Identification and Characterization of a New Family of Guanine Nucleotide Exchange Factors for the Ras-related GTPase Ral*

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Guanine nucleotide exchange factors (GEFs) are responsible for coupling cell surface receptors to Ras protein activation. Here we describe the characterization of a novel family of differentially expressed GEFs, identified by database sequence homology searching. These molecules share the core catalytic domain of other Ras family GEFs but lack the catalytic non-conserved (conserved non-catalytic/Ras exchange motif/structurally conserved region 0) domain that is believed to contribute to Sos1 integrity. In vitro binding and in vivo nucleotide exchange assays indicate that these GEFs specifically catalyze the GTP loading of the Ral GTPase when overexpressed in 293T cells. A central proline-rich motif associated with the Src homology (SH)2/SH3-containing adapter proteins Grb2 and Nck in vivo, whereas a pleckstrin homology (PH) domain was located at the GEF C terminus. We refer to these GEFs as RalGPS 1A, 1B, and 2 (Ral GEFs with PH domain and SH3 binding motif). The PH domain was required for in vivo GEF activity and could be functionally replaced by the Ki-Ras C terminus, suggesting a role in membrane targeting. In the absence of the PH domain RalGPS 1B cooperated with Grb2 to promote Ral activation, indicating that SH3 domain interaction also contributes to RalGPS regulation. In contrast to theRal guanine nucleotide dissociation stimulator family of Ras GEFs, the RalGPS proteins do not possess a Ras-GTP-binding domain, suggesting that they are activated in a Ras-independent manner.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF221098.

Materials and Methods

Plasmid Constructs—RalGPS 1A partial cDNA was obtained as a human expressed sequence tag (EST) clone (AA121710). Further sequence was obtained by 5’ RACE using nested primers and a Marathon-Ready™ human heart cDNA library (CLONTECH). ClaI and BsmI sites were incorporated immediately 5’ of the presumed start codon by polymerase chain reaction, and RalGPS 1A (ClaI-XhoI) was inserted naturally conserved region(s); RalGDS, Raf guanine nucleotide dissociation stimulator; RA, RalGDS/AF6 homology or Ras-associated domain; RID, Ral interacting domain; PIP3, phosphatidylinositol 1,4,5-trisphosphate; P13K, phosphatidylinositol 3-kinase.

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The abbreviations used are: GEF(s), guanine nucleotide exchange factor(s); PH, pleckstrin homology; SH, Src homology; RalGPS, Ral GEF with PH and SH3-binding domains; GAP(s) GTPase-activating protein(s); EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PMSF, phenylmethysulfonyl fluoride; GST, glutathione S-transferase; TIU, trypsin inhibitory units; DTT, dithiothreitol; kb, kilobase pair; PAGE, polyacrylamide gel electrophoresis; SCR(s), structurally conserved region(s); RalGDS, Raf guanine nucleotide dissociation stimulator; RA, RalGDS/AF6 homology or Ras-associated domain; RID, Ral interacting domain; PIP3, phosphatidylinositol 1,4,5-trisphosphate; P13K, phosphatidylinositol 3-kinase.

GDP from Ras, permitting GTP, which is present in molar excess in the cytoplasm, to associate with Ras (1, 2). This GTP-bound Ras adopts an active conformation and signals to downstream effector proteins such as Raf, phosphoinositide 3-kinase, and RalGDS (reviewed in Ref. 1). GTPase-activating proteins (GAPs) subsequently enhance the intrinsic GTPase activity of Ras resulting in its rapid return to the inactive GDP-bound state.

In addition to the classic Ha-, Ki-, and N-Ras proteins, ~15 other GTPases make up the Ras sub-group of the Ras superfamily. These include R-Ras, TC21/R-Ras2, M-Ras/R-Ras3, RalA and B, Rap1A and B, Rap2A and B, Rit, Rin, Rheb, Rhse, and Dex-Ras (1). Although the function of many of these GTPases remains to be determined, they are presumably all regulated by a similar GDP/GTP cycle to Ras. However, only a limited number of Ras GEFs have so far been identified that regulate Ras family GTPases; Sos, GRF and GRF for Ras, C3G, CalDAG1 and Epac for Rap1, and RalGDS family members for Ral (2–7).

