Ras association domain family 1C protein stimulates human lung cancer cell proliferation

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THE RAS ASSOCIATION DOMAIN family 1 (RASSF1) proteins have been identified as Ras effectors. The most extensively studied member of this family, RASSF1A, resides on chromosome 3p21.3, a region that often undergoes homozygous or heterozygous deletions and hypermethylation-induced suppression in many human cancers (5, 6, 13). RASSF1 encodes two major mRNA isoforms, RASSF1A and RASSF1C, derived by alternative promoter selection and alternative mRNA splicing (5, 6). The RASSF1A protein (340 amino acids) contains an amino-terminal diacylglycerol binding domain (C1 domain), an ataxia telangiectasia mutated (ATM) phosphorylation site, and a carboxy-terminal putative Ras association (RA) domain. The RASSF1C protein (270 amino acids) contains the ATM phosphorylation site and the RA domain but not the C1 domain (5, 6).

RASSF1A has been characterized as a tumor suppressor gene. RASSF1A is epigenetically inactivated by cytidine methylation in many human solid tumors. It has been reported that in 80–100% of lung cancer cell lines and tumors (1, 2, 5, 7), 49–62% of breast cancers (2, 8), 67–70% of primary nasopharyngeal cancers (8), 90% of hepatocellular carcinomas (21), 91% of renal cell carcinomas (9), and 70% of prostate cancers (11, 13), RASSF1A, but not RASSF1C, is inactivated. In addition, RASSF1A overexpression reduces colony formation, suppresses anchorage-independent growth, and inhibits tumor growth in nude mice (5, 9, 13). Recently, it has been shown that overexpression of RASSF1A inhibits cell growth by inducing G1/S phase cell cycle arrest and by blocking cyclin D accumulation (17). Studies of RASSF1A-knockout mice showed that RASSF1A−/− and RASSF1A+/− mice exhibit enhanced tumor multiplicity and tumor size compared with wild-type animals on exposure to the chemical carcinogens benzo(a)pyrene and urethane (19).

RASSF1C is the second major isoform encoded by RASSF1, and it differs from the RASSF1A isoform by the lack of the amino-terminal diacylglycerol binding domain. RASSF1C, unlike RASSF1A, is expressed in the majority of human solid tumors. RASSF1C has not been extensively studied, and little is known about its role in cellular growth. The majority of published literature indicates that RASSF1C has no tumor suppressor activity (5, 10, 13, 15, 17). We recently (3) identified RASSF1C as an insulin-like growth factor binding protein-5 interacting protein and showed that silencing of RASSF1C expression resulted in a significant decrease in osteosarcoma cell proliferation. In this paper we report on the growth-silencing effects of RASSF1C in lung cancer cells. We found that suppression of RASSF1C expression caused a significant decrease in cell proliferation of lung cancer cells, suggesting that RASSF1C is not a tumor suppressor gene.

MATERIALS AND METHODS

Cell culture. The human lung cancer cell line NCI H1299 was obtained from the American Type Culture Collection (ATCC). Cell culture was carried out as recommended by the ATCC.

RNA isolation and Northern blot analysis. Total RNA from human cancer cell lines was isolated with the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA).

Plasmid construction. The green fluorescent protein (GFP)-RASSF1A plasmid, which expresses RASSF1A as a green fluorescent fusion protein, was kindly provided by Dr. Wallace L. McKeehan (Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX). Each of the following plasmids was constructed as previously described (3). The yellow fluorescent protein (YFP)-RASSF1C plasmid expresses RASSF1C as a yellow fluorescent fusion protein. The Xi-RASSF1C plasmid over-
were transfected with small interfering RNA (siRNA)-258 sequence into the pSilencer system, expresses siRNA hairpins specific to RASSF1C mRNA. In each case, the control plasmid is the plasmid backbone without the gene or siRNA inserted into it.

