A Role for the RASSF1A Tumor Suppressor in the Regulation of Tubulin Polymerization and Genomic Stability

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ABSTRACT

The high frequency with which the novel tumor suppressor RASSF1A is inactivated by promoter methylation suggests that it plays a key role in the development of many primary human tumors. Yet the mechanism of RASSF1A action remains unknown. We now show that RASSF1A associates with microtubules and that this association is essential for RASSF1A to mediate its growth inhibitory effects. Overexpression of RASSF1A promotes the formation of stable microtubules, whereas a dominant-negative fragment of RASSF1A destabilizes microtubule networks. The RASSF1 protein is expressed as two main isoforms, 1A and 1C. The smaller 1C isoform also associates with microtubules but is less effective at stabilizing them. Because RASSF1A and RASSF1C localize to the mitotic spindle, we examined their effects upon genomic instability. RASSF1A and RASSF1C block activated Ras-induced genomic instability. However, a point mutant of RASSF1C, identified in human tumors, was severely defective for stabilizing tubulin and was unable to block the genomic destabilizing effects of Ras. Thus, we identify a role for RASSF1A/C in the control of microtubule polymerization and potentially in the maintenance of genomic stability.

INTRODUCTION

The RASSF1 gene is located at the 3p21.3 tumor suppressor locus (1) and is frequently inactivated by promoter methylation in a broad range of human tumors (2). RASSF1A is the founder member of a family of at least six related genes, several of which also demonstrate frequent epigenetic inactivation in human tumors (3). Exogenous expression of RASSF1 family proteins can promote cell cycle arrest and apoptosis (3–7). Moreover, their re-expression impairs the tumorigenic phenotype of a variety of human tumor cell lines (1, 3, 7, 8). Consequently, these observations suggest that RASSF1 family proteins may serve as tumor suppressors.

Examination of the primary amino acid sequence of the main splice forms of RASSF1, 1A and 1C, indicated that the proteins contain a Ras oncoprotein association domain. Experimental analysis showed that the RASSF1 Ras oncoprotein association domain can associate with activated Ras oncoproteins in vitro and when overexpressed in cells (5). RASSF1A also contains a cysteine rich domain. In the Ras effector Raf-1, a similar domain can also bind Ras and plays a key role in the activation of Raf-1 by Ras (9). However, it has been suggested that the physiological association of RASSF1 with Ras may be mediated via heterodimerization with other family members, rather than by direct interaction between Ras and RASSF1 (10).

RASSF1A and RASSF1C also contain a consensus phosphorylation site for the ataxia telangiectasia-mutated (ATM) kinase at RASSF1A residue serine 131 (7). ATM kinases have been found to play a key role in the response of cells to DNA damage and have been implicated in the regulation of cell cycle arrest, apoptosis, and the maintenance of genomic stability (11). Phosphorylation of RASSF1A on serine 131 appears to regulate some aspects of RASSF1A function, because phosphorylation-defective point mutants lose some of their growth inhibitory properties. Intriguingly, point mutations blocking the phosphorylation at serine 131 have been found in human tumors (7). Thus, RASSF1A/C could serve as a link between Ras and ATM-regulated pathways. The growth inhibitory properties of RASSF1 family proteins are not abrogated by oncogenic Ras, instead, they are activated by it (3–6, 10). Therefore, RASSF1 family proteins may play a key role in suppressing Ras-mediated oncogenesis by predisposing normal cells that develop an activated Ras pathway toward death rather than transformation. This hypothesis would explain the frequent loss of expression of RASSF1 proteins in tumor cell lines. Epigenetic inactivation of RASSF1A appears to be one of the most common molecular alterations found in human tumors (2), suggesting a particular importance for RASSF1A in the development of human cancers. Subversion of RASSF1 family activity may be an important prerequisite for the development of Ras pathway-dependent tumors. Yet the mechanism of action of RASSF1A remains uncharacterized.

