Involvement of the RASSF1A Tumor Suppressor Gene in Controlling Cell Migration

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Abstract

We have previously shown that RASSF1A associates with the microtubules. This association alters the microtubule dynamics and seems essential for RASSF1A tumor suppressive function. Mutant variants of RASSF1A that do not associate fully with the microtubules have reduced ability to stabilize them and cause cell cycle arrest. Here we show that overexpression of RASSF1A diminished the ability of A549 non–small cell lung cancer cells to migrate either through a transwell filter or to close a wound. In addition, we employed gene knockdown as well as mouse embryonic fibroblasts (MEFs) from Rassf1a knockout mice to analyze RASSF1A function in controlling cell motility. A549 cells stably transfected with RASSF1A exhibited increased cell-cell adhesion and less refractive morphology compared with controls. Conversely, RASSF1A knockdown in HeLa caused loss of cell-cell adhesion and a more refractive morphology. RASSF1A-depleted HeLa cells as well as Rassf1a+/− MEFs displayed increased cell migration that could be partly phosphatidylinositol 3-kinase dependent. Time-lapse microscopy showed the RASSF1A-depleted cells are highly motile with fibroblast-like morphology and diminished cell-cell adhesion. Staining of the cytoskeleton in RASSF1A-depleted HeLa cells and MEFs show marked differences in terms of microtubules outgrowth and actin stress fibers formation. This observation was associated with increased activation of Rac1 in RASSF1A-knockdown cells and the Rassf1a+/− MEFs. In addition, expression of a dominant-negative variant of Rac1 in the RASSF1A-depleted HeLa cells reduced their ability to form lamellipodia and other protrusions. These findings represent a novel function for RASSF1A, which may help explain its tumor suppression ability independently of its effects on cell cycle and apoptosis. (Cancer Res 2005; 65(17): 7653-9)

Introduction

Loss of RASSF1A expression by either promoter silencing or loss of heterozygosity is one of the most common events in human cancers. Frequent epigenetic inactivation of the RASSF1A promoter region has been detected in many common human tumors. In addition to its common inactivation, overexpression of RASSF1A in lung, kidney, and prostate cancer cell lines causes drastic reduction of tumorigenicity both in vitro and in vivo (1). Rassf1a knockout mice exhibit increased susceptibility to spontaneous and carcinogen-induced tumors (2). RASSF1A can delay the cell cycle at the G1-S border by association with the cyclin A transcription repressor, p120E4F (3). RASSF1A can also cause mitotic arrest at prometaphase through interaction with cdc20 and the inhibition of the anaphase-promoting complex (4). Proapoptotic actions of RASSF1A have been shown to be mediated through interaction with NORE1, MST1, and MST2 (5). We and others have shown that RASSF1A interacts with the microtubules (6–9). Overexpression of RASSF1A induces the stabilization of the microtubules as indicated by changes in their structure, resistance to nocodazole, and increased acetylation. The functional significance of RASSF1A association with the microtubules is highlighted by the inability of mutant forms of the gene product found in tumors to fully associate with them (6). A notable feature of RASSF1A interaction with the microtubules is causing them to exist in hyperstabilized circular bundles instead of polarized filaments with plus (growth) and minus (shrinkage) ends. The increased acetylation of microtubules and the loss of their polarity suggest that RASSF1A may also have a role in regulating cell migration and metastasis.

Materials and Methods

Cell lines and RNA interference. Cell lines used in this study were the non–small cell lung cancer (NSCLC) A549, HeLa, and mouse embryonic fibroblasts (MEFs) from wild-type or Rassf1a knockout mice (2). A549-RASSF1A cells were previously described (10). All cell lines were kept in DMEM, 10% FCS, l-glutamine-penicillin-streptomycin solutions and were routinely maintained at 37°C and 5% CO2. The double-stranded small interfering (siRNA) oligonucleotide targeting RASSF1A was synthesized by MWG Biotech and the sequences used were those published previously (11): Transfection was done using the oligofectamine reagent (Invitrogen, Paisley, United Kingdom) according to the manufacturer's recommended protocol for HeLa cells. Myc-N17Rac and L61Rac were kindly provided by Prof. L. Machesky (School of Biosciences, University of Birmingham, United Kingdom).

