RA-GEF-1, a Guanine Nucleotide Exchange Factor for Rap1, Is Activated by Translocation Induced by Association with Rap1-GTP and Enhances Rap1-dependent B-Raf Activation*

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We previously identified RA-GEF-1, a novel guanine nucleotide exchange factor (GEF) for Rap1 with the ability to associate with Rap1-GTP at its Ras/Rap1-associating (RA) domain. Because it possesses a PSD-95/Dlg/AZO-1 (PDZ) domain, it was also named PDZ-GEF. In this report, we have examined the role of the RA domain of this protein in Rap1-mediated cellular responses. A mutant of RA-GEF-1 (RA-GEF-1ΔRA) carrying a 21-residue deletion at its RA domain fully retains the in vitro GEF activity toward Rap1 but completely loses the Rap1 binding activity. In contrast, RA-GEF-1ΔRA, expressed in COS-7 cells, exhibits a 3-fold reduction in its in vivo GEF activity toward Rap1 compared with wild-type RA-GEF-1 as examined by the Rap1 pull-down assay. Correspondingly, when coexpressed with wild-type Rap1, RA-GEF-1ΔRA is unable to further activate B-Raf, whereas RA-GEF-1 stimulates B-Raf as efficiently as activated Rap1. Consistent with these observations, coexpression of activated Rap1 induces translocation of RA-GEF-1, which is otherwise located in the cytoplasm, to the perinuclear compartment, where Rap1 is also predominantly localized. This localization almost coincides with that of the Golgi apparatus, which was detected by anti-trans-Golgi-network 38 antibody. RA-GEF-1ΔRA fails to show the translocation. These results indicate that RA-GEF-1 defines a novel category of GEF that is translocated to a particular subcellular compartment by association with the GTP-bound form of a small GTPase and catalyzes activation of the GDP-bound form present in the compartment, thereby causing an amplification of cellular responses induced by the small GTPase.

Ras family small GTPases have been implicated as a molecular switch that directs cell proliferation and differentiation by cycling between GTP-bound and GDP-bound forms (1, 2). The GTP-bound form is active in that it directly binds to and activates specific effector molecules (3). The effector region of Ras family GTPases (amino acids 32–40 in human Ha-Ras) is involved in the interaction with effectors (1). The Ras-binding domain of the serine/threonine kinase Raf-1, one of the best characterized Ras effectors, interacts directly with the effector region of Ras in a GTP-dependent manner. On the other hand, the RA1 domain, which is also responsible for binding to Ras, was identified in several Ras effectors such as RalGDS and AF-6/Afadin (4). The tertiary structure of the Ras-binding domain of Raf-1 is similar to that of the RA domain of RalGDS, although no obvious homology was found in their amino acid sequences (5–7).

The Ras family consists of ~20 members, including Ha-Ras, Ki-Ras, N-Ras, Rap1A, Rap1B, Rap2A, Rap2B, R-Ras, R-Ras2/TC21, R-Ras3/M-Ras, RalA, and RalB. Among them, Rap1 was originally characterized as an antagonist of Ki-Ras-induced transformation and is thus termed Krev-1 as well (8). The ability of Rap1 to block transformation is likely ascribed to its competitive binding to Ras effectors because Rap1 shares the effector region with Ras and in fact associates with a subset of Ras effectors such as Raf-1 without stimulating their activities. This property of Rap1 is attributable to its greatly enhanced interaction with the cysteine-rich domain, a second Ras-binding site, of Raf-1 (9, 10). In mammalian cells, including fibroblasts, platelets, T and B lymphocytes, and neutrophils, Rap1 is activated in response to a diverse array of extracellular stimuli. Interleukin-2 gene transcription in T cells and insulin-induced mitogen-activated protein kinase activation in Chinese hamster ovary cells, for instance, are presumed to be regulated by both positive and negative actions on Raf-1 exerted by Ras and Rap1, respectively (2). However, Rap1 is rapidly activated after various stimulations without affecting the Ras signaling pathway (11, 12). Therefore, it is feasible that Rap1 also exerts its own physiological function other than the modulation of Ras-dependent pathways. In nerve growth factor-triggered signaling in PC12 pheochromocytoma cells, Rap1 is reported to be involved in B-Raf activation, leading to the sustained activation of extracellular signal-regulated kinases that is required for neuronal differentiation (13, 14). Rap1-dependent activation of B-Raf, but not Raf-1, was observed in COS-7 cells as well (10). Furthermore, the role of Rap1 in integrin-mediated leukocyte adhesion has recently been delineated. An active form of Rap1 potently induced the activation of integrins and subsequent cell aggregation (15, 16), whereas a dominant-negative form of Rap1 and GTPase-activating proteins for Rap1, Rap-GAP, and SPA-1 inhibited cell adhesion triggered by extracellular stimuli including T-cell receptor or CD3 ligation.

