RESEARCH ARTICLE

The tumor suppressor RASSF1A is a novel effector of small G protein Rap1A

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ABSTRACT

Rap1A is a small G protein implicated in a spectrum of biological processes such as cell proliferation, adhesion, differentiation, and embryogenesis. The downstream effectors through which Rap1A mediates its diverse effects are largely unknown. Here we show that Rap1A, but not the related small G proteins Rap2 or Ras, binds the tumor suppressor Ras association domain family 1A (RASSF1A) in a manner that is regulated by phosphorylation of RASSF1A. Interaction with Rap1A is shown to influence the effect of RASSF1A on microtubule behavior.

KEYWORDS RASSF1A, Rap1A, microtubule, vimentin, protein-protein interaction

INTRODUCTION

Rap1A (Krev-1) is a member of the Ras superfamily of small GTP-binding proteins and has highest homology to Ras. Rap1A behaves as an antagonist of oncogenic K-Ras activity in NIH3T3 cells (Kitayama et al., 1989) and of polyoma middle-T antigen in Rat-2 cells (Jelinek and Hassell, 1992). It inhibits transformation of human prostate cancer cells (Burney et al., 1994), hamster pancreatic adenocarcinoma

cells (Leach et al., 1998) and of ethylcarbamate induced lung adenomas in transgenic mice (Damak et al., 1996). However, Rap1A does not prevent Ras induced transformation in human HT1080, EJ30 or SW48 cells (Sato et al., 1994). Rap1A also plays a critical role in the regulation of normal morphogenesis in the eye disk and the ovary during embryo development (Hariharan et al., 1991; Asha et al., 1999). Recently, Rap1A has been implicated in integrin-mediated adhesion through RapL (Katagiri et al., 2003) and modulation of the actin cytoskeleton through RIAM (Lafuente et al., 2004). Beyond these targets, the downstream effectors through which Rap1A mediates its diverse effects are largely unknown.

The Ras association domain family 1A (RASSF1A) gene is localized at chromosome region 3p21.3, which frequently undergoes loss of heterozygosity in human solid tumors due to promoter methylation (Sekido et al., 1998; Dammann et al., 2000; Lerman and Minna, 2000). RASSF1A contains an amino-terminal cysteine-rich region, similar to the diacylglycerol-binding domain (C1 domain) found in the protein kinase C family of proteins and a carboxy-terminal Ras-association (RA) domain (Sekido et al., 1998; Dammann et al., 2000; Lerman and Minna, 2000; Dammann et al., 2005). The functional analysis of RASSF1A reveals its involvement in apoptotic signalling (Vos et al., 2000), microtubule stabilization (Liu et al., 2003; Dallol et al., 2004; Rong et al., 2004) and

G1–S progression (Shivakumar et al., 2002). RASSF1A gene knock-out mice are highly susceptible to various tumors (Tommasi et al., 2005). RASSF1A thus represents an epigenetically inactivated tumor suppressor in human carcinogenesis (Dammann et al., 2005).

In this study, we report that RASSF1A is a novel effector of Rap1A, linking Rap1A to microtubules. We also show specific and regulated interaction of RASSF1A with ectopically expressed activated Rap1A. This interaction appears to have robust effects on the intracellular distribution of the microtubule network.

RESULTS

Identification of RASSF1A as a Rap1A-interacting protein

X-ray crystallographic studies have shown that the highly acidic effector residues in the switch I region of Rap1A interact with the positively charged patch generated by the critical basic residues located in strands β 1, β 2, and the end of α 1 helix of RB/RA domain of the proteins cRaf and RalGDS (Nassar et al., 1995; Nassar et al., 1996; Huang et al., 1997). Furthermore, it has recently been shown that the novel effector RapL interacts with Rap1A through its RA domain and that the same critical basic residues within the RA domain of RapL play a key role in this interaction (Katagiri et al., 2003). Conserved domain neighborhood analysis performed on 84 RA/RB domains from 75 human protein entries of the SMART database (Table S1) identified RASSF1A as the most homologous domain neighbor of the RA domain of RapL (Fig. 1). Structural comparisons of the 3D models of RA domain of these proteins further suggested that the structure of the RA domain of RASSF1 was remarkably similar to the RA domain of RapL as indicated by a C α r.m.s.d (root mean square deviation) of 1.05 Å (Fig. 2A). The 3D structure-based primary amino acid alignment indicated that the specific basic residues in β 1, β 2 and α 1 strands known to be critical for interaction of Rap1A with its effectors (indicated as red arrows

in Fig. 2A) (Nassar et al., 1995; Nassar et al., 1996; Huang et al., 1997) were highly conserved in RASSF1A.

In vitro protein association assays demonstrated that Flag-RASSF1A specifically interacted with GST-Rap1A (Fig. 2B). To further assess the dependence of RASSF1A-Rap1A interaction on the activation state of Rap1A, the GST-Rap1A was loaded with either GDP- β -S or GTP- γ -S and binding reactions performed. It was found that Flag-RASSF1A preferentially interacted with GTP-Rap1A (Fig. 2C), suggesting that RASSF1A is a novel interactor of activated Rap1A.

