RASSF2 Is a Novel K-Ras-specific Effector and Potential Tumor Suppressor*

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The Ras family of oncoproteins is intimately involved in the regulation of a wide variety of biological processes. Although activated forms of Ras are frequently associated with oncogenesis, they may also provoke growth-antagonistic effects. These include senescence, cell cycle arrest, differentiation, and apoptosis. The mechanisms that underlie these growth-inhibitory activities are relatively poorly understood. Recently, two related novel Ras effectors, NORE1 and RASSF1, have been identified as mediators of apoptosis and cell cycle arrest. Both of these protein exhibit many of the properties normally associated with tumor suppressors. We now identify a novel third member of this family, designated RASSF2. RASSF2 binds directly to K-Ras in a GTP-dependent manner via the Ras effector domain. However, RASSF2 only weakly interacts with H-Ras. Moreover, RASSF2 promotes apoptosis and cell cycle arrest and is frequently down-regulated in lung tumor cell lines. Thus, we identify RASSF2 as a new member of the RASSF1 family of Ras effectors/tumor suppressors that exhibits a specificity for interacting with K-Ras.

Ras proteins regulate a wide range of biological processes by interacting with a broad assortment of effector proteins. Although activated forms of Ras are frequently associated with oncogenesis, they may also provoke growth-antagonistic effects. These include senescence, cell cycle arrest, differentiation, and apoptosis. The mechanisms that underlie these growth-inhibitory activities are relatively poorly understood. Recently, two related novel Ras effectors, NORE1 and RASSF1, have been identified as mediators of apoptosis and cell cycle arrest. Both of these proteins exhibit many of the properties normally associated with tumor suppressors. We now identify a novel third member of this family, designated RASSF2. RASSF2 binds directly to K-Ras in a GTP-dependent manner via the Ras effector domain. However, RASSF2 only weakly interacts with H-Ras. Moreover, RASSF2 promotes apoptosis and cell cycle arrest and is frequently down-regulated in lung tumor cell lines. Thus, we identify RASSF2 as a new member of the RASSF1 family of Ras effectors/tumor suppressors that exhibits a specificity for interacting with K-Ras.

The Ras family of oncoproteins is intimately involved in the regulation of a wide variety of biological processes (1–3). This versatility is facilitated by the ability of Ras proteins to interact with a broad range of heterologous effector proteins (1, 4–6). Although best known for their role in mitogenesis and oncogenesis, Ras proteins can also promote growth arrest and cell death (7–11). In contrast to the Ras pathways mediating mitogenesis and transformation, those mediating growth-antagonistic effects remain relatively poorly characterized.

RASSF1 has recently been identified as a potential tumor suppressor that can serve as a Ras effector (12, 13). RASSF1 can induce apoptosis or cell cycle arrest (12, 14) and is frequently down-regulated by promoter methylation during tumorigenesis (15). NORE1 is related to RASSF1 and can also induce a Ras-dependent apoptosis (16, 17). Like RASSF1, NORE1 is frequently down-regulated in primary tumors and tumor cell lines (17). Data base analysis suggests that there may be more members of this family. We identified in the GenBank™ data base a human RASSF1-like hypothetical protein that was originally designated KIAA0168 but now is being referred to as RASSF2. We cloned the gene and sought to determine whether RASSF2 is also a Ras effector/tumor suppressor of the RASSF1 family.

We now report that RASSF2 can bind directly to K-Ras in a GTP-dependent manner via the Ras effector domain. Therefore, RASSF2 demonstrates the basic characteristics of a Ras effector. The interaction of RASSF2 with Ras appears to be specific to K-Ras, as only a weak interaction could be detected with H-Ras.

Rather than promoting transformation, over-expression of RASSF2 inhibits the growth of lung tumor cells. RASSF2-mediated growth inhibition is enhanced by activated K-Ras and appears to involve both apoptosis and cell cycle arrest. Analysis of RASSF2 protein expression in a series of human lung tumor cell lines shows that the protein is frequently down-regulated. Thus, we show that RASSF2 is a new member of the RASSF1 family and shares the properties of being a potential Ras effector/tumor suppressor.

MATERIALS AND METHODS

Identification of RASSF2—RASSF2 was identified by performing a tblastn search of the GenBank™ data base using the Ras association (RA) domain of RASSF1 as a query. The human hypothetical protein KIAA0168 was identified as a potential RASSF1-like protein. This protein is now being referred to as RASSF2/Rasfadin (18), and we will conform to this convention. Sequences were aligned using ClustalW.

