FEBS Open Bio 4 (2014) 420–425

FEBS openbio

journal homepage: www.elsevier.com/locate/febsopenbio

Identification of a truncated kinase suppressor of Ras 2 mRNA in sperm

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ARTICLE INFO

Article history:
Received 3 December 2013
Revised 31 March 2014
Accepted 16 April 2014

Keywords:
KSR2
Scaffold
Sperm

ABSTRACT

The kinase suppressor of Ras 2 (KSR2) is a scaffold protein for the extracellular signal-regulated protein kinase (ERK) signaling pathway. KSR2 mediates germline *mpk-1* (*Caenorhabditis elegans* ERK) phosphorylation in *C. elegans* and has been implicated the regulation of meiosis. KSR2$^{-/-}$ mice exhibit metabolic abnormalities and are reproductively impaired. The role of KSR2 in meiosis and fertility in mice has yet to be elucidated. Here, we describe a novel truncated KSR2 mRNA identified in mouse testes (T-KSR2). Further analysis demonstrates T-KSR2 is specific to mouse testes and mature sperm cells. The detection of T-KSR2 may enhance our understanding of mechanisms controlling spermatogenesis and fertility.

1. Introduction

Kinase suppressor of Ras 1 and 2 (KSR1 and KSR2) are scaffold proteins for the Raf/MEK/ERK Mitogen Activated Protein Kinase (MAPK) signaling pathway [1–3]. KSR1$^{-/-}$ mice are overtly normal but resistant to Ras-induced tumors [4,5]. KSR2$^{-/-}$ adult mice are profoundly obese and insulin resistant [6,7]. Similarly, humans with KSR2 mutations have early onset obesity and severe insulin resistance [8]. The extent of murine KSR1 and KSR2 homology is contained within five conserved areas (CA). The N-terminus contains the CA1 domain, a domain unique to the KSR family; the CA2 domain is proline-rich and contains a Src homology 2 (SH2) domain; the CA3 domain is cysteine-rich and responsible for mediating the translocation of KSR protein to the plasma membrane; the CA4 is a serine/threonine-rich domain that contains the ERK binding motif; and the CA5 domain, which is located in the C-terminus end of KSR proteins, is a kinase-like domain and mediates the interaction with MEK [1]. In *Caenorhabditis elegans*, *ksr1* and *ksr2* are required for Ras-mediated signaling [3]. Although the two members coordinately regulate Ras signaling, these genes have distinct effects on fertility in *C. elegans*. Disruption of *ksr1* in *C. elegans* results in fertile offspring while *ksr2* disruption causes sterility. *ksr2* is specifically required for Ras-mediated signaling during germline meiotic progression in *C. elegans*. Without KSR2, oogenesis is arrested at the pachytene stage [3]. Proteins that interact with and phosphorylate KSR1 and KSR2 regulate Ras/MAPK activity to regulate *C. elegans* development. Nucleoside diphosphate kinase, NDK-1, regulates vulva development in *C. elegans* by direct physical interaction with KSR1 and KSR2 [9–11].

KSR1$^{-/-}$ mice are also fertile and develop normally, but do exhibit enlarged adipocytes, altered hair follicles, and modest defects in T cell activation [4,5,12]. However, KSR2 plays a larger role in reproduction, as male and female KSR2$^{-/-}$ mice exhibit impaired fertility [6]. KSR2$^{-/-}$ females begin estrous cycles later than WT females and have impaired mammary development, while KSR2$^{-/-}$ males have reduced sex drive and copulate infrequently (unpublished observations). These studies suggest KSR2 plays an important role in regulating fertility and metabolism in mammalian animals.

