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K-Ras4B proteins are expressed in the nucleolus: Interaction with nucleolin

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Abstract

Kirsten Ras4B (K-Ras4B) is a potent onco-protein that is expressed in the majority of human cell types and is frequently mutated in carcinomas. K-Ras4B, like other members of the Ras family of proteins, is considered to be a cytoplasmic protein that must be localized to the plasma membrane for activation. Here, using confocal microscopy and biochemical analysis, we show that K-Ras4B, but not H-Ras or the closely related K-Ras4A, is also present in the nucleoli of normal and transformed cells. Subcellular fractionation and immunostaining show that K-Ras4B is located not only in the cytoplasm, but also in the nucleolar compartment. Modification of a C-terminal hexa-lysine motif unique to K-Ras4B results in exclusively cytoplasmic forms of the protein. Nucleolin, a pleiotropic regulator of cellular processes, including transcriptional regulation, is also characterized by a nucleolar-like nuclear appearance. We show that K-Ras4B and nucleolin co-localize within the nucleus and that nucleolin physically associates with K-Ras4B. Inhibition of K-Ras4B/nucleolin association blocked nucleolar localization of K-Ras4B. Using siRNA to knockdown the expression of nucleolin eliminated the nucleolar localization of K-Ras4B and significantly repressed the activation of the well-characterized K-Ras4B transcriptional target Ap-1, but stimulated Elk1. These data provide evidence of a nucleolar localization of K-Ras4B and describe a functional association between K-Ras4B and nucleolin.

Keywords: K-Ras4B; Nucleolin; Interaction; Oncogenic

The family of Ras proteins works as a network of signal transduction pathways that transfer information from the extra-cellular environment to the nucleus. Ras proteins are cytoplasmic proteins that translocate to the plasma membrane where they transmit growth factor signals and drive cell proliferation [1]. The *ras* genes are the most frequently mutated in human cancers, and activated *k-ras* mutations are found in 90% of pancreatic, 50% of colon, and 30% of lung cancers [2]. The *k-ras* gene has two alternative fourth exon variants that result from differential splicing: exon 4A (K-Ras4A) is present in the viral *k-ras* oncogene, while exon 4B (K-Ras4B) is preferentially expressed in human cells [3,4]. Transcription of K-Ras4B in human cells is 10- to 20-fold higher than K-Ras4A [2,3,5].

The Ras proto-oncogene homologues H-Ras, N-Ras, and K-Ras are composed of 188–189 amino acids and share 100% homology within the first 85 amino acids. By comparison, the last 25 amino acids of the Ras proteins define a highly divergent region, the hyper-variable domain (HVD). The HVD region of K-Ras4B includes a continuous hexa-lysine domain of residues with a further six lysines distributed within the domain. The HVD domain is sufficient to direct the protein to the plasma membrane, a pre-requisite for transformation [4].

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Nucleolin is a pleiotropic protein that is ubiquitously expressed and plays a critical role in cell regulatory processes such as transcriptional regulation, cell cycle progression, and proliferation [6]. Nucleolin has been described as a nuclear protein with a speckled pattern of distribution, but recent studies have localized it to the plasma membrane where it acts as a shuttling protein between the cytoplasm and nucleus [7].

Recently, the mutant H-Ras^{Val12} and Rap-1 have been reported to be in the nucleus of transformed cells, although no function was ascribed to this localization [8,9]. Here, we demonstrate that K-Ras4B physically interacts with nucleolin, which results in a nucleolar localization, and that nucleolin affects the transcriptional regulatory activity of the protein.

Materials and methods

Cell culture and reagents. Normal human dermal fibroblast (NHDF) cells were cultured in the recommended media, FGM-2-BulletKit medium (Clonetics, Walkersville, MD), with 10% FCS and 2 mM L-glutamine. NIH3T3, Chang, and HEK293 cells were grown in DMEM with 10% FCS and 2 mM L-glutamine. H-Ras, K-Ras4B, Nucleolin, Histone H3, and VEGF antibodies were purchased from Santa Cruz Biotechnology. The K-Ras 2A and 2B antibodies (here referred as 4A and 4B) are specific for the corresponding proteins and guaranteed by the distributor not to cross-react with each other or with N-Ras or H-Ras. Additional characterization involved visual analysis of non-specific bands during Western studies, and peptide blocking analysis by both confocal and Western blotting (data not shown). Other sources of antibodies for Ras used in these studies were BD Pharmingen/Transduction Laboratories (San Diego, CA) and Upstate USA, Inc. (Charlottesville, VA). Anti-fibrillarin antibody was obtained from Cytoskeleton Inc. (Denver, CO). SMARTpools consisting of four individual siRNA duplexes targeted against nucleolin mRNA and a non-targeting siRNA control were obtained from Dharmacon RNA Technologies and used as recommended by the distributor. HEK293 cells were transfected with a FLAG-tagged wild-type (wt)-K-Ras4B (flag-wt-K-Ras4B) and with a FLAG-tagged K-Ras4B mutant 12 (flag-K-Ras mt12) expression plasmids, along with the indicated amounts of siRNA for 48 h and then analyzed by immunoblotting. The pShooter mammalian expression vectors were used to target flag-wt-K-Ras4B or flag-K-Ras mt12 proteins to the nucleus or to the cytoplasm by incorporating the full-length K-Ras4B proteins, including signal sequences, into recombinant proteins with specific targeting signals to direct them to the nucleus (pCMV-myc-nuclear-GFP, abbreviated pCMV-nuc-GFP) or to the cytoplasm (pCMV-myc-cyto-GFP, abbreviated pCMV-cyto-GFP) ([38], Invitrogen, CA, USA). To analyze whether the polybasic carboxy terminal domain of the K-Ras4B protein mediates nuclear localization, the hexalysine sequence (175-KKKKKK-180) present in flag-wt-K-Ras4B was mutated to obtain flag-K-Ras mt12. (mt12, 175-ILAILL-180) by introducing base substitution mutations into codons corresponding to basic amino acid residues resulting in codons corresponding to neutral amino acid residues (these amino acid changes altered the ability of the residues to bind negatively charged domains).

