RA-GEF, a Novel Rap1A Guanine Nucleotide Exchange Factor Containing a Ras/Rap1A-associating Domain, Is Conserved between Nematode and Humans*

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A yeast two-hybrid screening for Ras-binding proteins in nematode Caenorhabditis elegans has identified a guanine nucleotide exchange factor (GEF) containing a Ras/Rap1A-associating (RA) domain, termed Ce-RA-GEF. Both Ce-RA-GEF and its human counterpart Hs-RA-GEF possessed a PSD-95/DigAZO-1 (PDZ) domain and a Ras exchanger motif (REM) domain in addition to the RA and GEF domains. They also contained a region homologous to a cyclic nucleotide monophosphate-bind- ing domain, which turned out to be incapable of binding cAMP or cGMP. Although the REM and GEF domains are conserved with other GEFs acting on Ras family small GTP-binding proteins, the RA and PDZ domains are unseen in any of them. Hs-RA-GEF exhibited not only a GTP-dependent binding activity to Rap1A at its RA domain but also an activity to stimulate GDP/GTP exchange of Rap1A both in vitro and in vivo at the segment containing its REM and GEF domains. However, it did not exhibit any binding or GEF activity toward Ras. On the other hand, Ce-RA-GEF associated with and stimulated GDP/GTP exchange of both Ras and Rap1A. These results indicate that Ce-RA-GEF and Hs-RA-GEF define a novel class of Rap1A GEF molecules, which are conserved through evolution.

Ras proteins are small guanine nucleotide-binding proteins that serve as molecular switches in regulation of cellular proliferation and differentiation by cycling between the active GTP-bound and the inactive GDP-bound forms (for a review, see Ref. 1). In mammalian cells, the GTP-bound Ras exerts its action by physically associating with and activating effector proteins, such as the serine/threonine kinase Raf-1, through its effector region (amino acid residues 32–46 in human Ha-Ras). In addition to Raf-1 and its isoforms B-Raf and A-Raf, recent searches have identified a number of Ras effectors (or effecter candidates) that associate directly with Ras in a GTP-dependent manner (for a review, see Ref. 2). Two of them, RaI GDS3 and AF-6/Adafin, have been shown to possess homologous motifs of about 100 amino acids in their Ras-associating regions, termed RA domains (3). It has been shown that the RA domain of RaI GDS and the RBD of Raf-1 share a similar tertiary structure, the ubiquitin superfold (4–6).

Rap1A, another member of Ras family small GTP-binding proteins, possesses an identical effector region with that of Ras (for a review, see Ref. 7). Like Ras, Rap1A associates with Raf-1 when it is in a GTP-bound form. However, it fails to activate Raf-1 and, when overexpressed, even suppresses the Ras-dependent activation of Raf-1. Although conflicting reports exist, certain cellular responses, such as the interleukin-2 gene transcription in T cells and the insulin-induced mitogen-activated protein kinase activation in CHO cells, are presumed to be regulated by both the positive and negative actions on Raf-1 exerted by Ras and Rap1A, respectively (7). On the other hand, Rap1A activates B-Raf and may cooperate with Ras in regulation of B-Raf-mediated responses in some cell types (8, 9). In addition to Raf-1 and B-Raf, a majority of other Ras effector molecules are capable of associating with Rap1A as well, suggesting that both Ras and Rap1A are involved in a complex regulation of signaling networks downstream of them (7).

The upstream regulatory mechanisms of Ras and Rap1A appear also complex and await further clarification. The activities of Ras and Rap1A are regulated positively and negatively by specific GEFs and GAPs, respectively (1). The transition of Ras from its GDP- to GTP-bound form is stimulated by different types of GEFs such as Sos (10), RasGEFs (11, 12), and CalDAGGEFII/RasGRP (13, 14). Similarly, multiple GEFs acting on Rap1A have been identified, including C3G (15), Epac/ cAMP-GEF (16, 17), and CalDAGGEF (13). On the other hand, both Rap1A and Rap1A have very low intrinsic GTPase activities, and the activities are stimulated by GAPs. Neurofibromin and p120GAP were identified as specific GAPs for Ras (18), while Rap1A has specific GAPs, Rap1GAP (19) and the recently discovered Rap1GAPII (20).

