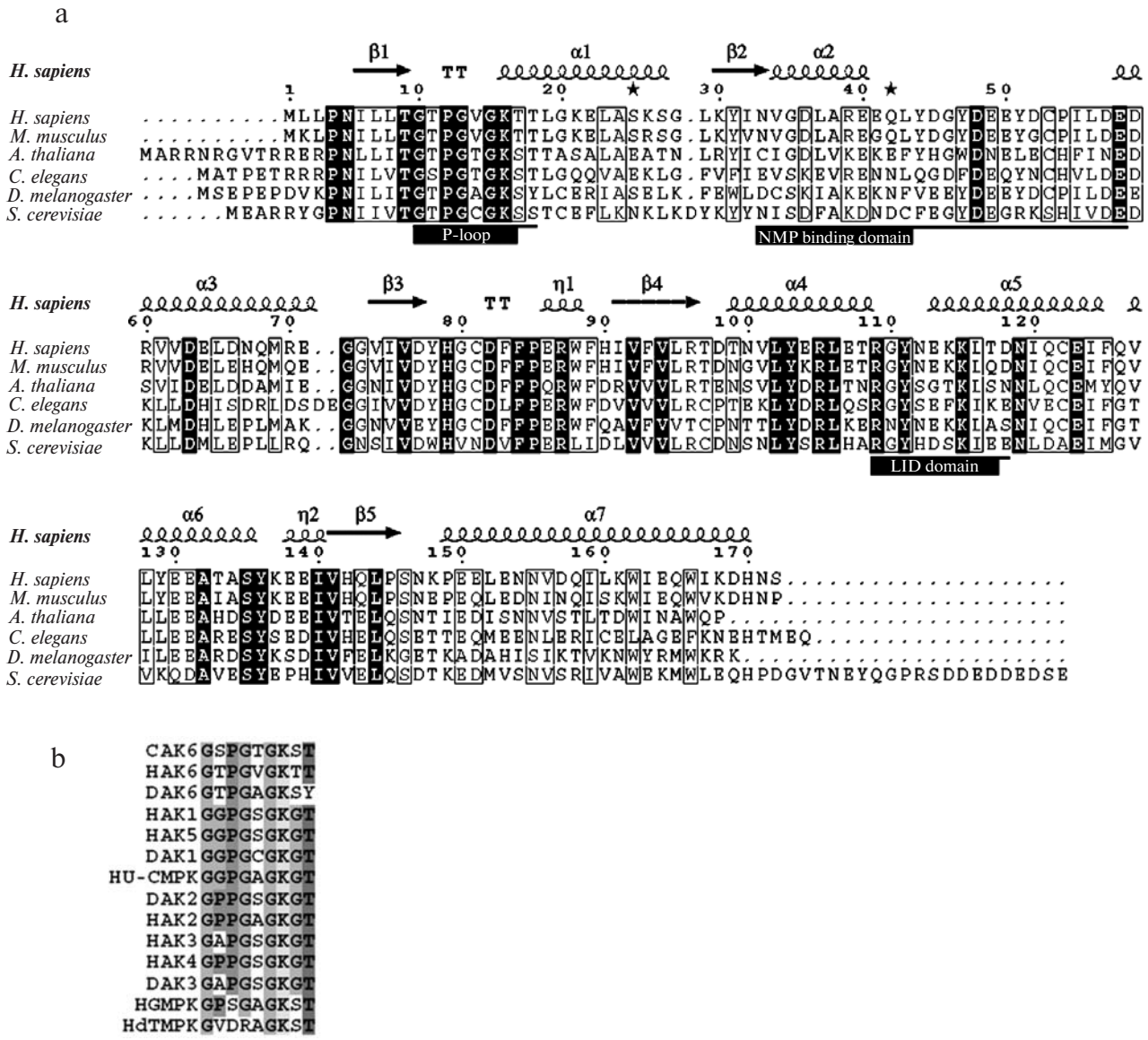

Fig. 1. Alignment of the amino acid sequences of the eukaryotic AK6 homologs. a) Multiple sequence alignment of some of the eukaryotic AK6 homologs. The alignment was performed using the program Clustal X [17]. The sequences searched using EMBL-EBI FASTA [18] are human (AAF14860), mouse (NP_081868), *Arabidopsis* (BAC42255), *Drosophila* (NP_610797), *Caenorhabditis elegans* (NP_496065), and *Saccharomyces cerevisiae* (NP_010115). The arrowhead indicates a mutation that may influence the substrate specificity. b) The conserved P-loop domain of DAK6 is aligned with AK1 (NP_000467), AK2 (NP_001616), AK3 (NP_057366), AK4 (P27144), AK5 (Q96EC9), AK6 (1RKB), U-CMPK (NP_057392), GMPK (NP_000849), and dTMPK (NP_036277) in human and AK1 (NP_524038), AK2 (NP_523836), and AK3 (NP_524312) in drosophila.

