RASAL3 preferentially stimulates GTP hydrolysis of the Rho family small GTPase Rac2

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Received May 11, 2018; Accepted June 28, 2018

DOI: 10.3892/br.2018.1119

Abstract. Members of the Ras superfamily of small G-proteins serve as molecular switches of intracellular signaling pathways. Rac2 is a Rho subfamily GTPase switch that is specifically expressed in hematopoietic cells and regulates AKT activation in cell signaling. Ras activating protein-like 3 (RASAL3) is the recently identified Ras GTPase activating protein (GAP) that is also specifically expressed in hematopoietic cells and stimulates p21ras GTPase activity. The restricted expression of both Rac2 and RASAL3 suggests that they may serve critical roles in hematopoietic cell signaling. Here in the present study demonstrates that the catalytic domain of RASAL3 may also be able to interact with Rac2 and stimulate its GTPase activity *in vitro*. By contrast, p50 rhoGAP molecules did not markedly affect Rac2 GTPase activity, but did accelerate the activity of other Rho GTPases, including Rac1, RhoA and Cdc42. Collectively, the present results indicate, seemingly for the first time, that GAP activity for Rac2 is regulated by the RasGAP family protein, RASAL3.

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Abbreviations: RASA1, RAS p21 protein activator 1; RASAL3, Ras activating protein-like 3; GAP, GTPase activating protein; SynGAP, synaptic GTPase activating protein; GST, glutathione S transferase; BCR/ABR, breakpoint cluster region/active bcr-related; ABL, Abelson murine leukemia viral oncogene homolog 1; CML, chronic myelogenous leukemia; IPTG, isopropyl β-D-1-thiogalactopyranoside; GSH, glutathione sepharose 4B

Key words: RASAL3, p50 rhoGAP, Rac2, GTP hydrolysis, GAP

Introduction

The Ras superfamily of small GTPases contains more than 170 members divided into 5 subfamilies, namely Ras, Rho, Rab, Ran and Arf (1-5). Upon ligand stimulation, the GTPases are activated by membrane-associated receptors and transmit signals to their downstream effectors, which regulate cell proliferation, differentiation, cytoskeletal regulation and vesicle trafficking (1,3).

Following signal transmission, the active GTP-Ras must become inactive GDP-Ras to cease signaling. However, the intrinsic GTPase activity of Ras proteins is weak, requiring Ras GTPase activating protein (RasGAP) for efficient conversion of GTP-Ras to GDP-Ras (6). RasGAP has emerged as a novel class of tumor suppressor protein and a potential therapeutic target for cancer (6,7). Therefore, it is important to identify the specific GAPs for each small GTPase. Although 14 GAPs for the Ras subfamily have previously been reported (6-10), the specific or redundant functions of these GAPs are unclear. The first RasGAP identified was p120 rasGAP, also known as RAS p21 protein activator 1 (RASA1). This protein is widely expressed, independent of cell type and tissue distribution (11,12). Subsequently, neurofibromatosis type 1 was identified as a RasGAP (13,14). The remaining 12 GAPs are affiliated with the GAP1 and synaptic GAP (SynGAP) family (8,9). Ras activating protein-like 3 (RASAL3) is a SynGAP family member that is predominantly expressed in hematopoietic cells, including Jurkat-T cells (15,16). Since RASAL3 is the most recently identified RasGAP, the specific role of this molecule in regulation of GTPase activity remains to be elucidated.

Among the Rho subfamily, which contains Rho, Rac and Cdc42 GTPases, Rac2 is specifically expressed in hematopoietic cells (17) and is known to be involved in chronic myelogenous leukemia (CML) via direct activation by the p210-breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL) fusion protein (18-20). Furthermore, knockout of Rac2 in mast cells resulted in defective AKT activation, followed by increased apoptosis upon agonist stimulation (18,21). To date the only GAP established for Rac2 is BCR/active bcr-related (ABR) in leukemia (22,23). Thus it

appears necessary to determine the presence of Rac2 GAP in normal hematopoietic cells, since GTP-Rac2 triggers the AKT pathway in cell survival signaling (18,20,21).

