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RASSF4/AD037 Is a Potential Ras Effector/Tumor Suppressor of the RASSF Family

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ABSTRACT

Activated Ras proteins interact with a broad range of effector proteins to induce a diverse series of biological consequences. Although typically associated with enhanced growth and transformation, activated Ras may also induce growth antagonistic effects such as senescence or apoptosis. It is now apparent that some of the growth-inhibitory properties of Ras are mediated via the RASSF family of Ras effector/tumor suppressors. To date, four members of this family have been identified (Nore1, RASSF1, RASSF2, and RASSF3). We now identify a fifth member of this group, RASSF4 (AD037). RASSF4 shows approximately 25% identity with RASSF1A and 60% identity with RASSF2. RASSF4 binds directly to activated K-Ras in a GTP-dependent manner via the effector domain, thus exhibiting the basic properties of a Ras effector. Overexpression of RASSF4 induces Ras-dependent apoptosis in 293-T cells and inhibits the growth of human tumor cell lines. Although broadly expressed in normal tissue, RASSF4 is frequently down-regulated by promoter methylation in human tumor cells. Thus, RASSF4 appears to be a new member of the RASSF family of potential Ras effector/tumor suppressors.

INTRODUCTION

Ras oncoproteins participate in the regulation of a broad range of normal biological processes, but activating point mutations in Ras are often associated with the development of human cancer (1). Ras functions by binding and modulating the enzymatic activity of a diverse array of effector proteins, many of which are potential oncoproteins themselves such as Raf kinases, RalGEFs, and phosphatidylinositol 3′-kinases (2–5). Consequently, Ras may be regarded as a master oncoprotein that can activate cascades of other oncoproteins to mediate transformation. However, it has now become apparent that activated forms of Ras can also induce growth antagonistic effects such as cell cycle arrest, senescence, and apoptosis (6–10). If Ras can antagonize growth in some circumstances, then it follows that some Ras effector pathways can operate to negatively regulate cell growth and survival. Indeed, this appears to be the case with the RASSF family of proteins (10, 11). To date, RASSF1 (12, 13), Nore1 [RASSF5 (14, 15)], and RASSF2 (16) have been shown to directly interact with Ras proteins with the characteristics of effectors. These proteins can induce cell death in a Ras-dependent manner. Moreover, these proteins are all frequently down-regulated during tumor development by promoter methylation (15–18). This suggests that one of the key characteristics of Ras-dependent tumors may be the subversion of growth-inhibitory Ras effector pathways.

We now identify a new member of the RASSF family, RASSF4 (also called AD037 in some early database entries). RASSF4 shows approximately 25% identity with RASSF1A and 60% identity with RASSF2. Here we show that human RASSF4 binds K-Ras in a GTP-dependent manner via the effector domain and synergizes with K-Ras to induce apoptotic cell death in 293-T cells. Expression of RASSF4 inhibits the growth of human tumor cells, and this effect is enhanced by the addition of a Ras CAA*X* membrane localization signal to the COOH terminus of RASSF4.

RASSF4 is broadly expressed in human tissues but is frequently down-regulated in human tumor cell lines and primary tumors. Down-regulation of RASSF4 expression correlates with the methylation of the promoter. Thus, we propose that RASSF4 is a new potential Ras effector/tumor suppressor of the RASSF family.

MATERIALS AND METHODS

Plasmids and DNA. RASSF4 was identified by TblastN searches of the expressed sequence tag (EST) database using sequences from the RASSF2 Ras association (RA) domain as probes. The EST clone (5727323) was purchased from the IMAGE consortium via the ATCC (Manassas, VA), and the gene was cloned by polymerase chain reaction (PCR) using the oligomers (5′-3′) ggagatctaccatgaaggaagactgtctgccg and cgcgaattcttacttggcctccaccagctg. A CAA*X* sequence from H-Ras was added by PCR using the 3′ oligonucleotide gcgaattcaggagagcacacacttgcagctcatgcacttggcctccaccagctgctc. The gene was cloned into expression vectors pEGFP and pHc-Red (Clontech, Palo Alto, CA), pcDNAF (19), and pBabe (20) as a *Bgl*II/*Eco*RI fragment. The RA domain of RASSF4 was cloned by PCR using the oligomers (5′-3′) ggagatctggtgaggcccagcgcatcc and gggaattcctagacttcccacgcccaagtcagc and cloned into pGEX2T (Pharmacia, Piscataway, NJ) as a *Bgl*II/*Eco*RI fragment. The pCGN K-Ras plasmids and pSensor system have been described previously (16). MST1 plasmids were a generous gift from J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA).

