Identification and Characterization of RA-GEF-2, a Rap Guanine Nucleotide Exchange Factor That Serves as a Downstream Target of M-Ras*

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The Ras family small GTPase Rap is regulated by an array of specific guanine nucleotide exchange factors (GEFs) in response to upstream stimuli. RA-GEF-1 was identified as a novel Rap GEF, which possesses a Ras/Rap1-associating (RA) domain. Here we report a protein closely related to RA-GEF-1, named RA-GEF-2. Like RA-GEF-1, a putative cyclic nucleotide monophosphate-binding domain, a Ras effector motif, a PSD-95/DlgA/ZO-1 domain, and an RA domain in addition to the GEF catalytic domain are found in RA-GEF-2. However, RA-GEF-2 displays a different tissue distribution profile from that of RA-GEF-1. RA-GEF-2 stimulates guanine nucleotide exchange of both Rap1 and Rap2, but not Ha-Ras. The RA domain of RA-GEF-2 binds to M-Ras in a GTP-dependent manner, but not to other Ras family GTPases tested, including Ha-Ras, N-Ras, Rap1A, Rap2A, R-Ras, RaL, Rin, Rit, and Rheb, in contrast to the RA domain of RA-GEF-1, which specifically binds to Rap1. In accordance with this, RA-GEF-2 colocalizes with activated M-Ras in the plasma membrane in COS-7 cells, suggesting a role of RA-GEF-2 in the regulation of Rap1 and Rap2, particularly in the plasma membrane. In fact, an increase in the level of the GTP-bound form of plasma membrane-located Rap1 was observed when co-expressed with RA-GEF-2 and activated M-Ras. Thus, RA-GEF-2 acts as a GEF for Rap1 and Rap2 downstream of M-Ras in the plasma membrane, whereas RA-GEF-1 exerts Rap GEF function in perinuclear compartments including the Golgi apparatus.

The Ras family small GTPase Rap1 participates in the regulation of a wide variety of cellular responses, including proliferation, differentiation, lymphocyte aggregation, T-cell anergy, and platelet activation (1, 2). In contrast, the physiological function of Rap2, a close relative of Rap1, remains largely unknown. Diverse effectors for Rap1, almost all of which are common to Ras effectors, have been identified, although their roles in individual signaling pathways remain obscure. For activation of the effectors, the interaction of the effector region of Rap1 (amino acids 32–40) with the Ras/Rap1-binding or RA domain of the effectors is important (3). In addition, the second Rap/Rap1-binding site identified in several Ras/Rap1 effectors, including Raf-1, B-Raf, and yeast adenyl cyclase, is required for proper effector activation (4–7). Suppression of Ki-Ras-induced transformation by overexpressed Rap1 is thought to be ascribed to tight binding to the second Rap/Rap1-binding sites of Ras effectors such as Raf-1 without stimulating their activities (5, 6).

Ras family GTPases cycle between GTP-bound active and GDP-bound inactive states, serving as a molecular switch of intracellular signaling (8, 9). Conversion between GTP- and GDP-bound states is controlled by GEFs and GTPase-activating proteins (8, 9). Particularly, GEFs enhance the formation of the GTP-bound active conformation in response to upstream signals mediated by various cell surface receptors. To date, various GEFs for Rap1 have been identified in mammalian cells. C3G binds to the adaptor protein Crk, being involved in tyrosine kinase-dependent activation of Rap1(10). Epa/cAMP-GEF is activated through direct association with cAMP, thereby stimulating Rap-dependent signaling (11, 12). Another Rap GEF, CalDAGGEF1, which contains calcium- and diacylglycerol-binding motifs, has a role in Rap activation in response to these second messengers (13). Additionally, we and other groups recently identified a novel type of the Rap GEF, RA-GEF-1 (also termed PDZ-GEF1, nRapGEP, or CNrasGEF), which exhibits GEF activity toward Rap1 and Rap2, but not Ha-Ras (14–18). RA-GEF-1 contains putative cNMP-binding, REM, PDZ, and RA domains as well as the GEF catalytic domain. We and others detected no specific cAMP/cGMP binding to the cNMP-binding domain (14–16, 18), although Pham et al. (17) reported cAMP binding to this domain and subsequent stimulation of Ras GEF activity. The RA domain of RA-GEF-1 binds to Rap1/GTP, suggesting that RA-GEF-1 plays an important role downstream of Rap1 as well (14). Indeed, the RA domain is required for translocation of RA-GEF-1 to the perinuclear compartments including the Golgi complex and for the full activation of Rap1, as evidenced by our recent obser-

* This work was supported by grants-in-aid for scientific research in priority areas and for scientific research (B) and (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†The abbreviations used are: RA, Ras/Rap1-associating; GEF, guanine nucleotide exchange factor; PDZ, PSD-95/DlgA/ZO-1; cNMP, cyclic nucleotide monophosphate; REM, Ras exchanger motif; RACE, rapid amplification of cDNA ends; EGFP, enhanced green fluorescence protein; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; Raf/DCS, Raf guanine nucleotide dissociation stimulator; RID, Rap1-interacting domain; RBD, Ras-binding domain; GST, glutathione S-transferase; MBP, maltose-binding protein; PLC, phospholipase C; GTPyS, guanosine 5’-3-O-(thio)triphosphate.