Although RASAL3 has been identified as a RasGAP (15,16), the level of GTPase activating activity *in vitro* was not as prominent compared to that of Rho GTPases including RhoA and Rac1. To determine the existence of a preferential target of RASAL3, the Rho subfamily of GTPases were examined. Rac2 was detected as a target GTPase for RASAL3 using an *in vitro* assay system. Overall it was demonstrated that RASAL3 may be a Rac2-selective GAP that has no apparent activity with Rac1. The current results may be useful to clarify the regulation mechanism of Rac2-mediated signaling at the cellular level.

Materials and methods

Reagents. Glutathione sepharose 4B (GSH) was obtained from GE Healthcare Life Sciences (Uppsala, Sweden). Reduced glutathione and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RhoGAP Assay Biochem kit (cat. no. BK105) containing H-Ras, RhoA, Rac1 and Cdc42, separate His-Rac2 protein (cat. no. RC02) and CytoPhos Reagent were purchased from Cytoskeleton, Inc. (Denver, CO, USA). Monoclonal anti-FLAG M2 antibody (cat. no. A2220) and 3X FLAG peptide (cat. no. F4799) were purchased from Sigma-Aldrich (Merck KGaA). Pre-stained molecular weight (MW) marker was purchased from Bio-Rad Laboratories, Inc., (Hercules, CA, USA).

Vector constructs. Full-length cDNA of human RASAL3 in pFLAG-CMV/hRASAL3, constructed by Professor Masahiro Fujii (Niigata University, Niigata, Japan) (16), were used as templates for polymerase chain reaction (PCR) amplification of RasGAP domain. For glutathione S transferase (GST) fusion construction, amplified cDNAs encoding the RasGAP domain of RASAL3 (amino acids 421-748) were ligated into the *Eco*RI/*Xho*I (Takara Bio, Inc., Otsu, Japan) restriction sites of pGEX-5X-1. For PCR amplification, the primers 5'-ACA GAA TTC GCG CGT CGC CTG CGC GTG-3' (forward) and 5'-ACA CTC GAG TCA CAT TGG CAC TGA CAC AAG-3' (reverse) were used for the RASAL3 GAP domain (underlined sequences represent the *Eco*RI and *Xho*I digestion sites, respectively). PCR amplification was performed using a PCR amplification kit (Takara Bio, Inc.; cat. no. R011) and involved an initial step of denaturation (94˚C for 5 min), followed by 35 cycles of denaturation (94˚C for 30 sec), annealing (57˚C for 30 sec) and extension (72˚C for 30 sec). Final extension was performed at 72˚C for 10 min.

Protein expression and purification. GST-RASAL3-GAP fusion proteins were expressed in *Escherichia coli* (*E. coli*) DH5α (Takara Bio, Inc.). Protein expression was induced with 200 μM IPTG overnight at room temperature (~18˚C). Ultrasonic-disrupted cell lysates were centrifuged at 12,000 x g and 4˚C for 20 min, and the supernatants were incubated with GSH beads to collect GST-fused proteins in Tris-Cl buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 2 mM β-mercaptoethanol). Fusion protein-bound beads were washed with the same Tris-Cl buffer, and the fusion proteins were eluted with 30 mM reduced glutathione solution in Tris-Cl buffer (pH 7.5). The purified proteins were concentrated using Amicon Ultra centrifugal filters (Merck KGaA, Darmstadt, Germany) at 4˚C. The resulting proteins were used for further experiments or immediately frozen in liquid nitrogen and stored at -70˚C. The purified GAP domain protein of p50 RhoGAP (also known as Cdc42 GAP) was supplied with the RhoGAP assay kit.