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1 The abbreviations used are: RA, Ras/Rap1-associating; GEF, guanine nucleotide exchange factor; cNMP, cyclic nucleotide monophosphate; PDZ, PSD-95/Dlg/AZO-1; HA, hemagglutinin; EGFP, enhanced green fluorescence protein; MBP, maltose-binding protein; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; RID, Rap1-interacting domain.

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Lipopolysaccharide-induced activation of integrins in macrophages also requires Rap1 (18). Mechanisms underlying the regulation of Rap1 remain largely unknown. Like Ras, Rap1 is believed to be activated by specific GEFs such as smgGDS (19), C3G (20), Epac/cAMP-GEF (21, 22) and CalDAGGEF1 (23). Recently, we identified a novel type of Rap1 GEF in humans (Hs-RA-GEF; referred to from here on as RA-GEF-1) and Caenorhabditis elegans (Ce-RA-GEF) (24). Other groups also reported a molecule identical to RA-GEF-1 and designated it PDZ-GEF1 (25), nRapGEF (26), and CNrasGEF (27). RA-GEF-1 contains the cNMP-binding, Ras exchange motif and PDZ and RA domains as well as the GEF catalytic domain. The RA domain of RA-GEF-1 associates directly with the GTP-bound form of Rap1, whereas it exhibits no detectable binding to Ha-Ras. On the other hand, RA-GEF-1 shows GEF activity toward Rap1 and Rap2, but not Ha-Ras. However, regulatory mechanisms of GEF activity remained obscure. We and others detected no specific cAMP/cGMP binding to the cNMP-binding domain of RA-GEF-1 (24–26), although Pham et al. (27) reported cAMP binding to this domain and subsequent stimulation of Ras GEF activity. Here we investigated the role of the RA domain in the regulation of RA-GEF-1. We show that a deletion mutation within the RA domain that virtually abolishes Rap1 binding significantly diminished GEF activity in the cell, whereas it did not affect GEF activity in vitro. Additionally, Rap1-dependent translocation of RA-GEF-1 to the perinuclear compartment was observed, which was totally abolished by the RA domain mutation. Hence, GEF activity of RA-GEF-1 in vivo is likely to be enhanced through the RA domain-mediated translocation to the perinuclear region, where Rap1 exerts its function.

**EXPERIMENTAL PROCEDURES**

**Materials—**Anti-Rap1A (sc-65) and anti-B-Raf (sc-166) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA (12CA5; Roche Molecular Biochemicals), anti-FLAG (M2; Sigma), anti-trans-Golgi-network 38 (Transduction Laboratories), and tetramethylrhodamine-conjugated goat anti-mouse IgG (T7262; Molecular Probes) antibodies were purchased from the indicated commercial suppliers. Horse heart peroxidase-conjugated goat anti-mouse immunoglobulin (NA9310) and anti-rabbit Ig (NA9340) antibodies were purchased from Amersham Pharmacia Biotec. [2,8-3H]cAMP and [8-3H]cGMP were purchased from Moravek Biochemicals. [γ-32P]ATP was purchased from Amersham Pharmacia Biotec.

