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Subcellular localization of adenosine kinase in mammalian cells: The long isoform of AdK is localized in the nucleus

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ABSTRACT

Two isoforms of adenosine kinase (AdK) have been identified in mammalian organisms with the long isoform (AdK-long) containing extra 20–21 amino acids at the N-terminus (NTS). The subcellular localizations of these isoforms are not known and they contain no identifiable targeting sequence. Immunofluorescence labeling of mammalian cells expressing either only AdK-long or both isoforms with AdK-specific antibody showed only nuclear labeling or both nucleus and cytoplasmic labeling, respectively. The AdK-long and -short isoforms fused at the C-terminus with *c-myc* epitope also localized in the nucleus and cytoplasm, respectively. Fusion of the AdK-long NTS to green fluorescent protein also resulted in its nuclear localization. AdK-long NTS contains a cluster of conserved amino acids (PKPKKLKVE). Replacement of KK in this sequence with either AA or AD abolished its nuclear localization capability, indicating that this cluster likely serves as a nuclear localization signal. AdK in nucleus is likely required for sustaining methylation reactions.

Introduction

Adenosine kinase (AdK) belongs to the ribokinase (RK) family of proteins and is the one of the most abundant nucleoside kinases in mammalian tissues [1,2]. This enzyme is well conserved among eukaryotic species both at sequence and structural level [3,4]. AdK knockout mice have been made and they showed a lethal phenotype indicating AdK is indispensible in eukaryotic organisms [5]. Adk is the first enzyme in the purine salvage pathway and catalyzes the phosphorylation of adenosine (Ado) to AMP, using ATP as a phosphate donor and produces ADP and AMP [1,6]. By performing this reaction, it controls intracellular and extracellular Ado concentration in the cell, which is a potent cardioprotective agent and neuromodulator [2,7,8]. Adenosine is also one of the obligate end products of all methylation reactions, which exhibits end product inhibition on the upstream reactions including various methyltransferases [1,5,9–11]. Therefore, besides its convention role in purine salvage, AdK also ensures the continuance of methylation reaction without impedance.

As a cardioprotective agents, Ado has been implicated in tissue-protective mechanism during and after instances of ischemia by activating adenosine receptors on the cell surface [2,8]. The AdK over-expression in neurons, which removes the inhibitory effect of Ado on neuronal excitability, was recently shown as the underlying mechanism for chronic epilepsies [12,13]. Because of the short half life of Ado in physiological fluids, there has been much interest in developing Ado inhibitors as they provide potential means of amplifying the beneficiary effects of Ado in both cardiovascular diseases and chronic epilepsies [13–15].

Two isoforms of AdK have been identified in various mammalian organisms and the recombinant proteins from both are functional and they show no differences in their biochemical or kinetic properties [3,4,16,17]. The two isoforms are identical except at the N-terminus where the long AdK isoform (AdK-long) contains extra 20–21 amino acids, which replace the first four amino acids in the short isoform (AdK-short) (see Fig. 1). Western blot analysis indicates that these two isoforms are differentially expressed in rat tissues [16]. However, there is no information available regarding possible significance of these two isoforms or differences in their functions. To date, the subcellular localization of AdK has not been experimentally determined. Most conventional programs for prediction of cellular localization (e.g. PSORT, BaCelLo) reveal no specific localization of these isoforms and thus they are both assumed to be present in the cytoplasm [18,19]. However, in this paper we show that in contrast to the cytoplasmic localization of AdK-short, the AdK-long isoform is localized in the nucleus and the extra 20–21 amino acids present at its N-terminus are capable of directing AdK as well as other proteins to the nucleus. The significance of nuclear localization of AdK is discussed.

Material and methods

Cell lines, cell culture conditions and plasmid constructs. The origin of various cell lines used in this study (viz. CHO, HeLa, HT-1080 and

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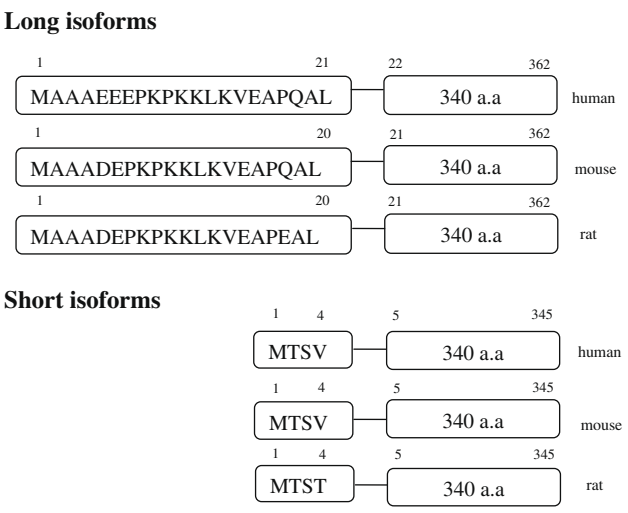

Fig. 1. Sequence characteristics of the short and long isoforms of AdK. The amino acid sequences of these two isoforms in human, mouse and rat are identical except for the sequence at the N-terminus region (NTS). The NTS of AdK-long in these species is highly conserved with complete conservation of the central basic cluster (PKPKKLK).