The catalytic domains of mammalian Ras GEFs share approximately 30% homology with each other and the Saccharomyces cerevisiae CDC25 (2, 8). In an attempt to identify the upstream GEFs and extracellular ligands responsible for the activation of Ras sub-family GTPases we searched the National Center for Biotechnology Information DNA sequence databases for novel cDNAs sharing catalytic domain homology with existing Ras family GEFs. We identified a family of GEFs that contain a catalytic/CDC25 homology domain, a proline-rich PXXP motif similar to SH3 domain binding motifs (9), and a C-terminal pleckstrin homology domain (10). Here we demonstrate that this family of GEFs promotes GTP loading of the Ral GTPase in vivo, binds to the SH3 domain-containing adapter proteins, Grb2 and Nck, and requires their PH domain for bioactivity. We refer to these exchange factors as RalGPS for Ral/PH/SH3-binding GEFs. The RalGDS family of Ras GEFs (consisting of RalGDS, Rgl, and Rgl2/Rlf) are downstream effectors of Rac (1). However, because of the lack of a Ras-binding RA (RalGDS/AF6 homology or Ras-associated) domain (11, 12) it is anticipated that this new Ral GEF family is regulated independently of Ras activation.

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into the pFLAG-CMV2 vector (Eastman Kodak Co.), creating FLAG-RalGPS 1A. Full-length human RalGPS 1B (KIAA0351) was obtained from Kazusa DNA Research Institute, Chiba, Japan. The FLAG-tagged construct was prepared by purifying the Xbal-SmaI fragment of RalGPS 1B and inserting it into the Xbal-SmaI site of pFLAG-CMV2 RalGPS 1B. The C-terminal fragment contains 392–528 amino acids for RalGPS 1A and B, respectively. The pHA-Dox construct was generated by inserting the fragment (ClaI-Ncol/blunt) into the pCI-Neo vector (Promega). The C-terminal fragment was then directionally ligated into the BamHI site of pFLAG-CMV2.

Ral, Rap2B, and Rheb were generously provided by D. Andres (University of Kentucky), J. de Gunzburg (Institut Curie, Paris, France), and G. Clark (NCI, National Institutes of Health), respectively. Rif and the pGEX-RID (Ral interacting domain from RalBP1, residues 648–677) were a gift from H. Hanafusa (Rockefeller University). SH3 domain-encoding plasminogen activator inhibitor type 2 (PAI-2) and the pCAAX vector (created by G. Clark, NCI, National Institutes of Health). RalGPS 1A/B-AP-CAAX was then directionally ligated into the BamHI site of pFLAG-CMV2.

RESULTS AND DISCUSSION

**RalGPS Represents a Novel Family of Putative Ras GEFs**—

The catalytic (CDC25 homology) domains of Ras family GEFs share ~30% homology with each other. Highest sequence homology is found within SCRs (structurally conserved regions 1–3) as defined in Ref. 8. Using a consensus SCR2 sequence supplemented with GRF1 residues at positions of low homology, we searched the EST database and identified several novel putative GEF sequences. One of these, AA121710, was obtained, sequenced, and found to encode the C-terminal portion of a CDC25 homology domain, as well as a proline-rich region similar to SH3 binding motifs, a pleckstrin homology domain, a stop codon, and a 3’ non-coding region (Fig. 1). This protein is referred to as RalGPS 1A. Using 5’ RACE we obtained the core catalytic domain (SCRs 1–3) but could not isolate SCR0 (also known as REM, Ras exchange motif or the conserved non-catalytic domain (15)), an additional N-terminal domain found in most GEFs and shown in the SosI crystal structure to play a role in maintaining the conformation of the catalytic domain (16). Subsequently an additional full-length sequence, KIAA0351 (accession number AB002349), which encoded a splice variant of RalGPS 1A, referred to subsequently here as RalGPS 1B, was entered in the NCBI database. The 5’ end of this clone extended beyond our 1A sequence and revealed a stop codon 5’ to a potential initiating ATG. This indicated that like the yeast GEF, BUD5, the RalGPS proteins do not contain the SCR0 domain (15). RalGPS 1B contained an extra 8-amino acid insertion within loop 3 of its PH domain, a shorter, alternate C terminus, and a unique 68-amino acid central portion that replaced 27 amino acids in RalGPS 1A (Fig. 1A). An additional mouse sequence, AA110466, appears to represent a novel family member, RalGPS 2 (Fig. 1B), because a closely related partial human sequence (AA252781) is also present in the database. The AA110466 partial cDNA was obtained and sequenced and found to similarly contain a PH domain (Fig. 1C) and PXXP motif.