DNA and Plasmids—RASSF2 was identified as IMAGE Consortium clone 22950 distributed by the American Type Culture Collection (ATCC, Manassas, VA). The RASSF2 coding region was PCR-cloned using oligomers 5’-ggcctgctgctgcagcgcaccaac and 3’-caattgtcagattgttgcttggttggc, which added a BamHI site to the 5’ end and an MfeI site to the 3’ end. After sequencing to confirm fidelity, the gene was cloned into pZIPHA, pCDNAF (17), and pEGFP (Clontech, Palo Alto, CA) as a BamHI/MfeI fragment. The RA domain of RASSF2 (nucleotides 535–789) was cloned as a BamHI/MfeI fragment into pMal (New England BioLabs, Beverly, MA) and pGEX2T (Amersham Biosciences). Activated K-Ras was cloned into the BamHI site of pCGNHA (19), and effector mutants were generated using a QuikChange kit (Stratagene, La Jolla, CA).

Ras Binding Assays—In vivo assays were performed by transfecting 293-T cells with 5 μg of each plasmid using LipofectAMINE 2000 (Invitrogen). After 48 h, the cells were lysed in modified radiolummmune precipitation assay buffer (20) and immunoprecipitated with HA beads (Sigma). The immunoprecipitate was then subjected to Western analysis with an anti-FLAG monoclonal (Sigma) to measure the co-precipitation of RASSF2. In vitro binding assays were performed using purified GST fusion protein of the RA domain of RASSF2 and purified K-Ras derived from baculovirus-infected Sf9 cells (a generous gift of D. Stokoe, University of California, San Francisco, CA). K-Ras protein was loaded with the GTP analog guanyl-5-imidodiphosphate (Sigma) by incubating in 100 mM Tris, pH 8, 50 mM NaCl, and 10 mM EDTA with

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Fig. 1. Alignment of RASSF2 with RASSF1 and NORE1. Amino acid comparison of human RASSF2 (GenBank™ NM_014737), human NORE1 (GenBank™ NM_031437), and human RASSF1A (GenBank™ AF102770). Alignments were generated using the ClustalW algorithm. Identical and similar amino acids within the RA domains are boxed and shaded in gray and dark gray, respectively. The percent identity between the RA domains of RASSF2 and RASSF1A is 28%, and the percent identity calculated between RASSF2 and NORE1 is 31%.

**RASSF2 Is a Novel Ras Effector**

RASSF2 was expressed in the HEK293 cell line and purified by affinity chromatography. The purified protein was then used to incubate with Sepharose beads in the presence of MgCl₂. After incubation, the beads were washed and the protein was eluted with MgCl₂. The eluted protein was then incubated with caspase-3 and fluorescent substrates. The fluorescence signal was measured and used to quantify the activity of caspase-3.

**Growth Inhibition Assays**—Cell lines were obtained from the ATCC. A549 human lung carcinoma cells were transfected with pCaspase3-Sensor and/or red fluorescent protein-RASSF2 for 24 h. Cells were then harvested, and the number of blue cells in random 40× fields was counted. The results were normalized to the number of blue cells in the control group.

**Fluorescence-activated Cell Sorting Analysis**—Cell cycle analysis was performed in 293-T cells transfected with pEGFP empty vector or pEGFP-RASSF2 using LipofectAMINE 2000 (Invitrogen). After 24 h, the cells were harvested and resuspended at a concentration of 1 × 10⁶ cells/ml. DNA content was determined using propidium iodide staining.

**RESULTS**

Sequence Comparison of Human RASSF2, RASSF1, and NORE1—Sequences were aligned using ClustalW (Fig. 1). The RA domain is boxed in Fig. 1 and shows 31% identity to that of RASSF1A and 33% identity to that of NORE1.

