Junichi Inokuchi · Misako Komiya · Iwai Baba
Seiji Naito · Takehiko Sasazuki · Senji Shirasawa

Deregulated expression of *KRAP*, a novel gene encoding actin-interacting protein, in human colon cancer cells

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Abstract We have identified a novel gene, designated *KRAP* (Ki-*ras*-induced actin-interacting protein), encoding a protein of 1,259 amino acids with coiled-coil regions and transmembrane regions, from the cDNA library of human colon cancer HCT116 cells, as one of the genes upregulated by activated Ki-*ras*. While *KRAP* was rarely expressed in normal colon epithelium, deregulated constitutive *KRAP* expression was observed in some other colon cancer cells. In normal tissues, *KRAP* was strongly expressed in pancreas and testis. Anti-*KRAP* polyclonal antibodies detected endogenous *KRAP* as the molecular size of M_r 180,000, and immunofluorescence microscopy and cytochalasin E treatment revealed that *KRAP* was clearly associated with the actin filaments. Furthermore, *KRAP* was localized as a membrane-bound form with extracellular regions. These results together suggested *KRAP* might be involved in the regulation of filamentous actin and signals from the outside of the cells.

Keywords *KRAP* · CS-1 · Ki-*ras* · Actin filaments · Cancer

J. Inokuchi · M. Komiya · I. Baba · S. Shirasawa (✉)
Department of Pathology, Research Institute,
International Medical Center of Japan,
1-21-1 Toyama, Shinjuku-ku,
Tokyo 162-8655, Japan
E-mail: sshirasawa@ri.imcj.go.jp
Tel.: +81-3-32027181
Fax: +81-3-32027364

J. Inokuchi · S. Naito
Department of Urology,
Graduate School of Medical Sciences,
Kyushu University,
3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan

T. Sasazuki
Research Institute,
International Medical Center of Japan,
1-21-1 Toyama, Shinjuku-ku,
Tokyo 162-8655, Japan

Introduction

Ras has been implicated in controlling cell proliferation, differentiation, and apoptosis (Barbacid 1987; Bos 1989; Almoguera et al. 1998; Bos et al. 1987; Forrester et al. 1987). We previously established HCT116-derived cells in which activated Ki-*ras* was disrupted through gene targeting (Shirasawa et al. 1993) and had reported the several critical molecular mechanisms of the activated Ki-*ras*-mediated signals in tumorigenesis utilizing these cells (Shirasawa et al. 1993; Allgayer et al. 1999; Okada et al. 1998; Rak et al. 1995; Baba et al. 2000; Tsunoda et al. 2002; Okumura et al. 1999). The cleavage signal-1 protein, CS-1, a protein of 249 amino acids reported to be detected as the molecular size of M_r 33,000 in Western blot using anti-CS-1 polyclonal antibodies, was originally identified as a sperm surface antigen involved in regulation of early cleavage of fertilized eggs (Javed and Naz 1992; Naz 1992). Here we have identified a novel gene, designated *KRAP* (Ki-*ras*-induced actin-interacting protein), including CS-1 sequences in the COOH terminal region. *KRAP* was overexpressed through the activated Ki-*ras*-mediated signals in HCT116 cells, and anti-*KRAP* polyclonal antibodies detected endogenous *KRAP* as a single band of the molecular size of M_r 180,000. Of great interest is that *KRAP* was colocalized with filamentous actin and was also localized at the cell surface.

Materials and methods

Isolation of differentially expressed genes

Isolation of differentially expressed genes between HCT116 and HKe3 cells was as described (Baba et al. 2000). *KRAP* cDNA was isolated by conventional plaque hybridization from the HCT116 cDNA library, and the full length of *KRAP* cDNA was determined by 5' rapid amplification of cDNA ends. Mouse *KRAP* cDNA was isolated from a mouse heart cDNA library using a human *KRAP* cDNA as a probe, and the full length of cDNA was determined as described above. DNA sequences were determined by ABI Prism Big Dye Terminator Cycle Sequencing (Applied Biosystems, Foster, CA, USA) with ABI 3100 sequencer.