Rap1A-RASSF1A interaction is regulated by phosphorylation of RASSF1A

In order to investigate whether RASSF1A phosphorylation could be induced in this model, 293T cells transfected with Flag-RASSF1A were treated with the cell permeable phosphatase inhibitor calyculin A. Cell lysates were prepared, resolved by SDS-PAGE and subjected to immunoblotting (IB) with RASSF1A antibody. Calyculin A treatment resulted in retardation of the electrophoretic mobility of RASSF1A on the SDS-PAGE (Fig. 3A, upper panel), indicative of the phosphorylation state of RASSF1A. Interestingly, calyculin A treatment also resulted in loss of Rap1A-RASSF1A interaction *in vitro* (Fig. 3A, middle panel), suggesting a RASSF1A phosphorylation dependent regulation of Rap1A-RASSF1A interaction. This dependence on the phosphorylation state of RASSF1A is a further validation of the specificity of this interaction.

Several putative phosphorylation sites were predicted by *in silico* analysis within RASSF1A; the sites S197 and S203, which are located within the RA domain of RASSF1A, have been established as novel protein kinase C (PKC) phosphorylation sites (Verma et al., 2008). In order to assess whether the specific phosphorylation of these two sites could modulate the interaction of RASSF1A with activated Rap1A, the 293T cells transfected with wild type or S197,

Figure 1. Neighbor Joining (NJ) tree showing the domain neighbors of RapL-RA, RIAM-RA and RalGDS-RA. The dendrogram is based on the multiple sequence alignments prepared by superimposing the primary amino acid sequences of 84 RA/RB domain entries of SMART (Table S1) on the alignment profile of 12 different high resolution 3D structures of RA/RB domains superimposed by Combinatorial Extension (CE) algorithm (Shindyalov and Bourne, 1998). The PDB structures used to generate the alignment profile were 1RAX:A (Solution structure of RalGDS-RA), 1LFD:A (Crystal structure of RalGDS), 1EF5:A (Solution structure of RGL), 1WXA:A (Solution structure of AF6-RA2), 1WGR:A (Solution structure of Grb7), 1GUA:B (Crystal structure of cRaf), 1C1Y:B (Crystal structure of cRaf), 2CS4:A (Solution structure of C12orf2), 1WXM:A (Solution structure of aRaf), 1WFY:A (Solution structure of RGS14-RA2) and 2BYF:A (solution structure of PLC-epsilon RA2). The codes for the color are as follows — Red: all the three critical residues are conserved (Identical or similar); Pink: 2 residues conserved; Green: 1 residue conserved; Gray: None of the critical residues are conserved; Blue: The proteins to which the RA/RB domain belongs. Consensus of critical residues at three positions is noted in brackets with the name of the protein. The proteins flagged with red arrows are the proteins that have an RA/RB domain that is known to interact with Rap1A as published before in the literature [RIAM: (Lafuente et al., 2004); RapL (Franke et al., 1997); (Katagiri et al., 2003)]. Clusters of domains (Cluster A–K) inferred from the tree are indicated. Three shaded blocks of clusters (Cluster A1, D3 and E) are the clusters of RA/RB domains that include at least one RA/RB domain which is known to interact with Rap1A. Protein shown with black arrow was selected as a candidate protein to further assess as a novel effector of Rap1A.

Figure 2. RASSF1A interacts with GTP-Rap1A. (A) 3D models, Cα superimpositions, sequence alignment and secondary structure assignments for the RA domains of RapL and RASSF1A. A 3D model of the RA domains of RapL and RASSF1A was generated using 1RAX:A (RalGDS-RA) as a template in alignment mode of SWISS-MODEL. Cα Superimposition of these models as indicated was generated using the Combinatorial Extension (CE) algorithm (Shindyalov and Bourne, 1998). All the models were drawn with MOLMOL (Koradi et al., 1996). Wire rendering of the superimposition was drawn with Cn3D (Hogue, 1997). The number of aligned Cα residues, % sequence identity (SI) and r.m.s. deviations for Cα traces are shown. Numbering at the top corresponds to the amino acid sequence of full length RapL-RA domain delineated from human RapL, Swiss-Prot accession number Q8WWW0. Letters marked with red arrows correspond to three critical residues in the RalGDS-RA domain (Huang et al., 1997). Positions of these residues in 3D models are shown with red arrows as well as in the alignment shown. The specific serine residues marked with black arrows are the PKC phosphorylation sites in RASSF1A (Verma et al., 2008). (B) RASSF1A interacts with Rap1A *in vitro*. HEK293 cells were transfected with or without the pCDNA3-Flag-RASSF1A expression vector (MATERIALS AND METHODS). At 24 h after transfection, the cells were harvested and lysed; equal amounts of cell lysates from untransfected or Flag-RASSF1A transfected cells as indicated were subjected to pull-down assay using Glutathion Sepharose Transferase (GST) fusion protein for Rap1A (GST-Rap1A) and subjected to immunoblot (IB) analysis using antibodies as indicated in the figure legend. (C) RASSF1A preferentially interacts with activated Rap1A *in vitro*. Glutathion Sepharose Transferase (GST) fusion protein for Rap1A (GST-Rap1A) in crude bacterial lysates was prepared and loaded with either GDP-β-S or with GTP-γ-S following the procedure described in MATERIALS AND METHODS. The lysate from Flag-RASSF1A transfected HEK293 cells was equally divided into two halves and each half was subjected to pull-down with the nucleotide loaded Rap1A as indicated. Bound protein was probed by western blotting for RASSF1A. Lanes L1 and L2 are the input lysates from Flag-RASSF1A transfected HEK293 cell used for pull-down by GDP or GTP loaded GST-Rap1A, respectively. These results are representative of 2 separate experiments.