KSR1 protein is expressed in the brain, spleen, bladder, ovary, testis, and lung. However, a variant form of KSR1, B-KSR1, has been identified in brain tissue [13,14]. KSR1 functions in mediating Ras-induced cell proliferation, cell transformation, and survival. B-KSR1, which has a longer CA4 domain and a truncated C-terminus relative to KSR1, is critical in mediating Ras-dependent signaling to promote neurite growth and to maintain neuronal differentiation [14]. The paralog *ksr2* gene was first discovered in *C. elegans* and was found to have two alternative spliced forms, with one variant having shorter CA1 and CA4 domains [3]. Human KSR2 was also found to have two alternative spliced forms that varied from the full-length 950 amino acids. One variant lacks the first 29 amino acids (hKSR2ΔN29) and the second identified

Abbreviations: KSR, kinase suppressor of Ras; ERK, extracellular signal-regulated protein kinase; T-KSR2, testes-kinase suppressor of Ras 2; MAPK, mitogen activated protein kinase; CA, conserved area; SH2, Src homology 2; RACE, rapid amplification of cDNA ends; NDK-1, nucleoside diphosphate kinase 1

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http://dx.doi.org/10.1016/j.fob.2014.04.004
2211-5463/

variant (hKSR2ΔCA1) lacks the CA1 domain. The hKSR2ΔCA1 cDNA clones were obtained from kidney and testes cDNA libraries. Northern blot analysis revealed hKSR2ΔCA1 mRNA expression in human brain and kidney tissue [15]. hKSR2ΔCA1 has been described as a regulator of proto-oncogene Cot-induced MAPK signaling. In mice, KSR2 protein is detected in the brain and pancreas [6,16]. A murine homolog of hKSR2ΔCA1 cDNA was detected in a mouse kidney cDNA library [15]. These data suggest that mouse KSR2 can be alternatively spliced and variant KSR2 expression is tissue-dependent.

In this study, we describe and characterize a truncated KSR2 mRNA in mouse testis. This variant form of KSR2 lacks the CA1 and CA2 domains, encoding a predicted 598 amino acids. We determined that this truncated mRNA leads to stable protein expression *in vitro*.

2. Materials and methods

2.1. Animals and tissue collection

Mice were housed in pathogen-free conditions and experiments were carried out under a protocol approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (University of Nebraska Medical Center, Omaha, NE). Mice were maintained on a 12 h light/dark schedule with free access to laboratory chow (*ad libitum*) and water. Mice were sacrificed by the administration of CO_2 followed by cervical dislocation. Tissues from 8 to 12 week old C57BL/6J mice were dissected, frozen on dry ice, and stored at −80 °C until used.

2.2. Sperm purification

Sperm were collected as previously described [17]. Briefly, the caudal epididymis was minced and the sperm were allowed to swim out in phosphate buffered saline (PBS). The sperm-containing PBS was gently aspirated and collected by centrifugation at 800×*g* for 5 min at room temperature. Sperm were then lysed in TRI reagent (Molecular Research Center Ins, TR118). Sperm were 99% pure as assessed by light microscope. To remove any potential somatic cells, the sperm were centrifuged at 800×*g* for 5 min and the pellet was treated with a hypotonic buffer (0.1% SDS, 0.5% Triton X100 in deionised water) for half hour, as previously described [18]. The sample was centrifuged at 600×*g* for 15 min at 4 °C. The supernatant was removed and the sample washed twice with PBS, then centrifuged at 600×*g* for 5 min at 4 °C.

2.3. RNA isolation and cDNA synthesis

Total RNA from mouse tissues was isolated with an RNeasy mini kit (Qiagen) according to the manufacturer's protocol with modification on the lysis step as previously described [19]. Blood samples were lysed in Tri reagent BD (MRC, TB126). Due to the low level of RNA in hypotonic buffer-treated sperm, yeast tRNA was added as carrier RNA during lysis. RNA was treated with DNase I (Ambion, AM1906) before cDNA synthesis. cDNA from total RNA was produced with M-MLV reverse transcriptase (Ambion, AM2043). PCR was done with Herculase II DNA polymerase (Agilent, 600675-5).