Cell Culture. 293-T cells were grown in Dulbecco's modified Eagle's medium/10% fetal bovine serum, and tumor cell lines were grown in RPMI 1640/10% fetal bovine serum supplemented with penicillin (100 units/mL)/streptomycin (100 μg/mL). Cells were incubated with 10% CO_2 at 37°C. Cell lines and primary tumors have been described previously (15, 16, 21, 22). Cells were transfected with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). *In situ*, trypan blue uptake assays were performed on 293-T human embryonic kidney cells (American Type Culture Collection, Manassas, VA). Cells were transfected with 5 μg of RASSF4 in the presence or absence of 50 ng of K-Ras12V. Seventy two hours after transfection, trypan blue was added at a final concentration of 0.04%. Dye uptake was quantified by counting the number of blue cells in three random ×40 fields in two separate assays. Apoptosis assays using the pSensor system were performed as described previously (16). 5-Aza-2′-deoxycytidine treatment was performed by plating 5 to 10 × 10^5 cells and allowing growth for 24 hours before the addition of 10 μmol/L 5-aza-2′-deoxycytidine (Sigma, St. Louis, MO). The medium (including 10 μmol/L 5-aza-2′-deoxycytidine) was changed 24 hours after seeding and then changed every 3 days. RNA was prepared 7 days after treatment using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Recombinant Protein Binding Assays. Preparation of RASSF4 glutathione *S*-transferase (GST) fusion proteins and Ras binding assays were performed as described previously (16). Purified, baculovirus-derived K-Ras was a generous gift of D. Stokoe (University of California San Francisco Cancer Center, San Francisco, CA). Ras was visualized using K-Ras–specific monoclonal antibody F234 (Santa Cruz Biotechnology, Santa Cruz, CA).

Bisulfite Modification and Methylation Analysis. Bisulfite DNA sequencing was performed as described previously (21). The *RASSF4* CpG island spanning the first noncoding exon was analyzed. The region was amplified from cell lines and tumors by PCR using the primers RASSF4 F

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Note: RASSF4 sequence is deposited as NP_114412.2 in Genbank.

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(5′-AGGATAYGATATATGTAGTGGTTTTTGGATT-3′) and RASSF4 R (5′-ATTATAACCCCTAAATTACTTAACAAAAATACCAAA-3′), where Y = C or T to ensure no bias toward methylated or unmethylated templates. PCR products were then digested with *Taq*I or *Bst*UI for 2 hours at 65°C and 60°C, respectively, to assay for methylation. PCR products were also concentrated and purified using QIAquick PCR purification columns (Qiagen) according to the manufacturer's instructions. Purified PCR products were sequenced directly using ABI BigDye cycle sequencing kit (Perkin-Elmer, Fremont, CA).

Expression Analysis by Real-Time Reverse Transcription-Polymerase Chain Reaction. Tumor cell lines were analyzed for *RASSF4* expression using real-time reverse transcription-PCR (RT-PCR) and RT-PCR. Quantitative real-time RT-PCR was performed as described previously (23). Quantified levels of TATA box-binding protein (*TBP*) transcripts were used as the endogenous RNA control, and each sample was normalized according to *TBP* content. Results, expressed as *N*-fold differences in target gene expression relative to the *TBP* gene (termed N^{target}), were determined by the following formula: $N^{\text{target}} = 2\Delta Ct_{\text{sample}}$, where the ΔCt value of the sample was determined by subtracting the average Ct value (cycle number) of the target gene from the average Ct value of the *TBP* gene. The N^{target} values of the samples were subsequently normalized such that the mean ratio of the normal breast samples would equal a value of 1. Primers used for real-time RT-PCR amplification were specific for exon 10 (5′-ACATTAAGTTTGAAATGCCG-GTGCT-3′) and junction exon 10/exon 11 (5′-GCAGGGCTTGGAACT-TCATGT-3′) to give an expected product size of 102 bp and for TBP-F (5′-TGCACAGGAGCCAAGAGTGAA-3′) and TBP-R (5′-CACATCA-CAGCTCCCCACCA-3′) to give a 132-bp product. PCR was performed using the SYBR Green PCR Core Reagents kit (Perkin-Elmer) under the following conditions: 95°C initial denaturation step for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 65°C for 1 minute. Experiments were performed with duplicates for each data point. The SE was <20%.

RT-PCR was performed using Ready-to-go RT-PCR reaction beads (Amersham Biosciences, Piscataway, NJ). Primers used for RT-PCR were specific for exon 2 (5′-GAAGACTGTCTGCCGAGTTC-3′) and exon 5 (5′-CTGTCT-GTGGAACTCTCAGC-3′) to give an expected product size of 362 bp. Two methylated colorectal carcinoma cell lines and two methylated breast carcinoma cell lines were treated with 10 μmol/L demethylating agent 5-aza-2′-deoxycytidine. Multiple tissue cDNA (MTC) panel I (Clontech) was used for expression analysis in normal tissues using the above-mentioned RT-PCR *RASSF4*-specific primers.

RESULTS

Sequence Alignment of RASSF4. RASSF4 was identified as a RASSF family member by TblastN searches of the EST database. Sequences were aligned with ClustalW (Fig. 1). The RASSF4 protein demonstrates approximately 60% identity with RASSF2 and 25% identity with RASSF1. The predicted RA domain is *shaded* in Fig. 1 and demonstrates 73% identity with that of RASSF2.