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vation that an RA domain mutation that abolishes Rap1 binding compromised RA-GEF-1-dependent activation of Rap1 in vivo (18).

Herein, we describe a novel member of the RA-GEF family, designated RA-GEF-2, whose structural features are intimately related to RA-GEF-1. Like RA-GEF-1, RA-GEF-2 exhibits GEF activity toward Rap1 and Rap2, but not Ha-Ras. However, unlike RA-GEF-1, the RA domain of RA-GEF-2 specifically binds to M-Ras-GTP, which causes the translocation of RA-GEF-2 to the plasma membrane, where M-Ras exists. Correspondingly, activation of Rap1 that localized in the plasma membrane was observed following coexpression of RA-GEF-2 and activated M-Ras.

**EXPERIMENTAL PROCEDURES**

Cloning of RA-GEF-2 and Construction of Mammalian Expression Plasmids—A genomic clone (AC004227) that encodes an open reading frame whose predicted amino acid sequence is highly homologous to RA-GEF-1 was identified through a search of the GenBank data base with the BLAST program. The predicted partial coding sequence in AC004227 was amplified from a cDNA library synthesized from human fetal brain mRNA (Invitrogen) using two gene-specific primers. Upstream and downstream sequences were obtained by 5′- and 3′-RACE using the same library by a Marathon cDNA amplification procedure (CLONTECH). The complete nucleotide sequence was confirmed by isolating and sequencing multiple clones, and the encoded protein was designated RA-GEF-2. The full-length RA-GEF-2 cDNA was subcloned into the mammalian expression vectors pFLAG-CMV2 (Sigma) and pcDNA3.1HisB (Invitrogen), generating pFLAG-CMV2-RA-GEF-2 and pcDNA3.1HisB-RA-GEF-2, respectively. The cDNA for RA-GEF-2 was subcloned into pFLAG-CMV2-RA-GEF-2 lacking the N-terminal portion of the RA domain (amino acids 749–779), which was constructed by the polymerase chain reaction and subcloned into pFLAG-CMV2, generating pFLAG-CMV2-RA-GEF-2. cDNAs for EGFP-tagged RA-GEF-2 and RA-GEF-2ΔR were constructed by the polymerase chain reaction and subcloned into the mammalian expression vector pcMV2, generating pCMV2-EGFP-RA-GEF-2 and pCMV2-EGFP-RA-GEF-2ΔR, respectively. cDNAs for wild-type and activated M-Ras were subcloned into pFLAG-CMV2, generating pFLAG-CMV2-M-Ras and pFLAG-CMV2-M-RasΔTM, respectively. The cDNA for HA-tagged M-RasΔTM was subcloned into the mammalian expression vector pEF-BOS (19), generating pEF-BOS-HA-M-Ras. The cDNA for Ras was kindly provided by Shintaro Iwashita (Mitsubishi Institute of Life Sciences, Japan, whose sequence was described above).

Cell Culture and Transfection—COS-7 cells were cultured in DMEM supplemented with 10% fetal calf serum. Expression plasmids were introduced into COS-7 cells by using GenePORTER (Gene Therapy System) or Superfect (Qiagen) according to the manufacturer’s protocol.

Northern Blotting—A human adult multiple tissue blot membrane containing 2 μg each of poly(A)+ RNA from various tissues (CLONTECH) was probed with a 32P-radiolabeled RNA probe (see Materials and Methods). Hybridization and washing were performed essentially as described (14). Briefly, COS-7 cells were transfected with pCMV2-EGFP-RA-GEF-2 and either pFLAG-CMV2-HA-Rap1A (14) or pFLAG-CMV2-HA-Ha-Ras (14). After serum starvation, cells were washed with phosphate-free DMEM and labeled with [35S]orthophosphate (7.4 MBq/ml of culture medium) for 4 h. FLAG-RA-GEF-2 and HA-Ha-Ras were immunoprecipitated from cleared cell lysates with anti-FLAG M2 resin and eluted with the peptide (Sigma). Guanine nucleotides bound to FLAG-Rap1A and FLAG-Ha-Ras were released by heating the eluate at 68°C for 20 min in denaturing buffer (14) and separated on a polyethyleneimine-cellulose plate. Radioactivities of GDP and GTP spots were quantified by using the BAS2000 bioimaging analyzer (Fuji, Tokyo, Japan). For analyzing the effect of M-Ras on GEF activity of RA-GEF-2 toward Rap1A in vitro, COS-7 cells were transfected with pFLAG-CMV2-Rap1A or FLAG-M-Ras and subjected to in vitro GEF assays. For examining the effect of M-Ras on GEF activity of RA-GEF-2 toward Rap1A in vivo, 6×His-M-Ras (purified from E. coli) or FLAG-M-Ras (purified from COS-7 cells) was preloaded with GTPγS and incubated at 4°C for 2 h with full-length FLAG-M-Ras purified from Si9 cells. Subsequently, the mixture was added to 4 pmol of preloaded Rap1A in a total volume of 200 μl, and GEF assays were performed as described above.