**Plasmid Construction—**The full-length human RA-GEF-1 cDNA (KIAA0013) was kindly provided by Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). pcDNA3.1/Hisc-Ra-GEF-1 was described previously (24). pcDNA3.1/Hisc-RA-GEF-1RA, which encodes a mutant (designated RA-GEF-1RA) containing an internal deletion (amino acids 606–626) of the RA domain, was generated by oligonucleotide-directed mutagenesis. For expressing EGFP-fused RA-GEF-1 and RA-GEF-1RA in mammalian cells, an EGFP cDNA was subcloned into pcDNA3.1/Hisc-RA-GEF-1 and pcDNA3/Hisc-RA-GEF-1RA, yielding pcDNA3.1/Hisc-EGFP-Ra-GEF-1 and pcDNA3.1/Hisc-EGFP-RA-GEF-1RA, respectively. pFLAG-CMV2-RA-GEF-1, pFLAG-CMV2-RA-GEF-1RA, pBlueBacIII-FLAG-RA-GEF-1, and pBlueBacIII-FLAG-RA-GEF-1RA were constructed by subcloning N-terminally FLAG-tagged RA-GEF-1 and RA-GEF-1RA coding sequences into pCMV2 (Sigma) and pBlueBacIII (PharMingen), respectively. Polypeptides corresponding to RA domains of RA-GEF-1 and RA-GEF-1RA (amino acids 539–709 and 539–688, respectively) were expressed as MBP-fusion proteins (MBP-RAWT and MBP-RAMUT, respectively) in Escherichia coli AG1 cells by using pMal-c2 (New England Biolabs). pEF-BOS-HA-Rap1AV12, pEF-BOS-HA-Rap1AV12ΔN, pH8-FLAG-B-Raf, pGEX2-MEK-His6, and pGEX-Kinase-negative extracellular signal-regulated kinase were overexpressed and purified from E. coli (10, 24).

**Cell Culture and Transfection—**COS-7 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Transfections were performed using Superfect (Qiagen) or GENEPORTER (Gene Therapy System) transfection reagents according to the manufacturer’s protocols.

**In Vitro Rap1 Binding Assay—**The post-translationally modified form of Rap1A was purified from Spodoptera frugiperda Sf9 cells infected with baculovirus overexpressing Rap1A as described previously (9). The in vitro binding assay was carried out by incubating 20 μl of antibody serum containing MBP-RaWT or MBP-RaWTΔN with guanosine 5′-O-(3-thiotriphosphate)- or guanosine 5′-O-(2-thiotriphosphate)-S-labeled Rap1A (10 μmol/L, 50,000 cpm/pmol) in 2% (v/v) glycerol, 75 mM HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl2, and 0.1% Lubrol PX. After incubation at 4 °C for 2 h, resin was washed, and bound proteins were eluted with buffer A containing 10 mM maltose and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblot detection was performed using anti-Rap1A antibody (sc-65) and enhanced chemiluminescence reagents (Roche Molecular Biochemicals). Two types of GEF Assay—RA-GEF-1 and RA-GEF-1RA were expressed in Sf9 cells and affinity-purified with agarose resin conjugated with the anti-FLAG antibody M2 (Sigma). GEF assays were performed as described previously (24). Briefly, 2 pmol of Rap1A loaded with [3H]GDP (5,000 cpm/pmol) was incubated with 1 μg of FLAG-RA-GEF-1 or FLAG-RA-GEF-1RA in 50 μl of reaction buffer containing 20 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 50 mM NaCl, 10 mM 2-mercaptoethanol, 0.5% (v/v) glycerol, 5 μg/ml GTProno, and 10 μg/ml TspGAPase. The reaction was terminated by the addition of 2 ml of ice-cold stop buffer containing 20 μM Tris-HCl, pH 8.0, 100 mM NaCl, and 5 mM MgCl2, and the reaction mixture was subjected to filtration through a nitrocellulose membrane (0.22-μm pore size). After washing with stop buffer, the membrane-trapped radioactivity was measured by liquid scintillation counting.