Fig. 1A–C Deregulated *Ki-ras*-induced actin-interacting protein (*KRAP*) mRNA expression in human cancer cell lines and normal tissues. **A** Twenty micrograms of total RNA of HCT116, HKE3, and HKE3-derived transfectants expressing activated *Ki-ras* (e3-MKRas#9 and e3-MKRas#14) were run in a 1% agarose-formaldehyde gel, then Northern blots were done using the coding region of *KRAP* (upper panel) and β -actin (lower panel) as probes. **B** Twenty micrograms of total RNA of each cancer cell line were run in a 1% agarose-formaldehyde gel, and Northern blots were done using *KRAP* (upper panel) and β -actin (lower panel) as probes. **C** Human endocrine system multiple tissue Northern (MTN) blot filter (Clontech) (left panel) and human MTN blot II (Clontech) (right panel), on which 2 μ g of polyA RNA from each tissue was loaded, hybridized with *KRAP* as probes

Northern blot

Total RNA of cell lines was isolated by ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. Total RNA (20 μ g) was separated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to nylon membrane (Pall, East Hills, NY, USA), as described (Baba et al. 2000; Tsunoda et al. 2002). Human endocrine multiple tissue Northern (MTN) blot filter and human MTN blot II filter were purchased from Clontech (Palo Alto, CA, USA) on which 2 μ g of polyA RNA from each tissue was loaded. These filters were hybridized with probes for human *KRAP* cDNA (full length of the coding region), and β -actin cDNAs, as described (Okumura et al. 1999).

Anti-KRAP polyclonal antibodies

Anti-KRAP polyclonal antibodies (FT017–2) were raised in rabbits using the polypeptide (amino acids 1,178–1,192 of human *KRAP*: VDAAEGAPEVVGPKS). Polyclonal antibodies were purified from serum by using protein-G Sepharose column.

Transfections and stable transfectants

COOH terminal hemagglutinin (HA)-tagged (Baba et al. 2000) *KRAP* cDNA was subcloned into pSI vector (Promega, Madison, WI, USA). Cells were transiently transfected using Polyfect (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Total cell lysates were extracted 24 h after transfection then subjected to immunoblot analysis using Western blots (Okumura et al. 1999). For stable transfectants, COOH terminal HA-tagged *KRAP* cDNA was subcloned into pMG vector (Invitrogen, San Diego, CA, USA), and the linearized pMG-KRAP-HA was transfected into NIH3T3 cells using Polyfect. Cells maintained in Dulbecco's modified eagle's medium (DMEM) with

10% fetal calf serum (FCS) were split after 2 days, and cells were selected with 100 μ g/ml of hygromycin B (Invitrogen, Carlsbad, CA, USA). Colonies surviving in selection media in 14 days were picked up under the microscope and expanded and characterized for the expression of *KRAP*-HA by Western blot using anti-HA antibody (3F10) (Roche, Mannheim, Germany).

Immunofluorescence staining

Cells were grown on collagen-coated glass coverslips in growth medium. Twenty-four hours after transfection, cells were fixed with 3.7% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. After blocking with 1.4% milk in PBS containing 0.1% saponin, cells were incubated with anti-HA antibody (3F10) in PBS containing 0.1% bovine serum albumin (BSA) and 0.1% saponin for 1 h at room temperature, followed by incubation with Alexa Fluor 488 anti-rat IgG and/or Alexa Fluor 594 phalloidin (Molecular Probes, Eugene, OR, USA) at a dilution 1:2,000 for 1 h at room temperature. For counterstaining the nucleic acids, cells were incubated with 100 μ g/mL of DNase-free RNase in 2 $\times$ SSC (0.3 M NaCl, 0.03 M sodium citrate, pH7.0) followed by incubation with 500 nM solution of propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) for 5 min at 37°C. Cells were covered with a drop of GEL/MOUNT (Biomedex, Foster City, CA, USA), viewed by confocal laser scanning microscopy (FLUOVIEW FV500, OLYMPUS). Pretreatment of cytochalasin E (Sigma, Deisenhofen, Germany) (100 ng/ml or 1.0 μ g/ml in DMEM with 10% FCS) was done 3 h before fixing.