FLAG-tagged RASAL3 proteins were purified from the lysates of HEK-293 cells (American Type Culture Collection, Manassas, VA, USA) that had been transiently transfected with the pFLAG-CMV/hRASAL3 vector. Transfection was performed according to the manufacturer's specification of a Lipofectamine 3000 transfection kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following lysate centrifugation at 12,000 x g and 4˚C for 20 min, anti-FLAG M2 affinity gel (Sigma-Aldrich; Merck KGaA) was added to the supernatant and further incubated on a shaker at 4˚C for 3 h. FLAG-RASAL3 was eluted by incubating the beads at 4˚C for 30 min with 200 ng/ml 3X FLAG peptide in Tris-buffered saline (pH 7.4).

In vitro binding assay. Purified His-Rac2 protein (2 μg) was mixed with 50 μl 1X GAP assay reaction buffer (Cytoskeleton, Inc.) in the presence of 50 μM GDP, GTP or GTPγS (Sigma-Aldrich; Merck KGaA) at 30˚C for 10 min. A 50 μl slurry of beads bound to GST-RASAL3-GAP fusion protein was added to each guanosine nucleotide-charged Rac2 GTPase aliquot. Each 100 μl reaction mixture was incubated at room temperature (~18˚C) for 30 min with gentle shaking. The beads were washed with an equal volume (100 μl) of 1X reaction buffer 3 times to remove unbound proteins. The bound proteins were resolved on an 8-16% gradient SDS-PAGE and visualized by Coomassie Brilliant Blue staining performed at room temperature for 1 h. Additionally, *in vitro* binding assays were performed between the GAP domain of RASAL3 and various small G-proteins (H-Ras, RhoA, Rac1, Rac2 and Cdc42). In this case, 1X reaction buffer was replaced by 50 mM Tris-Cl (pH 7.5) containing 150 mM NaCl and 20 μM GTP. The bound proteins were washed with the same buffer and resolved on SDS-PAGE. The quantities of GTPase pulled down with GST-RASAL3 GAP domain were analyzed by relative image density (Quantity One software ver. 4.6.9; Bio-Rad Laboratories, Inc.)

GAP activity assay. GAP activity assays were performed using the RhoGAP Assay Biochem kit, which contains GST-Rho-GAP fusion protein. Briefly, GTPase activity was measured by monitoring the free γPi release with or without purified GAP molecules via absorbance at 650 nm. Single turnover GTPase reactions (30 μl total volume per reaction) were initiated by the addition of 200 μM GTP. The reactions were performed at 37˚C for 15 min. Subsequently, 150 μl CytoPhos reagent was added to the reactions and incubated for a further 10 min at room temperature. The absorbance of the reactions was measured at 650 nm. The reaction time was controlled to 5, 10 or 15 min according to manufacturer's recommendations. Using each GTPase as a control, absorbance value was measured in the presence of GST alone.

Western immunoblotting. Purified FLAG-RASAL3 solution from pFLAG-CMV/hRASAL3 transfected HEK-293 cell lysates (minimal volume 10 μl) was resolved on 10% SDS-PAGE, and transferred to nitrocellulose membrane for 4 h at 200 mA current. The blot was blocked with 5% non-fat skim milk for 1 h at room temperature, and probed with monoclonal anti-FLAG M2 antibody (1,000x dilution in 5% skimmed milk) for 2 h at room temperature. Following extensive washing with Tris-buffered Tween-20 (50 mM Tris-Cl, 150 mM NaCl, 0.5% Tween-20, pH 7.5), the membrane was re-probed with hoarse-radish peroxidase conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature and visualized with an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. Experimental data are presented as the mean ± standard deviation and were analyzed by one-way analysis of variance and Student-Newman-Keuls post-hoc testing with SPSS 22.0 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate statistical significance.