It has now been shown that RASSF1A can associate with microtubules (12). We have also found that RASSF1A is a microtubule-associating protein (MAP). Moreover, we show that overexpression of RASSF1A stabilizes polymerized microtubules, whereas a dominant-negative (DN) fragment of RASSF1A destabilizes microtubule networks. Deletion mutagenesis of the RASSF1A protein demonstrates that the interaction with tubulin is essential for the growth inhibitory properties of RASSF1A. In mitotic cells, RASSF1A localizes to the spindle/centrosome and blocks the ability of activated K-Ras to provoke nuclear deformities characteristic of genomic instability. RASSF1 is expressed as two main isoforms, 1A and 1C. We found that the smaller 1C isoform also complexed with tubulin and inhibited genomic instability but was less effective than RASSF1A at stabilizing microtubules. However, a mutant form of RASSF1C, derived from a human tumor cell line, was unable to stabilize microtubules against nocodazole and lost the ability to block Ras-induced nuclear abnormalities. Thus, we reveal a potential role for RASSF1A proteins in the regulation of tubulin polymerization and the maintenance of genomic stability.

MATERIALS AND METHODS

Cell Lines. Experiments using COS-7, 293-T, human embryonic kidney cells, and human lung tumor cells were performed in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were obtained from the American Type Culture Collection.

Plasmids and Transfections. pcDNA3.1(+)/RASSF1A (a generous gift from Gerd P. Peifer, City of Hope Medical Center, Duarte, CA) was used as a template to amplify a 1023-bp cDNA using oligomers 5′-ggttctaggtcggggg-gagcctg and 3′-ggttcctaatgctttctgaccacagaa, which added a BamHI site to the 5′ end and an EcoRI site to the 3′ end. After sequencing to confirm fidelity, the gene was cloned into pcDNA3Flag (modified version of pcDNA3) and pEGFP-C1 (Clontech, Palo Alto, CA). The DN microtubule association (MA) domain of RASSF1A was generated using oligomers 5′-ggttcctaatgctttctgaccacagaa-
RASSF1 MODULATES TUBULIN POLYMERIZATION AND GENOMIC STABILITY

**RESULTS**

**RASSF1A Associates with Microtubules.** To elucidate the mode of action of RASSF1 proteins, we examined the location of EGFP-tagged RASSF1A in transfected H1299-human lung tumor cells.
Fluorescent microscopy revealed that RASSF1A localizes to the cytosol in a well-defined filamentous pattern (Fig. 1). Similar results were obtained in transfected COS-7 and 293-T cells (data not shown).

To determine the identity of the cytoskeletal component(s) associating with the RASSF1A protein, we performed a variety of costains with antibodies to cytoskeleton components including tubulin. Only tubulin colocalized with RASSF1A when transfected cells were examined under confocal microscopy (Fig. 1). To confirm that RASSF1A associated with tubulin, we performed immunoprecipitation studies using several tubulin isoforms and RASSF1A. 293-T cells were transfected with FLAG tagged RASSF1A. The cells were then lysed, and the lysate was clarified by centrifugation. Equal volumes of the lysate were then immunoprecipitated with antibodies to either β- or γ-tubulin. A mock immunoprecipitation with Sepharose A/G beads was used as a negative control. Tubulin isoforms β and γ coprecipitated with RASSF1A (Fig. 2A). We also examined the ability of endogenous RASSF1A to associate with the microtubules. For these studies, we used a human lung tumor cell line (H1437) that expresses high levels of RASSF1A protein. H1437 cells were lysed, and the resulting lysate was divided into halves. We immunoprecipitated one-half of the lysate with purified antibody to RASSF1 and immunoglobulin A/G beads and the other half with immunoglobulin A/G beads alone. The immunoprecipitate was then examined by Western blot for the presence of tubulin using an antibody to α-tubulin (Fig. 2B). Endogenous RASSF1A coprecipitated with endogenous tubulin.

The fluorescent microscopy experiments suggested that RASSF1A only associates with a subpopulation of the tubulin present in live cells.
cells, because some areas of tubulin appeared free of RASSF1A. To determine whether the RASSF1A was specifically associating with the polymerized fraction of the tubulin in the cell, we fractionated H1437 human lung tumor cell lysates to separate polymerized from unpolymerized tubulin. We then probed the fractions with an antibody to RASSF1A. The endogenous RASSF1A cosedimented only with the polymerized tubulin fraction (Fig. 2C).