203A double mutant (i.e., which cannot be phosphorylated on these specific sites) of Flag-RASSF1A were treated with phorbol 12-myristate 13-acetate (PMA) + calyculin A and the cell lysates prepared from these cells were employed to assess the interaction of RASSF1A with activated Rap1A by *in vitro* pull-down assay. The PMA + calyculin A induced phosphorylation of RASSF1A at S197 and S203 was monitored by phospho-RASSF1A S197 and phospho-RASSF1A S203 specific immune sera PPA545 and PPA548, respectively. The specificity of these phospho-specific antisera to the RASSF1A phosphorylation sites has been documented elsewhere (Verma et al., 2008). As

expected, the wild type RASSF1A, but not the AA mutant, was phosphorylated at these sites on PMA + calyculin A treatment (Fig. 3B). The *in vitro* GTP-Rap1A pull-down assay performed on these lysates demonstrated that upon PMA + calyculin A treatment, the wild type RASSF1A lost its interaction with activated Rap1A (Fig. 3C). It was also found that the AA mutant of RASSF1A, which is not phosphorylated at S197 and S203 in response to PMA + calyculin A treatment, also lost its interaction with activated Rap1A (Fig. 3C), indicating that RASSF1A phosphorylation at S197 and S203 was not responsible for regulating interaction with Rap1A. The specific phosphorylation site(s) within

Figure 3. Rap1A-RASSF1A interaction is regulated by phosphorylation of RASSF1A. (A) Retardation of the electrophoretic mobility of RASSF1A upon calyculin A treatment and influence on Rap1A-RASSF1A interaction. 293T cells grown to 90% confluence were transfected with the Flag-RASSF1A. At 24 h after transfection, the cells were treated with or without 100 nmol/L calyculin A for 30 min. A small fraction of the cell lysates prepared was resolved by SDS-PAGE and subjected to immunoblotting (IB) with RASSF1A antibody (upper panel). The rest of the lysates from calyculin A treated or untreated cells was subjected to pull-down assay using GTP loaded GST-Rap1A as probe. For this, the above lysate was incubated for 90 min with GTP loaded GST-Rap1A precoupled with glutathione Sepharose 4B (GSH) beads followed by 3 washes with MLB buffer. The protein bound to GSH beads was extracted with 2 × NuPAGE® SDS Sample Buffer and probed by western blotting for RASSF1A (middle panel). The input lysates are shown in the lower panel. (B) 293T cells grown to 90% confluence were transfected with the wild type Flag-tagged RASSF1A (WT) or with the AA double mutant of Flag-tagged RASSF1A. At 24 h after transfection, the cells were stimulated or not with 0.8 μmol/L PMA + 100 nmol/L calyculin A for 30 min followed by lysis in MLB buffer. The lysates were immunoblotted with Phospho-RASSF1A S197 (upper panel) and Phospho-RASSF1A S203 (lower panel) specific immune sera as indicated. (C) The lysates obtained in – B were incubated with GTP loaded GST-Rap1A precoupled with glutathione Sepharose 4B (GSH) beads for 90 min followed by 3 × washing with MLB buffer. The protein bound to GSH beads was extracted with 2 × NuPAGE® SDS Sample Buffer and loaded in gel along with input lysate followed by IB with RASSF1A antibody. These results are representative of 2 separate experiments.

RASSF1A, which are implicated in regulation of its interaction with GTP-Rap1A, remain to be identified.

RASSF1A interacts with Rap1A on a circular perinuclear network

Having established that RASSF1A can interact with activated Rap1A in a regulated manner, the subcellular localization of ectopically expressed EGFP-Rap1A and Flag-RASSF1A was

examined by confocal microscopy in transfected Cos-7 cells, which do not express endogenous RASSF1A. It was not possible to perform these experiments on the endogenous Rap1A since no antibody suitable for immunofluorescence was available. As suggested before (Liu et al., 2003; Dallol et al., 2004; Rong et al., 2004; Song et al., 2004), Flag-RASSF1A was present as a circular network in the cytoplasm and perinuclear region in Cos-7 cells (Fig. 4A), while the EGFP-Rap1A expressed alone was predominantly present in

punctate structures in the perinuclear region of these cells (Fig. 4A). Similar subcellular localization of endogenous Rap1A in BHK cells and in NIH3T3 cells (Pizon et al., 1994) or ectopically expressed Rap1A in Cos-1 has previously been demonstrated (Mochizuki et al., 2001). Interestingly, the co-expression of EGFP-Rap1A with Flag-RASSF1A led to a robust redistribution of EGFP-Rap1A from the punctate perinuclear structures to the circular perinuclear network where the EGFP-Rap1A and Flag-RASSF1A became strongly co-localized (Fig. 4A), indicating interaction of these proteins within cells.