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RASSF2   ----MDYSHQTSLVPCGQD----KYISKNELLLHLKTYNLYYE-----GQNLQLRHREEE
RASSF4   ---MKEDCLPSSHVPISDS----KSIQKSELLGLLKTYNCYHE-----GKSFQLRHREEE
RASSF1   MSGEPELIELRELAPAGRAGKGRTRLERANALRIARGTACNPTRQLVPGRGHRFQPAGPA

RASSF2   DEFIVEGLLNISWGLRRPIRLQMQDDNERIRPPP-----------SSSSWHSGCNLGAQG
RASSF4   GTLIIEGLLNIAWGLRRPIRLQMQDDREQVHLP-------------STSWMP----RRPS
RASSF1   THTWCDLCGDFIWGVVR-KGLQCAHCKFTCHYRCRALVCLDCCGPRDLGWEP---AVERD

RASSF2   TTLKPLTVPKVQISEVDAPPE-GDQMPSSTDSRGLKPLQEDTPQLMRTRSDVGVR---RR
RASSF4   CPLKEPSPQNGNITAQGPSIQPVHKAESSTDSSGPLEEAEEAPQLMRTKSDASCMS-QRR
RASSF1   TNVDEPVEWETPDLSQAEIEQKIKEYNAQINSNLFMSLNKDGSYTGFIKVQLKLVRPVSV

RASSF2   GNVRTPSDQRRIRRHRFSINGHFYNHKTSVFTPAYGSVTNVRINSTMTTPQVLKLLLNKF
RASSF4   PKCRAPGEAQRIRRHRFSINGHFYNHKTSVFTPAYGSVTNVRVNSTMTTLQVLTLLLNKF
RASSF1   PSSKKPPSLQDARRG--PGRGTSVRRRTSFYLPKD-AVKHLHVLSRTRARE VIEALLRKF

RASSF2   KIENSAEEFALYVVHTSGEKQ---KLKATDYPLIARILQGPCEQISKVFLMEKDQVEE
RASSF4   RVEDGPSEFALYIVHESGERT---KLKDCEYPLISRILHGPCEKIARIFLMEADLGVEVP
RASSF1   LVVDDPRKFALFERAERHGQVYLRKLLDDEQPLRLRLLAGPSDKALSFVLKEND------

RASSF2   YDVAQYIKFEMPVLKSFIQKLQEEEDREVKKLMRKYTVLRLMIRQRLEEIAETPATI
RASSF4   HEVAQYIKFEMPVLDSFVEKLKEEEEREIIKLTMKFQALRLTMLQRLEQLVEAK---
RASSF1   SGEVNWDAFSMPELHNFLRILQREEEEHLRQILQKYSYCRQKIQEALHACPLG----
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Fig. 1. Alignment of RASSF4, RASSF2, and RASFF1A. Sequences were aligned with ClustalW. The RA domain is *shaded*.