**In Vivo GEF Assay—** COS-7 cells were transfected with pFLAG-CMV2-RA-GEF-2 and either pEF-BOS-HA-Rap1A (14) or pEF-BOS-HA-Ha-Ras (14) by using the GenePORTER transfection reagent (Gene Therapy System). After transfection, cells were incubated in DMEM supplemented with 10% fetal bovine serum for 24 h and then serum-starved for another 16 h. After serum starvation, cells were harvested in phosphate-buffered saline and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2.5 mM MgCl2, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin). Cell lysates were cleared by centrifugation (15,000 × g for 15 min) and subjected to pull-down assays by the use of GST-RalGDS-RID (for Rap1A) or GST-Raf-1-RBD (for Ha-Ras). GTP-bound forms of HA-Rap1A and HA-Ha-Ras were detected by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-HA antibody (12CA5, Roche Molecular Biochemicals). Rap1A- or Ha-Ras-bound GDP/GTP ratios were measured essentially as described (14). Briefly, COS-7 cells were transfected with pCDNA3.1HisB-RA-GEF-2 and either pFLAG-CMV2-Rap1A (14) or pFLAG-CMV2-Ha-Ras (14). After serum starvation, cells were washed with phosphate-free DMEM and labeled with [35S]orthophosphate (7.4 MBq/ml of culture medium) for 4 h. FLAG-RA-GEF-2 and HA-Ha-Ras were immunoprecipitated from cleared cell lysates with anti-FLAG M2 resin and eluted with the peptide (Sigma). Guanine nucleotides bound to FLAG-Rap1A and FLAG-Ha-Ras were released by heating the eluate at 68°C for 20 min in denaturing buffer (14) and separated on a polyethyleneimine-cellulose plate. Radioactivities of GDP and GTP spots were quantified by using the BAS2000 bioimaging analyzer (Fuji, Tokyo, Japan). For analyzing the effect of M-Ras on GEF activity of RA-GEF-2 toward Rap1A in vivo, COS-7 cells were transfected with a combination of pEF-BOS-HA-Rap1A, pFLAG-CMV2-RA-GEF-2ΔR, and either pFLAG-CMV2-RA-GEF-2 or pFLAG-CMV2-RA-GEF-2ΔR by using the GenePORTER transfection reagent. In vitro GEF assays were performed as described above.

**In Vitro Association Assay—**Polypeptides corresponding to the RA domains and their flanking regions of RA-GEF-2 (amino acids 683–853) and RA-GEF-2ΔR (amino acids 683–822) were expressed as MBP fusion proteins (MBP-RA and MBP-RAΔUT, respectively) in E. coli by using pMal-c2 (New England Biolabs). The interaction of the RA domain of RA-GEF-2 with Ras family small GTPases was assessed essentially as described (14). For each binding reaction, small GTPase and its GTPγS or GDP were incubated at 4°C for 2 h with MBP-RA or MBP-RAΔUT (50 pmol) immobilized on amyllose resin in 100 μl of binding buffer (14). After extensive washing with binding buffer, bound proteins were eluted from the resin with 10 mM maltose and subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting.

**Subcellular Fractionation—** COS-7 cells cultivated in 20 culture plates (100-mm diameter) were harvested by centrifugation (600 × g for 5 min) and washed with phosphate-buffered saline. Preparation of the plasma membrane fraction was performed essentially as described (21). Cell lysates were resuspended to 0.25 mg STN (0.25 μg sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), homogenized using a Potter-Elvejem homogenizer, and centrifuged at 280 × g for 5 min. The supernatant was further centrifuged at 1,500 × g for 10 min, and the pellet was resuspended in 0.25 mM STN. 2 mM STN (2 mM sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM MgCl2) was added to adjust the sucrose concentration to 1.42 M. The suspension was transferred to a centrifugation
After centrifugation (82,000 × g for 1 h), the pellet at the 0.25 M/1.42 M interface was collected, resuspended in 0.25 M STM, and centrifuged at 1,500 × g for 10 min. The final pellet (the plasma membrane fraction) was resuspended in 150 l of lysis buffer and subjected to the pull-down assay. Alkaline phosphodiesterase (a marker enzyme for the plasma membrane) and mannosidase II (a marker enzyme for the Golgi apparatus) activities were determined as described by Storrie and Madden (22) to estimate the purity of the fractions.