Antibodies, immunofluorescence, and immunoblotting. Rabbit polyclonal anti-RASSF1 antibody was described previously (9). Mouse monoclonal anti-RAC1 antibody was from BD Biosciences (Cowley, United Kingdom). Rabbit polyclonal anti-AKT and phospho-AKT (p-AKT) were purchased from Cell Signaling (Hitchin, United Kingdom). Mouse monoclonal antibodies against α-tubulin (DM1A), myc-tag (9E10), and TRITC-Phalloidin were from Sigma (Poole, United Kingdom). Horseradish peroxidase (HRP)–conjugated Rabbit anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG secondary antibodies were purchased from DAKOCytomation (Ely, United Kingdom). Alexafluor-594-conjugated rabbit anti-mouse IgG was purchased from Molecular Probes (Paisley, United Kingdom). Visualizing α-tubulin was done as described previously (5). Filamentous-actin (F-actin) staining was achieved following the manufacturer's protocol. Western blotting was done according to standard procedures. For the detection of RASSF1A, HeLa cells were lysed at room temperature in HNE
buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L DTT, 0.1% Triton X-100, and Complete Protease inhibitors; Roche, Lewes, United Kingdom).

**Aggregation assay.** Cell aggregation assay was done as described previously (12). Briefly, cells were detached by 0.01% trypsin and 0.1% EDTA treatment. Cells (1 x 10^5) were suspended in 1 mL of HCMF buffer [100 mmol/L HEPES, 1% bovine serum albumin (BSA), and 100 units of DNase I in Hank’s solution] and placed on top of an agarose bed in each well of a 24-well plate placed on a rotating platform at 37°C and 5% CO_2. At each time point, cells were counted using a hemocytometer. The extent of aggregation was represented by the index N_t/N_0, where N_t is the number of remaining particles at the incubation time point (t) and N_0 is the initial number of particles.

**Wound-healing and transwell assays.** The transwell assay was done as described previously (13). Serum-starved cells were trypsinized and resuspended in DMEM containing 0.25% BSA and a total of 2 x 10^5 cells were added to the upper chamber of each well (Costar, Appleton Woods, Birmingham, United Kingdom; porosity, 8 μm) coated on the lower side with collagen I. Serum-free (HeLa) or 10% FCS (MEFs) in DMEM were added to the bottom chamber and cells were allowed to migrate overnight at 37°C. The nonmigrating cells were removed from the upper chamber with a cotton swap and the insert was stained with crystal violet solution. Migration was quantified by counting cells per five fields of view using 10x magnification. The extent of wound-healing was represented by the index N_t/N_0, where N_t is the number of remaining particles at the incubation time point (t) and N_0 is the initial number of particles.

**Rac1 activity assay.** Activation of Rac1 in vivo was determined as described previously with minor modifications (15). This assay uses the

**Figure 1.** Modulating RASSF1A expression affects cell migration and morphology. A, i, serum-starved vector-only or RASSF1A-stably transfected A549 cells were grown to confluence on a glass slide and a wound was introduced with a pipette tip. The slides were left in serum-free media o/n before photographing. ii, 2 x 10^5 cells treated as above were placed on the top chamber of a transwell filter and left to migrate o/n. The membrane inserts were stained in crystal violet stain and photographed at 10x magnification. iii, vector control or RASSF1A-expressing stable transfectants were photographed before use in the migration assay at 20x magnification. Examples of refractive cells with aberrant protrusions (black arrows). Increased cell-cell adhesion and the less refractive cells (white arrows). B, quantification of the relative migration of RASSF1A stably transfected A549 cells in relation to vector-only control.