**Transfection of cancer cell lines with plasmid DNA.** The NCI H1299 cell line was transfected with the YFP-RASSF1C, siRNA-RASSF1C, Xi-RASSF1C, and control plasmids. The cells were plated at 20,000 and 50,000 cells/well in RPMI medium with 10% calf serum in 24- and 6-well culture dishes, respectively. After 24 h, we transfected the cells with 1 μg/ml plasmid DNA, using either Effectene (Qiagen, Valencia, CA) or Lipofectamine (Invitrogen, Carlsbad, CA) as recommended. Forty-eight hours after transfection, cells were collected and were used for RNA extraction. Two hundred nanograms of total RNA were used to prepare cDNA with the Omniscript kit (Qiagen). One microliter of the reverse transcriptase reaction was used for PCR with HotStart master mix (Qiagen), and the PCR reactions were run with the following conditions: 95°C for 15 min, 95°C for 1 min, 60°C for 30 s, and 72°C for 30 s for 35 cycles.

**Western blot analysis of siRNA suppression of RASSF1C expression.** NCI H1299 cells overexpressing the HA-RASSF1C protein (previously transfected with the Xi-RASSF1C vector) were transfected with either siRNA oligos specific for RASSF1C or nontarget control siRNA oligos. At time zero, we replaced the medium and transfected the cells with 100 nM siRNA-RASSF1C or control plasmid and selected with G418 (800 μg/ml) for 2 wk. Cells then were stained with crystal violet dye, and cell colonies were counted. The colony number of NCI H1299 cells treated with siRNA-RASSF1C was significantly reduced compared with cells transfected with control plasmid, as determined by direct cell colony count. All experiments were repeated at least 3 times.

**Fig. 1.** Effect of suppression of RASSF1C expression in NCI H1299 lung cancer cells. A and B: NCI H1299 cells were transfected with small interfering RNA (siRNA)-RASSF1C (previously demonstrated to silence RASSF1C mRNA well; Ref. 3) or control plasmid. Cells were plated at 20,000 cells/well in 24-well plates 24 h before transfection. The cells were transfected with 2 μg/ml of plasmid DNA with the use of Effectene (Qiagen), and cells were incubated for 48 h before an Alamar blue (A) or [3H]thymidine (B) cell proliferation assay was carried out. Proliferation of cells treated with siRNA-RASSF1C was significantly reduced (P < 0.03 vs. vector control, t-test) compared with cells transfected with control plasmid. Values are means ± SE of 8 replicates. C: reverse transcriptase PCR analysis of RASSF1C expression from NCI H1299 lung cancer cells transfected with 2 μg/ml siRNA-RASSF1C or control plasmid. Total RNA was extracted from cells, and 200 ng of total RNA was used for reverse transcriptase reactions. One microliter of the reverse transcriptase reaction was used for PCR reactions with RASSF1C-specific primers and actin-specific primers (internal control). RASSF1C mRNA was significantly reduced in cells treated with siRNA-RASSF1C compared with control plasmid. D: NCI H1299 lung cancer cells were transfected with 2 μg/ml of Xi-RASSF1C [overexpressing hemagglutinin (HA)-RASSF1C] or control plasmid with the use of Lipofectamine as outlined in MATERIALS AND METHODS. Twenty-four hours later these cells were transfected with 100 nM of siRNA-RASSF1C or control siRNA oligos as described in MATERIALS AND METHODS and for an additional 18 h, and cells were analyzed by Western blotting for overexpression of HA-RASSF1C protein using the HA-tag antibody. The HA antibody detected a 37-kDa band in cells treated with control oligos but not in cells treated with siRNA-RASSF1C oligos. E: NCI H1299 lung cancer cells were seeded in 10-cm plates and treated with either 2 μg/ml of siRNA-RASSF1C or control plasmid and selected with G418 (800 μg/ml) for 2 wk. Cells then were stained with crystal violet dye, and cell colonies were counted. The colony number of NCI H1299 cells treated with siRNA-RASSF1C was significantly reduced compared with cells transfected with control plasmid, as determined by direct cell colony count. All experiments were repeated at least 3 times.

**Table 1.** Effect of suppressing or augmenting expression of RASSF1C on NCI H1299 cell proliferation was determined. Briefly, cells were treated with siRNA-RASSF1C (suppresses RASSF1C expression), Xi-RASSF1C (overexpresses RASSF1C), or control plasmid for 18 h. [3H]thymidine was then added, and cells were labeled for 6 h before cultures were

expresses RASSF1C as a hemagglutinin (HA)-tag fusion protein. The siRNA-RASSF1C plasmid, which was constructed by placing the small interfering RNA (siRNA)-258 sequence into the pSilencer system, expresses siRNA hairpins specific to RASSF1C mRNA. In each case, the control plasmid is the plasmid backbone without the gene or siRNA inserted into it.