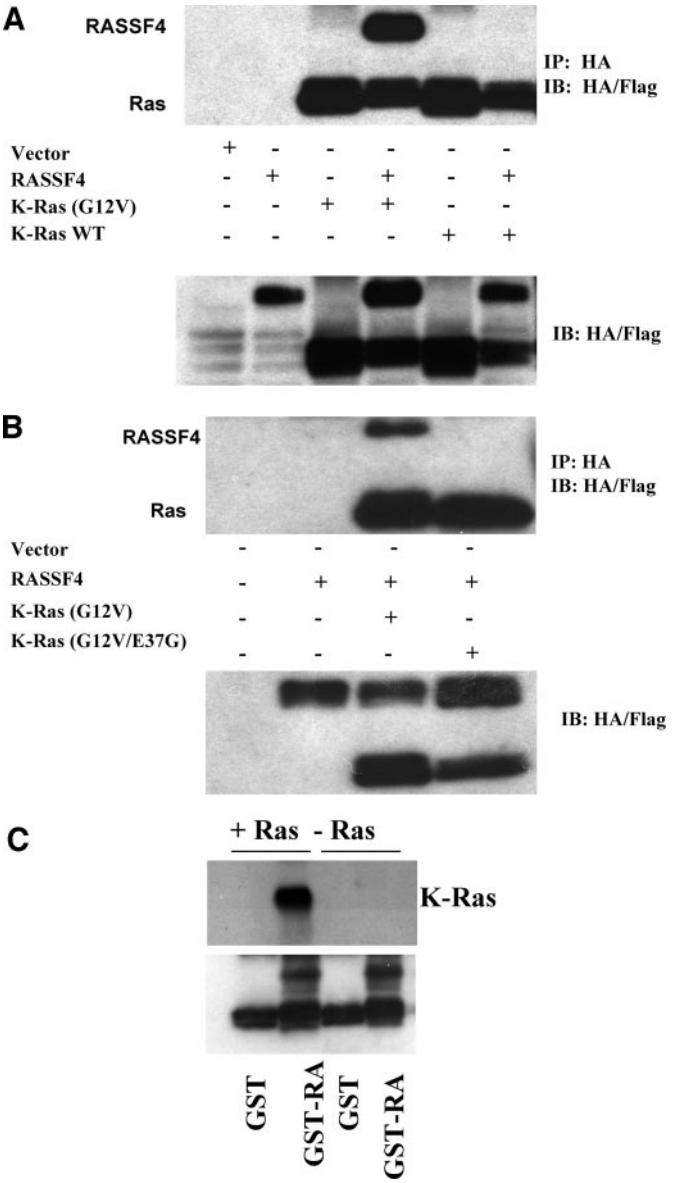


Fig. 2. *A*. RASSF4 binds activated K-Ras in cells. RASSF4 was transfected into 293-T cells with activated or wild-type K-Ras. The *bottom panel* is a Western blot of the lysates. The *top panel* is a HA immunoprecipitation followed by a Western blot with HA and FLAG. RASSF4 only precipitates with activated K-Ras. *B*. 293-T cells were transfected with RASSF4 and activated Ras or an effector mutant (E37G) of activated K-Ras. The *bottom panel* shows a Western blot of the lysates to confirm similar levels of expression of all of the recombinant proteins. The *top panel* shows that only the activated K-Ras with an intact effector domain can coprecipitate with RASSF4. *C*. RASSF4 interacts directly with activated K-Ras. A recombinant GST fusion protein of the RA domain of RASSF4 was prepared and used as an affinity reagent to precipitate farnesylated K-Ras loaded with GTP.

RASSF4 Binds Ras in a GTP-Dependent Manner via the Effector Domain. To determine whether RASSF4 might serve as a Ras effector, we cotransfected 293-T cells with FLAG-tagged RASSF4 and wild-type or activated K-Ras in a hemagglutinin (HA)-tagged vector and performed coimmunoprecipitation experiments. We found that RASSF4 could readily be precipitated with activated but not wild-type K-Ras (Fig. 2*A*). Thus, the interaction between Ras and RASSF4 requires that Ras be in the active, GTP-bound conformation. To confirm that the interaction was via the Ras effector domain, we used an effector mutant (24) of activated K-Ras (16). The binding of the effector mutant to RASSF4 was severely impaired (Fig. 2*B*).