**Additional GEF Assay**—Rap1A and Rap1AΔN (a 44-residue C-terminal fragment of human Ra1GDS, was described previously (24)). In vivo GEF activities of RA-GEF-1 and RA-GEF-1RA toward Rap1A were examined by the Ra1GDS-RID pull-down assay as described previously (24). Briefly, COS-7 cells (50% confluent) in 100-mm plates were cotransfected with pEF-BOS-HA-Rap1AV12 (1 μg) and either pFLAG-CMV2-RA-GEF-1 or pFLAG-CMV2-RA-GEF-1RA (2 μg) by using the GENEPORTER reagent. COS-7 cells transfected with pEF-BOS-HA-Rap1AV12 served as a positive control. After a 4-h incubation with the transfection mixture, cells were incubated in DMEM containing 10% fetal bovine serum for 26 h. Subsequently, cells were washed once with phosphate-buffered saline and starved for another 16 h in DMEM containing 0.1% fetal bovine serum. Cells were then harvested and lysed in 1 ml of lysis buffer A (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2.5 mM MgCl2, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin). Soluble cellular extracts (250 μl) were incubated with 5 μg of GST-Ra1GDS-RID immobilized on glutathione-agarose resin. After a 60-min incubation at 4 °C, resin was washed four times with lysis buffer A. Bound proteins were eluted with 10 mM glutathione and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblot detection was performed using anti-HA antibody (12CA5) and enhanced chemiluminescence reagents (Roche Molecular Biochemicals). Role of RA-GEF-1 in Rap1-mediated Responses (Roche Molecular Biochemicals).
anti-B-Raf (sc-166) antibodies, respectively, and enhanced chemiluminescence reagents (Roche Molecular Biochemicals).

Fluorescence Microscopy—COS-7 or Rat-1 cells were seeded on a 2-well chamber slide (Nalg Nunc) and transfected with pEBRBS-HA-Rap1AV12 (0.5 μg) in combination with either pDNA3.1/HisC-EGFP-RA-GEF-1 or pDNA3.1/HisC-EGFP-RA-GEF-1ΔRA (0.5 μg) by using the Superfect reagent. After a 4-h incubation with the transfection mixture, cells were incubated in DMEM containing 10% fetal bovine serum for 8 h. Subsequently, cells were washed once with phosphate-buffered saline and starved for another 16 h in DMEM containing 0.1% fetal bovine serum. After fixation with 4% paraformaldehyde and permeabilization with 0.2% Triton X-100, cells were stained with anti-HA (12CA5) and tetramethylrhodamine-conjugated goat anti-mouse IgG (T2762) antibodies. Subcellular localization of EGFP-RA-GEF-1, EGFP-RA-GEF-1ΔRA, and HA-Rap1AV12 was analyzed under a confocal laser microscope (MRC-1024; Bio-Rad).

In Vitro Association with cAMP and GMP—FLAG-RA-GEF-1 was purified from Sf9 cells as described above. GST-fused protein kinase A regulatory subunit I (GST-PKA-Rla) was prepared as described previously (24). The cAMP binding assay was performed essentially as described previously (24), with minor modifications. FLAG-RA-GEF-1 (1 μg) immobilized on anti-FLAG M2 resin or GST-PKA-Rla (0.5 μg) immobilized on glutathione-agarose resin was incubated in a 100-μl reaction mixture containing 10 mM potassium phosphate, pH 6.8, 150 mM NaCl, 1 mM EDTA, 100 μg/ml bovine serum albumin, 25 μM β-mercaptoethanol, and 500 ng [2,8-3H]cAMP (10,000 cpm/μmol) at 25 °C for 90 min with gentle shaking. After extensive washing, bound proteins were eluted with 0.2% SDS and counted for 3H label. The cGMP binding assay was carried out in a similar manner, except that [5-3H]cGMP (5,000 cpm/μmol) replaced cAMP.