In this report, we describe the isolation and characterization of a novel type of Rap1A GEF conserved between nematode
**Caenorhabditis elegans** and humans. It is clearly distinct from known Rap1A GEFs in that it contains a functional RA domain and is hence designated RA-GEF. The observed good structural conservation between the *C. elegans* form (Ce-RA-GEF) and the human form (Hs-RA-GEF) suggests their essential roles in biological processes of multicellular organisms.

**EXPERIMENTAL PROCEDURES**

**Cloning of Ce-RA-GEF**—The yeast two-hybrid screening for Ras-associating proteins in *C. elegans* has been performed as described in the previous report (21) by using a cDNA library provided by Dr. Robert H. Waterston (Baylor College of Medicine, Texas, TX) and expressed in a yeast two-hybrid system (22). The bait plasmid contained a partial cDNA insert of pACT5–7 (21, 22) (Fig. 1A). The yeast two-hybrid screening for Ras-associating proteins in *C. elegans* was performed as described previously (22). The 3′-primer corresponded to the sequence within the cDNA insert of a positive clone pACT5–7 (21, 22) (Fig. 1A). The nucleotide sequence of the PCR-amplified cDNA was confirmed by subcloning and sequencing multiple clones. A cDNA clone containing the full-length protein-coding sequence of *Hs-RA-GEF* was provided by Dr. Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan).

**In Vitro Rap/RA Association Assays**—The post-translationally modified forms of human Ha-Ras and Rap1A were purified from *Saccharomyces cerevisiae* expressing ras homologs and rap1A homologs, respectively (23, 24). A fragment of *Hs-RA-GEF* cDNA encoding amino acid residues 540–710, containing the RA domain, was amplified by PCR and cloned into pMal-c (New England Biolabs, Inc.) for expression as a GST fusion protein, MBP-Hs-RA-GEF-RA, in *Escherichia coli*. The *in vitro* association assay was carried out by incubating 20 μl of amylase resin carrying MBP-Hs-RA-GEF-RA with GDP/S or GTP/S-loaded Ha-Ras or Rap1A in a total volume of 20% buffer A (20 mM Tris/HCl, pH 7.4, 40 mM NaCl, 1 mM EDTA, 1 mM diethiothreitol, 5 mM MgCl₂, and 0.1% Lubrol PX). After incubation at 4 °C for 2 h, the resin was washed, and the bound proteins were eluted with buffer A containing 10 mM glutathione and subjected to SDS-polyacrylamide gel electrophoresis (12% gel) followed by Western immunoblot detection with anti-Ha-Ras monoclonal antibody F235 (Oncogene Science, Manhasset, NY) or anti-Rap1A polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as described previously (24, 25). For the quantitative *in vitro* association assays, Ha-Ras and Rap1A were loaded with γ-[35S]S(GTP/S (3,500 cpm/pmol) or [3H]GDP (1,100 cpm/pmol) and incubated with MBP-Hs-RA-GEF-RA as described above except that unlabeled GTP/S or GDP (0.1 mM) was added, respectively, and the incubation was included in the binding reaction. The eluates from amylase resin were counted for 35S or 3H label, respectively.

**In Vitro Association with cAMP and cGMP**—A fragment of *Hs-RA-GEF* cDNA encoding amino acid residues 123–243, encompassing the cNMP-binding domain, was amplified by PCR and cloned into pGEX-2T (Amersham Pharmacia Biotech) for expression as a GST fusion protein, GST-Hs-RA-GEF-cNMP. A full protein-coding sequence of human PKA regulatory subunit I (Amersham Pharmacia Biotech) for expression as a GST fusion protein, GST-PKA-RI, was amplified by PCR from a human brain cDNA library (CLONTECH, Palo Alto, CA) and cloned into pGEX-2T. The cAMP binding assay was carried out essentially as described (27). The two proteins were expressed in an adenylly cyclase-deficient *E. coli* strain CA8306 (26). The AMP binding assay was carried out essentially as described (27). GST-Hs-RA-GEF-cNMP or GST-PKA-RI (0.5 μg) each immobilized on glutathione-agarose resin was incubated in a 100-μl reaction mixture containing 10 mM potassium phosphate, pH 7.4, 6.8, 2 mM NaCl, 1 mM EDTA, 100 μg/ml bovine serum albumin, 25 mM 2-mercaptoethanol, and various concentrations of [35S]-cAMP (15,000 cpm/pmol) (Moravek Biochemicals Inc., Brea, CA) at room temperature for 90 min with gentle shaking. After incubation, the resin was washed, and the bound proteins were eluted with 10 mM glutathione and counted for 35S label. The cAMP-binding assay was carried out similarly as described above, except that [3H]-cAMP (5,700 cpm/pmol) (Moravek Biochemicals Inc.) replaced cAMP.