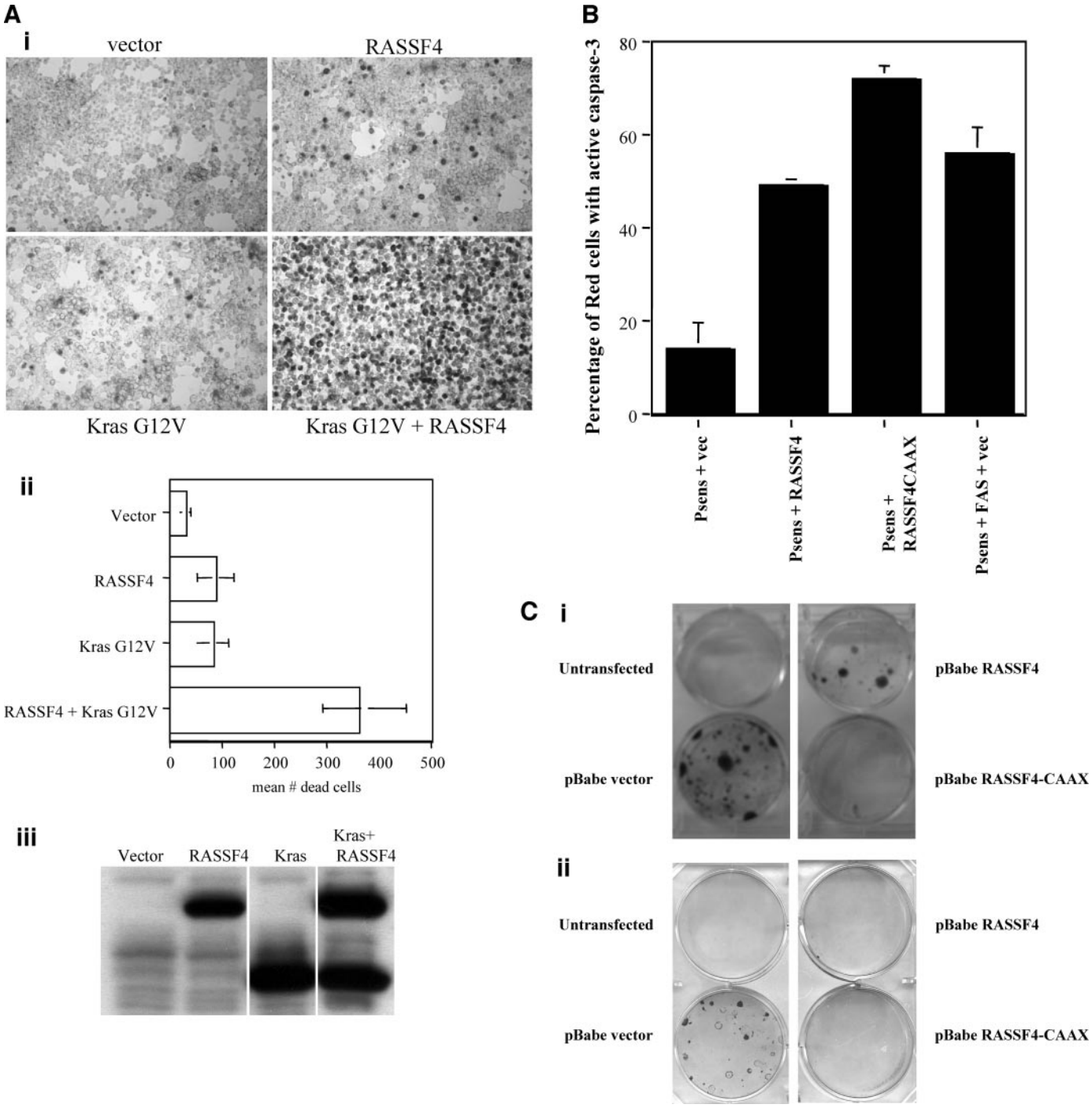


Fig. 3. *A*. RASSF4-mediated cell death is activated by K-Ras. 293-T cells were transfected RASSF4 ± activated K-Ras. Cell death was determined by trypan blue staining. *i*, a representative staining assay. *ii*, average of two separate assays. *iii*, expression levels of RASSF4 in the transfected cells. *B*. RASSF4 induces apoptosis when overexpressed in MCF-7 human breast tumor cells. MCF-7 cells were transfected with RASSF4 fused to red fluorescent protein and the EGFP-pSensor plasmid. RASSF4-positive cells were then scored after 16 hours for caspase-induced nuclear localization of the EGFP-pSensor protein by fluorescent microscopy. Results shown are the average of two separate assays. Fas serves as the positive control. *C*. RASSF4 inhibits human tumor cell growth. A549 human lung tumor cells (*i*) and MCF-7 human breast tumor cells (*ii*) were transfected with RASSF4 or RASSF4-CAA*X* in pBabe and selected in puromycin. Surviving colonies were stained after 2 weeks.

RASSF4 Binds K-Ras Directly. To determine whether the interaction between Ras and RASSF4 was direct, we prepared a GST fusion of the RA domain of RASSF4. The GST-RA fusion protein was then used as an affinity reagent in binding assays using purified, recombinant K-Ras protein derived from a baculovirus infection of Sf9 cells. The precipitate was then subjected to Western analysis using a K-Ras monoclonal antibody. GST-RASSF4 RA, but not GST, precipitated K-Ras. Thus, *in vitro*, the interaction of K-Ras and RASSF4 is direct (Fig. 2*C*). Levels of GST proteins used in the assays were determined by reprobing the blot with a GST antibody.

Activated Ras Stimulates RASSF4-Mediated Cell Death. To determine the biological effects of RASSF4 expression, we transfected RASSF4 into 293-T cells in the presence or absence of activated K-Ras. After 48 hours, the cells were stained with trypan blue to quantify cell death. RASSF4 promoted cell death and synergized

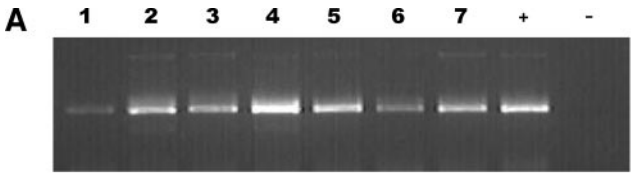
with activated K-Ras to kill cells (Fig. 3*A*, *i*). The average of two independent experiments is shown in the *bar graph* in Fig. 3*A*, *ii*. The protein levels of RASSF4 in the experiment were determined by Western blot and found to be similar (Fig. 3*A*, *iii*).

RASSF4 Can Induce Apoptosis in Human Tumor Cell Lines. To further examine the mechanism behind the growth-inhibitory properties of RASSF4, we performed apoptosis assays on transfected MCF-7 human breast tumor cells. Cells were transfected with pHc-Red RASSF4 and the indicator plasmid enhanced green fluorescent protein (EGFP)-pSensor. The EGFP-pSensor plasmid expresses a green fluorescent indicator protein that localizes to the nucleus on cleavage by caspases during apoptosis. Cells that expressed both RASSF4 and EGFP-pSensor were examined for the location of the Sensor indicator protein. RASSF4 induced an approximately 3-fold increase in the number of transfected cells exhibiting nuclear indicator protein and hence activated caspases (Fig. 3*B*). An established method of activating Ras effectors is to add a COOH-terminal CAA*X* membrane localization sequence to them (25). RASSF4-mediated caspase activation was further enhanced by the addition of a Ras-CAA*X* motif to the COOH terminus of RASSF4. Thus, RASSF4 can promote apoptosis in human tumor cells.

RASSF4 Inhibits the Survival of Tumor Cell Lines. To examine the effects of RASSF4 expression on the growth of tumor cells, the human lung tumor cell line A549 and the human breast tumor cell line MCF-7 were transfected with RASSF4 in the vector pBabe (20), and the cells were selected in puromycin. After 2 weeks, puromycin-resistant colonies were stained with crystal violet. Fig. 3*C* shows that RASSF4 inhibited colony formation. When we compared the wild-type RASSF4 and RASSF4-CAA*X* in the A549 lung tumor cells, we saw that the RASSF4-CAA*X* was more growth inhibitory than the wild-type protein.