Results and Discussion

Preparation of RASAL3 GAP proteins. RASAL3 includes pleckstrin homology and C2 domains close to its N-termini, while its GAP domain is located in the central portion of the molecule (Fig. 1A) (7). GST-RASAL3-GAP (421-748 aa) fusion protein expressed in *E. coli* were purified and resolved by 10% SDS-PAGE. In a typical induction system at 37˚C, the proteins were almost pelleted by centrifugation despite the fusion protein being efficiently induced in *E. coli* by 200 μM IPTG. The method was then modified as described in the 'Materials and methods'. A 64 kDa GST-RASAL3-GAP fusion protein was efficiently harvested by the modified methods (Fig. 1B).

GST-RASAL3 stimulates Rac2 GTPase in vitro. Recently, our group identified that a synthesized chemical directly bound to RASAL3 in Jurkat T-cell lysates (data to be published). During examination of RASAL3 GAP activity with chemical regulators, it was noted that the bacterially expressed GAP domain of RASAL3 reproducibly accelerated Rac2 GTPase activity (Fig. 2A).

Subsequently GST-RASAL3-GAP activity with H-Ras and Rho GTPases was examined. As depicted in Fig. 2A, RASAL3 did not markedly stimulate RhoA, Rac1 or Cdc2 GTPase activity, while it notably increased Rac2 GTPase activity. Therefore, RASAL3 was taken to preferentially stimulate Rac2, a Rho family GTPase, but not other Rho family GTPases. Although two recent reports demonstrated that RASAL3 stimulates p21ras GTPase activity (15,16), the present results indicated, seemingly for the first time, that RASAL3 preferentially stimulates GTP hydrolysis in Rac2. Using each GTPase as controls, the measured absorbance value did not change in the presence of GST alone (data not shown).

In the current assay system, RASAL3 increased H-Ras GAP activity ~2-fold, while it accelerated Rac2 GTPase activity >3.5-fold compared with Rac2 GTPase activity

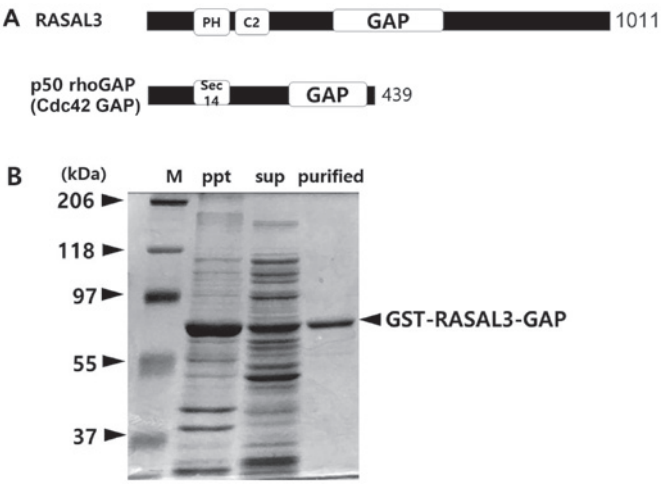

Figure 1. (A) Illustration of RASAL3 GAP molecule used in the experiments. The cDNA encoding RASAL3 GAP domain was amplified by polymerase chain reaction and fused with the *Eco*RI/*Xho*I sites of pGEX 5X-1. The resulting recombinant DNA was transformed into *E. coli*. The p50 rhoGAP (Cdc42 GAP) protein was supplied with the RhoGAP assay kit. (B) Bacterially expressed GST-RASAL3-GAP domain fusion proteins were induced with 200 μM IPTG and purified using a GSH slurry. Following treatment with reduced glutathione, GST-GAP fusion proteins were efficiently eluted, as apparent in the 'purified' lane, and visualized by Coomassie Blue staining. RASAL3, Ras activating protein-like 3; GAP, GTPase activating protein; E. coli, Escherichia coli; GST, glutathione S transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; GSH, glutathione sepharose 4B.

without RASAL3. This result suggests that RASAL3 may be a pivotal regulator of AKT-mediated signaling via Rac2 GTPase regulation in hematopoietic cells.