**In Vitro GEF Assays**—Fragments of Ce-RA-GEF cDNA, corresponding to amino acid residues 470–1300, and of Ha-RA-GEF cDNA, corresponding to residues 258–1147, were amplified by PCR, fused to a DNA fragment encoding the FLAG peptide, and cloned into pBluescript II (Stratagene, La Jolla, CA). The recombinant baculoviruses were transfected into the insect Sf9 cell line using the Insect transfection kit (Invitrogen) according to the manufacturer’s instructions. Briefly, COS-7 cells (50% confluent) in 100-mm plates were cotransfected with pEF-BOS-Ha-Ras and either pcDNA3.1His-C-Hs-RA-GEF or pcDNA3.1His-C-Rap1A or pcDNA3.1His-C-Vac for incubation, and the insect cells were washed and starved for another 24 h in the same medium containing 10% fetal bovine serum and antibiotics. The cDNA-expressing baculovirus was amplified from the insect cells by incubation with 5 μg of pcDNA-RGDS immobilized on glutathione-agarose resin. After incubation for 60 min at 4 °C, the resin was washed four times with the same buffer, and the bound proteins were eluted with 10 mM glutathione and subjected to SDS-polyacrylamide gel electrophoresis (12% gel) followed by Western immunoblot detection with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals). The cDNAs encoding Hs-RA-GEF and Rap1A were expressed in *Escherichia coli* and purified as GST fusion proteins. A full protein-coding sequence of human PKA regulatory subunit I (Amersham Pharmacia Biotech) for expression as a GST fusion protein, GST-PKA-RI, was immobilized on glutathione-agarose resin. After incubation for 60 min at 4 °C, the resin was washed four times with the same buffer, and the bound proteins were eluted with 10 mM glutathione and subjected to SDS-polyacrylamide gel electrophoresis (12% gel) followed by Western immunoblot detection with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals). For the same experiment, the cells employed for the GEF activity on Ha-Ras except that pEF-BOS-HA-RA-Ha-Ras was transfected in place of pEF-BOS-HA-Rap1A, the cell lysates were incubated with MBP-Hs-RA-GEF-RA to pull down Ha-Ras, and the bound proteins were eluted with 10 mM maltose.

We also analyzed the effect of Ha-Ras-GEF expression on Rap1A- and Rap1A-bound GDP/GTP ratios. Cotransfections of COS-7 cells were performed as described above except that pEF-BOS-HA-Rap1A and pEF-BOS-FLAG-Ha-Ras were used instead of pEF-BOS-HA-Rap1A and pEF-BOS-HA-RA-Ha-Ras, respectively. After serum starvation, the cells were washed twice with phosphate-free Dulbecco’s modified Eagle’s medium and incubated in 4 ml of the same medium supplemented with 0.5 μM of [32P]orthophosphate (Amersham Pharmacia Biotech) for 4 h. The cells were lysed in 0.75 ml of lysis buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1% Nonidet P-40, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 1 μl leupeptin (28), and 800 μl of the clarified lysate was incubated with 5 μg of pEF-BOS-HA-RA-Ha-Ras transfected in place of pEF-BOS-HA-Rap1A, the cell lysates were incubated with MBP-Hs-RA-GEF-RA to pull down Ha-Ras, and the bound proteins were eluted with 10 mM maltose. We also analyzed the effect of Ha-Ras-GEF expression on Rap1A- and Rap1A-bound GDP/GTP ratios.