**Figure 2.** RASSF1A controls cell aggregation and migration. Control siRNA-treated HeLa cells or RASSF1A siRNA-treated HeLa cells were serum starved o/n 72 hours after transfection. The following day, protein was extracted and equal amounts were run on SDS-PAGE. The membrane was probed with RASSF1 polyclonal antibody that seems to detect RASSF1A and RASSF1C simultaneously. Equal loading was verified by reprobing the membrane with β-actin. Band intensity was quantified using NIH Image software.
ability of a glutathione S-transferase (GST) fusion protein corresponding to the p21-binding domain (residues 67-150 amino acids; referred to here as GST-PAK1-PBD) of human PAK1 to specifically bind the active GTP-bound and not the inactive GDP-bound forms of Rac1 and CDC42. Briefly, 72 hours after transfection, control or RASSF1A-depleted/Rassf1a−/− cells were placed in medium containing 0.1% fetal bovine serum. Twenty-four hours later, cells were washed in cold PBS and lysed in Rac1 lysis buffer [25 mmol/L HEPES (pH 7.5), 150 mmol/L sodium chloride, 1% NP40, 0.25% sodium deoxycholate, 10% glycerol, 10 mmol/L magnesium chloride, complete protease inhibitors]. The cleared lysates were incubated with GST-PAK1-PBD agarose beads (Sigma) for 30 minutes at 4°C, leaving an aliquot for measuring the levels of total Rac1. The bound proteins were eluted in protein sample buffer and characterized by Western blotting using monoclonal anti-Rac1 antibody. Rac1 activity was determined by the densitometric quantification of the pulled-down Rac1-GTP levels and normalizing it to the levels of total Rac1. The bound proteins were eluted in protein sample buffer and characterized by Western blotting using monoclonal anti-Rac1 antibody. Rac1 activity was determined by the densitometric quantification of the pulled-down Rac1-GTP levels and normalizing it to the levels of total Rac1 detected in the sample lysate. Relative Rac1 activity was determined by normalizing the Rac1 activity of Rassf1a−/− MEFs and RASSF1A-siRNA-transfected cells to the levels of their respective controls.

**Results**

**Overexpression of RASSF1A inhibits cell migration and changes cell morphology.** To investigate the importance of RASSF1A in controlling cell migration, we used stably transfected clones of the A549 NSCLC cell line expressing RASSF1A or vector-only control. We tested the ability of the RASSF1A derivative cells to migrate in wound-healing and transwell assays. After 16 hours, the vector-only control cells closed the wound completely (Fig. 1A). In comparison, the RASSF1A-expressing cells failed to migrate significantly toward the gap (Fig. 1A, i). Haptotactic migration toward collagen was also significantly impaired in the RASSF1A-expressing cells compared with control with at least 3-fold reduction in migration ability (P < 0.05; Fig. 1A, ii and B). We have previously shown that RASSF1A-expressing clones of A549 have a slower cell cycle and increased sensitivity to staurosporine-induced apoptosis compared with the vector-only cells (10); therefore, we have incubated all cell lines used in serum-free media 24 hours before using them in migration assays. Examination of the A549 stably transfected clones under phase-contrast microscope show marked differences in morphology and intercellular adhesion between the vector control and the RASSF1A-expressing A549 clones (Fig. 1A, iii). The vector control cells seem scattered, more refractive, and with a significant number of cellular protrusions. The RASSF1A-expressing cells seemed less refractive and do not possess noticeable or long cellular protrusions. A fraction of these cells may also be undergoing apoptosis. In addition, the RASSF1A-expressing cells showed a marked increase in intercellular adhesion compared with the vector control.

**Down-regulation of RASSF1A changes cell morphology and reduces cell-cell adhesion.** To test whether the down-regulation of RASSF1A can cause the converse effects seen with its overexpression, we used RASSF1A knockdown using oligos that have been previously validated (11). We routinely obtain an average of 60% knockdown of RASSF1A protein in HeLa cells (Fig. 2). To obviate any cell cycle effects due to RASSF1A knockdown, cells were serum-starved for 24 hours before performing cell migration analysis. Following serum withdrawal, RASSF1A-depleted HeLa cells exhibited drastic morphologic changes compared with control cells (Fig. 3A, i). RASSF1A knockdown caused loss of the colonial morphology and reduced cell-cell adhesion. In addition, Rassf1a−/− MEFs exhibited an increased number of spindle-shaped cells compared with Rassf1a+/+ MEFs following serum withdrawal (Fig. 3A, ii). The differences in aggregation ability between RASSF1A-depleted cells and control cells were determined. The RASSF1A-depleted cells have significantly reduced ability to aggregate compared with control cells over a 2-hour period (P < 0.05 at t = 60 minutes and t = 120 minutes; Fig. 3B).