Surface biotinylation and immunoprecipitation

Biotinylation of cell-surface proteins was done as described (Baba et al. 2000). The surface-biotinylated lysates of the transfected HCT116 cells (4×10^7) were immunoprecipitated with 2 μ g/ml of

Fig. 2 Amino acids alignment of human and mouse Ki-ras-induced actin-interacting protein (KRAP). Transmembrane and coiled-coil regions are indicated by *dotted* and *solid lines*, respectively

Fig. 3 Activated *Ki-ras*-induced actin-interacting protein (KRAP) expression. Anti-KRAP rabbit polyclonal antibodies detected the endogenous KRAP at the molecular size of M_r 180,000 (arrow). HCT116, HKe3, and HKe3-MKRas#9 cells were grown at exponential growth conditions; 293T cells were transfected with pSI-KRAP-hemagglutinin (HA) or empty vector, and then whole cell lysates were run in a 6% SDS-PAGE

anti-HA antibody for overnight at 4°C, then protein G-Sepharose was added for 2 h. Protein G-Sepharose complexes were boiled for 10 min, and the supernatants were run in a 6% SDS-PAGE. Western blots were done using streptavidin-horseradish peroxidase (SA-HRP) (Amersham Biosciences, Piscataway, NJ, USA) and anti-HA antibody (3F10).

Results

To identify the genes located downstream of the activated *Ki-ras*-mediating signals, a PCR-based cDNA subtraction library was constructed between HCT116 cells and HCT116-derived activated *Ki-ras*-disrupted clone cells (HKe3) through homologous recombination (Shirasawa et al. 1993). The *KRAP* cDNA fragment was detected from this subtracted library, and the full length of *KRAP* cDNA was isolated from the HCT116 cDNA library (Baba et al. 2000). To confirm the differential expression of *KRAP* between HCT116 cells and HKe3 cells, Northern blot analysis was done under exponential condition. Northern blot detected the 5.2 kb-major *KRAP* transcript and the other minor 6.0 kb-*KRAP* transcript (Fig. 1). *KRAP* was strongly expressed in HCT116, whereas it was rarely observed in HKe3 (Fig. 1A). Furthermore, HKe3-stable transfectants expressing the activated *Ki-ras* (e3-MKRas#9, e3-MKRas#14) (Baba et al. 2000) evidently expressed *KRAP* (Fig. 1A). Northern blots revealed that *KRAP* expression was rarely evident in the normal colon epithelium, whereas it was strongly expressed in several kinds of human colon cancer cells (Fig. 1B). On the other hand, *KRAP*

expression in human normal tissues was strongly observed in pancreas and testis (Fig. 1C).

Human *KRAP* cDNA was isolated from the HCT116 cDNA library and the full length of *KRAP* cDNA was determined by 5' rapid amplification of cDNA ends (AB116937). *KRAP* encodes a hypothetical protein of 1,259 amino acids (a.a.) with the transmembrane (a.a.870–890) and coiled-coil regions (a.a. 992–1,038) (Fig. 2). To identify a mouse homologue of human *KRAP*, mouse cDNA library was screened using human *KRAP* cDNA fragment as a probe, and mouse *KRAP* cDNA encoding a protein of 1,252 a.a. was cloned (AB120565). Mouse *KRAP* cDNA had 81% identity with human *KRAP* at the nucleotides level and 86% similarity at the amino acid level (Fig. 2).

Anti-KRAP polyclonal antibodies (FT017–2) raised in rabbits using the peptides of the COOH terminal region of *KRAP* (a.a.1,178–1,192), recognized a single band of the molecular size of about M_r 180,000 in HCT116 and e3-MKRas#9, but barely in HKe3 (Fig. 3). The 293T cells were transiently transfected with *KRAP*-HA, HA-tagged in the COOH terminal region of *KRAP*, and *KRAP*-HA was evidently detected by FT017–2 at almost the same molecular size of *KRAP* detected in HCT116 cells, but was not detected in the nontransfected 293T cells. Furthermore, any processed *KRAP* was not detected in other human cancer cell lines examined by FT017–2 (data not shown).