RESULTS

RA Domain Mutation That Abolishes the Association with Rap1A—RA-GEF-1 acts as a GEF for Rap1 and Rap2 (24–27), but its RA domain efficiently associates with Rap1A-GTP (24) and not with Rap2A-GTP (data not shown) in vitro. However, the role of the RA domain in the regulation of GEF activity remained unknown. To clarify this point, we first tried to identify a mutation in the RA domain that abolishes the binding activity. Arg-20 of RalGDS is critical for the association between RalGDS-RID and Rap1, as indicated by the finding that the R20A mutation of RalGDS completely eliminated the binding activity (6). Although this Arg residue is conserved in the RA domain of RA-GEF-1 (Arg-611), RA-GEF-1 carrying the R611A mutation still retained a residual binding activity toward Rap1A-GTP (data not shown). Subsequently, we deleted 21 N-terminal amino acids (amino acids 606–626) of the RA domain by oligonucleotide-directed mutagenesis (Fig. 1A), and the association of MBP-RAWT and MBP-RAΔmut with GDP-bound and GTP-bound forms of Rap1A was examined (Fig. 1B). Whereas MBP-RAWT associated with Rap1A in vitro in a GTP-dependent fashion, as we described previously (24), MBP-RAΔmut exhibited no detectable binding to Rap1A.

Effect of the RA Domain Mutation on GEF Activity of RA-GEF-1 in Vitro—To examine the effect of the RA domain mutation on GEF activity, we assayed the GEF activities of full-length wild-type RA-GEF-1 and its RA domain mutant (RA-GEF-1ΔRA). Both proteins were expressed with a FLAG tag in Sf9 cells and purified to near homogeneity by using anti-FLAG M2 resin. As shown in Fig. 2A, both of these proteins exhibited virtually the same enzymatic activity to stimulate GDP release from Rap1A. Therefore, the internal deletion in the RA domain does not interfere with the intrinsic activity of the GEF domain. It has been reported that binding of a ligand to the regulatory domain of certain GEFs, such as Epac/cAMP-GEF (21, 22) and Asef (28), modulates GEF activity. To examine the possibility of ligand induction of GEF activity in vitro, FLAG-RA-GEF-1 and FLAG-RA-GEF-1ΔRA were incubated with guanosine 5′-O-(3-thiotriphosphate)-loaded Rap1A, and reaction mixtures were subjected to in vitro GEF assays. FLAG-RA-GEF-1 and FLAG-RA-GEF-1ΔRA preincubated with or without guanosine 5′-O-(3-thiotriphosphate)-loaded Rap1A exhibited no significant differences in their GEF activity toward Rap1A (data not shown).

Effect of the RA Domain Mutation on GEF Activity of RA-GEF-1 in Vivo—GEF activities of RA-GEF-1 and RA-GEF-1ΔRA in vivo were examined by pull-down assays for Rap1A-GTP. RA-GEF-1 and RA-GEF-1ΔRA were expressed as FLAG-tagged proteins in COS-7 cells in combination with HA-tagged wild-type Rap1A (HA-Rap1AWT). Cell lysates prepared from transfectants were incubated with immobilized GST-RalGDS-RID, and bound HA-Rap1AWT-GTP was quantitated by immunoblotting (Fig. 2B). Coexpression of FLAG-RA-GEF-1WT caused a 6-fold increase in the amounts of the GTP-bound form of HA-Rap1AWT, which were almost equivalent to those of constitutively active HA-Rap1A (HA-Rap1AV12). In contrast, the GTP-bound Rap1A level in FLAG-RA-GEF-1ΔRA-expressing cells was ~3-fold lower than that in cells expressing FLAG-RA-GEF-1WT.