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**Alamar blue assay.** The effect of suppressing or augmenting expression of RASSF1C on NCI H1299 cell proliferation was determined. Briefly, cells were treated with siRNA-RASSF1C (suppresses RASSF1C expression), Xi-RASSF1C (overexpresses RASSF1C), or control plasmid for 48 h, and cell proliferation was measured by the Alamar blue assay as previously described (3).

**[3H]thymidine incorporation.** Cells were treated with siRNA-RASSF1C (suppresses RASSF1C expression), Xi-RASSF1C (overexpresses RASSF1C), or control plasmid for 18 h. [3H]thymidine was then added, and cells were labeled for 6 h before cultures were
experiments were repeated at least 3 times. Cancer cells transfected with siRNA-RASSF1C showed a significant decrease \((P < 0.01)\) in cell proliferation compared with cells transfected with control plasmid, as judged by Alamar blue and \(^{3}H\)thymidine incorporation assays (Fig. 1, A and B). To confirm that the inhibitory effect of RASSF1C siRNA on cell number correlated with RASSF1C mRNA, we measured RASSF1C mRNA levels in cultures treated with siRNA-RASSF1C or control plasmid. Figure 1C shows that transient transfection with siRNA-RASSF1C reduced RASSF1C mRNA levels by 50–70% in NCI H1299 cells. We also treated cells expressing the HA-tagged RASSF1C protein with siRNA-RASSF1C oligos or nontarget control siRNA oligos to evaluate the effect of siRNA on RASSF1C protein. Figure 1D shows that siRNA-RASSF1C reduced HA-RASSF1C protein expression compared with nontarget siRNA oligos, as judged by Western blot analysis using HA-tag antibody. In addition, we examined the effects of silencing RASSF1C on lung cancer cell colony number after 2 wk of selection with G418. The number of NCI H1299 cells treated with siRNA-RASSF1C was significantly less compared with cells treated with control plasmid, as judged by direct counting of cell colonies (Fig. 1E). Cell viability was not affected by RASSF1C siRNA treatment, as judged by trypan blue staining (data not shown).

Overexpression of RASSF1C in lung cancer cells. To further elucidate the function of RASSF1C, we carried out transient RASSF1C overexpression studies in NCI H1299 lung cancer cells. For overexpressing RASSF1C in cells, we used a HA-tagged RASSF1C expression plasmid to transfect cells as outlined in MATERIALS AND METHODS. Western blots using anti-HA tag antibody verified that RASSF1C was overexpressed in the cell cytoplasm (Fig. 3A). Importantly, overexpression of RASSF1C significantly increased lung cancer cell proliferation (Fig. 2, A and C), as demonstrated by Alamar blue and \(^{3}H\)thymidine cell proliferation assays.

FACS analysis of lung cancer cells overexpressing RASSF1A or RASSF1C. We expressed GFP alone, RASSF1A as a fusion protein with GFP, or RASSF1C as a fusion protein with YFP in NCI H1299 lung cancer cells. GFP alone was diffusely and evenly distributed throughout the cell cytoplasm (Fig. 3A), whereas GFP-RASSF1A fusion protein (Fig. 3B) appeared on fibers and YFP-RASSF1C (Fig. 3C) was perinuclear. The cell cycle profiles of NCI H1299 cell lines transiently transfected with GFP (Fig. 1A), GFP-RASSF1A (Fig. 1B), and YFP-RASSF1C (Fig. 1C) plasmids were determined by FACS analysis. RASSF1A overexpression is associated with an increase of cells in the G0/G1 phase and a decrease of cells in the G2/M and S phases of the cell cycle (Fig. 3B). In contrast, RASSF1C overexpression is associated with a decrease in the number of cells in the G0/G1 phase and an increase of cells in the G2/M and S phases of the cell cycle (Fig. 3C) compared with cells expressing the control GFP plasmid \((P < 0.05)\) (Fig. 3A). Consistent with our findings, overexpression of GFP-RASSF1A has also been previously shown to arrest cancer cells in the G1 phase (14). These data suggest that overexpression of RASSF1C and RASSF1A has different effects on the cell cycle of the lung cancer cell line NCI H1299.