RASSF4 Is Frequently Inactivated by Promoter Methylation in Human Tumor Cells. Real-time RT-PCR analysis showed that *RASSF4* is expressed in normal breast, lung, and kidney tissue (Fig. 4*A*).

RASSF4 is related to the RASSF1 and Nore1 tumor suppressors. The expression of RASSF1A and Nore1 is frequently down-regulated by a process of promoter methylation (18, 19). Consequently, we sought to determine whether the promoter of *RASSF4* was also the subject of epigenetic inactivation during the process of transformation. Combined bisulfate restriction analysis (COBRA) was performed on a series of human tumor cell lines and primary tumor samples. The results are summarized in Fig. 4*B* and show that the promoter of *RASSF4* is frequently methylated in primary tumors and tumor cell lines. No methylation was detected in normal samples. Direct sequencing analysis of the promoter of *RASSF4* in a range of tumor cell lines was used to confirm the methylation (Fig. 5*A* and *B*). Examination of the expression status of *RASSF4* in kidney and lung tumor cell lines by real-time RT-PCR showed that methylation (*M*) of the *RASSF4* promoter correlated with a loss of expression of the gene (Fig. 5*C* and *D*). Examination of breast tumor cell lines and primary breast tumor samples also showed that methylation correlated with reduced *RASSF4* expression (Fig. 5*E* and *F*). To confirm that promoter methylation was the basis for the down-regulation of *RASSF4*, we treated a series of tumor cell lines with the demethylating agent 5-Azacytidine (AzaC). RT-PCR analysis showed that in each case, expression of the gene was enhanced after treatment (Fig. 6). Thus, promoter methylation contributes to the frequent loss of RASSF4 expression in tumor cells.



B

Tumor Type	Cell lines	Primary tumours
Breast	15/28 (54%	10/37 (27%)
SCLC	8/25 (32%)	6/29 (21%)
NSCLC	7/14 (50%)	6/27 (22%)
Kidney (RCC)	4/9 (44%)	ND

Fig. 4. *A*. *RASSF4* is broadly expressed in normal tissue. RT-PCR of a multiple tissue cDNA panel was used to assess the relative expression levels of *RASSF4* in different tissues. *Lane 1*, heart; *Lane 2*, brain; *Lane 3*, placenta; *Lane 4*, lung; *Lane 5*, liver; *Lane 6*, skeletal muscle; *Lane 7*, pancreas. *B*. The *RASSF4* promoter is frequently methylated during transformation. A summary table of the percentage of tumor cell lines and primary tumor samples that scored positive for promoter methylation by COBRA is shown. *ND*, not determined.

DISCUSSION

The versatility of Ras oncoproteins in modulating growth processes stems from the ability of Ras oncoproteins to interact with a broad range of heterologous effector proteins. These effector proteins often contain a conserved RA domain. Activated forms of Ras oncoproteins are usually associated with loss of growth control and tumorigenic transformation. However, it has become increasingly apparent that Ras (and other oncogenes) have the ability to activate both growth-enhancing and growth-inhibiting pathways (10, 26, 27). These contrasting activities suggest that the activation of powerful oncogenes such as Ras can promote conflicting biological processes in a potential tumor cell. To successfully manifest transforming activity, the growth-inhibiting pathways controlled by Ras in normal cells must be subverted in some manner, allowing the transforming pathways to dominate.

The recent discovery of the RASSF family of Ras effectors allows an explanation of at least some of the growth-inhibitory actions of Ras. RASSF1, Nore1 (RASSF5), and RASSF2 mediate a Ras-dependent cell cycle arrest and apoptotic death (12, 15, 16, 28, 29). Moreover, these proteins are frequently down-regulated by promoter methylation in human tumors and tumor cell lines (15–18). A fourth member of the family, RASSF3, has been identified but remains uncharacterized (30).

By using a bioinformatics-based approach to detect novel RA domain-containing proteins, we have now identified a fifth member of this class of protein, RASSF4. RASSF4 bears closest homology to RASSF2, showing approximately 60% identity overall. RASSF4 lacks the cysteine-rich domain (CRD) present in RASSF1A and Nore1/RASSF5. It also lacks the putative ATM phosphorylation site present before the RA domain in RASSF1A (28). However, RASSF4 does contain the SARAH motif present in the COOH terminus of RASSF1A and other family members (31).

Like the other family members (13–16, 19), RASSF4 can bind Ras directly in a GTP-dependent manner via the effector domain. Consequently, it has the potential to serve as a Ras effector. RASSF4 synergized with activated K-Ras to kill cells and a CAA*X*ed form of RASSF4 exhibited enhanced growth-inhibitory properties in human tumor cell lines. Adding a Ras-CAA*X* membrane localization signal to an effector of Ras is an established method of activating the function of the effector by simulating the membrane recruitment by Ras (25). These results suggest that activated K-Ras binds and activates RASSF4 to stimulate its growth-inhibitory properties.

