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RKIP regulates MAP kinase signaling in cells with defective B-Raf activity

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ABSTRACT

MAP kinase (MAPK) signaling results from activation of Raf kinases in response to external or internal stimuli. Here, we demonstrate that Raf kinase inhibitory protein (RKIP) regulates the activation of MAPK when B-Raf signaling is defective. We used multiple models including mouse embryonic fibroblasts (MEFs) and primary keratinocytes from RKIP- or Raf-deficient mice as well as allografts in mice to investigate the mechanism. Loss of B-Raf protein or activity significantly reduces MAPK activation in these cells. We show that RKIP depletion can rescue the compromised ERK activation and promote proliferation, and this rescue occurs through a Raf-1 dependent mechanism. These results provide formal evidence that RKIP is a bona fide regulator of Raf-1. We propose a new model in which RKIP plays a key role in regulating the ability of cells to signal through Raf-1 to ERK in B-Raf compromised cells.

1. Introduction

The mitogen-activated protein kinases (MAPKs) MAPK1 and MAPK3 (also known as ERK2 and ERK1, respectively) play a central role in cell proliferation, migration, differentiation and apoptosis by regulating immediate-early and delayed gene expression [1]. The upstream signaling pathway (RAS–MAPKKK–MAPKK) links extracellular and intracellular signals to MAPKs, and is regulated at multiple levels. Therefore, it may not be surprising that dysregulation of this pathway leads to various diseases including cancer [2] and developmental disorders [3]. While mammalian cells have three MAPKKKs (B-Raf, Raf-1, and A-Raf), previous studies have shown that two Rafs, B-Raf and Raf-1, can form a heterodimer with increased activity relative to their homodimers or monomers [4,5]. Thus their interaction is thought to be biologically significant for activation of the MAPKK, MEK, in the cell. In addition, association of B-Raf and/or Raf-1 with the scaffold protein KSR1 also modulates activation of MEK [6–8]. These studies underscore the importance of multiplex protein interactions in the regulation of this pathway.

Several proteins including Raf kinase inhibitory protein (RKIP) negatively regulate MAPK signaling [9]. RKIP belongs to the phosphatidylethanolamine binding protein (PEBP) family in prokaryotic and eukaryotic organisms [10]. Our previous work demonstrated that the direct binding of RKIP to Raf-1 inhibits Raf-1 activation via the blockade of two key activation sites on Raf-1: Ser338 and Tyr341 phosphorylated by PAK and Src, respectively [11]. The association between RKIP and Raf-1 is negatively regulated by PKC-mediated phosphorylation of RKIP (Ser153) [12]. Although RKIP also modulates other signal pathways including G protein-coupled receptors (GPCRs) and nuclear factor-κB (NF-κB) signal transduction cascades in addition to the MAPK pathway [13,14], it has been unclear whether RKIP plays a physiologically and biologically significant role in modulating these signaling pathways.

Despite the implicated role of RKIP in the regulation of the MAPK pathway, a previous study reported that RKIP deficient mice reproduced and lived normally [15]. To provide more definitive answers to the question of whether RKIP plays a regulatory role in the MAPK pathway in normal cells, we have also generated RKIP knockout mice and isolated MEFs that can be manipulated in culture. Furthermore, we have set up genetic crosses between Braf and RKIP mutant mice to address the role of RKIP in the regulation of Raf-1. We show that while loss of B-Raf significantly reduces MAPK activation in wild type MEFs, loss of RKIP can rescue the compromised ERK activation in B-Raf-deficient MEFs through a Raf-1-dependent mechanism. These results are confirmed in primary keratinocytes isolated from mice lacking either or both Braf and RKIP, as well as MEF allografts in mice. Our studies uncover the role of RKIP in regulating the ability of Raf-1 to compensate for B-Raf deficiency in cells.

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2. Experimental procedures

2.1. ES cell line, generation of chimeras and genotyping

The gene-trapped ES cell line AQ0005 with disruption of RKIP expression was obtained from The Welcome Trust Sanger Institute (GT allele: Pebp1 Gt(AQ0005)Wtsi).

The approach used to generate RKIP−/− mice is similar to that described previously [15]. C57BL/6 blastocyst stage embryos were injected with AQ0005 ES cells (RKIP+/−), and then the injected blastocysts were transferred to pseudopregnant CD1 mice for further development. Chimeric mice were mated to C57BL/6 and agouti offspring were genotyped. Germline transmission of the gene-trapped allele was determined by PCR analysis of tail DNA. The PCR primers used for RKIP genotyping were reported previously: (a) 5′-GAG CCC TGG CCG GTC TCC CTT GTC CCA AAC TTT-3′; (b) 5′-TGA GGA CTC CCT GGC CTC CAG ACA AGT AGA TCC-3′; and (c) 5′-GAC TTC CGT GTC CGG ATG ATA GAT AGC CTC TCC-3′. Primers a+b produce approximately 600 bp PCR fragments for detecting the RKIP gene trapped allele, and primers a+c produce 978 bp PCR fragments for detecting the RKIP wild-type allele. The homozygous RKIP-deficient mice appear normal and breed without any overt problems in 129Sv/B6 hybrid backgrounds as well as in a congenic C57BL/6J background (>N10).