RESULTS

Cloning of RA-GEF-2—Through a BLAST search of the GenBank data base, we identified a genomic clone (AC004227) encoding an open reading frame closely related to RA-GEF-1, designated RA-GEF-2. Two gene-specific primers designed on the basis of the AC004227 sequence were employed to isolate a partial cDNA clone encoding RA-GEF-2. Subsequently, the full-length cDNA was reconstructed from 5′- and 3′-RACE products obtained from a human fetal brain cDNA library. The putative start codon matched the Kozak consensus sequence and was preceded by in-frame stop codons. The full-length open reading frame was composed of 4,530 nucleotides, encoding a protein of 1,509 amino acids, and was divided into four contiguous genomic clones (AC005218, AC005576, AC004227, and AC004622 from the N terminus to the C terminus) derived from chromosome 5. RA-GEF-1 and RA-GEF-2 were highly homologous in five domains identified by ISREC ProfileScan (76.2, 80.0, 71.4, 81.6, and 85.9% identity in cNMP-binding, REM, PDZ, RA, and GEF domains, respectively), whereas both N-terminal and C-terminal regions were rather divergent (Fig. 1). Three regions (named structurally conserved regions 1–3), which are highly conserved among diverse Ras/Rap GEFs, were also identified in the GEF domain of RA-GEF-2 (Fig. 1C).

Compared with RA-GEF-1, RA-GEF-2 has 145-amino acid extension at its N terminus, but lacks the C-terminal 102 amino acid residues. In particular, the C-terminal region was least homologous (47.5% identity).

Tissue Distribution of RA-GEF-1 and RA-GEF-2—We next examined mRNA levels for RA-GEF-1 and RA-GEF-2 in various human tissues using a multiple tissue blot membrane (Fig. 2). Probes derived from divergent regions were used under stringent hybridization conditions to compare the distribution of the transcripts. A single band of 8 kilobases was detected for both probes. The RA-GEF-2 transcript was abundant in heart, brain, placenta, lung, and liver, but barely detectable in skeletal muscle, kidney, and pancreas, whereas RA-GEF-1 was expressed in all tissues tested.

GEF Activity of RA-GEF-2—A FLAG-tagged RA-GEF-2 fragment (amino acids 403–1276) containing the GEF domain was
expressed in Sf9 cells and purified to near homogeneity. By using this protein, in vitro GEF activities toward various Ras family proteins were examined. RA-GEF-2 showed GEF activity toward Rap1A and Rap2A, but not toward other Ras family members including Ha-Ras, N-Ras, M-Ras, R-Ras, RalA, Rin, Rit, and Rheb, as determined by [3H]GDP-releasing assays (Fig. 3A). [35S]GTP·S-binding assays were also performed for Rap1A and Rap2A, revealing that GEF activity toward Rap2A was higher than that toward Rap1A (Fig. 3B). Next, in vivo GEF activities of RA-GEF-2 toward Rap1A and Ha-Ras were examined by pull-down assays (Fig. 3C). Rap1A-GTP and Ha-Ras-GTP were affinity-precipitated by GST-RalGDS-RID and GST-Raf-1-RBD, respectively, from COS-7 cells that expressed increasing amounts of RA-GEF-2 and either Rap1A or Ha-Ras, and then were detected by immunoblotting. In parallel with the in vitro activity, RA-GEF-2 caused the accumulation of Rap1A-GTP, but not Ha-Ras-GTP. RA-GEF-2-dependent increase in the level of Rap1A-GTP, but not Ha-Ras-GTP, was also shown by measuring the ratio of Rap1A- or Ha-Ras-bound GDP and GTP (Fig. 3D). Collectively, RA-GEF-2, like RA-GEF-1, acts as a specific GEF for Rap1 and Rap2.

Association of M-Ras with RA-GEF-2 through the RA Domain—The RA domain of RA-GEF-1 binds to Rap1A in a GTP-dependent manner (14), suggesting that the RA domain of RA-GEF-2 may also bind to Rap1A-GTP. To test this possibility, we employed in vitro association assays using an MBP fusion construct, named MBP-RAWT, consisting of the RA domain and its flanking region of RA-GEF-2. Unexpectedly, MBP-RAWT bound to Rap1A only weakly compared with the binding of the RA domain of RA-GEF-1 to Rap1A, and this binding was GDP-independent (Fig. 4A). Thus, we next examined the binding activity of MBP-RAWT to a variety of Ras family proteins. Among Ras family members tested, only M-Ras associated with MBP-RAWT in a GTP-dependent manner, and virtually no binding was detected for other Ras family proteins, such as Ha-Ras, N-Ras, Rap2A, R-Ras, RalA, Rin, Rit, and Rheb (Fig. 4A). Quantitative analyses further confirmed that the association between MBP-RAWT and M-Ras was dose- and GTP-dependent (Fig. 4B). Additionally, the binding of M-Ras-GTP was abolished when a 31-amino acid deletion was introduced within the RA domain, suggesting that these highly conserved residues are crucial (Fig. 4C).