Examination of the mechanism by which RASSF4 promotes cell death showed that RASSF4 activates caspases in human tumor cells, suggesting that the cell death is apoptotic. The proapoptotic effects of Nore1 have been ascribed to the interaction of Nore1 with MST1

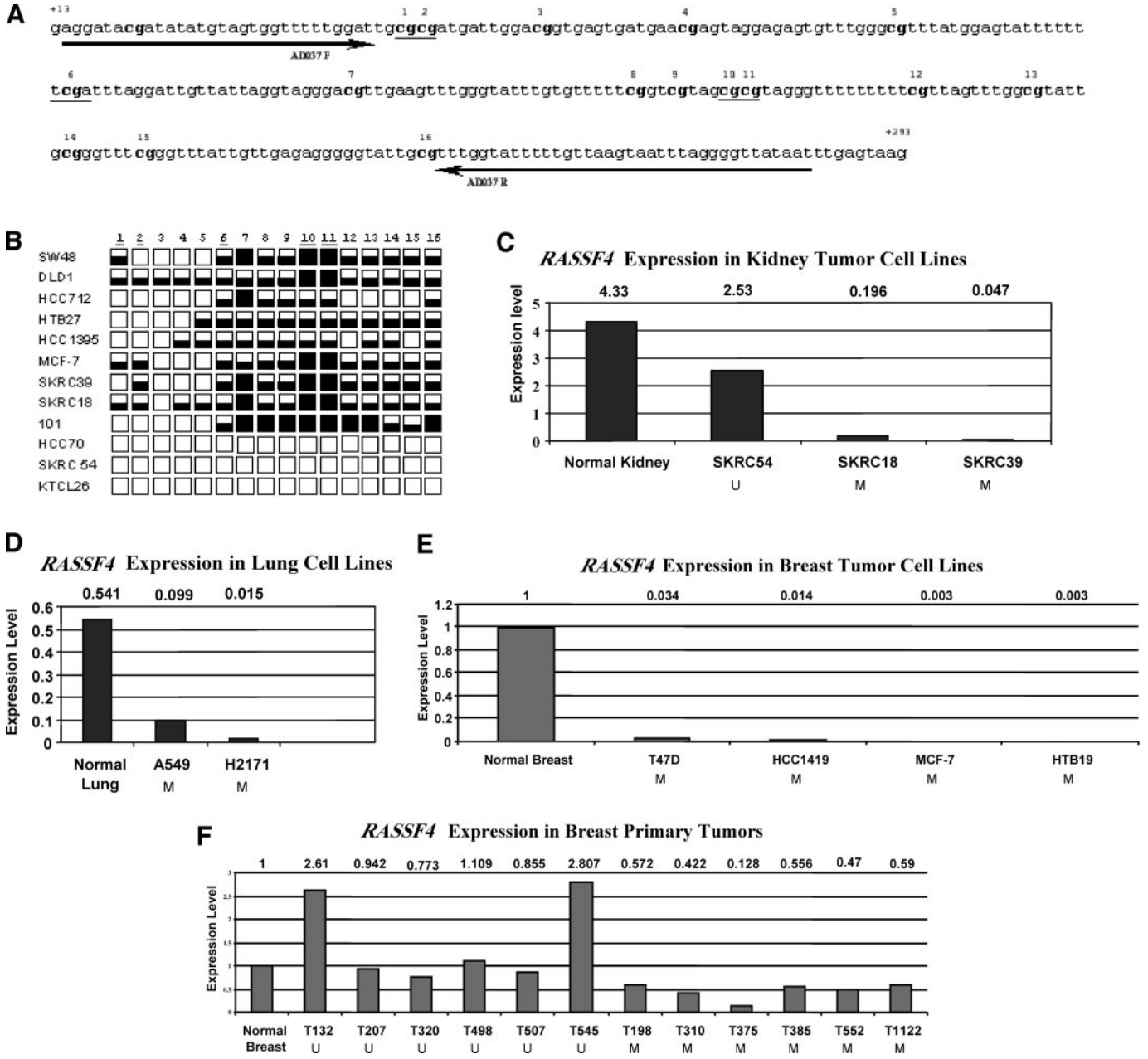


Fig. 5. Direct sequencing confirms hypermethylation of the *RASSF4* CpG island in human tumor cell lines. *A*. The sequence shows bisulfite modified *RASSF4* CpG island predicted promoter region. Primers used for PCR amplification are *labeled*. CG dinucleotides within the amplified region are *numbered*, and the location of the first and last nucleotides relative to the transcription start site of *RASSF4* (AF260335) are indicated. *Underlined sequences* and *numbers* indicate *Taq*I and *Bst*UI sites that can be used to detect methylation by restriction digest. A distinct pattern of hypermethylation was observed in methylated cell lines with frequent hypermethylation seen at CpG dinucleotides 6 through 16. *B*. Direct sequencing of COBRA PCR products showing the extent of methylation across the *RASSF4* CpG island in tumor cell lines. Colorectal tumor lines, SW48 and DLD1; breast tumor lines, HCC712, HTB27, HCC1395, HCC70, and MCF-7; kidney tumor cell lines, SKRC54, SKRC39, SKRC18, and KTCL26; SCLC cell line, 101. Each *box* represents the methylation status of each CG dinucleotide; ■, methylated; ◪, partial methylation; □, unmethylated. *C* and *D*. *RASSF4* CpG island hypermethylation corresponds with down-regulated or absent *RASSF4* expression in lung and kidney tumor cell lines. Real-time RT-PCR expression data showing *RASSF4* expression levels from several methylated (*M*) and unmethylated (*U*) tumor cell lines. Each panel gives levels of *RASSF4* mRNA transcript relative to internal control TBP. Results are the average of two assays. SE was <20%. Indicated *below* each column is the methylation status as determined by COBRA and sequencing. *M*, methylated; *U*, unmethylated. *C*, kidney tumor cell lines and normal kidney; *D*, lung tumor cell lines (A549 and H2171) and normal lung. *E* and *F*. Promoter methylation correlates with reduced expression of *RASSF4* in human breast tumor cell lines (*E*) and primary breast tumors (*F*). *M*, methylated; *U*, unmethylated. Expression levels were expressed in comparison with a normalized to a value of 1 obtained from the average of four normal breast samples. SE was <20%.