It is known that Rap1A, Rap2 and Ras share a 100% identical core effector region (see Bos et al., 2001). It is possible therefore that these proteins share some of their effectors. Indeed, it has been shown that many of the Ras effectors such as cRaf, AF6 and RalGDS are shared by Rap1A (though with differential affinities) (Herrmann et al., 1996; Vetter et al., 1999; Boettner et al., 2000; Zhang et al., 2005). In order to test whether RASSF1A is a specific effector of Rap1A compared to Rap2 and Ras, the subcellular localization of Flag-RASSF1A in Cos-7 cells expressing EGFP-Rap2 or EGFP-Ras was examined by confocal microscopy. Unlike EGFP-Rap1A, which is redistributed from punctate perinuclear structures to the circular perinuclear network along with Flag-RASSF1A, EGFP-Rap2 or EGFP-Ras was neither redistributed nor co-localized with Flag-RASSF1A (Fig. 4A). These observations were further substantiated by examining the *in vitro* association of Flag-RASSF1A with the GTP- γ -S loaded GST-Rap1A, GST-Rap2 and GST-Ras proteins. It was found that RASSF1A selectively interacted with GTP-Rap1A. No binding was detected with either GST beads alone (negative control), GTP-Rap2 or GTP-Ras (Fig. 4B), suggesting that RASSF1A was a specific effector of Rap1A and it was not shared either by Rap2 or Ras. Together, these results demonstrate that RASSF1A selectively interacts with activated Rap1A *in vitro* and *in vivo*; and ectopically expressed Flag-RASSF1A and EGFP-Rap1A co-localize on a circular perinuclear network in Cos-7 cells.

Rap1A-RASSF1A interaction plays a role in the intracellular distribution of microtubules

Previous studies have shown that RASSF1A associates with microtubules in the interphase cells (Liu et al., 2003; Dallol et al., 2004; Rong et al., 2004; Song et al., 2004). In order to confirm this finding, Cos-7 cells transfected with Flag-tagged RASSF1A were immunostained for Flag-RASSF1A and α -tubulin, and analyzed by confocal microscopy. It was found that RASSF1A indeed co-localized with microtubules (Fig. 5). In addition, it was observed that ectopic expression of RASSF1A in Cos-7 cells appeared to cause rearrangement of the microtubules into circular rings, whereas the untransfected or vector transfected cells showed the microtubules

Figure 4. RASSF1A is a specific effector of Rap1A. (A) Cos-7 cells transfected with the indicated vector (corresponding to legend on the right side of each panel) were immunostained using specific antibody indicated inside the panels and analyzed using confocal microscopy. Flag-RASSF1A was visualized using Flag-M1 antibody (Rabbit polyclonal, Sigma-Aldrich). Nuclei are counterstained with DAPI. Two-color superimposed images (Merge) of each panel are shown as indicated. The scale bar is equivalent to 20 μm. (B) The crude bacterial lysates containing GST-Rap1A, GST-Rap2 or GST-Ras proteins were treated with GTP- γ -S following the procedure described in MATERIALS AND METHODS. Equal amounts of Flag-RASSF1A transfected Hek293 cell lysate were incubated with a GTP- γ -S loaded, active forms of GST-Rap1A, Rap2 or Ras pre-bound to glutathione Sepharose 4B (GSH) beads in MLB buffer; the GSH beads alone served as a negative control. Following 90 min incubation at 4°C, beads were pelleted and washed three times with ice-cold MLB buffer. Protein was eluted from the beads in 2 × Laemmli buffer and probed by western blotting for RASSF1A as indicated.

radiating from a microtubule-organizing center (MTOC) (Bailly and Bornens, 1992) (Fig. 5). This phenotype is consistent with microtubule staining in cell lines with or without endogenous RASSF1A expression as suggested before (Dallol et al., 2004). Together, this data confirms that RASSF1A is a microtubule associating protein and has an effect on the intracellular distribution of the microtubule network. Since RASSF1A was found here as a Rap1A-interacting protein, we further sought to investigate the effects of Rap1A on the distribution pattern of microtubules. To explore this, EGFP-Rap1A transfected Cos-7 cells were immunostained for α -tubulin. It was found that similar to RASSF1A, ectopic expression of EGFP-Rap1A also induced microtubule circularization in Cos-7 cells and loss of the MTOC (Fig. 5).

Figure 5. Co-localization of RASSF1A and Rap1A and their effect on the microtubule network. Cos-7 cells transfected with the indicated vector (corresponding to legend on the right side of each panel) were immunostained using specific antibody indicated inside the panels and analyzed using confocal microscopy. Flag-RASSF1A was visualized by Flag-M1 antibody and endogenous microtubules by the α -tubulin antibody. Microtubules (red) radiating from a microtubule-organizing center (MTOC) (white arrows) are shown. Two-color/multi-color superimposed images (Merge) of each panel are shown as indicated. MTOC (wherever is indicated with white arrows). Each panel represents a mixture of transfected and non-transfected cells. The scale bar is equivalent to 20 μ m.