The Association of RASSF1A with Tubulin Is Essential for Its Growth Inhibitory Properties. To determine the role of the microtubule-binding activity in RASSF1A function, we mapped the site of the MA domain of RASSF1A to a small stretch of amino acids between residues 120 and 185 by deletion mutagenesis. This region is shared by RASSF1A and RASSF1C. Deletion of this region rendered RASSF1A unable to associate with microtubules, promoting a diffuse, partially nuclear staining (Fig. 3A). The ability of this mutant to induce growth inhibition/cell death was then examined. Fig. 3B shows that the MA domain deletion mutant RASSF1AΔ120–185 is severely defective for the ability to promote cell cycle arrest of 293-T cells. Moreover, when the deletion mutant was coexpressed with the wild-type protein, it partially inhibited cell death, as measured by trypan blue vital dye staining of the transfected cells. (Fig. 3C). In comparison, the isolated F1-MA domain fragment strongly inhibited the ability of RASSF1A to kill cells (Fig. 3C). Thus, the isolated F1-MA domain appears to serve as a DN for RASSF1A.

RASSF1A Modulates Tubulin Stability. To determine the effects of RASSF1A on microtubule stability, we examined the effects of the depolymerizing agent nocodazole on tubulin in the presence or absence of RASSF1A. We found that RASSF1A profoundly stabilized microtubules in the presence of nocodazole (Fig. 4A). In the absence of RASSF1A, the nocodazole disrupted microtubules to give a punctate cytoplasmic staining pattern. However, in the presence of RASSF1A, the microtubule structure remained largely intact. This assay was a long-term assay, scored after 24 h of drug exposure. At this time point, approximately 90% of the control cells demonstrated loss of polymerization, whereas approximately 90% of the RASSF1A-positive cells retained the polymerized structures. We then examined the microtubule stability in the transfected cells by Western blotting with an antibody for acetylated tubulin (Fig. 4B). Acetylation of tubulin has been shown to be a marker for stabilized, polymerized microtubules (13). Cells transfected with RASSF1A but not the tubulin associated protein MAP4 demonstrated elevated levels of stabilized, acetylated microtubules. To determine the effect of Ras on this system, we performed similar experiments including activated K-Ras. Although K-Ras 12v alone had no effect on the level of acetylated tubulin, it enhanced the level of acetylation induced by RASSF1A (Fig. 4C). The average densitometric volume of the acetylated tubulin and RASSF1A bands from two separate experiments are shown in the table.

We then expressed the isolated MA domain of RASSF1A (F1-MA) and found that it caused a destabilization of the microtubule network of H1299 human lung tumor cells. This result is shown in Fig. 5, bottom panel, in which the tubulin colocalizes with the Red-F1-MA domain in a punctate cytoplasmic distribution, reminiscent of nocodazole treatment. Thus, excessive RASSF1A activity enhances tubulin polymerization, and inhibition of RASSF1A activity inhibits tubulin polymerization.

RASSF1A Inhibits Ras-Induced Genomic Instability. We and others have found that RASSF1A localizes to the mitotic spindle (12) and can complex with the centrosome component γ-tubulin (Fig. 2A). Defects in spindle regulation have been shown to lead to genomic instability (14), and consequently, we became curious to see whether RASSF1A might influence genomic stability. Activated Ras expression has been associated with genomic instability, giving rise to polyploidy, aneuploidy, and derangements of the nuclear structure (15–18). Using DAPI to stain nuclei, we found that expression of red fluorescent activated K-Ras in 293-T cells caused profound aberrations in nuclear structure. Most notably, we observed the formation of cells with deformed, multiple nuclei and micro-nuclei (Fig. 6A, panel
However, cotransfection of GFP-RASSF1A blocked the ability of oncogenic K-Ras to promote genomic instability because cotransfected cells exhibited normal nuclei. (Fig. 6A, panel iii). In contrast, the MA deletion mutant of RASSF1A (1AΔ120–185) was unable to block the Ras-induced nuclear deformities (Fig. 6A, panel iv). With this mutant, the DAPI staining is almost unnecessary because the mutant protein localizes to the nucleus. The results are quantified in Fig. 6B. We obtained similar results in H1299 human lung tumor cell lines (data not shown).

