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Raf kinase inhibitory protein knockout mice: Expression in the brain and olfaction deficit

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Abstract

Raf kinase inhibitory protein (RKIP-1) is involved in the regulation of the MAP kinase, NF-κB, and GPCR signaling pathways. It is expressed in numerous tissues and cell types and orthologues have been documented throughout the animal and plant kingdoms. RKIP-1 has also been reported as an inhibitor of serine proteases, and a precursor of a neurostimulatory peptide. RKIP-1 has been implicated as a suppressor of metastases in several human cancers. We generated a knockout strain of mice to further assess RKIP-l's function in mammals. RKIP-1 is expressed in many tissues with the highest protein levels detectable in testes and brain. In the brain, expression was ubiquitous in limbic formations, and homozygous mice developed olfaction deficits in the first year of life. We postulate that RKIP-1 may be a modulator of behavioral responses.

Keywords: RKIP; PEBP; Gene trap; Knockout mouse; Brain; Behavior; Olfaction

1. Introduction

Raf kinase inhibitory protein (RKIP), also known as phosphatidylethanolamine binding protein (PEBP) [3], was first documented as an important regulator of the MAP kinase (MAPK) signal transduction pathway, whose role is to rapidly convert extracellular signals into activation of transcription factors [6,20,32,38]. To date, RKIP-1 is the only known cellular inhibitor of Raf kinase.

Other signaling pathways are also influenced by RKIP-1. For example, the inhibition of RKIP-1 enhances NF-κB-induced transcription while over-expression reduces it [40]. In addition, RKIP-1 impinges on GPCR signaling by controlling the activity of the G-protein coupled receptor kinase-2 (GRK2) [21,23].

The functions of RKIP-1 in a variety of organisms are diverse and include inhibition of carboxypeptidase Y in yeast [5], an immunoprotective function in nematodes [12], odorant binding and detection in *Drosophila* [12], and control of shoot growth and flowering in plants [33]. In mammals, signaling proteins are often linked to disease states [22], and recent work indicates that RKIP-1 is a metastasis suppressor [7,13,19,31]. Although localized primarily in the cytoplasm, it is also the primary source of the extracellular hippocampal cholinergic neurostimulatory peptide (HCNP) [30].

Here, we report the generation of a knockout strain of mice. These mice were derived from ES cells carrying a gene trap in intron 1 of RKIP-1. We found that mice homozygous for the mutated allele are viable and appear normal up to 10 months of age. However, they develop an olfaction deficit, a phenotype that correlates with the expression pattern of the gene in the brain.

2. Materials and methods

2.1. ES cells

The ES cell line AQ0005 carrying the pGT01xr vector was obtained from The Welcome Trust Sanger Institute.

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2.2. Generation of mice

All breeding and procedures were carried according to NIH Guide for the Use and Care of Laboratory Animals and institutional regulations at Brown University animal facility. The AQ0005 ES cells were grown until 90% confluency and tripsinized before injection. E3.5 blastosysts were derived from C57BL/6-*Tyr*$^{c\text{-}Brd}$, female mice and injected with 12–20 ES cells. The injected blastocysts were implanted into the uteri of day 2.5 pseudo-pregnant females for generation of chimeras. Eight to ten injected embryos were implanted per uterine horn. The chimeras were mated with C57BL/6-*Tyr*$^{c\text{-}Brd}$ females to obtain Fl progeny. The strain carrying the germ line transmitted allele (designated *RKIP1*$^{Gt(pGT01xrBetageo)1Jkl}$ and referred to hereafter to as the minus allele) was maintained on a mixed C57BL/6-129Ola background.

2.3. Genotyping

Genomic DNA was prepared from ES cells or tail biopsies by incubation in 200 μl of lysis buffer (20 mM NaCl, 50 mM Tris–HCl, pH 8.8, 10 mM EDTA, 1.5% sodium dodecyl sulfate and l mg/ml of proteinase K). DNA was extracted with phenol/chloroform (1:1), centrifuged 4 min at 10,000 RPM, transferred to a new tube, extracted three times with chloroform, and precipitated with 500 μl of ethanol. The DNA pellet was washed twice with 80% ethanol and dissolved in 10 mM Tris–HC1, pH 7.5. PCR was performed using primers b + f for detecting the gene trap allele, and primers b + g for detecting wild-type allele (Fig. 1A). A 600 bp product was produced with primers b + f, while primers b + g produced a 900 bp PCR band. The PCR consisted of an initial incubation at 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 60 s. The amplified DNA was resolved on 2% agarose gels.