Given that Rap1A and RASSF1A individually could modulate the intracellular distribution of the microtubules and that RASSF1A interacts with Rap1A *in vitro* and *in vivo*, the effect of the Rap1A-RASSF1A association on the intracellular distribution of the microtubule network was investigated. Cos-7 cells transiently transfected with the expression vectors carrying EGFP-Rap1A and Flag-tagged RASSF1A were co-stained for Flag-tag and α -tubulin to detect RASSF1A and microtubules, respectively. Fig. 5 (e–h) shows a field of cells representing different populations of Cos-7 cells including: untransfected cells, EGFP-Rap1A transfected cells, and cells that are co-transfected with EGFP-Rap1A + Flag-RASSF1A. As expected, the untransfected or vector transfected cells exhibited a normal pattern of microtubules with radial arrays emanating from the MTOC and extending towards the cell periphery (Fig. 5). In the Flag-RASSF1A transfected Cos-7 cells the microtubule network was circularized. Interestingly, the circularized microtubule network was strongly retracted around the nucleus in the cells that express both, the EGFP-Rap1A and Flag-RASSF1A, leaving much of the cell body devoid of a microtubule network (Fig. 5). Taken together, this data suggests that in Cos-7 cells ectopically expressed Rap1A or RASSF1A leads to the circularization of the microtubule network and that co-expression of both Rap1A and RASSF1A induces bundling of circularized microtubules around the nucleus where the Rap1A-RASSF1A complex becomes co-localized.

It has been shown that RASSF1A has microtubule stabilizing properties (Shivakumar et al., 2002; Liu et al., 2003; Dallol et al., 2004; Rong et al., 2004). This was further confirmed here by confocal microscopy of Flag-RASSF1A transfected, nocodazole treated Cos-7 cells along with appropriate controls. Nocodazole is a microtubule-specific agent that binds to the subunits of microtubules and prevents their polymerization (Downing, 2000). Treatment of Cos-7 cells with nocodazole caused dispersal of microtubules in empty vector transfected or untransfected cells (Fig. 6). However, in Flag-RASSF1A-expressing cells, microtubules still maintained their modified network structure (Fig. 6). Flag-RASSF1A and EGFP-Rap1A cotransfected, nocodazole treated Cos-7 cells displayed an intact but retracted perinuclear-network of microtubules, where Flag-RASSF1A and EGFP-Rap1A co-localized (Fig. 6). Thus, RASSF1A stabilizes microtubules and on interaction with Rap1A, this stabilization is retained albeit in an altered, nuclear retracted form.

RASSF1A and Rap1A influence the cellular processes that are directly or indirectly dependent on intracellular microtubule organization

The microtubule network plays an important role in the maintenance of cellular structures including the microfilament network (Liao and Gundersen, 1998; Prahlad et al., 1998;

Figure 6. RASSF1A stabilizes microtubules against disruption by nocodazole treatment and the influence of Rap1A. Cos-7 cells transfected with the empty vector (a, b) or the expression vectors encoding Flag-RASSF1A (c, d), EGFP-Rap1A (e, f) or both (g, h, i) were treated with 20 $\mu\text{mol/L}$ nocodazole for 1 h followed by immunostaining and analysis by confocal microscopy (LSM510, Carl Zeiss Jena). Flag-RASSF1A was visualized by a Flag-M1 antibody and the endogenous microtubule network by an α -tubulin antibody. Two-color/multi-color superimposed images (Merge) are shown as indicated. The scale bar is equivalent to 20 μm .

Chou et al., 2007) and the Golgi (Thyberg and Moskalewski, 1985). To determine whether the RASSF1A + Rap1A induced alteration in microtubule organization influences these processes, the distribution of vimentin and localization of a Golgi marker were assessed. Upon ectopic expression of Flag-RASSF1A or co-expression of both the EGFP-Rap1A + Flag-RASSF1A in Cos-7 cells, vimentin was found to be retracted in perinuclear bundles (Fig. 7). However, ectopic expression of EGFP-Rap1A alone did not have a visible effect on the distribution pattern of vimentin (data not shown). Similarly, the ectopic expression of Flag-RASSF1A or EGFP-Rap1A led to the partial dispersal of Golgi complex, and their co-expression enhanced this effect resulting in complete fragmentation and dispersal of Golgi complex (Fig. 8). Taken together, these findings suggested that RASSF1A + Rap1A influence the cellular processes that are directly or indirectly dependent on microtubule organization.

DISCUSSION

Rap1A is a small G protein implicated in an array of biological processes ranging from cell proliferation, differentiation and adhesion to embryogenesis. The effectors through which Rap1A might mediate its diverse functions are largely unknown with the exception of two recent studies correlating Rap1A with integrin-mediated adhesion through RapL (Kata-giri et al., 2003) and RIAM (Lafuente et al., 2004). The present

study has shown that the tumor suppressor RASSF1A is a candidate novel effector of Rap1A. As typical for most small G proteins, the activation state of Rap1A regulates its interaction with RASSF1A. Additionally, this interaction was also dependent on the phosphorylation state of RASSF1A. Treatment of cells with calyculin A, an inhibitor of the activity of protein phosphatases PP1 and PP2A, led to retardation of the electrophoretic mobility of RASSF1A protein in SDS-PAGE, indicative of calyculin A induced phosphorylation of RASSF1A. Furthermore, calyculin A treatment led to inhibition of the *in vitro* association of RASSF1A with activated Rap1A. The regulation of this interaction is indicative of a functional association.