**RESULTS**

**Cloning and Structures of Ce-RA-GEF and Hs-RA-GEF**—In a previous report (21), we carried out a yeast two-hybrid screening for *C. elegans* proteins associating with Ras (encoded by the let-60 gene in this organism) and isolated a novel phosphoinositide-specific phospholipase C, PLC210. PLC210 contained two tandemly arranged RA domains, one of which was associated in vitro with Ras directly in a GTP-dependent manner. In addition to PLC210, the same screening has identified an

**FLAG-Ha-Ras** was transfected in place of pEF-BOS-HA-Rap1A, the cell lysates were incubated with MBP-Hs-RA-GEF-RA to pull down Ha-Ras, and the bound proteins were eluted with 10 mM maltose. We also analyzed the effect of Ha-Ras-GEF expression on Rap1A- and Rap1A-bound GDP/GTP ratios.

**RESULTS**

**Cloning and Structures of Ce-RA-GEF and Hs-RA-GEF**—In a previous report (21), we carried out a yeast two-hybrid screening for *C. elegans* proteins associating with Ras (encoded by the let-60 gene in this organism) and isolated a novel phosphoinositide-specific phospholipase C, PLC210. PLC210 contained two tandemly arranged RA domains, one of which was associated in vitro with Ras directly in a GTP-dependent manner. In addition to PLC210, the same screening has identified an
other novel protein encoded by 10 overlapping partial cDNA clones. The longest clone, pACT5–7, encoded a protein truncated at both its N and C termini (Fig. 1A). A cDNA coding for the upstream sequence was isolated by the spliced leader sequence PCR. A putative initiator ATG was identified in this cDNA as that matching the Kozak consensus sequence (31) and preceded by in-frame stop codons. Also, a BLAST search (32) of GenBank™ entries identified a C. elegans expressed sequence tag clone yk17d8.3 coding for the 3'9-portion of this protein. A composite cDNA encoding the full-length protein consisting of 1,470 amino acid residues was reconstructed by joining the three cDNAs. The deduced Ce-RA-GEF protein contained, from the N terminus to the C terminus, a cNMP-binding domain, a REM domain, a PDZ domain, a RA domain and a GEF domain, all of which were predicted based on their sequence homology to the corresponding functional domains already characterized (Fig. 1, A and B). A BLAST search of the GenBank™ data base identified a cDNA encoding an uncharacterized 1,499-amino acid human protein (gene name KIAA0313; accession number AB002311), containing all of these domains in the same order as in Ce-RA-GEF, and the protein was termed Hs-RA-GEF (Fig. 1, A and B).