TRIZOL reagent (Invitrogen, USA) and examined by a 1.2% agarose gel electrophoresis. The cDNA of the AK6 homolog (Accession No. NM_136953) in *D. melanogaster* (we named it DAK6 based on its sequence similarity to human AK6) was amplified by RT-PCR (AccessQuick™ RT-PCR System; Promega, USA) using the following primers, 5′-ggaattccatatgtcagaaccagagccaga (the underline indicates the *Nde*I site) and 5′-cgggatcct-tattttcttttccacatacg (the underline indicates the *BamH*I site). The amplified cDNA fragment was examined and purified by 1.2% agarose gel and then inserted into pET28a vector through the *Nde*I and *BamH*I sites. The positive clone was confirmed by PCR and DNA sequencing, followed by transformation into *Escherichia coli* expression strain BL21(DE3).

The vectors of N-terminal and C-terminal green fluorescent protein (GFP) fusion DAK6 were created by using the following primers: gfp-DAK6-F: 5′-ccg-gaattcatgttgcttccgaacatcc (the underline indicates the *EcoR*I site) and gfp-DAK6-R: 5′-ccgggatccagagttat-gatctttgatcc (the underline indicates the *BamH*I site). The resulting PCR products were digested with *EcoR*I and

*Bam*HI and ligated into vectors pEGFP-C2 and pEGFP-N3 (Clontech, USA) that cut with the same restriction enzymes.

Protein expression and purification. The recombinant protein of DAK6 was expressed in *E. coli* strain BL21(DE3). The expression and solubility of the recombinant protein was investigated first by following the procedure of our previous study [8]. Briefly, the protein was induced by 0.5 mM isopropyl β-thiogalactopyranoside (IPTG) (Calbiochem, USA) after A_{600} value of the cultures reached 0.6. Cells were collected by centrifugation, resuspended in 15 ml of TN buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.8), and sonicated using a JY92-II sonic disintegrator (Ningbo, P. R. China) (200 W, on for 5 sec, off for 15 sec; 90 cycles). A 1-ml aliquot of lysate was taken and centrifuged at 15,000 rpm for 30 min. The recombinant protein in the supernatant or pellet was analyzed by SDS-PAGE. The control sample was produced under the same conditions without adding IPTG. Different culture temperatures, i.e. 37, 30, and 18°C, were examined for the optimal expression of the soluble protein.

For large-scale protein purification, 1 liter of culture was prepared and centrifuged at 5000 rpm for 10 min. The harvested cells were resuspended in TN buffer (pH 7.8) containing 5 mM imidazole and disrupted by sonication as described above. The lysate was clarified by centrifuging at 18,000 rpm for 30 min at 4°C, and the supernatant was applied to a Ni-affinity column equilibrated with TN buffer. The column was washed with TN buffer containing 50 mM imidazole, and $(His)_6$-tagged recombinant protein was eluted using TN buffer containing a linear gradient of 50-500 mM imidazole. The fractions were checked by SDS-PAGE and those containing target protein were pooled and concentrated in a Centricon PL-20 (Millipore, USA). Then the concentrated protein was applied to a Hiload Superdex-75 gel filtration column (Amersham Pharmacia Biosciences, Sweden) equilibrated with TN buffer (20 mM Tris, 150 mM NaCl, pH 7.8) to further improve the purity of the recombinant protein. The peak fractions from the elution were pooled and concentrated in a Centricon PL-20 and then checked by SDS-PAGE. The concentration of the recombinant protein was measured with a Bio-Rad Protein Assay kit (Bio-Rad Pacific Ltd, USA).

Enzyme assays. Using the purified recombinant protein, enzymatic activity of DAK6 was measured following the method described previously [9]. [α-^{32}P]AMP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were purchased from Furui Company (China). A phosphoryl transfer assay was performed with [γ-^{32}P]ATP as the phosphate donor and non-radioactive nucleoside monophosphates (Sigma, USA) as substrates. The reaction solution that contained 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 2 mM NMP, 1 μCi/μl [γ-^{32}P]ATP, and 50 ng of tested protein in a total volume of 50 μl was incubated at 37°C for 20 min and then stopped by heating at 70°C for 2 min. The reaction products were examined by thin-layer chromatography on polyethyleneimine-cellulose F chromatography sheets (Merck, USA), which were autoradiographed using a storage phosphor screen (Amersham Pharmacia Biosciences). A reaction mixture without DAK6 was used as negative control to subtract the background; a commercially available AK1 (Sigma) was used as positive control. Screening of phosphate donor was performed with [α-^{32}P]AMP as substrate and non-radioactive nucleoside triphosphate as phosphate donor following the procedure described above.