Intracellular localization of Rap1A and RASSF1A was investigated by confocal microscopy. As reported before, Rap1A was present in perinuclear punctate structures in Cos-7 cells (Mochizuki et al., 2001) and RASSF1A was found to co-localize with microtubules (Liu et al., 2003; Dallol et al., 2004; Rong et al., 2004; Song et al., 2004). Ectopically expressed Rap1A or RASSF1A led to rearrangement of the microtubules into circular rings, which appeared to result in the loss of the radial array of microtubules and that of the MTOC. Co-expression of RASSF1A and Rap1A in Cos-7 cells led to robust re-localization of Rap1A from perinuclear punctate structures to circular perinuclear rings where it became strongly co-localized with RASSF1A. This also led to the retraction of microtubules to perinuclear rings, leaving a

Figure 7. Perinuclear retraction of vimentin upon ectopic expression of Flag-tagged RASSF1A and co-expression of Flag-RASSF1A and EGFP-Rap1A. Cos-7 cells transfected with the empty vector (a, b) or the expression vector encoding Flag-RASSF1A (c, d) or Flag-RASSF1A + EGFP-Rap1A (e, f, g) (as indicated in the legend on right side of each panel) were immunostained and analyzed by confocal microscopy (LSM510, Carl Zeiss Jena). Flag-RASSF1A was visualized by a Flag-M1 antibody and vimentin by a vimentin monoclonal (Santa Cruz). Nuclei in c and d were counterstained by DAPI. Multi-color superimposed images (Merge) are shown as indicated. The scale bar is equivalent to 20 μm.

Figure 8. Ectopic expression of Flag-RASSF1A leads to disintegration of the Golgi complex; influence of EGFP-Rap1A. Cos-7 cells transfected with the empty vector (a, b) or the expression vector encoding Flag-RASSF1A (c, d), EGFP-Rap1A (e, f) or both (g, h, i) (as indicated in the legend on right side of each panel) were immunostained as indicated and analyzed by confocal microscopy. Flag-RASSF1A was visualized by a Flag-M1 antibody (Rabbit polyclonal, Sigma-Aldrich), and the Golgi complex by the Golgin-97 antibody. Nuclei were counterstained with DAPI with color/multi-color superimposed images (Merge) are shown as indicated. The scale bar is equivalent to 20 μm.

substantial part of the cell body devoid of a microtubule network. Thus, it is evident that RASSF1A links Rap1A to microtubules and Rap1A-RASSF1A interaction plays a role in microtubule organization.

The microtubule dependence of the structural organization of Golgi complex has long been established (Robbins and Gonatas, 1964; Thyberg and Moskalewski, 1985, 1999). In view of the fact that ectopic expression of either Flag-RASSF1A or EGFP-Rap1A or co-expression of these two has robust effects on intracellular distribution of the microtubule network, the effect of RASSF1A and Rap1A on the integrity of Golgi complex was investigated. It was found that the ectopic expression of Flag-RASSF1A or EGFP-Rap1A led to the partial dispersal of Golgi complex, and the co-expression of these two enhanced this effect resulting in complete fragmentation and dispersal of Golgi complex (Fig. 8).

Several alternative subcellular localizations have been proposed for Rap1A. It is reported that Rap1A is predominantly present at intracellular membranes in the perinuclear region, at endocytic and exocytic vesicles and also at the plasma membrane (Pizon et al., 1994). Rap1A is localized to secretory granules in platelets (Lapetina et al., 1989) and neutrophils (Maridonneau-Parini and de Gunzburg, 1992) and translocates from the granules to the plasma membrane after stimulation of these cells (Maridonneau-Parini and de Gunzburg, 1992; Quinn et al., 1992; D'Silva et al., 1998). In fibroblasts, Rap1A is mainly localized in either the medial-Golgi compartment or the early and late endosomes (Béranger et al., 1991; Pizon et al., 1994). There is one report suggesting that an exogenously expressed constitutively active form of Rap1A is localized in the nucleus in NIH3T3 fibroblasts and that growth factors stimulate Rap1A translocation from the cytoplasm to the nucleus in squamous carcinoma cells (Mittra et al., 2003). The RASSF1A dependent microtubule recruitment of Rap1A as a perinuclear network has been demonstrated for the first time in this study. Notably, Epac1, which is an exchange factor for Rap1A, has been shown to interact with the microtubule associated protein(s) MAP1A and MAP1B in Cos cells (Gupta and Yarwood, 2005; Borland et al., 2006). MAPs and RASSF1A-assisted physical proximity of Epac1 and Rap1A on microtubules could lead to the rapid onset of mechanisms that lead to Epac1 mediated Rap1A activation and downstream signaling cascade(s).