**SCRs 1–3** were originally defined primarily using yeast Ras GEF sequences (8). Alignment with subsequently identified mammalian GEF sequences has revealed two other regions of high homology that are indicated as SCRs 4 and 5 in Fig. 1B. Interestingly, SCR4, a highly conserved I/VNF motif has been reported to sit within a small hydrophobic groove formed by the SCR0 domain in the SosI crystal structure (16). Because SCR0 is absent from RalGPS, it is not surprising that charged residues (ENE) occupy the place of the INF motif of Sos1 (Fig. 1B). Further, RalGPS family members do not have the extension in SCR3 characteristic of the RabGDS family of Ras GEFs (Fig. 1B).

**Splice Variants of RalGPS 1 Are Differentially Expressed**—

A 1.5-kb XbaI-HindI fragment from RalGPS 1A was radiolabeled with [γ-32P]ATP and used to probe the human Northern blot (CLONTech) as described previously (13). 293T human embryonic kidney cells were cultured as described (13). SH3 binding assays were performed essentially as described (14).

**Protein Binding Assay**—

Thirty 100-mm dishes of 70% confluent 293T cells were transfected with 7.5 μg of FLAG-RalGPS 1A/B using 15 μg of NoveFECT/dish (Vennon Nova Inc.) following the manufacturer’s directions. After 72 h, cells were lysed (1 ml per dish) in 20 mM Tris-HCl, pH 7.4, 1% IGEPAL (Sigma), aprotinin (0.05 TIU/ml), 1 mM EDTA, 100 mM NaCl, 0.05 μM protease inhibitors, and then incubated for 1 h at 4 °C. The cell lysate containing most of the RalGPS 1A/B coding region was used to probe a human multi tissue Northern blot to examine mRNA expression. Three major bands were detected (Fig. 1A): a 12-kb doublet (+56), because a closely related partial human sequence (AA252781) is also present in the database. The AA110466 partial cDNA was obtained and sequenced and found to similarly contain a PH domain (Fig. 1C) and PXXP motif.

**Levels of RalGPS 1B expression** were determined by M2 anti-FLAG antibody using ECL reagents (Amerham Pharmacia Biotech).

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**Miscellaneous**—A 1.5-kb XbaI-HindI fragment from RalGPS 1A was radiolabeled with [γ-32P]ATP and used to probe the human Northern blot (CLONTech) as described previously (13). 293T human embryonic kidney cells were cultured as described (13). SH3 binding assays were performed essentially as described (14).

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for RalGPS 1A and B, we first assessed their ability to bind a panel of nucleotide-free GTPases. Because GEFs bind with high affinity to Ras proteins in the absence of Mg\(^{2+}\)/GTP or GDP and stabilize the nucleotide-free state (2), epitope-tagged RalGPS 1 proteins expressed in 293T cells were incubated with GST-Ras fusion proteins that had been stripped of GDP in the presence of EDTA. As shown in Fig. 3A, nucleotide-free Ral but not other GTPases or GST bound to both RalGPS 1 splice variants.

To confirm that the interaction of RalGPS with Ral resulted in nucleotide exchange we examined the ability of RalA to be loaded with GTP in vivo following cotransfection with known and putative Ral GEFs. For these assays we took advantage of the fact that the putative Ral effector RalBP1 binds preferentially to Ral-GTP versus Ral-GDP to specifically extract activated Ral from cell lysates (18, 19). As can be seen in Fig. 3B, there was little Ral-GTP present in serum-starved 293T cells. However, following co-transfection with the RalGDS family member Rif or with RalGPS 1B, Ral-GTP levels were increased by an equivalent amount, indicating that RalGPS was indeed a Ral GEF. Because a trace of Rap1A binding to RalGPS was detected in some assays (see Fig. 3A), we examined the Rap1 protein nucleotide exchange specificity of RalGPS. Using a GST fusion protein containing the Ras/Rap1-GTP-binding domain of cellular Rap1 to precipitate activated Rap1A from cell lysates we found that cotransfection with RalGPS 1B failed to activate Rap1A under conditions where the known Rap1 GEF, C3G, promoted significant accumulation of Rap1A-GTP in vivo. Taken together, the data in Fig. 3, A and B indicate that...
RaLGPS is a highly specific Ral GEF. We have not confirmed that RaLGPS 2 is also a Ral GEF, but given the high degree of sequence conservation with RaLGPS 1A/B within its catalytic domain (Fig. 1B) this seems likely.