Plasma Membrane Translocation of RA-GEF-2 through the Binding to M-Ras—RA-GEF-1 is translocated to the perinuclear compartments, including the Golgi apparatus, upon interaction with Rap1A-GTP (18). Thereafter, RA-GEF-1 serves as an amplifier of Rap1A signaling by yielding the GTP-bound form of Rap1A through the action of the GEF domain (18). In view of the observations that the RA domain of RA-GEF-2, unlike that of RA-GEF-1, specifically binds to M-Ras-GTP, it is feasible that RA-GEF-2 colocalizes with M-Ras in the cell and plays a role downstream of M-Ras. As an initial step to clarify this point, localization of Rap1A, M-Ras, and RA-GEF-2 was examined by immunofluorescence microscopy. Although Rap1A was localized mainly in the perinuclear region as reported previously (23–26), small amounts of Rap1A were detectable in the plasma membrane (Fig. 5A). In contrast, M-Ras was localized mainly in the plasma membrane (Fig. 5B). Both RA-GEF-2 and RA-GEF-2ΔRA were distributed to the cytoplasm, when expressed alone in serum-starved cells (Fig. 5B). Upon coexpression of M-Ras12V, significant amounts of RA-GEF-2 were conveyed to the plasma membrane and colocalized with M-Ras12V (Fig. 5C). In contrast, RA-GEF-2ΔRA did not colocalize with M-Ras in the plasma membrane, indicating that the interaction mediated by the RA domain is critical. Ha-Ras is also localized in the plasma membrane, but does not bind to the RA domain of RA-GEF-2 (Fig. 4A). As expected, RA-GEF-2 was not translocated to the plasma membrane, when coexpressed with Ha-Ras12V (Fig. 5E).

Effect of M-Ras Binding on GEF Activity in Vivo and in Vitro—Given that RA-GEF-2 is an effector for M-Ras, M-Ras...
showed no significant effect on GEF activity. The addition of post-translationally modified M-Ras also exhibited GEF activity toward Rap1A in a dose-dependent manner, which remained totally unaffected following the addition of M-Ras on GEF activity of RA-GEF-2. Thus, we next examined the effect of translational modifications of M-Ras may be critical for the regulation of RA-GEF-2. Considering that post-translational modifications of Ras may modulate the activity of RA-GEF-2. To this end, the effect of M-Ras on GEF activity of RA-GEF-2 was assessed. Full-length recombinant RA-GEF-2 was purified from Sf9 cells, and its GEF activity was measured by [3H]GDP releasing assays in the presence and absence of M-Ras·GTP. Full-length RA-GEF-2 exhibited GEF activity toward Rap1A in a dose-dependent manner, which remained totally unaffected following the addition of the GTP-bound form of M-Ras purified from E. coli (Fig. 6A). Considering that post-translational modifications of Ras and Rap1 are required for the activation of their effectors, such as Raf kinases (4–6) and yeast adenyl cyclase (7, 20), post-translational modifications of M-Ras may be critical for the regulation of RA-GEF-2. Thus, we next examined the effect of post-translationally modified M-Ras purified from COS-7 cells. The addition of post-translationally modified M-Ras also showed no significant effect on GEF activity in vitro (Fig. 6B).

To further analyze the role of M-Ras, RA-GEF-2-dependent increase in the level of Rap1A-GTP was examined by pull-down assays following coexpression of M-Ras71L. We measured the level of Rap1A-GTP in the plasma membrane fraction in addition to that in the total cellular extract because a subset of RA-GEF-2 translocated to the plasma membrane when coexpressed with M-Ras71L (Fig. 5C). The purity of the plasma membrane fraction was assessed by measuring activities of alkaline phosphodiesterase (a plasma membrane marker) and mannosidase II (a Golgi marker). The specific activity of alkaline phosphodiesterase was increased by 6.2-fold in the plasma membrane fraction compared with that in the total cellular extract, whereas that of mannosidase II was decreased by 2.5-fold. When precipitated from the total cellular extracts, the Rap1A-GTP level was slightly decreased upon coexpression of M-Ras71L, whereas RA-GEF-2ΔRA-dependent Rap1A-GTP formation was unaffected by M-Ras71L (Fig. 6C). In marked contrast, RA-GEF-2-dependent increase in the level of Rap1A-GTP in the plasma membrane fraction was enhanced when M-Ras71L was coexpressed (Fig. 6D). Considering that the plasma membrane fraction used in our assays may include some contamination of the Golgi fraction, Rap1A-GTP formation in the plasma membrane upon coexpression of M-Ras71L may actually be more remarkable. Taken together, RA-GEF-2 translocates to the plasma membrane through the interaction with M-Ras·GTP, thereby serving as a GEF specific to plasma membrane-located Rap1A.