Effect of the RA Domain Mutation on B-Raf Kinase Activation in Response to RA-GEF-1—We next examined B-Raf activation following expression of RA-GEF-1 and its RA domain mutant on the basis of previous reports that Rap1-GTP activates B-Raf (10, 13, 14). To this end, FLAG-RA-GEF-1 or FLAG-RA-GEF-1ΔRA was expressed in combination with FLAG-B-Raf and either HA-Rap1AWT or HA-Rap1AV12 in COS-7 cells. FLAG-B-Raf was immunoprecipitated from total cellular extracts and subjected to kinase assays as described under "Experimental Procedures." As shown in Fig. 3, expression of HA-Rap1AWT resulted in only a 2-fold increase in kinase activity of FLAG-B-Raf. As expected, FLAG-B-Raf coexpressed with HA-Rap1AV12 showed significantly increased activity.

FIG. 1. Deletion in the RA domain of RA-GEF-1. A, schematic representation of the structure of RA-GEF-1 and its RA domain mutant. REM, the Ras exchanger motif. B, in vitro associations of wild-type and deletion mutant RA domains of RA-GEF-1 with Rap1A. Six pmol of guanosine 5′-O-(3-thiotriphosphate)-loaded Rap1A (T) or guanosine 5′-O-(2-thiotriphosphate)-loaded Rap1A (D) was incubated with 50 pmol of MBP-RAWT or MBP-RAΔmut immobilized on amylase resin at 4 °C for 2 h. After extensive washing, bound proteins were eluted with 10 mM maltose. Rap1A present in eluates (Bound Rap1A) and the 1:10 aliquot of Rap1A used for the assay (Input of Rap1A) were visualized by immunoblotting with anti-Rap1A antibody (sc-65).
FIG. 2. GEF activities of RA-GEF-1 and RA-GEF-1RA toward Rap1A in vitro and in vivo. A, in vitro GEF assays. [3H]GDP-loaded Rap1A was incubated with FLAG-RA-GEF-1 (Δ), FLAG-RA-GEF-1RA (Δ), or buffer (○) in the presence of unlabeled GTP for the indicated periods. [3H]GDP remaining bound to Rap1A was measured and expressed as a percentage of the value at the zero time point. Mean values obtained from three independent experiments are shown with S.E. B, in vivo GEF activities of FLAG-RA-GEF-1 and FLAG-RA-GEF-1RA as measured by pull-down assays. Cellular extracts were prepared from COS-7 cells expressing the indicated proteins and incubated with 5 μg of GST-RalGDS-RID immobilized on glutathione-agarose resin at 4 °C for 60 min. HA-Rap1A associated with GST-RalGDS-RID (top panel) was detected by immunoblotting with anti-HA antibody (12CA5). FLAG-RA-GEF-1, FLAG-RA-GEF-1RA, HA-Rap1AWT, and HA-Rap1AV12 in cellular extracts were visualized by immunoblotting with anti-FLAG (M2) (middle panel) and anti-HA (12CA5) (bottom panel) antibodies, respectively. Pull-down assays were performed three times, giving equivalent results.

When coexpressed with FLAG-RA-GEF-1 and HA-Rap1AWT, FLAG-B-Raf also exhibited increased kinase activity equivalent to that induced by HA-Rap1AV12. In marked contrast, when coexpressed with FLAG-RA-GEF-1ΔRA and HA-Rap1AWT, the activity of FLAG-B-Raf was similar to that induced by HA-Rap1AV12 alone. These results parallel the lower level of HA-Rap1A GTP within the cell expressing HA-Rap1AV12 in cellular extracts were visualized by immunoblotting with anti-HA antibody (12CA5), or buffer (○) in the presence of unlabeled GTP for the indicated periods. [3H]GDP remaining bound to Rap1A was measured and expressed as a percentage of the value at the zero time point. Mean values obtained from three independent experiments are shown with S.E. B, in vivo GEF activities of FLAG-RA-GEF-1 and FLAG-RA-GEF-1RA as measured by pull-down assays. Cellular extracts were prepared from COS-7 cells expressing the indicated proteins and incubated with 5 μg of GST-RalGDS-RID immobilized on glutathione-agarose resin at 4 °C for 60 min. HA-Rap1A associated with GST-RalGDS-RID (top panel) was detected by immunoblotting with anti-HA antibody (12CA5). FLAG-RA-GEF-1, FLAG-RA-GEF-1RA, HA-Rap1AWT, and HA-Rap1AV12 in cellular extracts were visualized by immunoblotting with anti-FLAG (M2) (middle panel) and anti-HA (12CA5) (bottom panel) antibodies, respectively. Pull-down assays were performed three times, giving equivalent results.