The influence of Rap1A-RASSF1A on microtubules is particularly notable given the importance of microtubules in many cellular processes including cell division, vesicular traffic, migration and apoptosis (Brinkley, 1997; Mollinedo and Gajate, 2003; Liu et al., 2007). RASSF1A has also been shown to interact with the pro-apoptotic Sterile20-related (Ste20-related) kinase MST1 (mammalian Sterile20-like 1) via its SARAH domain (Khokhlatchev et al., 2002). It is possible that Rap1A-RASSF1A association modulates microtubule behavior through MST1. Interestingly, it has recently been shown that the Rap1A effector RapL, which is related to

RASSF1, can associate with MST1 and regulate the localization and kinase activity of MST1 (Katagiri et al., 2006). In addition, the *Drosophila* RASSF1A ortholog (dRASSF1) has been shown to restrict the activity of the *Drosophila* ortholog of MST1 and MST2 (Hpo) by competing with Sav for binding to Hpo (Polesello et al., 2006). It would be intriguing to explore whether the *Drosophila* Rap1A ortholog (Dras3) also interacts with dRASSF1 and whether this interaction influences the dRASSF1-mediated inhibition of Hpo activity and the microtubule network.

In summary, our present studies have provided valuable information on the selective interaction of the tumor suppressor RASSF1A with Rap1A. The molecular function of this interaction has been examined. It has also been shown that the regulated interaction of Rap1A with RASSF1A plays an important role in microtubule organization.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies to Rap1A (rabbit polyclonal) and Vimentin (mouse monoclonal) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Other antibodies used were: Pan-Ras (mouse monoclonal) from Upstate Biotechnology (Upstate, Lake Placid, NY); RASSF1A (mouse monoclonal, ebiosciences, San Diego, CA, USA); α -tubulin (mouse monoclonal), β -tubulin (mouse monoclonal) and Flag-M1 (rabbit polyclonal) from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). The antibody Golgin-97 (mouse monoclonal), ProLong[®] Gold antifade reagent with 4'-6-Diamidino-2-phenylindole (DAPI) and secondary antibodies Alexa Fluor[®] 488 Donkey Anti-Rabbit and Alexa Fluor[®] 555 Donkey Anti-Mouse used for immunofluorescence were from Invitrogen (Invitrogen, Paisley, UK). The Cy5 Donkey Anti-Rabbit IgG was from Jackson ImmunoResearch Laboratories, Inc. (Jackson ImmunoResearch, Europe). Phospho-RASSF1A S197 and phospho-RASSF1A S203 specific immune sera PPA545 and PPA548, respectively, were produced by us as described elsewhere (Verma et al., 2008). Affinity purified horseradish peroxidase (HRP) coupled mouse and rabbit secondary antibodies, ECL reagent and glutathione Sepharose 4B beads were purchased from GE Healthcare (GE Healthcare, Buckinghamshire, UK). GDP- β -S, GTP- γ -S and Phosphatase inhibitor cocktail 1 and 2 were from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Enzyme T4 DNA ligase, transfection reagent Lipofectamine[™] 2000 and Zero Blunt TOPO PCR Cloning Kit were from Invitrogen (Invitrogen, Paisley, UK). RapidTrans[™] competent cells used for transformation were purchased from Active Motif (Active Motif, California, USA). All other common reagents were from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). The cell culture media were obtained from Cancer Research UK Media Production (CRUK, London, UK).

Plasmid constructs

The cDNA of the RA/RB domains of RalGDS and Raf, subcloned in pGex4T3 vector (kindly provided by Dr. J. L. Bos, Utrecht University, The Netherlands) (Franke et al., 1997) were used to express RalGDS-RA and Raf-RBD, respectively, as GST-tagged fusions. To

create a bacterial expression plasmid for Glutathion Sepharose Transferase (GST) fusion protein for Rap1A (GST-Rap1A), the cDNA encoding full-length Rap1A (Human Rap1A, Swiss-Prot accession No. P62834) was PCR amplified from pRK5-HA-Rap1A (a gift from Dr. J. de Gunzburg, Institut Curie-Centre de Recherche and Inserm, Paris) and subcloned into the pGex4T3 bacterial expression vector (GE Healthcare, Buckinghamshire, UK). The constructs pCAGGS-EGFP-Rap1A (encoding for amino acid residues 1–184 of Swiss-Prot accession No. P62834), pCAGGS-EGFP-Rap2 (encoding for amino acid residues 1–183 of Swiss-Prot accession No. P10114) and pCAGGS-EGFP-H-Ras (encoding for amino acid residues 1–189 of Swiss-Prot accession No. P01112) were kindly provided by Dr. M. Matsuda (National Cardiovascular Center Research Institute, Japan). To create the bacterial expression plasmid for Glutathion Sepharose Transferase (GST) fusion protein for Rap2 (GST-Rap2) and Ras (GST-Ras), the cDNAs encoding for full-length Rap2 and Ras were PCR amplified from the above vectors (pCAGGS-EGFP-Rap2 and pCAGGS-EGFP-H-Ras, respectively) and subcloned into the pGex4T3 bacterial expression vector (GE Healthcare, Buckinghamshire, UK). pCDNA3-Flag-RASSF1A (encoding for full-length human RASSF1A protein) (Armesilla et al., 2004) was a gift from Dr. L. Neyses (University of Manchester, UK). The plasmid construct pCDNA3-Flag-RASSF1A (S197A–S203A) was created by PCR based site-directed mutagenesis (Stratagene, CA, USA) using pCDNA3-Flag-RASSF1A as a template as described elsewhere (Verma et al., 2008). All the constructs were sequence verified. Plasmid DNA was prepared by standard methods using Qiagen Plasmid Maxi Kits.