**Down-regulation of RASSF1A promotes cell migration.** The effect of down-regulating RASSF1A expression on cell migration was investigated using wound-healing and transwell assays.

![Figure 3](image-url)
RASSF1A-depleted HeLa cells were seeded to confluence and serum was withdrawn overnight. On the following day, cells were trypsinized and 2 x 10^5 cells were added to the top chamber of a transwell filter and allowed to migrate overnight towards collagen I in serum-free media. RASSF1A depletion caused a significant increase in the number of migrating cells relative to control transfected cells (P < 0.05). Rassf1a^-/- MEFs also exhibited increased cell motility and migration towards serum compared with Rassf1a^+/+ MEFs (P < 0.05; Fig. 4B). A role for phosphatidylinositol 3-kinase (PI3K) in mediating the increase in cell migration is indicated by the ability of LY294002, a PI3K inhibitor, to abrogate this increase. (Fig. 4B). In addition, activation of the PI3K pathway is also suggested by the detection of increased phosphorylation of AKT in the RASSF1A-depleted cells (Fig. 4C). Time-lapse microscopy of HeLa cells with RASSF1A knockdown showed that within 10 hours, cells exhibit highly motile cell membrane protrusions (filopodia/lamellipodia) extending towards other neighboring cells but without achieving stable cell-cell adhesion. The RASSF1A-depleted cells were also more motile throughout the incubation period without obvious differences in cell divisions (Fig. 5; Supplementary Movies).

**RASSF1A has the ability to modulate cytoskeleton dynamics.**
To test the effect of RASSF1A knockdown on microtubules, we stained the RASSF1A-depleted HeLa cells or the Rassf1a^-/- cells with an antibody against α-tubulin. As shown in Fig. 6, depletion of RASSF1A or loss of Rassf1a caused increased microtubule outgrowth and their extension towards the cell cortex. The increase in microtubules outgrowth was the opposite of what is observed when RASSF1A is overexpressed in mammalian cells. We have stained the RASSF1A-depleted HeLa or the Rassf1a^-/- cells with TRITC-conjugated phalloidin to reveal the F-actin organization in these cells. As shown in Fig. 6, RASSF1A depletion is associated with increased presence of polymerized stress fibers.

Rac1 activation modulates the actin cytoskeleton and subsequently controls the formation of cellular protrusions. As shown in Fig. 7A, RASSF1A-depleted HeLa cells and Rassf1a^-/- MEFs exhibit increased detectable levels of active Rac1-GTP, relative to controls. To show that Rac1 is actively involved in mediating the observed RASSF1A knockdown phenotype, we expressed Rac1 mutants in siRNA-treated cells and observed the effects on cellular protrusions. RASSF1A knockdown cause about 90% of cells to exhibit lamellipodia and other protrusions. In contrast, only 2% of the control treated cells exhibit a motile phenotype. When transfected with N17Rac1 dominant-negative mutant, only 30% of the RASSF1A-depleted cells expressing Rac1 retained cellular protrusions. RASSF1A depletion and its effect on inducing cellular protrusions was mirrored by the ability of a dominant-active Rac1 ability to induce such changes in control siRNA-treated cells (Fig. 7B-C).

**Discussion**
We have previously shown that the association of RASSF1A with the microtubules is central to its function and disruption of this association may promote tumorigenesis. Overexpression of RASSF1A in various cell lines caused the formation of stable circular and acetylated microtubules. This phenotype is the reverse to what is normally observed in migrating cells. Motile and migrating cells usually exhibit a polarized microtubule cytoskeleton with centrosomes and most microtubules are oriented toward the...
leading edge (16). In this study, we provide evidence that RASSF1A negatively controls cell migration. Conversely, siRNA-mediated down-regulation of RASSF1A increased cell migration in a PI3K-dependent manner. MEFs from Rassf1a−/− mice also exhibit similar levels of increased motility and migration potential.

