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Differential expression of adenylate kinase 4 in the context of disparate stress response strategies of HEK293 and HepG2 cells

Fanzhi Kong [a,1,2], Bert Binas [a,1], Jae Hee Moon [a], Sung Soo Kang [a], Hyo Joon Kim [a,b,*]

[a] Division of Molecular & Life Science, College of Science & Technology, Hanyang University, Ansan, Republic of Korea
[b] SJ Biomed Inc., 604 HBI, Hanyang University, Ansan, Republic of Korea

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ABSTRACT

Adenylate kinase isozyme 4 (AK4) belongs to a family of nucleotide monophosphate kinases involved in energy metabolism. Recently, AK4 was reported to play a role in protection from stress: In HEK293 cells, hypoxia increases AK4 expression but does not affect proliferation or viability, while RNA interference (RNAi) directed against AK4 inhibits proliferation and promotes death. By contrast, we show here that HepG2 cells showed much higher AK4 levels, which decreased under hypoxia along with markedly reduced cell proliferation and increased cell death. Nevertheless, RNAi directed against AK4 inhibited cell proliferation and caused death in both cell types, although cell cycle parameters were affected only in HepG2 cells. Hence reductions of AK4 levels were always associated with cell death. These results extend the notion of a stress-protective function of AK4 to a novel physiological context and show that AK4-mediated stress protection is not limited to one particular death scenario. Our data also allow the hypothesis that the different basal AK4 levels reflect different basal stress levels, causing alternative responses to additional stress.

Introduction

The adenylate kinase (AK[3]) proteins constitute are structurally and functionally related enzyme family [1] and are known best for their role in maintaining homeostasis of nucleotide pools through catalysis of the phosphoryl-transfer reaction Mg*N*TP + AMP ↔ Mg*N*DP + ADP (*N* = A or G) [2]. Eight AK isozymes (AK1- AK8), which are all encoded by separate genes (Ref. [3], and references therein), have been identified in human tissues. AK1 and AK6 are short forms while the long forms include AK2, AK3 and AK4. AK5, AK7 and AK8 are novel members of the human AK family, with two functional short-form domains [4]. AK1 is the major cytosolic isoform, AK2 is found in the mitochondrial intermembrane space, and AK3 and AK4 are located in the mitochondrial matrix [5,6]. AK4 contains an *N*-terminal mitochondrial import sequence that remains uncleaved after import into the mitochondrial matrix [7]. AK5 is cytosolic or both cytosolic and nuclear depending on the transcript variants, and is, in contrast to most other AKs, expressed almost exclusively in brain [3,8]. No expression profile has been reported for AK6, AK7, and AK8 [4,9,10].

Interestingly, only AK1, the dominant cytosolic isoform, exhibits a high enzymatic activity *in vitro* [5], which led to the idea that some AKs may fulfill functions other than maintaining nucleotide homeostasis. For example, we have previously shown that AK2 mediates apoptosis through the formation of an AK2-FADD-caspase-10 complex, a pathway that may be involved in tumorigenesis [11]. Mutation or deficiency of human AK2 causes reticular dysgenesis [12] or a profound hematopoietic defect associated with sensorineural deafness [13].

The purpose of the present study was to shed light on the biological role of human AK4 (GTP: AMP phosphotransferase, EC 2.7.4.10). AK4, which was originally named AK3 [14], is expressed predominantly in kidney, liver, heart, and brain [5,6]. It consists of 223 amino acids and was first isolated from bovine heart [15], and the human cDNA was later cloned based on homology [16]. Unlike AK1, but similar to AK2, AK3, and the long variant AKs, AK4 shows only a very low enzyme activity *in vitro*, but uniquely amongst the AKs, AK4 accepts GTP as a phosphoryl group donor [1].

Large-scale genomic and proteomic studies have shown that AK4 gene expression changes under various stress conditions [17–20]. Hypoxia-responsive elements (HREs) could regulate gene expression under hypoxia [21–23]. Significantly increased AK4 protein levels have been detected during development, in cultured cells exposed to hypoxia, and in an animal model of amyotrophic

* Corresponding author. Address: Division of Molecular & Life Science, College of Science & Technology, Hanyang University, 55 Hanyangdaehak-ro, Sangrok-gu, Ansan, Gyeonggi-do 426-791, Republic of Korea. Fax: +82 31 418 7606.
E-mail address: kimhj104@hanyang.ac.kr (H.J. Kim).
[1] These authors contributed equally to this work.
[2] Present address: College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing 163319, China.
[3] *Abbreviation used*: AK, adenylate kinase.

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lateral sclerosis, a neurodegenerative disease in which oxidative stress is implicated [24–27]. Furthermore, artificial reduction of AK4 expression led to reduced cell proliferation and an increased rate of death, while an artificial increase protected the cells against H_2O_2 [24]. These observations lead to the idea that AK4 has a non-enzymatic protective role [24].

Here, we studied the expression and biological role of AK4 in HepG2 cells. Our data provide further support for a stress-protective function of AK4 and reveal through a comparison with HEK293 cells how this function is subordinated under the cellular and environmental context.

Materials and methods

Materials

The anti-AK4 antibody (SJB3–36) was from SJ Biomed, Inc. (Ansan, Korea). The anti-Gapdh (2D4A7) and anti-HIF-1α (3C144) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The lentiviral vectors MISSION pLKO.1-puro, MISSION non-target shRNA control (SHC002), and MISSION control vector purified DNA TurboGFP™ (SHC003) were purchased from Sigma (St. Louis, MO, USA). The virus particles were packaged by MACROGEN Inc. (Seoul, South Korea).