2.4. Rapid amplification of cDNA 5′ ends (5′ RACE)

Ten micrograms of total RNA was used as template for the First-Choice RLM-RACE kit (Ambion, AM1700). Briefly, RNA was treated by Calf Intestine Alkaline Phosphatase (CIP), followed by Tobacco Acid Pyrophosphatase (TAP) and adaptor ligation. A no TAP (–TAP) control was used to ensure the 5′ RACE products are from full-length mRNA. A primary and a nested PCR were performed with 5́ RACE adapter primers provided by the kit (modified by changing the *BamH1* restriction enzyme site into an *EcoR1 site*) and gene specific reverse primers. The PCR products were cloned into the vector pcDNA3.1(−) and several clones were isolated for sequencing.

Primer sequences (*Kpn1* and *EcoR1 sites* in bold, PYO tag in Italic):

Gene-specific RACE inner primer R460: 5′-GAT TAT CCA CAG AGG AGA CCC **GGT ACC** GG-3′
Gene specific RACE outer primer R490: 5′-GTC AGA CTC TCC CCA AAA CC-3′
KSR2 F610: 5′-CC **GAA TTC** CAA CCT CCG AGA ACG AAG AG-3′
KSR2 Rstop: 5′-GC **GGT ACC** TCA CAG CTC TGC AGA CTT CCA GAA ATG TCC-3′
T-KSR2 5UTR: 5′-CG **GAA TTC** AAT GTA TCA GGC GCT TTG CCG AAC AC-3′
KSR2 F9: 5′-CGA AAA GCG AAG AGC AGC AAC-3′
KSR2 R207: 5′-CG **GAA TTC** GGC TGG TAG GAC AGA AGT GC-3′
GAPDH F: 5′-AGG CCG GTG CTG AGT ATG TC-3′
GAPDH R: 5′-TGC CTG CTT CAC CAC CTT CT-3′
PYO-KSR2 R: 5′-GC **GGT ACC** TCA *CTC CAT TGG CAT GTA CTC CAT CTC CAT TGG CAT GTA CTC CAT* CAG CTC TGC AGA CTT CCA GAA ATG TCC-3′
Protamine-1 F: 5′-AGC AAA GCA GGA GCA GAT G-3′
Protamine-1 R: 5′-GGC GAG ATG CTC TTG AAG TC-3′
E-cadherin F: 5′-CAG CTC CTT CCC TGA GTG TG-3′
E-cadherin R: 5′-TGC ACC CAC ACC AAG ATA CC-3′
c-kit F: 5′-AAC GAT GTG GGC AAG AGT TC-3′
c-kit R: 5′- CCT CGA CAA CCT TCC ATT GT-3′

2.5. Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were passaged every 2–3 days. Both T-KSR2 and full-length KSR2 were infused with polyoma virus-derived (PYO) epitope tag and were cloned into pcDNA3(−) vectors. Two copies of the PYO epitope tag (amino acids MEYMPME) were included in the 3′ primer immediately upstream of the stop codon. Transfections were performed utilizing Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol.

2.6. Western blots

Western blots were done as described previously [20] with slight modifications. Briefly, cells were lysed in buffer containing 1% Igepal, 20 mM Tris (pH 8), 137 mM NaCl, 10% glycerol, 10 μg/ml aprotinin, 20 nM leupeptin, 0.5 mM sodium orthovanadate, 2 mM EDTA, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. Primary PYO antibody was obtained from cultured hybridoma cells as described previously [21]. Proteins were detected using Odyssey imaging system (LI-COR Biosciences).

3. Results

3.1. A truncated KSR2 transcript in testes

By analyzing a number of 5′ RACE products from testes, an alternate KSR2 mRNA (T-KSR2) (GenBank: KJ719253) was detected exclusively in testes (Fig. 1A). The predicted first exon of T-KSR2 (exon1t) resides within intron 5 of full-length KSR2, contains a