Transfection assays. Transfection assays were carried out in HeLa cells as described previously [2]. Briefly, 2 μl pEGFP-DAK6 plasmid was diluted in 250 μl Dulbecco's modified Eagle medium (DMEM), mixed gently with Lipofectamine 2000 (Invitrogen) in 250 μl DMEM, and incubated at room temperature for 20 min. Then the medium was discarded, the cells were mulched with the mixture and incubated at 37°C and 5% CO_2 for 4 to 6 h. The medium was supplemented to a final volume of 2 ml. Cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature, mounted by Mowiol, and visualized with a fluorescent microscope 24 h post-transfection. 4,6-Diamidino-2-phenylindole (DAPI) (1 μg/ml in Mowiol) was used to stain DNA to visualize the nucleus.

RESULTS

Cloning of DAK6 in *E. coli*. Total RNA was extracted from *D. melanogaster*. The cDNA of the gene was obtained, which was consistent with its predicted length, 525 bp (Fig. 2a). The expression vector for DAK6, pET28a-DAK6, was constructed by insertion of the cDNA into pET28a vector through *Nde*I and *Bam*HI sites, and verified by DNA sequencing. An N-terminal $(His)_6$-tag is convenient for protein purification.

Protein expression and purification. The recombinant DAK6 was examined in *E. coli* BL21(DE3) strain as described in "Materials and Methods". The protein showed an obvious band corresponding to the predicted molecular weight compared to the control sample without IPTG induction. Optimization of the expression conditions indicated that DAK6 was expressed well in BL21(DE3) at 30°C for 4 h, for obtaining the maximum amount of soluble protein.

The cells of 1 liter of IPTG-induced bacterial culture were collected for large-scale protein purification. The recombinant protein was purified in two steps using a Ni-affinity column and a Superdex-75 column as described above. SDS-PAGE analysis of the purified protein showed only one band corresponding to the predicted molecular weight for the target protein (Fig. 2b). The concentration of the purified protein estimated by the

Bio-Rad Protein Assay Kit showed that about 15 mg recombinant protein with at least 99% purity could be obtained from 1 liter of culture.

Polymerization of DAK6. According to our study on human AK6, the protein exists as a complex of dimer and monomer due to the three cysteines in the protein [10]. Since DAK6 contains four cysteine residues, in which Cys49 and Cys77 are conserved in the AK6 family (Fig. 1a), it is likely that they can form dimer or polymers due to the formation of disulfide bridges between molecules. The result of gel filtration chromatography showed that two peaks can be observed in the elution profile of DAK6, which represented dimer and monomer, respectively (Fig. 2c). The non-reducing SDS-PAGE analysis also revealed the presence of DAK6 dimer (Fig. 2d). A possible explanation is that DAK6 formed dimer due to the formation of intermolecular disulfide bonds. These results indicated that the purified proteins existed in both dimer and monomer forms.

DAK6 shows the enzymatic activity of an adenylate kinase. In order to investigate the enzyme activity of DAK6, different substrates—AMP, CMP, TMP, UMP, and GMP—were screened with the phosphate donor [γ-^{32}P]ATP. Commercial AK1 from chicken (Sigma) was used as positive control. According to the results (Fig. 3a, upper panel), AMP was the best phosphate acceptor of all tested NMPs, CMP also showed significant activity,