S131F Mutant RASSF1A Retains the Ability to Stabilize Microtubules. Point mutations have been found in RASSF1 in human tumor cells that inactivate a putative site of phosphorylation by ATM kinase (7). In the RASSF1A isoform, the mutation is at residue 131 where a serine is mutated to a phenylalanine. This location falls within the minimal MA domain of RASSF1, and so we examined the mutant to determine the effect of mutation on the interaction of RASSF1A with tubulin. RASSF1A S131F exhibited tubulin association and stabilizing properties similar to the wild-type protein. Both wild-type and mutant proteins were stable in nocodazole-treated cells. (Fig. 7A, panels i and ii). Western analysis of acetylated tubulin confirmed this result (Fig. 7B).

Fig. 5. The MA domain of RASSF1A (F1-MA in red) when coexpressed with GFP-tubulin (green) is able to destabilize the microtubule network in H1299 cells.

Fig. 6. RASSF1A inhibits genomic instability in K-Ras-12V expressing cells. A, 293-T cells coexpressing the following. i, phcRed vector and GFP vector. Normal nuclei are stained with DAPI. ii, phcRed-K-Ras12V and GFP-tubulin. Malformed, unstable nuclei are identified with the arrow. iii, phcRed K-Ras 12V and EGFP-RASSF1A. DAPI-stained nuclei retain normal structure. iv, phcRed K-Ras 12V and EGFP-1A(Δ120–185). Again, malformed nuclei are illustrated with arrows. B, quantification of two assays. SE was less than 20%.

Fig. 7. The S131F mutant of RASSF1A retains the ability to stabilize tubulin. 293-T cells were transfected with EGFP RASSF1A or the 1A S131F mutant. The cells were treated with nocodazole and the effects on microtubule stability determined visually (A) and by Western analysis of acetylated tubulin levels (B). WT, wild type.
and mutant protein decorated microtubules equally when expressed as GFP-fusion proteins (Fig. 7A). Furthermore, polymerized tubulin was retained to the same degree in nocodazole-treated cells transfected with wild type or RASSF1A S131F, as measured by the presence of acetylated tubulin (Fig. 7B). We were also unable to discern any obvious loss of ability of the RASSF1A S131F mutant to block Ras-induced genomic instability using the DAPI stain technique described in Fig. 6 (data not shown).

**RASSF1C and RASSF1CS61F Are Impaired in Their Ability to Stabilize Microtubules.** As noted previously, the RASSF1 protein exists in two main splice forms, 1A and a smaller 1C protein. To determine whether the 1C isoform was able to stabilize microtubules similar to the 1A isoform, we examined the effects of the wild-type RASSF1C isoform and an S61F mutant form of the 1C protein on microtubule structure. The RASSF1C-S61F (1C-S61F) mutant is equivalent to the S131F mutation of the larger RASSF1A isoform. Both the wild-type RASSF1C protein and the 1C-S61F mutant retained the ability to associate with microtubules (Fig. 8A, top panels). However, when the GFP fusion-transfected cells were treated with nocodazole, the 1C-S61F mutant was unable to stabilize microtubules, whereas the wild-type RASSF1C appeared to give an intermediate phenotype compared with RASSF1A (Fig. 8A, bottom panels). Moreover, Western analysis of polymerized (acetylated) tubulin in RASSF1A/C-transfected cells that had been treated with nocodazole showed that both the wild-type and mutant RASSF1C were defective for tubulin stabilization (Fig. 8B).

**RASSF1C and Genomic Instability.** Using the DAPI stain technique to study nuclear structure, we examined the effects of RASSF1C wild type and 1C-S61F on Ras-induced genomic instability. Although the wild-type RASSF1C protein could still block Ras-induced nuclear deformities, the 1C S61F mutant could not (as shown in Fig. 9A and quantified in Fig. 9B).

**DISCUSSION**

Epigenetic inactivation of the RASSF1A tumor suppressor is one of the most common molecular changes associated with the development of human cancer (2). Restoration of RASSF1A expression in tumor cell lines impairs their tumorigenicity (8, 19). This evidence suggests that loss of RASSF1A function plays an important role in the development of many human cancers, yet its mode of action remains unknown.