Cell culture, transfections and treatments

HEK293 (human embryonic kidney cells), 293T (human embryonic kidney cell's variant with SV40 Large T-antigen) and green monkey kidney lines Cos-7 (all obtained from CR-UK research services) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂. Transfections were performed using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The transfections and treatments were performed in six well plates. After stimulation the cells were washed once with ice cold PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na₂HPO₄, 1.47 mmol/L KH₂PO₄, pH 7.4) and lysed in MgCl₂ containing lysis buffer (MLB) [25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 10% glycerol, 1% Nonidet P-40, 2 × protease inhibitor tablets, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 μmol/L aprotinin, 1 μmol/L leupeptin, 1 μmol/L Pepstatin, 1 mmol/L Na₃VO₄, 1 mmol/L Na₂MoO₄, 4.46 mg/mL Na₄P₂O₇, 25 mmol/L β-glycerophosphate, 1 × phosphatase inhibitor cocktail 1, 1 × phosphatase inhibitor cocktail 2 (both cocktails from Sigma-Aldrich, St. Louis, MO)]. Cell lysis was performed on ice for 30 min and the cell lysates were cleared by centrifugation at 13,500 rotation/min for 30 min at 4°C and used immediately for further assays.

Pull-down assay for Rap1A and Ras activation

Glutathion Sepharose Transferase (GST) fusion proteins (GST-RalGDS-RA and GST-Raf-RBD) (Franke et al., 1997) were purified by standard procedures and the quantity and quality were checked by

SDS-PAGE. Pull-down assays for Rap1 and Ras were performed as described before (Franke et al., 1997).

In vitro loading of Rap1 with GTP-γ-S and *in vitro* Rap1A/Rap2/Ras binding assays

The cell lysates containing endogenous Rap1A and/or ectopically expressed EGFP-Rap1A were incubated with 100 μmol/L GTP-γ-S (or 1 mmol/L GDP-β-S as a negative control) at 30°C for 30 min with agitation to allow the loading of GTP-γ-S on Rap1A present in the lysates. Loading was stopped by placing the tubes on ice and adding 60 mmol/L MgCl₂. For Rap1A/Rap2/Ras binding assays bacterial lysates containing approximately 5 μg of GST-Rap1A, GST-Rap2 or GST-Ras protein (or GST alone, as a negative control) were incubated with 100 μmol/L GTP-γ-S (or 1 mmol/L GDP-β-S as a negative control) at 30°C for 30 min with agitation to allow the loading of GTP-γ-S. Loading was stopped by placing the tubes on ice and adding 60 mmol/L MgCl₂. The suspensions were then incubated with glutathione Sepharose 4B beads. Following a 1 h incubation at 4°C, beads were pelleted and washed three times with ice-cold MLB buffer. These beads were incubated for 90 min at 4°C with cell lysates containing ectopically expressed Flag-tagged RASSF1A. Beads were then washed three times with MLB buffer and subjected to immunoblot (IB) analysis using antibodies to RASSF1A as indicated in the figure legends.

Immunofluorescence

Cos-7 cells were transfected with plasmids as indicated in the figure legends. 16 h post-transfection the cells were trypsinized and seeded at 60% confluence on acid washed glass coverslips. Cells were allowed to grow for an additional 16 h. The cells were washed in PBS, fixed with 3.7% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. The coverslips were then treated with 1% BSA in PBS for 30 min at RT followed by incubation with an appropriate dilution of primary antibody prepared in 1% BSA. After washing in PBS, the coverslips were incubated in the dark with fluorescent dye-conjugated secondary antibody for 45 min at RT. Finally, the coverslips were washed in 3 × PBS and 1 × water and mounted onto glass slides using ProLong[®] Gold antifade reagent with DAPI (Invitrogen, Paisley, UK). The slides were left at RT overnight to allow the mountant to set and the images were acquired using an upright laser scanning confocal microscope (LSM 510 Carl Zeiss Jena) equipped with a 64 × /1.4 Plan-Apochromat Oil Ph3 objective. EGFP and Alexa Fluor[®] 488 were excited with the 488 nm line of an Argon laser, Alexa Fluor[®] 555 with the 543 nm line and Cy5 with the 633 nm line in HeNe laser. Each image represents a single 0.38 μm 'Z' optical section and each confocal image is representative of at least four separate experiments.

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ABBREVIATIONS

GST, Glutathione Sepharose Transferase; GST-Rap1A, Glutathione Sepharose Transferase (GST) fusion protein for Rap1A; IB, immunoblot; MTOC, microtubule-organizing centre; NJ, neighbour joining; RASSF1A, Ras association domain family 1A; RA, Ras-association

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