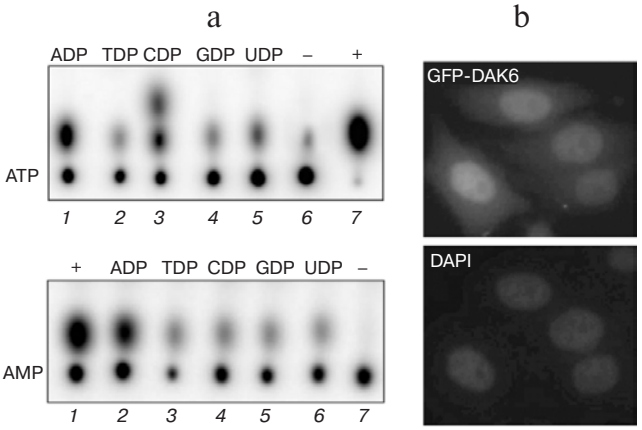

Fig. 3. Characterization of DAK6 by enzymatic activity assays and subcellular localization. a) Enzyme assays of DAK6 by TLC blotting. The upper panel shows the screening of substrate specificity of DAK6. Lanes: *1-5*) nucleoside monophosphates AMP, TMP, CMP, GMP, and UMP, respectively; *6*) negative control; *7*) positive control (AK1). The lower panel shows the screening of phosphate donor. DAK6 catalyzed phosphorylation of radiolabeled AMP with different phosphate donors. Lanes: *1*) positive control (AK1); *2-6*) nucleoside triphosphates ATP, TTP, CTP, GTP, and UTP, respectively; *7*) negative control. b) Subcellular localization of DAK6 in HeLa cell via fluorescence microscopy. HeLa cells were transfected by pEGFP-DAK6 and visualized with a fluorescent microscope. Strong nuclear fluorescence was observed. DAPI staining of the DNA indicated nuclear localization of DAK6.

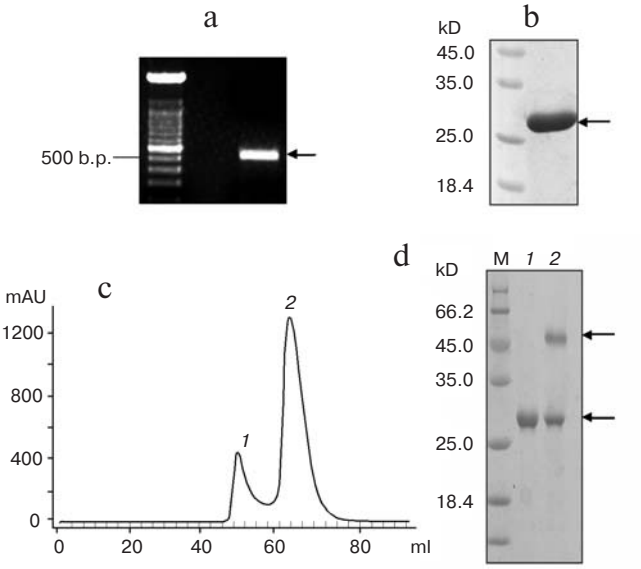

Fig. 2. Cloning and expression of DAK6. a) The cDNA of DAK6 was obtained by RT-PCR. A 1.5% agarose gel is shown. b) The protein was purified to near homogeneity through Ni^{2+} affinity column and Superdex-75 column. c) The polymerization of DAK6 was characterized via analytical gel filtration chromatography. Peak *1* corresponds to dimers and peak *2* corresponds to monomers. d) SDS-PAGE analysis of DAK6 protein collected from peak *1* under reducing condition (lane *1*) and non-reducing condition without adding DTT (lane *2*). Lane M, protein molecular weight markers.

which is similar to HAK6 (human AK6) and CAK6 (*C. elegans* AK6). The preferred phosphate donors were further examined by testing ATP, TTP, CTP, GTP, and UTP, using [α-^{32}P]AMP as substrate. The results showed that ATP was the best phosphate donor (Fig. 3a, lower panel).

DAK6 localized to the nucleus of HeLa cells. Since human AK6 and the AK6 homologs of yeast (Fap7) and *C. elegans* localize to the cell nucleus [2, 9, 11], DAK6 was tested here to see whether it exhibits similar localization. pEGFP-DAK6 that contain an N-terminal GFP fused DAK6 was transfected into HeLa cells and visualized under a fluorescence microscope. Figure 3b showed that DAK6 was mainly localized in the nucleus, which was verified by staining the nucleus with DAPI. A similar observation for C-terminal GFP fused DAK6 was obtained as well (data not shown).

DISCUSSION

Thus far, six AK isozymes, AK1 to AK6, have been identified in mammals; they contribute to the homeostasis of cellular adenine nucleotide composition and nucleotide metabolism. Among them, the newly discovered AK isozyme AK6 has some special features, but is far from well investigated. In *D. melanogaster* only AK1, AK2, and AK3 have been identified [3]. DAK2 was well