**Binding of K-Ras to RASSF2**—To determine whether Ras and RASSF2 could interact, we first performed co-transfection experiments in 293-T cells with FLAG-tagged RASSF2 and HA-tagged activated and wild type K-Ras. After 48 h, cells were lysed and immunoprecipitated with HA-Sepharose beads (Sigma) for 1 h. After washing, the immunoprecipitate was subjected to Western analysis using an anti-FLAG antibody. Figure 2a (top) shows that RASSF2 preferentially precipitates with activated K-Ras. The relative quantities of Ras and RASSF2 in the lysate were determined by immunoblotting the lysate with HA and FLAG (Fig. 2a, bottom). We next determined if the interaction required the effector domain of Ras by using a similar strategy with a series of effector mutants of K-Ras.
RASSF2 precipitating with activated H-Ras were barely detectable. Protein levels in the lysate are again shown at the bottom of Fig. 2c.

To confirm that the K-Ras-RASSF2 interaction is direct, we prepared recombinant GST fusion protein of the RA domain of RASSF2. This was used as an affinity reagent to precipitate a solution of purified, farnesylated K-Ras4B loaded with a non-hydrolyzable GTP analog. K-Ras was identified by Western analysis using sc-135 anti-K-Ras polyclonal antibody (Santa Cruz Biotechnology). Levels of GST and GST-F2RA were confirmed by Western analysis with polyclonal antiserum specific for GST (Santa Cruz Biotechnology).

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domain (data not shown). Therefore, RASSF2 binds directly to K-Ras in a GTP-dependent manner via the effector domain.

**RASSF2 Expression Inhibits Cell Growth**—RASSF2 was cloned into a selectable expression vector, pZIPHA, and transfected into A549 human lung carcinoma cells. Cells were selected for 2 weeks and then stained with crystal violet (Fig. 4). Cells transfected with RASSF2 failed to generate colonies, whereas the vector alone gave a virtually confluent plate.

As we were unable to generate stable cell lines expressing RASSF2, we resorted to transient transfections to determine the effects of Ras on RASSF2-mediated growth inhibition. 293-T cells were transfected with RASSF2 activated K-Ras and examined after 48 h (Fig. 5). At this time point, modest growth inhibition was detected with the RASSF2 alone, but this was dramatically enhanced by the presence of activated K-Ras. Interestingly, H-Ras had little effect on this activity. To determine whether the growth inhibition by RASSF2 was apoptotic in nature and whether the presence of activated Ras enhanced this process, we transfected 293 cells with RASSF2 activated K-Ras. Trypan blue was added after 72 h, and dye uptake was quantitated. RASSF2 promotes a modest cell death that is enhanced in the presence of activated K-Ras4B, in situ trypan blue staining of K-Ras12V, RASSF2, K-Ras12V + RASSF2, and FAS. Dye uptake is enhanced when RASSF2 is co-expressed with activated K-Ras. b, quantification of the results in panel a.

**DISCUSSION**

Although Ras oncoproteins are powerfully transforming in many cellular systems (2, 5, 21), they may also provoke a variety of growth-antagonistic effects including senescence (9), apoptosis (10, 11), and cell cycle arrest (22). This capacity to induce transformation or death is not unique to Ras but has also been described for other key oncoproteins such as c-MYC (23, 24). Thus, certain critical oncoproteins provide a close...
these proteins have the potential to serve as tumor suppressors. Loss of function of these growth-inhibitory Ras-controlled pathways may prove as critical as gain of function of conventional Ras-controlled pathways during tumor development. Bioinformatic data base analysis suggests that there may be several other members of the NORE1-RASSF1 family that remain uncharacterized. We have now cloned and characterized RASSF2 to determine whether it too serves as a Ras effector/tumor suppressor.

Examination of the Ras binding properties of RASSF2 showed that it would readily associate with farnesylated K-Ras in a GTP-preferential manner. Moreover, the interaction was direct as the purified RA domain bound purified, farnesylated K-Ras4B. K-ras is the most commonly activated ras gene in human tumors (25) and is the only ras gene that is essential for embryonic development (26). Thus, it appears that K-Ras protein is functionally different from the other Ras isoforms and has a special role in transformation and development. It has been hypothesized that the reason for the special properties of K-Ras is that there may be certain Ras effector proteins that are particularly critical to tumorigenesis and development that preferentially associate with K-Ras (27). However, although differences in effector binding between the Ras isoforms have been reported (28), these involved only a 2–3-fold difference in binding activity. Here we see an almost complete preference for K-Ras over H-Ras. Thus, RASSF2 could play a key role in mediating some of the unique biological properties of K-Ras.