To elucidate the localization of *KRAP* in the cells, NIH3T3-derived stable transfectants expressing *KRAP*-HA (NIH3T3-KRAP-HA#1) were stained with anti-HA antibody. *KRAP*-HA seemed to be colocalized with phalloidin-specific dye for F-actin in NIH3T3-KRAP-HA#1 (Fig. 4A), indicating that *KRAP* is colocalized with actin filaments. Cytochalasin E, an epoxide-containing metabolite of *Aspergillus clavatus*, is a compound with diverse activities on cellular function, including inhibition of actin polymerization and glucose transport (Udagawa et al. 2000). To elucidate the interaction of *KRAP* with the actin filaments, treatment with cytochalasin E was done. Phalloidin staining indicated that disruption of actin filaments by cytochalasin E occurred in a dose-dependent manner, and the interaction of *KRAP* with phalloidin gradually disappeared (Fig. 4B, C). These results together suggested that *KRAP* is associated with actin filaments in NIH3T3 cells.

To determine the localization of *KRAP* in cancer cells, COS7 cells and HCT116 cells were transfected with *KRAP*-HA and were stained with anti-HA and PI, indicating that *KRAP* is not localized in the nucleus (Fig. 4D). In COS7 cells, *KRAP* seemed to be localized near the cell membrane and the leading edge of the cells (Fig. 4E). In HCT116 cells, *KRAP* seemed to be also localized near the membrane regions (Fig. 4F). To elucidate whether *KRAP* exists as cell-surface protein, surface biotinylation was done, indicating that *KRAP* has the outer membrane region in HCT116 cells (Fig. 5).

Fig. 4A–F Colocalization of *Ki-ras*-induced actin-interacting protein (KRAP) with actin filaments. **A** A NIH3T3-derived stable transfectant expressing human KRAP hemagglutinin (HA) (NIH3T3-KRAP-HA#1) was established. Cells were fixed and stained with anti-HA antibody (*green*) and phalloidin (*red*). Superimposed images are also shown (*merge*). NIH3T3-KRAP-HA#1 cells were pretreated with 100 ng/ml (**B**) and 1 μ g/ml (**C**) of cytochalasin E in growth medium for 3 h. Cells were washed three times with PBS and stained with anti-HA antibody (*green*) and phalloidin (*red*). Superimposed images are also shown (*merge*). **D** COS-7 cells were transfected with pSI-KRAP-HA, and cells were grown on collagen-coated glass coverslips in growth medium. 24 h after transfection, cells were fixed and stained with anti-HA (*green*) and propidium iodide (PI) (*red*). Superimposed images are also shown (*merge*). COS7 cells (**E**) and HCT116 cells (**F**) were transfected with pSI-KRAP-HA and were stained with anti-HA antibody (3F10). COS7 cells were also stained with PI

Fig. 5 Ki-*ras*-induced actin-interacting protein (KRAP) as cell-surface protein. HCT116 cells were surface-biotinylated and then immunoprecipitated with anti-hemagglutinin (HA) antibody. The immunoprecipitated products were detected by anti-HA antibody (*left*) and streptavidin-horseradish peroxidase (SA-HRP) (*right*). Arrow indicates biotinylated KRAP

Discussion

We have identified a novel gene, designated *KRAP* (Ki-*ras*-induced actin-interacting protein), encoding a protein of 1,259 amino acids with coiled-coil and transmembrane regions, from the cDNA library of human colon cancer HCT116 cells, as one of the genes upregulated by activated Ki-*ras*.