Cell culture

HepG2 (KCLB, No. 88065) and HEK293 (KCLB, No. 21573) cells were obtained from the Korean Cell Line Bank (Seoul, South Korea) and cultured in Dulbecco Modified Eagle's Minimal essential medium supplemented with 10% FBS, 1% penicillin/streptomycin (all from WelGENE, Daegu, South Korea) and 0.1% gentamycin (Invitrogen, Carlsbad, CA, US).

Hypoxia treatment

HepG2 and HEK293 cells were seeded into 60 mm^2 culture plates and incubated for 24 h in humidified atmosphere containing 5% CO_2 at 37 °C. For the hypoxia treatment, cells were transferred into a modular incubation chamber (Billups-Rothenberg, San Diego, CA, USA) with 5% CO_2 and 1% O_2 balanced with N_2 at 37 °C for the indicated periods of time. Normoxic control cells were incubated under the regular condition.

Microarray analysis

Cells were cultured under regular or hypoxic conditions for 12 h. Total RNA was extracted using TRI REAGENT (MRC, OH, USA) and further processed by GenoCheck Co., Ltd. (Ansan, South Korea). Briefly, the RNA was converted to Cy3-labeled cDNAs. The cDNAs were placed on a Roche NimbleGen Human whole genome 12-plex array (Roche NimbleGen, Inc., Madison, WI, USA) and covered by a NimbleGen H12 mixer (Biomicro System, Inc., Madision, WI, USA). After hybridization, the arrays were analyzed using an Axon GenePix 4000B scanner with associated software (Molecular Devices Corp., Sunnyvale, CA, USA). Gene expression levels were calculated with NimbleScan Version 2.4 (Roche NimbleGen). Fold change filters included the requirement for at least 2-fold increases or decreases of gene expression compared to the normoxic condition.

Short hairpin RNA interference

To test the efficiency of transduction, MISSION control vector purified DNA TurboGFP™ was first infected into HepG2 or HEK293 cells and maintained without selection. For experimental transductions, HepG2 cells were plated into 12-well plates at 5×10^4 cells per well before transduction. On the second day, the cells were infected with same titer virus with 8 μg/ml polybrene (Sigma). 6 h post-infection, the medium was replaced with fresh medium containing 10 μg/ml puromycin (Sigma). Cells were allowed to grow for 5–7 days, then passaged, and used for analysis after ~2 weeks.

Western blotting

The cells were directly lysed in gel sample buffer, separated under reducing conditions on 12% SDS–polyacrylamide gels, and subjected to immunoblotting using primary antibodies, and detection was made using HRP conjugated goat anti-mouse IgG (Cell Signaling, Danvers, MA, USA) and ECL (GE healthcare, Wilmington, MA, USA) as substrate for HRP. Densitometric analysis of bands was performed using National Institutes of Health ImageJ Version 1.45 software.

Quantitive RT-PCR

Total RNA was isolated from cells with Trizol reagent (Sigma) and reverse-transcribed with the DiaStar™ RT kit (SolGent, Daejeon, South Korea) using oligo-dT primers. Quantitive RT-PCRs were carried out in the presence of SYBR Green (Takara, Otsu, Japan), and the reactions were monitored using an ABI 7500 real-time instrument (Applied Biosystems Inc., MA, USA). Reactions were carried out in triplicate with –RT controls, and the mRNA levels of interest were normalized to the levels of β-actin mRNA and compared between samples by calculating the delta–delta Ct values. The following primers (5′ to 3′) were used: AK4: gcaatggcttccaaactc/tcggcttgtcctaatgtc, T_m = 52 °C; β-actin: tgacaggatgcagaaggaga/cgctcaggaggagcaatg, T_m = 55 °C; MCL1 (S + L): tcggcccggcgagagatagg/tccgggagtctggcgtgagg, T_m = 60 °C; MCL1L: agcgacggcgtaacaaactggg/acacctgcaaaagccagcagca, T_m = 62 °C.

Cell proliferation assay

5×10^4 Trypan Blue-excluding cells/well were seeded into 12-well plates. At the indicated time points, total cell numbers were counted in triplicate (three wells per time point, and each well counted three times), and results are given as means plus/minus standard errors.

Assessment of cell survival

Cell survival was accessed by Trypan Blue exclusion (dye-excluding = living cells; non-excluding = dead cells) or by using the MTT assay [28]. Briefly, 1×10^5 Trypan Blue-excluding cells/well were seeded into a 24-well plate, and at the indicated time points, 100 μl of 4 mg/ml MTT (Sigma) were added and the incubation continued for 3 h. The medium was then replaced with 500 μl dimethyl sulfoxide (Sigma), and after another 30 min of incubation (37 °C with shaking), optical densities were read in triplicate in 96-well plates at 540 nm with a Power WaveTM XS spectrophotometer (Biotek Instruments, Winooski, VT, USA).

Colony formation assay

Equal numbers of Trypan Blue-excluding HepG2 cells were seeded into a 6-well plate (5×10^4 cells per well), and five days later the cells were fixed with methanol (10 min), stained with Crystal Violet (30 min), and washed with distilled water. The number of colonies formed over a 5-day period was determined from counting 10 random fields.