The GAP domains of RasGAP and RhoGAP are structurally related (24), and their GTPase tertiary folding patterns appear to be similar (6,25). In particular, an arginine residue at position 789 in RASA1 or position 305 in p50 rhoGAP is reportedly capable of penetrating into the active site of each corresponding GTPase (25,26). However, the current results suggested that RasGAP (RASAL3) may stimulate GTP hydrolysis of Rac2, while RhoGAP (p50 rhoGAP) did not exhibit any GAP activity with H-Ras. Therefore, it is conceivable that Ras and Rac2 have structural similarities in their active site. Although Rac1 and Rac2 exhibit >92% amino acid sequence homology (27), RASAL3 activated Rac2 but not Rac1, suggesting somewhat different active site structures.

The p50 rhoGAP GAP domain exhibited highest GAP activity for RhoA and Cdc42, while it exhibited no activity for H-Ras (Fig. 2B). The p50 rhoGAP domain increased Rac2 GTPase activation by <2-fold, while GTPase activation of RhoA, Rac1 and CDC42 increased ~4-, 3.5- and 3-fold, respectively. These results suggest that p50 rhoGAP stimulates GTPase activity of only the Rho family small G-proteins. Furthermore, it is consistent with the report that human p50 rhoGAP has *in vitro* specificity only for Cdc42, Rac1 and RhoA (5).

Complete RASAL3 exhibits a similar activity to the GST-GAP domain. To clarify any difference in GAP activities between the GAP domain alone and the intact GAP molecule, intact RASAL3 was expressed and purified from HEK-293 cells using the pFLAG-CMV/hRASAL3 vector (16). The

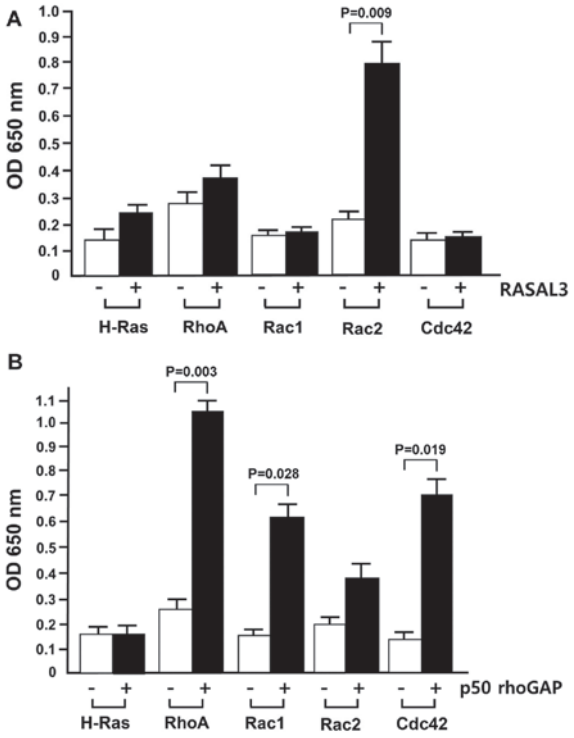

Figure 2. Comparisons of GAP activity between small G-proteins. GTP hydrolysis was determined by measuring the amount of released γPi. Following reaction, the γPi level was measured in the presence of CytoPhos reagent from absorbance at 650 nm. Hydrolysis of GTP by the indicated small G-proteins was performed either in the presence or absence of (A) GST-RASAL3 and (B) GST-p50 rhoGAP at 37˚C for 15 min. The CytoPhos reagent was added to the reactions and incubated for a further 10 min at room temperature, after which the absorbance at 650 nm was measured. Means ± standard deviation of three independent experiments are represented. GAP, GTPase activating protein; GST, glutathione S transferase; RASAL3, Ras activating protein-like 3.