RASSF1A depletion in HeLa caused a drastic change in cell morphology. HeLa cells used in this study normally maintain highly organized cell-cell adhesion. Depletion of RASSF1A in these cells led to loss of cell-cell contacts and cell scattering. The cobblestone-like appearance of these cells was replaced by a spindle-like, fibroblast-like morphology. This morphologic change represents one of the hallmarks of an epithelial-to-mesenchymal transition. Hence, down-regulation of RASSF1A may potentially promote epithelial-to-mesenchymal transition during the transformation process. The loss of cell-cell contact may have been a contributing factor towards the increased ability of RASSF1A-depleted cells to migrate.

We have found that PI3K inhibitors abrogated the increase in cell migration. The PI3K pathway plays a very important role in controlling cell migration. In addition, it was previously shown that RASSF1A depletion can cause the accumulation of cyclin

Figure 5. Time-lapse microscopy of RASSF1A-depleted cells. A, time-lapse series were obtained by photographing the cells in serum-free media at 20× magnification every 15 minutes over a 10-hour period. Representative still images taken every 60 minutes. B, tracks generated by cell movement in control or RASSF1A-depleted HeLa cells in addition to the comparison of the average cell speed over a 10-hour period.

Figure 6. Down-regulation or loss of RASSF1A modulates the cytoskeleton. The microtubules and F-actin in control/RASSF1A-depleted cells or the Rassf1a+/−/Rassf1a−/− MEFs were visualized using an antibody against α-tubulin and TRITC-conjugated phalloidin, respectively.
D1 in HeLa cells (11). PI3K activation of AKT subsequently lead to the p-AKT counteracting the inhibitory actions of GSK3β on cyclin D1 levels (17). We have found that LY294002 can block the increase in cyclin D1 levels seen following RASSF1A knockdown in HeLa cells (Fig. 4C). In addition to the increased levels of p-AKT in RASSF1A-depleted cells, there was an increased level of Rac1-GTP in RASSF1A-depleted or Rassf1a−/−/− cells. The mechanisms by which RASSF1A depletion can cause such observations are unknown. Modulating microtubule dynamics has been shown to affect the activation of Rho GTPases and consequently the formation of lamellipodia and filopodia and cell contraction. It has been shown that in fibroblasts, inducing microtubules growth by nocodazole washout can activate Rac1 GTPase leading to the polymerization of actin in the lamellipodia protrusions (18). Rac1 activation in turn can promote MT plus end growth and turnover (19). Therefore, it is difficult to determine whether RASSF1A knockdown can affect microtubule outgrowth that in turn can modulate the levels of active Rac1 or activate the PI3K pathway independently of its effects on the microtubules.

In this study, we show that the siRNA-mediated knockdown is specific for RASSF1A with no noticeable effects on RASSF1C levels. In addition, RASSF1C, in comparison with RASSF1A, has reduced ability to associate with and stabilize the microtubules (9). That is reflected by its reduced ability to induce G2-M arrest (8). Therefore, RASSF1A is expected to be the main isoform controlling cell migration through its greater ability to control microtubule stabilization.

Recent reports suggest that RASSF1A inactivation is associated with increased tumor aggressiveness. This may reflect, in light of the findings in this study, the increased migratory and invasive potential of cells that have lost RASSF1A expression. For example, RASSF1A hypermethylation is associated with aggressive phenotype of neuroblastoma tumors that are associated with decreased survival (20). Advanced grade and stage prostate tumors exhibit a greater degree of RASSF1A hypermethylation than lesser grade/ stage tumors (21, 22).

This study reports a novel function of RASSF1A. In addition to its reported ability to control different stages of the cell cycle...
and the induction of apoptosis, we show how RASSF1A expression is required to regulate microtubule-mediated cell-cell adhesion, epithelial morphology, and cell motility. This regulation can be mediated through the PI3K pathway and increased activation of Rac1. It will be very interesting therefore to further investigate the mechanisms by which RASSF1A can induce such drastic changes on the microtubules and the cytoskeleton in general.

References

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