**DISCUSSION**

The cDNA encoding a novel Rap1 GEF closely related to RA-GEF-1, designated RA-GEF-2, was isolated from a human fetal brain library. Although RA-GEF-1 and RA-GEF-2 show a striking sequence homology in their RA domains, the binding specificity is completely different. The RA domain of RA-GEF-1 interacts with Rap1-GTP, leading to the translocation of RA-GEF-1 to the perinuclear compartments including the Golgi apparatus (14). In marked contrast, the RA domain of RA-GEF-2 binds to M-Ras, but not Rap1, in a GTP-dependent manner. Upon binding, RA-GEF-2 translocates to the plasma membrane, where it may act as a GEF specific to plasma membrane.
RA-GEF-2 or FLAG-RA-GEF-2 were expressed with or without H9004 Rap1A GTP in the total cellular extract. HA-Rap1A and either FLAG-GEF-2 were added to [3H]GDP-preloaded Rap1A, and [3H]GDP remaining bound to Rap1A was precipitated from extracts of the plasma membrane fraction. HA-Rap1A was precipitated from extracts of the plasma membrane fraction. RA in 1/10 aliquots of the extracts was also monitored by immunoblotting.

**Effect of M-Ras on Rap1 GEF activity of RA-GEF-2.** A, effect of unmodified M-Ras on Rap1 GEF activity in vitro. 6×His-M-Ras was purified from *E. coli* and preloaded with GTP·S. Various amounts of full-length FLAG-RA-GEF-2 were incubated with (closed square) or without (open square) 6×His-M-Ras·GTP·S (50 pmol). Subsequently, the mixture was added to [3H]GDP-preloaded Rap1A, and in vitro GEF assays were performed. Radioactivities of [3H]GDP remaining bound to Rap1A are shown. Results are shown as the mean ± S.E. (n = 3). B, effect of post-translationally modified M-Ras on Rap1 GEF activity in vitro. FLAG-M-Ras was purified from COS-7 cells and preloaded with GTP·S. Full-length FLAG-RA-GEF-2 (5 pmol) was incubated with or without FLAG-M-Ras·GTP·S (20 pmol). Subsequently, the mixture was added to [3H]GDP-preloaded Rap1A, and in vitro GEF assays were performed. [3H]GDP remaining bound to the protein was quantitated and expressed as the percentage of the values in the absence of RA-GEF-2. Representative results of three independent experiments performed in duplicate are shown. C, effect of M-Ras on the level of Rap1A-GTP in the total cellular extract. HA-Rap1A and either FLAG-RA-GEF-2 or FLAG-RA-GEF-2RA were expressed with or without FLAG-M-Ras71L. The GTP-bound form of HA-Rap1A was precipitated from total cellular extracts by the use of GST-RalGDS-RID and detected by immunoblotting using anti-HA antibody. The amounts of HA-Rap1A, FLAG-RA-GEF-2, and FLAG-RA-GEF-2RA in 1/10 aliquots of the extracts were also monitored by immunoblotting. D, effect of M-Ras on the level of plasma membrane-localized Rap1. In fact, M-Ras71L-dependent increase in the level of the GTP-bound form of plasma membrane-localized Rap1 was observed when coexpressed with RA-GEF-2 (Fig. 6D). However, total amounts of Rap1GTP in cells expressing Rap1 and RA-GEF-2 were decreased in the presence of M-Ras71L (Fig. 6C). This may be ascribed to the translocation of RA-GEF-2 to the plasma membrane that may cause sequestration of RA-GEF-2 from a major population of Rap1, which is not localized in the plasma membrane.

It should be noted that we observed no significant increase or decrease in GEF activity in vitro upon the association of the RA domain of RA-GEF-2 with M-Ras·GTP (Figs. 6, A and B). Likewise, in vitro GEF activity of RA-GEF-1 is thought to remain unchanged upon the binding to Rap1-GTP at its RA domain on the basis of the analysis of an RA domain-deletion mutant (18). Rather, it is feasible that Rap1 binding promotes the translocation of RA-GEF-1 to specific subcellular compartments, such as the Golgi apparatus, where RA-GEF-1 enhances the formation of Rap1-GTP (18). By analogy with this mechanism, translocation of RA-GEF-2 to the plasma membrane may be a crucial event in signal transduction through M-Ras and RA-GEF-2.