To investigate the mechanism of action of RASSF1A, we tagged the protein with EGFP and examined its subcellular localization. RASSF1A appeared to associate with polymerized tubulin. We confirmed this by cosedimentation and coimmunoprecipitation assays of the endogenous proteins. Indeed, RASSF1A seemed to promote microtubule stabilization, because RASSF1A-transfected cells retained microtubule networks after challenge with nocodazole and exhibited high levels of acetylated tubulin, a marker of stable, polymerized microtubules. This action was specific to

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**Fig. 8.** RASSF1C and 1C-S61F are defective for microtubule stabilization. 293-T cells were transfected with EGFP-RASSF1C or the 1C-S61F mutant. A, like RASSF1A, 1C and 1C S61F associate with microtubules. B, in the presence of nocodazole, 1C is impaired but retains some ability to stabilize tubulin. However, the 1C 61F mutant is completely defective. C, 1C and 1C-S61F are less able to stabilize levels of polymerized, acetylated tubulin than RASSF1A. IB, immunoblot. Top panel, a Western blot of acetylated tubulin; bottom panel, the relative expression levels of the RASSF1 proteins. Assay shown is representative of three separate assays.

**Fig. 9.** A, RASSF1C but not RASSF1C S61F can block activated K-Ras induced genomic instability. 293-T cells coexpressing the following.

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4249
RASSF1A, because the tubulin-associating protein MAP4 had no such effect. Moreover, a RASSF1A DN fragment was highly effective at blocking tubulin polymerization. Consequently, we propose that RASSF1A can modulate tubulin stability. The association with tubulin is essential for the growth inhibitory properties of RASSF1A, because the deletion mutant lacking the tubulin association domain lost growth inhibitory activity.

RASSF1 is expressed as two main isoforms, 1A and 1C. The 1C isoform can also inhibit cell growth (5), but in our hands, it is less effective than RASSF1A. Examination of the 1C isoform showed that it too associated with microtubules, but it was less effective at stabilizing them against nocodazole than 1A. Point mutations have been found in RASSF1 in human tumor cells that inactivate a putative site of phosphorylation by ATM kinase (7). In the 1A isoform, this mutation had little obvious effect on the association with or stabilization of tubulin. However, 1C-S61F was almost completely defective for stabilizing microtubules against nocodazole. Thus, it seems that the 1C isoform may be the most sensitive to regulation by ATM phosphorylation.

Aberrant spindle function can induce checkpoints leading to cell cycle arrest and cell death (20–22). Failure of these checkpoints can lead to genomic instability and transformation (23). We hypothesized that a tumor suppressor that could modulate tubulin stability and localize to the mitotic spindle might be able to influence spindle function and therefore genomic instability. Experimentally, genetic instability can be rapidly induced by activated Ras (15–18). We introduced activated Ras into 293-T or H1299 cells, we readily observed nuclear malformations indicative of genomic instability, including micro-nuclei, multi-nuclei, and chromatin bridges. (Fig. 6). The nuclear aberrations could be abolished by cotransfecting wild-type RASSF1A or RASSF1C. However, a deletion mutant of RASSF1A that could not bind tubulin was unable to suppress the effects of Ras. This implies that loss of RASSF1A function sensitizes cells to genomic instability.

This hypothesis was supported by our observations with the RASSF1C S61F mutant. This mutant has the ATM phosphorylation site abolished (7) and was unable to suppress the Ras-induced genomic instability. ATM kinase has long been held to play a role in the control of checkpoints and genomic stability (24, 25), and loss of ATM function in humans leads to predisposition to cancer (26). Thus, RASSF1A/C may be one of the checkpoint proteins controlled by ATM.

During these studies, we saw no clear colocalization of RASSF1A and K-Ras12v in the cells. This is despite the facts that they can be coimmunoprecipitated (data not shown) and that K-Ras has previously been detected associated with tubulin (27). This suggests first that the blockage of Ras-mediated genomic instability by RASSF1A is not due to simple titration of Ras. Second, it suggests that only a small fraction of the K-Ras molecules associate with RASSF1A in cells.

Thus, we demonstrate a role for RASSF1A/C in modulating tubulin polymerization and, potentially, genomic instability. Several other well-known tumor suppressors have now been shown to associate with tubulin and modulate genomic instability, such as BRCA1 (28, 29) and APC (30, 31). It will be interesting to determine whether there is any functional link between these tumor suppressors and RASSF1.

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