As we had found some difficulty in showing stable association of RASSF2 with H-Ras, we manufactured the three classic Ras effector mutants, E37G, T35S, and Y40C (29) in K-Ras4B12V. Only the T35S mutant retained any ability to interact with RASSF2, and this was rather weak. Thus, the interaction of RASSF2 and K-Ras4B is via the Ras effector domain. Hence, RASSF2 meets the basic requirements for a potential Ras effector. Ras effector mutants have been used as tools to identify the Ras pathways that are critical for tumor development (30, 31). Interpreting the results of this type of study should now take into account the potential loss of interaction with tumor suppressor effectors, such as RASSF2, as well as the loss of interaction with oncoprotein effectors, such as Raf-1.

The mechanism of action of the RASSF1 family of proteins remains under investigation. Whereas RASSF1 and NORE1 have been shown to promote apoptosis in some cell types (12, 16, 17), Shivakumar et al. (14) suggest they cause cell cycle arrest, not apoptosis, in others. Consequently, we decided to examine both parameters for RASSF2. We found that RASSF2 promoted the activation of caspase-3 in COS-7 cells. A similar effect was observed in 293-T cells (data not shown), but the results were much clearer in the COS-7 cell type due to their flatter morphology. Thus, RASSF2 can promote apoptosis. To examine cell cycle effects, we used fluorescence-activated cell sorting analysis of GFP-RASSF2 expressing 293-T cells. These fast cycling cells showed a 20% decrease in the G2/M component in three separate experiments. This suggests that the cells are tending to arrest in G0/G1. Thus, RASSF2 can promote apoptosis and cell cycle arrest.

A potential mechanism for NORE1-induced apoptosis was recently identified by Khokhlatchev et al. (16) when it was demonstrated that NORE1 binds to the pro-apoptotic kinase MST1. However, they were unable to demonstrate that NORE1 activated the MST1 kinase activity (in fact, it appears to inhibit it), and so although this is an intriguing observation, the role of MST1 kinase in NORE1/RASSF1/RASSF2-mediated cell death remains unclear.

Analysis of RASSF2 mRNA levels in normal tissue shows considerable differential expression. Interestingly, some of the
tissues which show the lowest levels of RASSF2 expression (heart, kidney, and skeletal muscle) give among the highest levels of RASSF1 expression in an identical blot (12). Both genes show detectable levels of mRNA in normal lung tissue.

Analysis of RASSF2 expression in a panel of lung tumor cell lines showed that the protein is expressed at modest levels in a normal human bronchial epithelia cell line but fell to undetectable levels in most of the tumor cell lines examined. Intriguingly, one cell line, 441, showed very high levels of RASSF2 expression. This suggests that either there is a mutation in the

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**Fig. 8.** RASSF2 promotes cell cycle arrest. Cells were transfected with pEGFP vector or pEGFP-RASSF2. 50,000 GFP-positive cells were examined for each analysis, and the results shown here are the mean of three independent experiments. CV values were below 8%. A 20% decrease in the G2/M phase was observed with RASSF2, suggesting that the cells are prone to arrest in G0/G1.

**Fig. 9.** Expression of RASSF2 mRNA in normal tissue. Northern analysis was performed using a commercially available normal multitissue mRNA blot. RASSF2 is highly expressed in brain, placenta, peripheral blood, and lung. *Skel. Mus.*, skeletal muscle; *Small Intes.*, small intestine; *Periph. Bld.*, peripheral blood.

**Fig. 10.** RASSF2 protein expression is frequently down-regulated in human lung tumor cell lines. A RASSF2-specific polyclonal antibody to RASSF2 was prepared and used to examine a series of lung tumor cell lines by Western analysis. A normal human bronchial epithelia (NHBE) cell line was used as a positive control. Most lung tumor cell lines were negative for expression. The blot was reprobed with proliferating cell nuclear antigen (PCNA) as a loading control.
RASSF2 that renders it non-growth-inhibitory or that there is a mutation in a downstream component of the RASSF2 pathway that makes this cell line resistant to RASSF2-mediated growth inhibition. These possibilities are currently being investigated.

In summary, we now identify a third member of the RASSF1-NORE1 family that preferentially binds K-Ras with the characteristics of an effector. RASSF2 inhibits the growth of tumor cells, and its growth-inhibitory properties are enhanced by activated K-Ras. RASSF2 promotes both cell cycle arrest and apoptosis; consequently, its down-regulation may play a key role in the development of cancer.

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