GFR/MR-GEF is another member of the RA domain-containing Rap1 GEF family (27, 28). The RA domain of GFR/MR-GEF specifically interacts with the GTP-bound form of M-Ras in vivo and in vitro, suggesting that GFR/MR-GEF as well functions downstream of M-Ras (28). Interestingly, primary structures of RA domains of different structures of GFR/MR-GEF are much less homologous between RA-GEF-2 and GFR/MR-GEF (19.7% identity) compared to the homology between RA-GEF-1 and RA-GEF-2 (81.6% identity), although both RA-GEF-2 and GFR/MR-GEF, but not RA-GEF-1, exhibit specific binding ability toward M-Ras·GTP. GFR/MR-GEF-dependent increase in the total Rap1-GTP level within the cell was suppressed when coexpressed with activated M-Ras, which is similar to our results described in Fig. 6C, and thus negative roles for M-Ras and GFR/MR-GEF in the regulation of the Rap1 pathway were suggested (28). However, considering our observation that the level of the GTP-bound form of plasma membrane-localized Rap1 in RA-GEF-2-expressing cells was significantly increased upon coexpression of the activated form of M-Ras, it is likely that in vivo GEF activity of GFR/MR-GEF may also be up-regulated by M-Ras·GTP at the plasma membrane.

Another example of a molecule that possesses both RA and GEF domains, thereby regulating Ras family-mediated signaling via subcellular translocation, is PLCε. PLCε was originally isolated as a Ras effector, which in fact was activated by Ras in a liposome-based reconstitution system as well as in cotransfected cells (29–31). Presumably, PLCε is an effector for Rap1 as well because the RA domain of PLCε associates with Rap1 in a GTP-dependent manner, and epidermal growth factor stimulation induces PLCε to translocate to the perinuclear region where Rap1 is colocalized (29). Our recent characterization of the CDC25 homology domain of PLCε revealed that this domain acts as a GEF for Rap1 and is required for prolonged activation of the Rap1 pathway particularly in the perinuclear region (32). These observations suggest a mechanism whereby PLCε acts not only as a Ras/Rap1-regulated PLC, but also as a signal amplifier that continuously generates the GTP-bound form of Rap1 in a specific subcellular compartment.

In addition to RA and GEF domains, RA-GEF-2 contains putative cNMP-binding and PDZ domains (Fig. 1). Neither cAMP nor cGMP binds to the putative cNMP-binding domain of RA-GEF-1 (14–16, 18). Likewise, RA-GEF-2 interacted with neither cAMP nor cGMP, and thus the function of this domain remains totally unknown. The PDZ domain is responsible for protein-protein interaction, leading to the formation of a functional signaling complex at specific subcellular sites (33). To date, binding partners of PDZ domains of RA-GEF-1 and RA-GEF-2 have not been identified. Considering the totally different subcellular localization of RA-GEF-1 and RA-GEF-2 upon

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the binding to GTPases through the RA domains, their PDZ domains may interact with a distinct set of proteins.

Although little is known regarding the physiological function of M-Ras, it is feasible that M-Ras may direct signaling pathways downstream of cell surface receptors because M-Ras is regulated by GEFs such as Sos1 and Ras-GRF1, which are known to act downstream of a wide variety of receptors (34). Moreover, an activated form of M-Ras displays transforming potential (34, 35), implying that M-Ras, like Ha-, Ki-, and N-Ras, may be involved in the modulation of cell growth and differentiation. In fact, an activated mutant of M-Ras interacts with the Ras effector AF6 (34). However, M-Ras does not bind to multiple other known Ras effectors, such as Raf-1 and phosphoinositide 3-kinase (34), and therefore signaling pathways downstream of M-Ras remain to be clarified. Our findings that the Rap-specific GEF RA-GEF-2 serves as an effector of M-Ras in the plasma membrane may provide insights into the understanding of downstream pathways of M-Ras. Although speculative, RA-GEF-2 may be implicated in the activation of Rap1 extracellular signal-regulated kinase signaling pathways through Rap1 because Rap1 activates B-Raf in various types of cells (6, 36, 37). Future studies will reveal the role of a novel signaling cascade involving M-Ras and Rap1 in receptor-mediated cell responses.

Acknowledgment—We are grateful to Shintaro Iwashita for the Ras-cDNA.