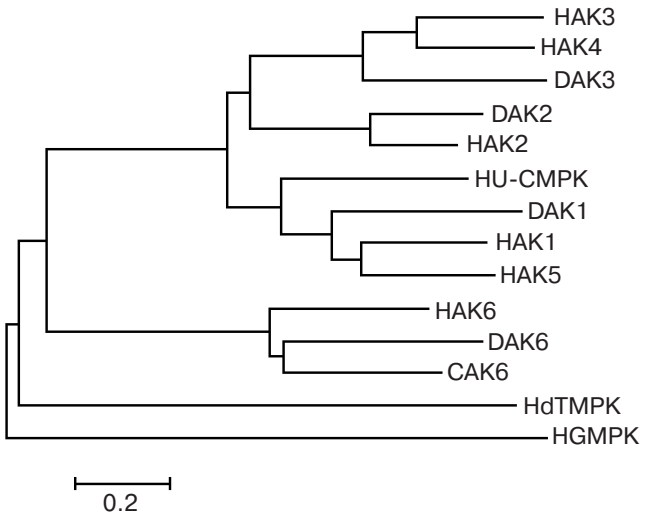

Fig. 4. An unrooted phylogenetic tree was constructed on the basis of sequence alignments of AK isoforms including HAK1 (NP_000467), HAK2 (NP_001616), HAK3 (NP_057366), HAK4 (P27144), HAK5 (Q96EC9), HAK6 (1RKB), DAK1 (NP_524038), DAK2 (NP_523836), DAK3 (NP_524312), HU-CMPK, and CAK6. The phylogenetic tree shows that the protein in drosophila belongs to the AK6 family.

investigated and thought to function in mitochondrial energy metabolism [7]. For further understanding the nucleotide metabolism in this model organism, we performed this study to try to find a new adenylate kinase from drosophila that may be related to human AK6.

Our phosphoryl transfer assay showed that the homolog of human AK6 in *D. melanogaster* does have adenylate kinase activity. ATP is the best phosphate donor for DAK6; mammalian AK1, AK2, and AK5 also preferentially use ATP as the phosphate donor [1], which may due to the existence of the conserved P-loop in all NMPKs. Also, the preference of the phosphate acceptors in DAK6 is similar to that of human AK6 and its homolog in *C. elegans* [2, 9]: in addition to AMP that is shown to be the preferred substrate, CMP is also a good substrate for all AK6s, which is different from other AK isozymes. This substrate specificity of AK6s indicates conserved function lies in different species.

Due to their critical roles in cells, various isozymes of adenylate kinase have been found in mitochondria, cytosol, and nucleus [2, 9, 12, 13]: AK1 localizes in cytosol [3, 14], AK2 localizes in mitochondrial intermembrane space [15], both AK3 and AK4 localize in mitochondrial matrix [5, 6, 14], and AK5 is found in the cytosol [4]. In contrast to all other AK isozymes, the newly discovered AK6 homologs from *Homo sapiens* (HAK6), yeast (Fap7p), and *C. elegans* (CAK6) show a dominantly nuclear localization [2, 9, 11], which suggests that AK6s have a common feature of nuclear subcellular localization and may function in nucleotide metabolism of the nucleus. In this study, our results show that DAK6 is mainly localized in the nucleus (Fig. 3b), but we failed to find the nuclear localization sequence (NLS) in DAK6 as in HAK6, which is different from CAK6 and yeast Fap7p that contain putative NLS at their N-termini. A possible explanation for nuclear localization of human AK6 is proposed from the examination of its crystal structure: the acidic domains on the surface of the protein may be important [2]. We also did structure homology building of *D. melanogaster* based on the human AK6 crystal structure (data not shown), and the same acidic domain was found just at the same part of the protein. Therefore we proposed that the high percentage of the acidic amino acids that are conserved among AK6 families may explain this nuclear localization.

From the results of enzymatic activity and subcellular localization (Fig. 3), accompanied with the sequence alignment (Fig. 1) and phylogenetic analysis (Fig. 4), we conclude that DAK6 should belong to the novel nuclear localized AK6 family and is the first identified nuclear adenylate kinase in *D. melanogaster*.

Information obtained so far indicates that AK is associated with the mechanism of efficient transfer of high-energy phosphate within the cell. As a recently identified AK isozyme, AK6, its real biological function is still undefined. To date, in the AK6 family only human AK6 and its homologs in yeast (Fap7) and *C. elegans* (CAK6) have been identified. Fap7 is involved in the oxidative stress response of *S. cerevisiae* [11], and Granneman et al. report that Fap7 functions as an NTP-dependent molecular switch in 18S rRNA maturation [16]. Knockdown of CAK6 gene in *C. elegans* results in the suppression of worm growth [9]. The identification of the nuclear localized AK6 homolog in *D. melanogaster* in this study suggests that in cooperation with DAK2 that mainly functions in mitochondrial energy metabolism, DAK6 might mainly function in nuclear energy metabolism. Further efforts on this newly identified AK6 from various organisms will help us to determine their biological functions.

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