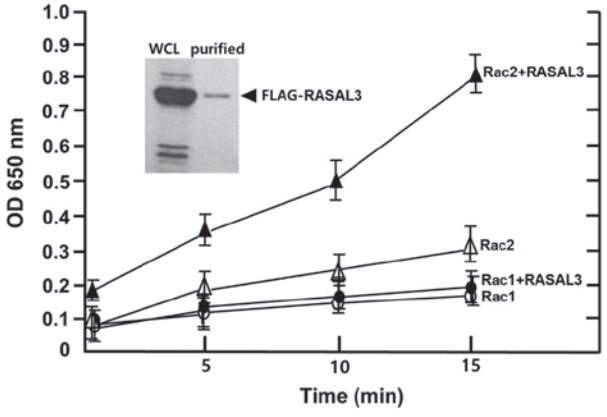

Figure 3. GAP assay using full-length RASAL3 protein. The GTPase activating activity of Rac1 and Rac2 was examined in the presence or absence of whole RASAL3 protein, which was prepared using the pFLAG-CMV/hRASAL3 vector. Means ± standard deviation of three independent experiments are represented. The panel within the graph represents an image of western immunoblotting with anti-FLAG antibody (MW was determined as 120 kDa). RASAL3, Ras activating protein-like 3; GAP, GTPase activating protein; MW, molecular weight; WCL, whole cell lysate.

GTPase activity of Rac1 and Rac2 was measured in the presence and absence of FLAG-RASAL3 at certain time intervals. As depicted in Fig. 3, Rac1 GTPase activity did not increase in the presence of FLAG-RASAL3, while Rac2 GTPase activity was markedly accelerated in the presence of FLAG-RASAL3. The stimulation rate was similar to that of GST-RASAL3-GAP (Fig. 2A), suggesting that both the FLAG-RASAL3 and GST-RASAL3-GAP domain have similar activating activity *in vitro*. The absorbance increased several-fold (~3.5-fold after 15 min) in a time-dependent manner in the presence of FLAG-RASAL3. This contrasts the results of Rac1, which exhibited only basal level changes even in the presence of FLAG-RASAL3 (Fig. 3). Therefore, specific GTP hydrolysis of Rac2 in hematopoietic cells may be potently stimulated by RASAL3.