REFERENCES

1. Bos, J. L. (1998) EMBO J. 17, 6776–6782
2. Bos, J. L., de Rooij, J., and Reedquist, K. A. (2001) Nat. Rev. Mol. Cell. Biol. 2, 369–377
3. Ponting, C. P., and Benjamin, D. R. (1996) Trends Biochem. Sci. 21, 422–425
4. Hu, C.-D., Kariya, K., Tamada, M., Akasaka, K., Shirouzu, M., Yokoyama, S., and Kataoka, T. (1995) J. Biol. Chem. 270, 30274–30277
5. Hu, C.-D., Kariya, K., Kotani, G., Shirouzu, M., Yokoyama, S., and Kataoka, T. (1997) J. Biol. Chem. 272, 11702–11705
6. Okada, T., Hu, C.-D., Jin, T.-G., Kariya, K., Yamawaki-Kataoka, Y., and Kataoka, T. (1999) Mol. Cell. Biol. 19, 6057–6064
7. Shima, F., Okada, T., Kido, M., Sen, H., Tanaka, Y., Tamada, M., Hu, C.-D., Yamawaki-Kataoka, Y., and Kataoka, T. (1999) Mol. Cell. Biol. 19, 6057–6064
8. Lowry, D. R., and Williamsen, B. M. (1993) Annu. Rev. Biochem. 62, 851–891
9. Katz, M. E., and McCormick, F. (1997) Curr. Opin. Genet. Dev. 7, 75–79
10. Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsui, H., Hatase, O., Takahashi, H., Kurata, T., and Matsuda, M. (1995) Mol. Cell. Biol. 15, 6746–6753
11. de Rooij, J., Zwartbruij, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A., and Bos, J. L. (1998) Nature 396, 474–477
12. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) Science 282, 2275–2279
13. Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13278–13283
14. Liang, Y., Kariya, K., Hu, C.-D., Shibatahoge, M., Goshima, M., Okada, T., Watarai, Y., Gao, X., Jin, T.-G., Yamawaki-Kataoka, Y., and Kataoka, T. (1999) J. Biol. Chem. 274, 37815–37820
15. de Rooij, J., Boeninck, N. M., van Triest, M., Cool, R. H., Wittinghofer, A., and Bos, J. L. (1999) J. Biol. Chem. 274, 38125–38130
16. Ohitsuka, T., Hata, Y., Ide, N., Yasuda, T., Inoue, E., Inoue, T., Mizoguchi, A., and Takai, Y. (1999) Biochem. Biophys. Res. Commun. 265, 38–44
17. Pham, N., Cheghakov, L., Koch, C. A., de Hoog, C. L., Moran, M. F., and Rotin, D. (2000) Curr. Biol. 10, 555–558
18. Liao, Y., Satoh, T., Gao, X., Jin, T.-G., Hu, C.-D., and Kataoka, T. (2001) J. Biol. Chem. 276, 28478–28483
19. Mizushima, S., and Nagata, S. (1990) Nucleic Acids Res. 18, 5222
20. Kuroda, Y., Suzuki, N., and Kataoka, T. (1993) Science 259, 683–686
21. Hubbard, A. L., Wall, D. A., and Ma, A. (1983) J. Cell Biol. 96, 217–229
22. Storrie, B., and Madden, E. A. (1990) Methods Enzymol. 190, 207–225
23. Berzanger, F., Goud, B., Tavitian, A., and de Gunduz, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1606–1610
24. Wienecke, R., Maize, J. C., Shorainjead, F., Vaas, W. C., Reed, J., Bonifacino, J. S., Resau, J. H., de Gunduz, J., Yeung, H. S., and DeClue, J. E. (1996) Oncogene 14, 913–923
25. Matsubara, K., Kishida, S., Matsura, Y., Kitayama, H., Noda, M., and Kikuchi, A. (1999) Oncogene 18, 1303–1312
26. York, R. D., Molliver, D. C., Grewal, S. S., Stenberg, P. E., McCleskey, E. W., and Stork, P. J. (2000) Mol. Cell. Biol. 20, 8069–8083
27. Ichiba, T., Hoshi, Y., Eto, Y., Tajima, N., and Kuraishi, Y. (1999) FEBS Lett. 457, 85–89
28. Rebhun, J. P., Castro, A. F., and Quilliam, L. A. (2000) J. Biol. Chem. 275, 34901–34908
29. Song, C., Hu, C.-D., Masago, M., Kariya, K., Yamawaki-Kataoka, Y., Shibatahoge, M., Wu, D., Satoh, T., and Kataoka, T. (2001) J. Biol. Chem. 276, 2752–2757
30. Lopez, I., Mak, E. C., Ding, J., Hamm, H. E., and Lomasney, J. W. (2001) J. Biol. Chem. 276, 2752–2765
31. Kelley, G. G., Reks, S. E., Ondrak, J. M., and Smrekova, A. V. (2001) EMBO J. 20, 743–754
32. Jin, T.-G., Satoh, T., Liao, Y., Song, C., Gao, X., Kariya, K., Hu, C.-D., and Kataoka, T. (2001) J. Biol. Chem. 276, 30301–30307
33. Pawson, T., and Scott, J. D. (1997) Science 278, 2075–2080
34. Quilliam, L. A., Castro, A. F., Rogers-Graham, K. S., Martin, C. B., Der, C. J., and Bi, C. (1999) J. Biol. Chem. 274, 23850–23857
35. Kimmelman, A., Tolkacheva, T., Lorenzi, M. V., Osada, M., and Chan, A. M. (1997) Oncogene 15, 2675–2685
36. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Cameron, J., and Stork, P. J. (1997) Cell 89, 73–82
37. York, R. D., Yao, H., Dillon, T., Elling, G. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. (1998) Nature 392, 622–626
Identification and Characterization of RA-GEF-2, a Rap Guanine Nucleotide Exchange Factor That Serves as a Downstream Target of M-Ras
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J. Biol. Chem. 2001, 276:42219-42225.
doi: 10.1074/jbc.M105760200 originally published online August 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105760200

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