Direct Association of Hs-RA-GEF with Rap1A but Not with cAMP and cGMP—Ce-RA-GEF associated with human Ha-Ras and Rap1A in addition to LET-60 but not with human R-Ras, RalA, RhoA, Cdc42, and Rac1 as judged by the two-hybrid assay using the clone pACT5–7 (data not shown). However, direct and GTP-dependent association of its RA domain with Ras and Rap1A could not be tested in vitro, since an MBP fusion protein of this RA domain was found to be insoluble when expressed in E. coli. On the other hand, a similar fusion protein derived from Hs-RA-GEF, MBP-Hs-RA-GEF-RA, was found to be soluble and could be used for the in vitro association assay with Ha-Ras and Rap1A. As shown in Fig. 2B, the association of the immobilized MBP-Hs-RA-GEF-RA with Ha-Ras was barely detectable and was independent of the guanine nucleotide configuration. In contrast, it associated efficiently with Rap1A, and the association exhibited a clear GTP dependence (Fig. 2A). In agreement with this, quantitative analyses with the radiolabeled Ha-Ras and Rap1A indicated that MBP-Hs-RA-GEF-RA was capable of specific association with the GTP-bound form of Rap1A but not with the GTP-bound form of Ha-Ras or the GDP-bound form of Rap1A (Fig. 2C). The amount of the bound Rap1A increased almost linearly to the concentration of 500 nM and reached to the level where about 35% of the input RA domain established the association. Unavailability of a higher concentration preparation of Rap1A
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FIG. 2. Direct association of the RA domain of Hs-RA-GEF with Rap1A. A, 6 pmol of GTPγS-loaded Rap1A (T) or GDPγS-loaded Rap1A (D) were incubated with the indicated amounts of MBP-Hs-RA-GEF-RA (MBP-RA) or MBP alone (MBP) immobilized on amylose resin, and the bound proteins were eluted with 10 mM maltose as described under "Experimental Procedures." Rap1A in the eluate (Bound Rap1A, upper panel) and 1/5 aliquot of Rap1A used in the assay (Input of Rap1A, lower panel) were detected by Western immunoblotting with the anti-Rap1A antibody. B, the in vitro association assays were carried out as in A except that 10 pmol of Ha-Ras were incubated with MBP-Hs-RA-GEF-RA and that the anti-Ha-Ras antibody was used for Western immunoblotting. The assays in A and B were performed three times, giving equivalent results. C, 25 pmol of MBP-Hs-RA-GEF-RA immobilized on amylose resin were incubated with increasing amounts of Rap1A loaded with [γ-32P]GTPγS (●) or [3H]GDP (○), or of Ha-Ras loaded with [γ-32P]GTPγS (×) as described under "Experimental Procedures." The bound Rap1A and Ha-Ras proteins were quantitated by counting for 32P or 3H label. Mean values obtained from two independent experiments performed in duplicate are shown with S.E. values.

FIG. 3. Absence of the cAMP/cGMP binding activity in the putative cNMP-binding domain of Hs-RA-GEF. GST-Hs-RA-GEF-cNMP (○) or GST-PKA-R1α (●) (0.5 μg each) immobilized on glutathione-agarose resin was examined for in vitro association with increasing concentrations of cAMP as described under "Experimental Procedures." The association of GST-Hs-RA-GEF-cNMP with cGMP was also measured similarly (×).

precluded the assessment of the dissociation constant for Rap1A.

Next, we tested the ability of Hs-RA-GEF to bind cAMP or cGMP in vitro. GST-Hs-RA-GEF-cNMP, encompassing the putative cNMP-binding domain, was immobilized on glutathione-agarose and examined for association with increasing concentrations of radiolabeled cAMP and cGMP as described under "Experimental Procedures." In contrast, the full-length Hs-RA-GEF tagged with the Xpress peptide was expressed in COS-7 cells together with the HA-tagged Rap1A. The cell lysate was incubated with the immobilized GST-RalGDS-RID, which contained the RA domain of RalGDS and associated specifically with the GTP-bound form of Rap1A but not with its GDP-bound form (28). As shown in Fig. 5A, the lysates of cells expressing Hs-RA-GEF contained an increased amount of the GTP-bound HA-Rap1A compared with the lysate of cells expressing HA-Rap1A alone. In contrast, when the lysate of cells expressing HA-Ras tagged with the Xpress peptide was expressed in COS-7 cells together with the FLAG-tagged Rap1A or Ha-Ras, and the cells were metabolically labeled with [32P]orthophosphate. The FLAG-tagged Rap1A and HA-Ras were immunoprecipitated from the cell lysates with the anti-FLAG antibody, the bound guanine nucleotides were separated, and the radioactivities associated with GDP and GTP were quantified (Fig. 5C). More than 2-fold increase was observed in the Rap1A-bound GTP concomitant with the coexpression of Hs-RA-GEF (Fig. 5C, lanes 2 and 3), whereas no
increase was observed in the Ha-Ras-bound GTP (Fig. 5C, lanes 4 and 5). These results unambiguously demonstrated that Hs-RA-GEF has a GEF activity toward Rap1A but not toward Ha-Ras.