The PH Domain of RaLGPS Is Required for Biological Activity—PH domains are typically involved in protein-protein or protein-lipid interactions (10). For example, the PH domains of the β-adrenergic receptor kinase and GRF bind to the β subunits of heterotrimeric G proteins (20), whereas most PH domains associate with the polyphosphoinositides, phosphatidylinositol-4,5-bisphosphate (PIP2) and phosphatidylinositol-1,4,5-trisphosphate (PIP3). Although full-length RaLGPS 1A was expressed poorly in 293T cells, RaLGPS 1B includes a consensus phosphorylation site for the cyclic AMP-dependent protein kinase (KKVSI (28), underlined in Fig. 1C). GEFS that regulate Rho family GTPases (22, 23). It has been reported that the Tiam-1 N-terminal PH domain binds to PIP2 (21) (21). Comparison of the RaLGPS PH domain with that of other molecules demonstrated strongest identity with the N-terminal PH domains of Tiam-1 and the Drosophila melanogaster Still life (Fig. 1C), GEFS that regulate Rho family GTPases (22, 23). It has been reported that the Tiam-1 N-terminal PH domain binds to PIP2 (21) and is required for membrane association and biological activity of this Rac GEF (23). Therefore, we deleted the PH domain from RaLGPS 1B (RaLGPS-PH) and examined the consequence of this modification on in vitro GEF activity.

As shown in Fig. 3C, this deletion completely abrogated the ability of overexpressed RaLGPS 1B to activate Rac in vitro. To address whether the PH domain might be responsible for interaction of the GEF with the plasma membrane, it was replaced with the C-terminal 18 residues of Ki-Ras that we and others have previously reported will target heterologous proteins to the plasma membrane (24, 25). Upon addition of this Ras CAAX sequence to RaLGPS-PH, we rescued its ability to promote Ral-GTP accumulation in vivo (Fig. 3C), suggesting that the role of the PH domain was indeed membrane targeting. It has previously been established that Ras is responsible for the recruitment of RaLGDS family members to the plasma membrane and that this membrane targeting is essential for GEF activity (26, 27). The RA domain responsible for RaLGDS association with Ras-GTP (11, 12) is absent from the RaLGPS family and is presumably replaced in RaLGPS by the PH domain. We have not established whether the sequence difference between RaLGPS 1A and B in loop 3 of the PH domain affects their ligand specificity or function. However, the sequence of RaLGPS 1B includes a consensus phosphorylation site for the cyclic AMP-dependent protein kinase (KKVSI (28), underlined in Fig. 1C). Introduction of a negatively charged phosphate group at this site could inhibit binding of the PH domain to PIP2. Although full-length RaLGPS 1A was expressed poorly in 293T cells, RaLGPS 1A-PH-CAAX activated Ral as effectively as its 1B counterpart.

The RaLGPS 1 Proline-rich Motif Binds to Grb2 and Nck and Facilitates Adapter Protein-mediated Activation of Ral—As shown in Fig. 4A, the proline-rich motif present in RaLGPS 1A/B bears sequence homology with the SH3 binding motifs of the Grb2 and Nck adapter protein targets, Sos1 and PAK. In addition to the core PXPF motif, there are surrounding proline residues to form a left-handed P3 helix plus adjacent arginine residues that help orient the peptide and confer binding specificity (9). Indeed the proline-rich region in RaLGPS matches consensus sequences derived for Grb2 and Nck SH3 binding peptides by phage-displayed random peptide libraries (9, 14). To examine the ability of RaLGPS 1 to bind to SH3 domain-containing proteins in vitro, FLAG-tagged RaLGPS 1B was