RESULTS

Silencing of RASSF1C in lung cancer cells. NCI H1299 lung cancer cells transfected with siRNA-RASSF1C showed a significant decrease \((P < 0.01)\) in cell proliferation compared with cells transfected with control plasmid, as judged by Alamar blue and \(^{3}H\)thymidine incorporation assays (Fig. 1, A and B). To confirm that the inhibitory effect of RASSF1C siRNA on cell number correlated with RASSF1C mRNA, we measured RASSF1C mRNA levels in cultures treated with siRNA-RASSF1C or control plasmid. Figure 1C shows that transient transfection with siRNA-RASSF1C reduced RASSF1C mRNA levels by 50–70% in NCI H1299 cells. We also treated cells expressing the HA-tagged RASSF1C protein with siRNA-RASSF1C oligos or nontarget control siRNA oligos to evaluate the effect of siRNA on RASSF1C protein. Figure 1D shows that siRNA-RASSF1C reduced HA-RASSF1C protein expression compared with nontarget siRNA oligos, as judged by Western blot analysis using HA-tag antibody. In addition, we examined the effects of silencing RASSF1C on lung cancer cell colony number after 2 wk of selection with G418. The number of NCI H1299 cells treated with siRNA-RASSF1C was significantly less compared with cells treated with control plasmid, as judged by direct counting of cell colonies (Fig. 1E). Cell viability was not affected by RASSF1C siRNA treatment, as judged by trypan blue staining (data not shown).

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Fig. 3. Fluorescence-activated cell sorting (FACS) analysis of lung cancer cells overexpressing RASSF1A or RASSF1C. Histograms show the cell cycle profile of NCI H1299 cells transiently expressing RASSF1A or RASSF1C. NCI H1299 cells transfected with green fluorescent protein (GFP; A), GFP-RASSF1A (B), or yellow fluorescent protein (YFP)-RASSF1C (C) were collected and analyzed after 48 h of incubation. Cells were fixed and stained with propidium iodide, and % of cells in the G0/G1, G2/M, and S phases was determined by FACS analysis (n = 3). YFP-RASSF1C expression in NCI H1299 resulted in a different cell cycle profile compared with GFP-RASSF1A, suggesting that RASSF1C overexpression has effects on the cell cycle distinct from those of RASSF1A. The values are means ± SE of 3 replicates. FL2-H, relative fluorescent intensity; N, nucleus.
RASSF1C, unlike RASSF1A, does not upregulate N-cadherin 2 and transglutaminase 2 gene expression in lung cancer cells. To establish that RASSF1C has different functions compared with that of RASSF1A, we determined the effects of overexpressing RASSF1C on two known RASSF1A target genes, N-cadherin 2 (CDH2) and transglutaminase 2 (TGM2). In lung cancer cells, RASSF1A has been shown to upregulate CDH2 and TGM2 expression (1). As expected, transient overexpression of RASSF1A in NCI H1299 cells increased CDH2 and TGM2 expression (Fig. 4). However, transient overexpression of RASSF1C did not affect the expression of CDH2 and TGM2, suggesting that these two genes are not RASSF1C targets (Fig. 4). These findings provide additional evidence that RASSF1C has gene targets that are distinct from those of RASSF1A and hence further suggest that RASSF1C is not a tumor suppressor gene.