kinase (29). We found that RASSF4 also binds MST-1 when the two proteins are exogenously expressed (data not shown). Thus, MST1 may also play a role in the action of RASSF4.

The RASSF1A and Nore1 tumor suppressors are frequently inactivated in human tumors by promoter methylation (17, 18). If loss of function of RASSF4 also plays a role in the development of human tumors, we might expect that it too would be down-regulated during tumorigenesis. COBRA showed that the promoter region of *RASSF4* was subject to methylation in between 32% and 54% of the tumor cell lines and between 21% and 27% of the primary samples. Thus, the *RASSF4* promoter is often subjected to methylation during cellular transformation, and this is not just a phenomenon associated with *in vitro* cell culture. Expression analysis of *RASSF4* in cell lines and primary breast tumors showed that the methylation correlated with reduced expression of *RASSF4*. Moreover, a demethylating agent could restore *RASSF4* expression in several human tumor cell lines.

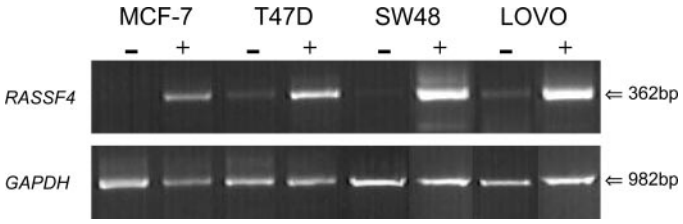


Fig. 6. *RASSF4* expression is restored after treatment with a demethylating agent. RT-PCR from two methylated breast (MCF-7 and T47D) and two methylated colorectal (SW48 and LoVo) tumor cell lines before (−) and after 5-aza-2′-deoxycytidine treatment (+).

Consequently, it appears that, like other members of the *RASSF* family, *RASSF4* is frequently down-regulated by promoter methylation in human tumor cells.

The same primary lung tumor samples analyzed for *RASSF4* were also examined for *RASSF1A* promoter methylation (21). No significant correlation could be determined, perhaps because the sample was not large enough. The *RASSF1A* promoter is methylated in approximately 70% to 80% of primary small-cell lung cancers (SCLCs) and 30% to 34% of non–small-cell lung cancers [NSCLCs (21)], suggesting that *RASSF1A* methylation is more important for the development of SCLC than NSCLC. In contrast, the *RASSF4* promoter was essentially equally methylated in approximately 21% of NSLCs and SCLCs. Several of the cell lines analyzed for *RASSF4* expression have also been examined previously for *RASSF1* or *Nore1* expression. Although the sample is too small to draw strong conclusions, DLD-1, SKCR18, and SW48 cells are down-regulated for *RASSF4* and *Nore1*. Furthermore, A549 and HCC1395 cells are down-regulated for *RASSF4* and *RASSF1A* (18, 32).

When we restored the expression of RASSF4 by transfecting a RASSF4 expression construct into RASSF4-negative tumor cells, such as MCF-7 cells, we observed a potent inhibition of growth. Thus, RASSF4 can antagonize the growth of transformed cells. However, this effect was tumor cell dependent because the human lung tumor cell line H1299 was completely resistant to RASSF4-mediated growth inhibition (data not shown). This observation may give some clues as to the mechanism of action of RASSF4 because H1299 cells (unlike MCF-7 cells) are known to be defective for p53. We are currently attempting to determine whether the biological effects of RASSF4 are p53 dependent.

The coding sequence of *RASSF4* was examined for potential inactivating mutations, but none were detected (data not shown). Interestingly, the gene locus of *RASSF4* (10p11.21) has been found to be a site of loss of heterozygosity in primary prostate tumors (33). It is notable that primary prostate tumors have previously been shown to exhibit a high degree of *RASSF1A* promoter methylation (34). The loss of expression of *RASSF4* during tumorigenesis implies that, like the other *RASSF* family members, *RASSF4* plays an important role in the genesis of human tumors.

In summary, we have cloned and characterized a fourth member of the *RASSF* family and shown that it too exhibits the properties of a Ras effector/tumor suppressor that may play an important role in the development of human cancer.

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