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Full-length RA-GEF-1 Associates with Neither cAMP nor cGMP—Previously, we could not detect the binding of cAMP or cGMP to the isolated cNMP-binding domain of RA-GEF-1 (24). In this study, we tested whether full-length RA-GEF-1 is able to associate with either cAMP or cGMP. FLAG-RA-GEF-1 purified from Sf9 cells was immobilized on anti-FLAG M2 resin and examined for the association with [3H]cAMP and [3H]cGMP. The binding assay was performed at a lower salt concentration than that in the previous study (24) as described under “Experimental Procedures.” Full-length RA-GEF-1 also exhibited no detectable binding to cAMP or cGMP (Fig. 5). Furthermore, no binding activity was detected under high salt conditions as described previously (24) (data not shown).

DISCUSSION

Diverse roles for Rap1 in cell signaling imply intricate mechanisms whereby Rap1 activity is strictly regulated by upstream signals. Three distinct signaling pathways mediated by cAMP, calcium, and diacylglycerol, respectively, have been implicated in Rap1 activation as shown by studies using second messenger analogues and specific inhibitors (2). Indeed, various types of GEFs for Rap1, which may play a role for Rap1 regulation in individual signaling pathways, have recently been identified. Epac/cAMP-GEF contains a cAMP-binding site through which cAMP increases GEF activity toward Rap1 (21, 22). On the other hand, calcium- and diacylglycerol-binding domains were found in another Rap1 GEF, CalDAGGEFI (23). C3G also belongs to the Rap1 GEF family regulating Rap1 in response to tyrosine...
Role of RA-GEF-1 in Rap1-mediated Responses

A novel type of Rap1 GEF, RA-GEF-1, was cloned recently by our group and others (24–27). RA-GEF-1 consists of multiple domains, including the cNMP-binding, Ras exchanger motif, PDZ, RA, and GEF domains, whose functions remain to be clarified. The cNMP-binding domain of RA-GEF-1 exhibits sequence homology to those of Epac/cAMP-GEF and the regulatory subunit of protein kinase A, except that the PRAA motif is missing. We and other groups observed no significant binding of cAMP and cGMP (24–26), whereas one group described cAMP binding to this domain and a cAMP-dependent increase in the Ras-GTP level within CNrasGEF (RA-GEF-1)-expressing cells (27). However, the binding efficiency seemed very low, although the stoichiometry of cAMP binding was not clearly shown (27). Hence, it seems unlikely that cAMP directly regulates RA-GEF-1 in an allosteric manner. In this study, we further confirmed that full-length recombinant RA-GEF-1 as well as the isolated cNMP-binding domain does not show any significant cAMP or cGMP binding activity. RA-GEF-1 is unique in that it contains both the Rap1 GEF domain and the RA domain that binds Rap1-GTP, suggesting that it plays a role both upstream and downstream of Rap1. A possible mechanism for the regulation of GEF activity upon binding of Rap1 to the RA domain is a ligand-induced conformational change as proposed for Epac/cAMP-GEF (21, 22). However, binding of Rap1 to the RA domain did not affect GEF activity in vitro, and therefore an allosteric effect may not be plausible. In contrast, as shown in this study, the RA domain contributes to direct the subcellular localization of RA-GEF-1, leading to the augmented activation of Rap1 at specific subcellular locations, presumably as shown in this study, the RA domain contributes to direct the subcellular localization of RA-GEF-1, leading to the augmented activation of Rap1 at specific subcellular locations, presumably.