**DISCUSSION**

Very little is known about the role of RASSF1C in cellular growth, compared with the much better-studied RASSF1A. Recent reports have shown that overexpression of RASSF1C inhibits the growth of some ovarian cancer cell lines (20) and the prostate cell line LNCaP (12), suggesting that RASSF1C may be a tumor suppressor. In this study, we have studied the effects of silencing RASSF1C expression on lung cancer cell growth. We have demonstrated that reduction of RASSF1C mRNA (Fig. 1C) and protein (Fig. 1D) levels in NCI H1299 cells was correlated with a significant decrease in cell growth compared with control cells that express RASSF1C (Fig. 1, A and B). The reduction in RASSF1C expression was not associated with apoptosis, as judged by trypan blue staining (data not shown). Our findings in the present study are consistent with our findings when RASSF1C was silenced in a number of osteosarcoma cell lines including TE85, MG63, and U2 cells (3). It should be noted that the silencing effects of RASSF1C on TE85 cells that express both the RASSF1A and RASSF1C isoforms are very similar to those observed in cell lines that express only the RASSF1C isoform (MG63 and U2 cells), suggesting that RASSF1A does not modulate the effects of RASSF1C on cell proliferation (3). On the basis of these findings we suggest that RASSF1C is a growth-stimulating factor unlike RASSF1A, which is a known tumor suppressor.

To further study the function of RASSF1C in lung cancer cells, we transiently overexpressed RASSF1C. Transient overexpression of RASSF1C increased lung cancer cell proliferation (Fig. 2, B and C), as demonstrated by the Alamar blue and [3H]thymidine cell proliferation assays, suggesting further that RASSF1C is not a tumor suppressor. It should be noted that a recent study showed that regulated overexpression of RASSF1C inhibited the growth of prostate cancer (LNCaP) and renal cell carcinoma (KRC/Y) cells (12). The different effects of RASSF1C overexpression in NCI H1299, LNCaP, and KRC/Y cells suggest that RASSF1C may have tissue- or cell-specific functions or that when RASSF1C is overexpressed it may mimic the effects of RASSF1A in certain cell types (such as LNCaP and KRC/Y; Ref. 12).

RASSF1A and RASSF1C have identical sequences except at their respective amino termini. It is possible that the proteins could have similar or overlapping functions, or they could have distinct roles. One approach that we have taken to further elaborate the functional differences of these two RASSF1 isoforms is to study their molecular targets. For example, we investigated the effect of RASSF1C overexpression on gene targets that are known to be under the regulation of RASSF1A in the lung cancer cell line NCI H1299 (1). If RASSF1C does not regulate known RASSF1A gene targets, then RASSF1C and RASSF1A may modulate novel gene targets that have diverse functions. In this regard, we tested the effect of transiently overexpressing RASSF1C on two RASSF1A gene targets, CDH2 and TGM2. We found that RASSF1C does not increase expression of CDH2 and TGM2 target proteins regulated by RASSF1A (Fig. 3; Ref. 1), suggesting that RASSF1C may modulate different target genes.

We think that RASSF1C has a different and important role from that of RASSF1A, based on several important findings previously reported and presented in this report. First, unlike RASSF1A, the RASSF1C promoter is not epigenetically inactivated by methylation, and it is expressed in the majority of human solid tumors including breast, lung, prostate, osteosarcoma, and other types of cancers tested to date (5, 11, 13, 18, 19). Second, RASSF1A expression has been shown to inhibit the accumulation of cyclin D1 (CD1), whereas RASSF1C has no effect on CD1 expression (17). Third, the scaffold protein connector enhancer of KSR (CNK)1, which suppresses tumor cell growth and promotes apoptosis, binds to both RASSF1A and RASSF1C (16). This interaction is required for CNK1 to associate with mammalian sterile 20 kinase 1. Coexpression of CNK1 with RASSF1A enhances CNK1-induced apoptosis, whereas RASSF1C has no effect on CNK1-induced apoptosis (16). Fourth, silencing of RASSF1C expression in lung and osteosarcoma cancer cells causes a consistent and significant reduction in cell proliferation (Fig. 1; Ref. 3). We also have preliminary data that silencing RASSF1C expression in breast and prostate cancer cells also significantly reduces cell prolif-
eration (data not shown). Fifth, unlike RASSF1A, RASSF1C overexpression does not inhibit cancer cell growth (Fig. 2, B and C). These findings suggest that RASSF1A and RASSF1C may play antagonistic roles in tumor cell growth. Indeed, a careful balance of RASSF1A and RASSF1C isoform expression may be critical to determining the neoplastic potential of a cell. This makes it critically important to better understand the expression and function of RASSF1C in cancer cells.

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GRANTS

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