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**Fig. 3.** RaLGPS 1 is a Ral GEF that requires membrane targeting to function in vivo. A, the indicated GST-Ras fusion proteins (20 µg) were stripped of nucleotide and used to precipitate FLAG-tagged RaLGPS 1A or B from 293T cell lysates. Bound GEFS were detected by Western blotting with M2 anti-FLAG antibody. B, empty vector or vectors encoding RaLGPS 1B, the Ra GEF, Rif, or the Ral1 GEF, C3G, were cotransfected into 293T cells with vectors encoding FLAG-tagged RalA or Ral1A as indicated. Following overnight serum starvation, cells were lysed, and RaLGTS and RapA-GTP and RapLA-GTP were extracted by incubation with the GSTase-binding domains of RaB1P and cellular Rap1, respectively. Stimulation of Rap1-GTP accumulation was determined by Western blotting with anti-FLAG antibody. Lower panels demonstrate equal GSTase transfection levels under each condition by Western blotting cell lysates for FLAG Ral or Rap1. C, 293T cells were transfected with vectors encoding FLAG-tagged Ral1 or Rap1. 

**Fig. 4.** The RaLGPS 1 PXPF motif is required for Grb2 and Nck SH3 domain binding and regulation. A, alignment of proline-rich motifs that bind to the N-terminal Grb2 or central Nck SH3 domains, B, the ability of FLAG-RaLGPS 1B to bind to SH3 domains in vitro was determined by incubating 293T cell lysates with equal amounts of glutathione-agarose bead-immobilized GST fusion proteins containing full-length Grb2, the SH3 domains of Nck, the isolated SH3 domains from p120 Ras GAP, Abl, Src, and phospholipase Cγ, or the N-terminal SH3 domains from Crk and p67phox. Beads were washed, and associated RaLGPS was detected by Western blotting. C, to determine whether RaLGPS could interact with SH3-containing adapter proteins in vivo, 293T cells were cotransfected with empty vector or vector encoding FLAG-RaLGPS 1B, HA-Grb2, or HA-Nck as indicated. Following immunoprecipitation with anti-HA antibody, associated adapter proteins were detected by blotting with anti-HA antibody. Lower panels demonstrate expression levels of RaLGPS, Grb2, and Nck in cell lysates. HC and LC indicate heavy and light chains, respectively, of the M2 antiFLAG antibody. D, RaLGPS 1PH is activated by Grb2. 293T cells were cotransfected with plasmids encoding FLAG-RalA, RaLGPS-ΔPH, and Grb2 as indicated. After serum starving overnight cell lysates were prepared, and RaLGTS-GTP was precipitated using GST-RalBIP (10 µg) and detected by Western blotting. All data are representative of at least three independent experiments.
expressed in 293T cells and cell lysates incubated with GST fusion proteins containing the SH3 domains of various proteins. As seen in Fig. 4B, RalGPS 1B was precipitated by SH3 domains from Grb2, Nck, Src, and PLCγ but not those of p120 Ras GAP, Crk, Abl, or p67 phox. To determine whether the interactions with adapter proteins might occur in vivo, 293T cells were co-transfected with plasmids encoding FLAG-tagged RalGPS 1B and HA-tagged Grb2 or Nck. Following lysis and precipitation with anti-FLAG antibody, both Grb2 and Nck were found to coprecipitate with RalGPS 1B (Fig. 4C).

It is well documented that Grb2 contributes to the recruitment of the Ras GEF, Sos1, to the plasma membrane, leading to Ras activation (2) and that the Sos PH domain also plays a role in this process (29). Association of Grb2 with Sos may also alleviate negative allosteric control imparted by the Sos C terminus (25, 30). Although over-expression of Grb2 did not enhance the ability of recombinant RalGPS to activate Ral in vivo (Fig. 4), we rationalized that the adapter protein/GEF interaction likely contributes to the physiological regulation of RalGPS. Because RalGPS did not activate Ral following removal of the GEF’s PH domain, we examined whether over-expression of Grb2 could negate the ability of this ΔPH mutant to activate the Ras pathway (27), but similarly to the Ras GEF, Sos1 (30), RalGPS 1 nucleotide exchange activity is regulated in vivo by a PH domain and a Grb2-binding PXPh motif. Although Ca²⁺ has been reported to activate Ral in a Ras-independent manner (31) there is no indication from their primary sequences that RalGPS 1A/B could respond to Ca²⁺ elevation. Why nature has created so many Ras GEFs and how they are differentially regulated will be the topics of future investigation.

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