RA-GEF-1 colocalizes with Rap1A in the perinuclear region. A, COS-7 cells expressing EGFP-Rap1A \( \text{V12} \) were analyzed under a confocal microscope. Green fluorescence of EGFP is shown in a, and staining with anti-trans-Golgi-network 38 (1:200 dilution) and tetramethylrhodamine-conjugated goat anti-mouse IgG (T2762; 1:100 dilution) antibodies is shown in b. c is a merged image of a and b. B, COS-7 cells expressing the following proteins were analyzed under a confocal microscope: EGFP-RA-GEF-1, a; EGFP-RA-GEF-1 \( \text{V12} \), b; EGFP-RA-GEF-1 and HA-Rap1A \( \text{V12} \), c–e; and EGFP-RA-GEF-1 and HA-Rap1A \( \text{V12} \), f–h. Green fluorescence of EGFP is shown in c–e and f. C, Rat-1 cells expressing the following proteins were analyzed under a confocal microscope: EGFP-RA-GEF-1, a; EGFP-RA-GEF-1 \( \text{V12} \), b; EGFP-RA-GEF-1 and HA-Rap1A \( \text{V12} \), c–e; and EGFP-RA-GEF-1 \( \text{V12} \) and HA-Rap1A \( \text{V12} \), f–h. Green fluorescence of EGFP is shown in a–c and f. Staining with anti-HA (12CA5; 1:100 dilution) and tetramethylrhodamine-conjugated goat anti-mouse IgG (T2762; 1:100 dilution) antibodies is shown in d and g. e and h are merged images.

Fig. 5. Full-length RA-GEF-1 does not bind to cAMP and cGMP. FLAG-RA-GEF-1 was immobilized on anti-FLAG M2 resin and examined for in vitro association with \(^{3}H\)cAMP and \(^{3}H\)cGMP. GST-PKA-RIs was used as a positive control for cAMP binding. Binding data are expressed as pmol bound cAMP or cGMP/1 pmol protein. Mean values obtained from three independent experiments are shown with S.E. values.

kinase-type receptor-mediated signals through interaction with the adaptor protein Crk (20).

For instance, the serine/threonine kinase Raf-1 translocates to the plasma membrane upon binding to Ras (34). Once targeted to the plasma membrane, Raf-1 becomes phosphorylated at multiple residues and activated (34). For the activation of Raf-1, however, conformational alteration caused by Ras-GTP binding is also considered to be crucial in addition to membrane recruitment (34, 35). Phospholipase C, a recently identified RA domain-containing phospholipase, is also activated through binding to Ras and Rap1 and subsequent translocation to the plasma membrane and the perinuclear region, respectively (33). Thus, a dual specificity RA domain like that of phospholipase C may play a pivotal role in switching and coordinating two signaling pathways mediated by distinct small GTPases by...
differentially distributing the effector. In addition, a molecule closely related to RA-GEF-1, named GFR/MR-GEF, was recently identified as a Rap1 GEF (36, 37). The overall structural features of GFR/MR-GEF are similar to those of RA-GEF-1, and the RA domain specifically interacts with M-Ras-GTP. Although the mechanism remains to be clarified, the MR-GEF-dependent accumulation of Rap1-GTP within the cell was abrogated by coexpression of the activated form of M-Ras (37).

In addition to the RA domain, the PDZ domain may serve to define the subcellular localization of RA-GEF-1. The PDZ domain of PSD95, for example, binds to a specific motif at the C terminus of several signaling molecules, such as the N-methyl-D-aspartate receptor and K+ channels (38). Through this interaction, a multimolecular signaling complex is composed at a specific site in the cell (38). Thus, it is conceivable that the subcellular localization of RA-GEF-1 is also regulated, at least in part, by the PDZ domain, although the binding partner is currently unidentified. Additional studies will reveal the precise mechanisms whereby the subcellular localization of Ras family GTPases and their signaling components is determined. Additionally, roles of Ras family GTPases specific to their subcellular localization will be clarified.

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