2.2. Cell culture for MEFs and cell lines

Mouse embryonic fibroblasts (MEFs) were derived from embryos at embryonic day 12.5 (E12.5) [16]. After removal of the head and internal organs, embryos were rinsed with phosphate-buffered saline (PBS), and minced using 3 ml syringe and 18 G needle for 10 times. The MEFs were maintained in Dulbecco minimal Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U/ml of penicillin/streptomycin. Primary MEFs were immortalized using a retrovirus encoding a temperature-sensitive SV40 large T-antigen (T^{ts}) [17]. Virus and cells were co-incubated in 8 μg/ml polybrene for 4–24 h and immortalized cells were grown out by continuous culture at 33 °C, the permissive temperature for the T^{ts}, in DMEM containing 10% FBS, 50 U/ml of penicillin/streptomycin, 200 μg/ml G418. T^{ts}-immortalized MEFs were then serum-starved at 39 °C, the non-permissive temperature, in DMEM overnight prior to EGF treatment. Other cells such as 293-T, non-T^{ts}-immortalized MEFs, and primary MEFs were grown in DMEM with 10% FBS, 50 U/ml of penicillin/streptomycin at 37 °C in a standard CO_2 incubator, and serum-starved at 37 °C in DMEM overnight up to 24 h prior to treatment.

2.3. Generation of B-Raf−/−; RKIP−/− mice and isolation of primary mouse keratinocytes

To obtain RKIP−/−; B-Raf−/− keratinocytes we mated RKIP−/− mice with epidermis-restricted B-Raf knockout induced by a keratin 5-promoter driven Cre recombinase (*K5Cre*; $Braf^{flox/flox}$ mice; hereafter referred to as $Braf^{\Delta/\Delta ep}$) [18] on a 129SV background.

Primary mouse keratinocytes were isolated from adult mice as previously described [19], plated at a density of ~2×10^6 cells/60 mm plate, cultured in low-calcium MEM (Sigma) containing 1 μg/ml insulin (Sigma), 2 ng/ml EGF (Roche), 2 μg/ml transferrin (Sigma), 10 μM phosphoethanolamine (Sigma), 10 μM ethanolamine (Sigma), 0.36 μg/ml hydrocortisone (Calbiochem), glutamine (Invitrogen, Glutamax-I), penicillin/streptomycin (Invitrogen) and 8% chelated FBS (BioRad, Chelex 100 Resin). The cells were serum-starved overnight in medium containing 1% FBS without growth factors before stimulation.

2.4. Stable short hairpin RNA (shRNA) and RKIP rescue cell lines

The RKIP shRNA retroviral construction and stable cell lines generation were described previously [11]. Briefly, the puromycin-based constructs with RKIP shRNA and vector control were used for transient transfection or transfected into the Phoenix-Ampho packaging cell line for virus production. To make stable cell lines, cells were exposed three to five times to virus-containing supernatant, and stable populations were obtained by selection and subsequent maintenance with 2 μg/ml puromycin. RKIP rescue MEFs were made by transducing immortalized RKIP−/− MEFs with the retrovirus vector pCLE-HA-RKIP.

2.5. Knockdown of Raf-1 or B-Raf by small interfering RNA (siRNA)

siRNAs specific to rat, mouse or human Raf-1 and B-Raf were obtained from Dharmacon Inc. and transfected at a concentration of 50 nM using Nucleofector reagent V (Amaxa, Inc.) by electroporation. 48 h post-transfection, cells were serum-starved and then left unstimulated or treated with EGF (10 ng/ml).

2.6. Immunoreagents and chemicals

Phospho-ERK1/2 antibody (Cat no: 9101) and ERK1/2 antibody (Cat no: 9107) were purchased from Cell Signaling. Raf-1 antibody (Cat no: sc133) and B-Raf antibody (Cat no: sc-5284) and goat anti-mouse IgG-HRP (Cat no: sc-2005) were purchased from Santa Cruz. RKIP rabbit polyclonal antibody was described in our previous report [12]. β-actin antibody (Sigma, Cat no:A5441), anti-rabbit IgG-HRP (GE Healthcare, Cat no: NA934V), α-tubulin (Santa Cruz, Cat no: sc-58667), Goat anti-Mouse IRdye 800CW (Li-COR, Cat no: 926-32210), Goat anti-Rabbit IRdye 680 (Li-COR, Cat no: 926-32221) were also used.

2.7. Immunoblotting

Total cellular protein was extracted from cells with Radio Immuno Precipitation Assay (RIPA) lysis buffer containing non-ionic (NP-40 or Triton X-100) and ionic (sodium dodecylsulfate and sodium deoxycholate) detergents (USB or Sigma) and the protease inhibitors (Calbiochem, Cat no: 539134), and protein concentration was determined with the modified Bradford assay (Biorad). 20–30 μg total protein was separated on 12% bis/tris SDS-PAGE gels and transferred to nitrocellulose membranes (BioRad) using a semi-dry apparatus. Nitrocellulose membranes were blocked in Odyssey blocking buffer: 1× PBS (1:1) (Li-COR, Cat no: 927-40000), incubated at 4 °C with primary antibodies with various dilutions overnight (RKIP 1:1000; phospho-ERK 1:1000, total ERK 1:2000; B-Raf 1:500; Raf-1 1:500), incubated with secondary antibodies Goat anti-Mouse IRdye 800CW (1:8000) or goat anti-rabbit IRdye 680 (1:8000), and detected and quantified using the Li-COR Odyssey system. For those antibodies that are not well compatible with Odyssey system, the regular blocking buffer 5% non-fat dry milk, and enhanced chemiluminescence detection system (Amersham) were used for the immunoblotting. Total ERK, α-tubulin or β-actin were used as loading control. PageRuler Prestained Protein markers (Fermentas, Cat no: SM0671) were used as molecular weight standards.

2.8. Ethics statement

All procedures involving mice were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC) under the protocol #1196. Mice were housed under the standard care in one of the AALAC-accredited SPF facilities at the University of Chicago. All efforts were made to minimize suffering.