Although Ki-*ras* mutation was detected in colon cancer cell lines, including HCT116, DLD-1, HCT-15, LS180, LoVo, and SW620 (Shirasawa 1993), *KRAP* was not overexpressed in LoVo cells, suggesting that other signals, in addition to the activated Ki-*ras*-mediated signals, might be necessary for the overexpression of *KRAP*. On the other hand, the colon cancer cells without Ki-*ras* mutation, including WiDr and Colo201 (Shirasawa 1993), expressed evident expression of *KRAP*, suggesting that other signals also could induce deregulated *KRAP* expression in human colon cancer cells. NIH3T3-derived stable transfectants expressing *KRAP* did not show tumorigenicity in nude mice nor did they show soft agar colony formation (data not shown), suggesting that overexpression of *KRAP* itself might not be sufficient for tumorigenicity and that other additional signals might be necessary for tumorigenicity. However, deregulated expression was observed not only in colon cancer cells but also in other kinds of cancer cell lines (data not shown), suggesting that *KRAP* might play critical roles in tumorigenesis through collaboration with other signals.

Mouse *KRAP* cDNA had 81% identity with human *KRAP* at the nucleotides level and 86% similarity at the amino acid level, indicating that *KRAP* was highly conserved between human and mouse. COOH terminal regions (residues of a.a.1013–1259) of *KRAP* had been reported as the CS-1 (Javed and Naz 1992). CS-1 was originally identified as a sperm surface antigen of the molecular size of M_r 33,000, and anti-CS-1 polyclonal antibodies inhibited the early cleavage of fertilized eggs without affecting binding between sperm and egg or pronuclear formation (Javed and Naz 1992; Naz 1992).

We prepared the anti-*KRAP* polyclonal antibodies (FT017–2) raised in rabbits using the peptides of the COOH terminal region of *KRAP*, which recognized a single band of the molecular size of about M_r 180,000 in HCT116 cells, and processed *KRAP* was not detected in other human cancer cell lines examined. Although there is a possibility that CS-1 is located in the sperm surface as the molecular size of M_r 33,000, all these results together suggested that *KRAP* is an actual protein as the molecular size of about M_r 180,000.

XCS-1, *Xenopus* homologue of CS-1, was reported to be detected on the membrane and in the nucleus of *Xenopus* blastomeres, on the mitotic spindle in mitotic cells, and on the centrosomes in interphase cells (Nakamura et al. 2000). Immunofluorescence microscopy revealed that neither *KRAP* nor CS-1-HA, HA-tagged in the COOH terminal region of the original CS-1 (Javed and Naz 1992), was localized in the nucleus in HCT116 cells, COS-7 cells, or NIH3T3 cells (data not shown). On the other hand, immunofluorescence microscopy and cytochalasin E treatment revealed that *KRAP* was clearly associated with the actin filaments in NIH3T3 cells. Furthermore, immunofluorescence microscopy and surface biotinylation revealed that *KRAP* exists as a membrane-bound form with the extracellular region in HCT116 cells. All these results together suggest that the localization of *KRAP* in the cells might vary depending on the cell types or other signals involved in the regulation of *KRAP*.

One of the critical processes in cancer developments is migration that requires the continuous, coordinated formation and disassembly of adhesions. These processes are complex and require a coordinated interaction of actin (Pollard 2003) or actin-interacting proteins, signaling molecules, structural proteins, integrins, adaptor molecules, and microtubules (Webb et al. 2002). Colocalization of *KRAP* with actin filaments and localization at the cell membranes with extracellular region suggest that *KRAP* might be involved in regulation of actin filaments and signals from the outside of the cells.

We have demonstrated previously that activated Ki-*ras* is involved in the upregulation of *c-myc*, *VEGF*, the urokinase-type plasminogen activator receptor, *epiregulin*, and *mig-6*, all of which are critically involved in tumorigenesis. Here we have identified a novel actin-interacting protein, *KRAP*, evidently induced by acti-

vated Ki-*ras*-mediated signals in human colon cancer HCT116 cells. Further elucidation of KRAP function in regulation of cytoskeleton and migration and KRAP-mediated signaling pathways should shed light on the molecular mechanisms of tumorigenesis.

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