**DISCUSSION**

The recent discovery of multiple forms of Rap1A GEF has revealed an unexpected complexity of Rap1A regulation. Each of the Rap1A GEF molecules has been shown to possess distinct regulatory elements: C3G contains proline-rich regions that associate with SH3-containing adaptor proteins (15); Epac/cAMP-GEF contains a binding site for cAMP (16, 17); and CalDAGGEFI contains domains that bind Ca\(^{2+}\) and diacylglycerol (13). The presence of the RA domains clearly distinguishes Ce-RA-GEF and Hs-RA-GEF from these known Rap1A GEF molecules. In addition, the PDZ domains, which are found in a number of proteins localized at cellular junctions (33), represent another unique structural feature of RA-GEFs, although the function of these PDZ domains remains to be clarified.

On the other hand, Ce-RA-GEF and Hs-RA-GEF share some elements with the other Rap1A GEF proteins. REM domains are found in all the GEF proteins acting on Rap1A and GTP-binding proteins (34). In agreement with previous reports on the other GEFs (34, 35), fragments of both Ce-RA-GEF and Hs-RA-GEF carrying the REM domains but lacking the REM domains were inactive in the in vitro GEF assays, suggesting that the REM domains are required for the catalytic activities.\(^2\)

In addition, both Ce-RA-GEF and Hs-RA-GEF possess the putative cNMP-binding domains, whose amino acid sequences exhibit a considerable homology to the cAMP-binding domain of Epac/cAMP-GEF (Fig. 1B). Epac/cAMP-GEF was reported to be activated directly by cAMP (16, 17). However, the cNMP-binding domain of Hs-RA-GEF failed to exhibit any binding to both cAMP and cGMP. This may be explained by specific amino acid substitutions found in the cNMP-binding domains of both Ce-RA-GEF and Hs-RA-GEF. A PRAA motif is present in both the slow and fast cAMP-binding pockets of the two PKA regulatory subunits and is also conserved in Epac/cAMP-GEF (res-

\(^2\) Y. Liao and T. Kataoka, unpublished observations.
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The RA domain of Hs-RA-GEF was found to associate with Rap1A but not with Ras. This kind of binding specificity of the RA domain is not unprecedented; theRalGDS RA domain was reported to associate with Rap1A much more strongly than with Ras (38). These binding specificities are presumably determined by the nature of certain amino acids within or flanking the RA domains. More importantly, the GEF activity of Hs-RA-GEF also exhibits clear specificity for Rap1A, whereas Ce-RA-GEF has GEF activity toward both Ras and Rap1A. In this line, it may be interesting to note that CalDAGGEFII/RasGRP, a Ras-specific GEF, and CalDAGGEFI, a Rap1A-specific GEF, share an identical domain organization with each other, suggesting that they might have diverged from a common ancestral protein. Hs-RA-GEF might have acquired the specificity toward Rap1A in the course of evolution from an ancestral GEF protein that may have exhibited no selectivity toward Ras and Rap1A. Ce-RA-GEF may be a direct descendant of this ancestral protein, still retaining the original substrate specificity. This raises an interesting possibility that there may exist another isoform of Hs-RA-GEF that is specific for Ras in humans.

The unique and remarkable feature of Hs-RA-GEF is that it possesses two domains that are capable of interacting with different forms of Rap1A; the RA domain associates with the GTP-bound form, whereas the GEF domain uses the GDP-bound form as its substrate. The role of the RA domain in a physiological function of Hs-RA-GEF remains to be clarified. One possibility is that Hs-RA-GEF is translocated to a Rap1A-containing membrane compartment through association with the GTP-bound Rap1A and catalyzes activation of other GDP-bound Rap1A molecules present in the compartment, thereby causing an amplification or a sustained activation of the Rap1A molecules present in the compartment, thereby stabilizing the GTP-bound Rap1A and catalyzes activation of other GDP-containing membrane compartment through association with the Rap1A GEF and catalyzes activation of other GDP-containing membrane compartment through association with Rap1A. One possibility is that Hs-RA-GEF is translocated to a Rap1A-containing membrane compartment through association with the GTP-bound Rap1A and catalyzes activation of other GDP-bound Rap1A molecules present in the compartment, thereby causing an amplification or a sustained activation of the Rap1A-mediated cellular responses. Experiments with Hs-RA-GEF molecules carrying RA domain mutations that alter its Rap1A-binding property may provide further insights into this possibility.