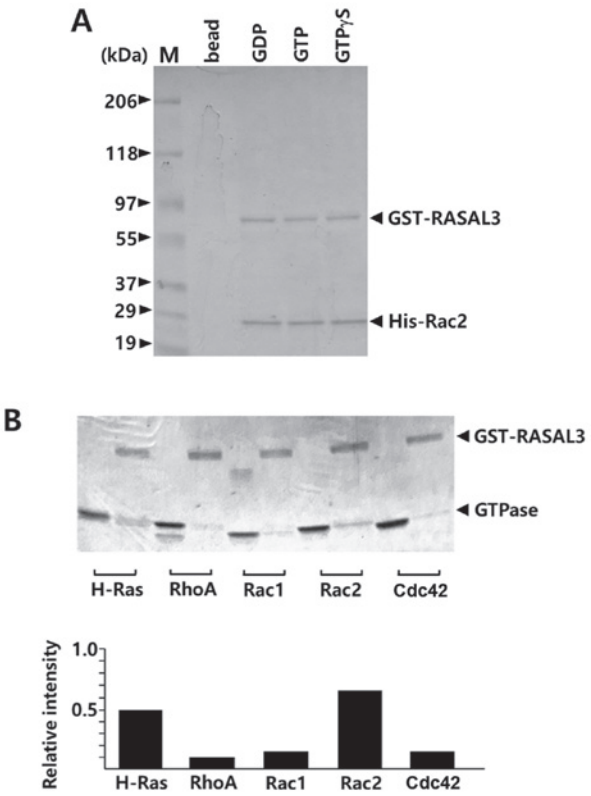

Figure 4. *In vitro* binding assay between RASAL3 GAP domain and Rac2. (A) Purified His-Rac2 proteins (2 μg each) and slurries of GST-RASAL3-GAP beads (50 μl) were mixed with 50 μl 1X GAP assay reaction buffer in the presence of 20 μM GDP, GTP or GTPγS at room temperature for 30 min. The bound proteins were washed and resolved on SDS-PAGE and stained with Coomassie Blue. (B) *In vitro* bindings between the GST-RASAL3-GAP and each small G-protein were independently performed with 50 mM Tris-Cl (pH 7.5) containing 150 mM NaCl and 20 μM GTP at room temperature for 30 min (upper). The quantities of GTPase pulled down with GST-RASAL3 GAP domain were expressed by relative image density, which was normalized against each GTPase alone (lower). RASAL3, Ras activating protein-like 3; GAP, GTPase activating protein; GST, glutathione S transferase.

Rac2 interacts with GST-RASAL3-GAP. To investigate whether the GAP domains of RASAL3 interact with Rac2, an *in vitro* binding assay was performed with GST-GAP fusion proteins. In GST pull-down experiments, Rac2 directly associated with GST-RASAL3-GAP (Fig. 4A). GST-RASAL3-GAP did not exhibit any difference in affinity to either GDP-Rac2 or GTP-Rac2. Generally, GTP-loaded small G-proteins exhibit higher affinity to GAP domains than GDP-loaded proteins (1). In this context, the current results may suggest that the preloaded GTP was

efficiently degraded to GDP during binding in the presence of GST-GAP molecules at high concentration (50 μl). Therefore, the Rac2 fractions pulled down with GST-RASAL3-GAP were likely Rac2 alone or GDP-Rac2. In addition, it was examined whether the GAP domain of RASAL3 binds to other G-proteins including H-Ras, RhoA, Rac1 and Cdc42 in the presence of 20 μM GTP. As presented in Fig. 4B, the Rac2 band exhibited the strongest intensity among the G-proteins pulled down with GST-RASAL3-GAP. Rac2 exhibited marginally higher affinity than H-Ras for the GST-RASAL3 GAP domain, while other Rho subfamily GTPases exhibited seemingly basal level affinities. This result is consistent with the result of Fig. 2A, which overall suggests that the RASAL3 GAP domain preferentially stimulates Rac2 GTPase activity.

In conclusion, in the present study, it was suggested that RASAL3 may be responsible at least in part for controlling AKT-mediated survival signaling in hematopoietic cells via Rac2 GTPase regulation. Although results were obtained *in vitro*, the current findings may provide insight into the regulation mechanism of Rac2-AKT signaling at the cellular level, since the only GAP for Rac2 identified to date is BCR/ABR in Philadelphia chromosome-positive leukemia (22,23). Patients with CML have lymphocytes containing the Philadelphia chromosome, caused by gene fusion between BCR and ABL. Rac GTPase in such lymphocytes has been revealed to be the fully activated form (GTP-Rac) compared with in normal lymphocytes (GDP-Rac) (28). Furthermore, it was demonstrated that CML phenotype was significantly attenuated by depletion of Rac2 in a mouse model (28), suggesting that Rac2 is a crucial regulator in CML disease. In this sense, it is conceivable that RASAL3, as a Rac2 inactivating protein, may be a possible target for CML disease.

Acknowledgements

The authors are grateful to Professor Masahiro Fujii (Niigata University, Niigata, Japan) for providing the pFLAG-CMV/hRASAL3 construct.

Funding

No funding was received.

Availability of data and materials

The analyzed data sets obtained during the study are available from the corresponding author on reasonable request.

Authors' contributions

YS and YWK analyzed GAP activity. HK, NS and TSK purified GST fusion protein. JHC purified FLAG-RASAL3 proteins from HEK-293 cells. TKK and JSC were primarily responsible for writing of the manuscript. JSC contributed to overall design, acquisition and interpretation of data. All authors approved the manuscript submission.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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