2.4. Staining of tissues and embryos

Tissues and embryos were dissected out from deeply anesthetized (ketamine) adult animals, placed in cold 1 × PBS buffer and fixed for 1–2 h in 2% paraformaldehyde (Sigma) depending on the size. Tissues were then washed three times in PBS for 20 min each time. Staining for *β-geo* was done in 2 mM $MgCl_2$-0.01%, deoxyxholate-0.02% NP-40-100 mM phosphate buffer (pH.8.0), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 1 mg/ml of X-Gal. After staining, the tissues and embryos were washed in PBS and stored in 4% PFA at 4 °C. Brains were isolated, post-fixed for 2 h in 5% formaldehyde at 4 °C, and embedded in 3% low melting temperature agarose (Invitrogen) pre-cooled to 37 °C. After 10 min at 4 °C, vibratome sections were made and used immediately for X-Gal staining.

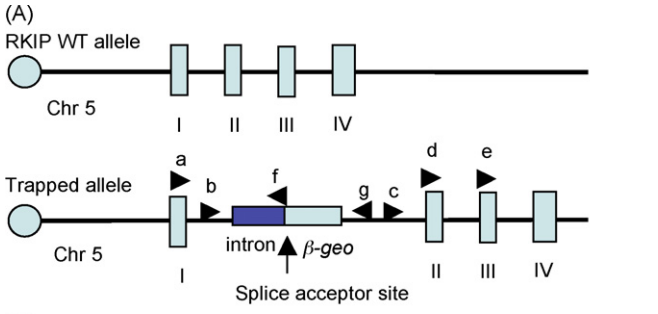

Fig. 1. Genomic structure of RKIP-1 locus and the identity of gene trapped ES cells. (A) Genomic structure of the RKIP gene and the gene trapped allele. (B) Primers used to verify integration site of the gene trap at RKIP-1 locus. (C) Gel electrophoresis of PCR products obtained using AQ0005 DNA as a template and different combinations of primers.

2.5. Western blots

Primary cultures of tail fibroblasts were established [35] and protein extracts prepared using RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% NP40) supplemented with protein inhibitors (1 mM PMSF, 1 mM Na_3Vo_4, 1 mM NaF). For preparation of tissue extracts, 10 mg of tissue was homogenized on ice in 450 μl of RIPA buffer supplemented with protein inhibitors. The lysate was then centrifuged for 40 min, 16,000 × *g* at 4 °C, and the collected supernatant was centrifuged a second time for 15 min at 16,000 × *g* at 4 °C. The concentration of protein was determined using the Biorad D/C protein assay kit. The extract was mixed with Laemli sample buffer (containing SDS and β-mercaptoethanol), 50 μg of total protein per lane were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to immobilon-P, and probed with rat anti-RKIP antibodies (Upstate Biotechnology). After probing with a secondary antibody (HRP-conjugated goat-anti rabbit IgG, Jackson Immunoresearch, Inc.) the filters were incubated with a chemiluninescent substrate and detected by exposing the blot to KODAK Biomax film.

2.6. Real time qPCR

Total cellular RNA was extracted with the Trizol reagent (Invitrogen) and transcribed into cDNA using random hexamer primers and the TaqMan (r) kit from Applied Biosystems. qPCR was performed using the SYBR green system from Applied Biosystems according to provided instructions and analyzed in the Prism 7700 sequence detector (Applied Biosystems). All reactions were performed in duplicate and glyceraldehyde phosphate dehydrogenase (GAPDH mRNA) was used as an internal standard. Default cycling parameters and threshold values, provided by Applied Biosystems for the Prism 7700 instrument were used. Relative abundance of RKIP mRNA was calculated using the ΔΔCT method provided by Applied Biosystems using the abundance of GAPHD mRNA as an internal standard to correct for sample recovery and experimental variability between samples.

2.7. Behavioral tests

For the odor object recognition test, mice (50% C57BL/6,50% 129Ola) between 2 and 4 months ($n = 18$ per group) or 5 and 8 months of age ($n = 13$ per group) were selected randomly. Food and water were available *ad libitum*. The age distribution between the groups under comparison was always similar. The odor object (cheddar cheese) recognition test was performed essentially as described [8]. Briefly, the experimenter was blind to the genotypes of the investigated mice. The piece of cheese was hidden under the bedding in one corner of the clean testing arena. The mouse was placed in the opposite corner of the arena and positioned diagonally from the hidden odor food object. The time that elapsed to uncover the food was recorded in each case. The cut off time point was set at 20 min. Mice were maintained on a 12 h day/night light cycle and tests performed during the dark phase.

RKIP-1$^{-/-}$ ($n = 26$) and wild-type ($n = 28$) mice were also tested on a sensory discrimination task that included visual, tactile, and olfactory problems. For these experiments Fl mice were backcrossed three times to C57BL/6 and then intercrossed to generate testing animals. Twenty-six mice were tested at 3.5–4 months of age and 28 mice were tested at 5–5.5 months of age. Tested mice included males ($n = 30$) and females ($n = 24$). In this task, mice were presented with two clay pots one of which contained a hidden food reward (candy sprinkle, Sweet Toppings Carousel Mix, Betty Crocker). Mice were required to learn which pot contained the food reward. Three problems (pairs of pots) were presented. Stimulus pots were distinguished by digging medium inside (green or