Immunofluorescence and confocal microscopy. The cellular distribution of the proteins studied was examined with the antibodies indicated. Cells were transfected with expression vectors for flag-wt-K-Ras4B and flag-K-Ras mt12. The transfected or non-transfected cells were serum-starved for 24 h before confocal microscopy analysis. Cells grown on poly-L-lysine-coated coverslips were washed with PBS and fixed with 10% formalin (Sigma). Coverslips were washed with PBS and blocked with 10% normal goat serum in TBST (0.1 M Tris–HCl, pH 8.0, 0.15 M NaCl, and 0.5% Triton X-100) for 60 min, followed by incubation with primary antibody overnight at 4 °C. The indicated antibodies were added to a final concentration of 1μg/ml, unless otherwise specified. Blocking peptide was added to the antibody at a final concentration of 10 μg/ml. Following incubation at 4 °C for 30 min, 150 μl was applied to each sample and then were incubated overnight at 4 °C in the dark. After washing the slides, the appropriate FITC- and rhodamine-labeled secondary antibodies (Molecular Probes, Eugene, OR) were added and incubated for 60 min at room temperature. The coverslips were mounted on slides using Gel/Mount (Biomeda, Foster City, CA). The confocal images were made using a Zeiss 310 confocal microscope with a HeNe laser with 543-excitation for rhodamine, argon laser with 488-excitation for FITC, and a coherent UV laser with 364-excitation for DAPI. The images and stored parameter files for intensity, pinhole aperture, contrast, attenuation, etc., were held constant and identical for all images. Files were stored to disk and printed using a HP Laser Jet 4500. Co-localization of red and green produces orange-yellow, while red, green, and blue combine to produce a pale yellow.

Pixel quantification. The relative amounts of fluorescent intensity were then measured quantitatively using the Optimas 5.0 image analysis program (Bothell, WA). Measurements were done as previously described [37]. Areas of the samples were evaluated and a cursor drawing delineating the region of interest was made and called a mask. That region/mask was then applied to the corresponding fluorescent image and the fluorescence intensity of that area was measured.

Immunoprecipitation and Western blot analysis. Immunoprecipitations were carried out using Dynabeads according to the manufacturer's instructions (Dynal, New York City, NY). Cells were lysed in immunoprecipitation assay buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing phosphatase and protease inhibitors at 4 °C for 30 min, following by centrifugation to remove cell debris. Samples (1.5 mg) were incubated with beads coupled with a monoclonal anti-Flag antibody (Sigma) or corresponding polyclonal antibody for 4 h with horizontal rotation at 4 °C. Beads were washed at least five times and resuspended in SDS loading buffer. Immune complexes were resolved by electrophoresis on 4–20% gradient SDS–PAGE gels and transferred to membranes. Membranes were probed by Western blotting. The species-specific control antibodies used as needed were rabbit polyclonal IL-5 or tuberin, goat polyclonal IL8 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or rabbit or mouse IgG (Sigma, San Louis, MO). Immunoblots were visualized using an ECL system (Amersham Biosciences).

Transient transfection assays. All transfections used the FuGENE 6 Transfection Reagent following the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN) with a ratio of DNA to FuGENE 6 of 1 mg DNA to 3 ml reagent. Transfections lacking the vectors of interest contained an equal amount of the 'empty' expression vector. After overnight incubation, the cells were then analyzed for luciferase activity. β-Galactosidase activity and protein determination were used to correct for transfection efficiencies. Luciferase assays were carried out using a kit from Promega and following the manufacturer's instructions (Madison, WI).

Cell fractionation and nucleolar purification. Cell fractionation and nucleolar purification were carried out as indicated [10]. For protocol details, see http://www.lamondlab.com/f5nucleolarprotocol.htm. Following protein quantification, the samples were analyzed by SDS–PAGE and Western blot analysis.

Expression vectors and mutants. The human cytomegalovirus (CMV) major immediate-early promoter was used to initiate expression of the full-length K-Ras4B recombinant proteins with specific targeting signals to direct them to the nucleus (pCMV-myc-nuclear-GFP, abbreviated pCMV-nuc-GFP) or to the cytoplasm (pCMV-myc-cyto-GFP, abbreviated pCMV-cyto-GFP). M7 is a cDNA construct encoding 5′-flag sequence (DYKDDDDK) and K-Ras4B (Accession NP004976) in a mammalian expression vector. It was amplified by PCR, using pfu Turbo polymerase (Stratagene, La Jolla, CA) and the ATCC cDNA clone as template. The fragment was inserted into *Xho*I and *Kpn*I sites of the pCI expression vector (Promega, Madison, WI). K-ras encodes a protein of 188 amino acids with six consecutive lysine residues at 175KKKKKK180 for the wild-type M7 construct. Amino acid substitution mutants were also made. M12 is