LM (TK^-)) has been described in our earlier work [20]. All of the cells were grown in α-MEM supplemented with 5% fetal bovine serum at 37 °C in a 95% humid air–5% CO_2 atmosphere. The full-length sequences for AdK-long and AdK-short isoforms were PCR amplified using cDNA from HT-1080 cells with PCR primers based on known sequences (Accession No. NM_006721 and NM_001123). Resulting PCR products were cloned and sequenced to confirm that the sequences had no errors. These sequences were re-amplified with PCR primers containing appropriate restriction sites (HindIII/XhoI) and a c-terminal *c-myc* tag and ligated into pcDNA3.1 vector (Invitrogen). A construct containing the NTS (MAAAEEEPK PKKLKVEAPQLKR) from human AdK-long and enhanced green fluorescent protein (eGFP) was constructed by PCR amplification from a previously described eGFP-containing plasmid [21]. Site-directed mutagenesis of KK (in the NTS) to AA and AD was carried out using the "Quikchange" kit (Stratagene).

Cell transfection and fluorescence microscopy. These studies were carried out with Chinese Hamster Ovary (CHO) cells. Transient transfections were performed by plating 2×10^5 cells in 35 mm tissue culture dishes (containing acid washed 22 × 22 mm cover glass) 24 h prior to the experiment, such that the cells were about 60–70% confluent at the time of experiment. On the day of transfection, the original growth medium was replaced with serum-free medium and DNA transfection was carried out using Lipofectamine-2000 reagent (Invitrogen) as described in earlier work [21]. Column purified DNA (1–2 μg in 200 μl) was mixed with 4 μl of Lipofectamine-2000 and allowed to incubate with cells at room temperature for 20 min. After 6 h of incubation at 37 °C in CO_2 incubator, the medium was replaced with serum-containing medium and the dishes were returned to the incubator. Expression of the fluorescent protein was observed at 24, 48, 72 h post-transfection. The eGFP fluorescence in live cells was visualized by mounting the coverslip on a glass slide in a drop of medium. The cells were observed and photographed within a time frame of 5–10 min to minimize changes in cell morphology.

A rabbit polyclonal antibody to human recombinant AdK was raised in our lab by immunizing New Zealand White rabbits (Charles River). For immunofluorescence labeling, cells growing on coverslips were fixed by immersion in 100% methanol at −20 °C for 10 min. After blocking nonspecific signals by incubation with 3% BSA in TBS, the cells were incubated in 1:100 dilutions of the primary antibodies (viz. rabbit polyclonal antibody to AdK or a mouse monoclonal antibody to c-Myc (Cat. No. 2Q329; Santa Cruz Biotechnology) for 2 h at 37 °C. After rinsing the coverslips three times with TBS they were incubated in 1:200 dilutions of secondary antibodies (viz. anti-mouse or anti-rabbit Alexa Fluor 488; Molecular Probes) for 2 h at 37 °C. After further rinsing, the coverslips were mounted on glass slides in 90% glycerol. Photographs were taken on an Olympus BX51 microscope equipped with REITGA Exi (Qimaging) digital camera and Northern Elite software.

Results

Differential expression and subcellular localization of the two AdK isoforms

To investigate the subcellular localization of AdK in mammalian cells a polyclonal antibody against human recombinant AdK was raised. Using this antibody, the presence of AdK-antibody cross-reactive protein(s) in a number of mammalian cell lines was studied (Fig. 2A). The cell lines used in these studies included WT Chinese hamster ovary (CHO) cells, HeLa cells—a human epithelial cell line derived from cervix, HT-1080 cell line—a human epithelial cell line from connective tissue and mouse LM (TK^-) cells, a fibroblastic cell line of connective tissue. As seen, in HeLa and CHO cell lines, the antibody to AdK specifically detected a single band of ∼40 kDa, which corresponded to the long isoform of AdK. In contrast to these two cell lines, in the human HT-1080 and mouse LM (TK^-) cell lines, the antibody showed cross-reactivity with two closely related bands of ∼40 kDa and ∼38 kDa (Fig. 2A), which corresponded to the long and short isoforms of AdK, respectively. Except for these bands, no other cross-reactive bands were observed in these experiments, indicating that this antibody is highly specific in terms of its recognition of AdK. Although, difference in expression of the two AdK isoforms has been reported in rat tissues [16], this is the first report on their differential expression in mammalian cell lines.

In view of the differential expression of the AdK isoforms in these cell lines, it was of much interest to determine the subcellular localization of AdK in them by means of immunofluorescence. The results of these studies are shown in Fig. 2B. As seen, in HeLa and CHO cell lines, which expressed only the AdK-long isoform, immunofluorescence labeling with AdK-antibody was mainly observed in the nucleus (Fig. 2B: panels b and d). In contrast, in HT-1080 and LM (TK^-) cell lines expressing both isoforms, labeling was observed both in the nucleus as well as in cytoplasm (Fig. 2B: panels a, c). These results indicated that while the AdK-long is localized in the nucleus, the short isoform of AdK remains in the cytoplasm. Because these two isoforms differ from each other only in their NTS and AdK-long has a longer NTS (Fig. 1), this strongly suggested that this NTS is likely involved in its nuclear localization.

Confirming that the NTS in AdK-long serves as a nuclear localization signal

The subcellular localization of the two AdK isoforms was initially examined using various online programs for predicting subcellular localization of proteins including BaCelLo, CELLO, and PSORT [18,19,22]. None of these programs predicted nuclear localization of the AdK-long isoform, or that the NTS in this sequence has nuclear localization capability. However, a weak prediction (p = 43.5%) for its nuclear localization was made by the κ-NN program [23]. However, NLS sequences are quite diverse and one cannot rely upon these programs for correctly predicting or identify different NLS [24,25].