Ral is a ubiquitously expressed Ras-like small GTPase. Several guanine nucleotide exchange factors for Ral have been identified, including members of the RalGDS family, which exhibit a Ras binding domain and are regulated by binding to RasGTP. Here we describe a novel type of RalGEF, RalGEF2. This guanine nucleotide exchange factor has a characteristic Cdc25-like catalytic domain at the N terminus and a pleckstrin homology (PH) domain at the C terminus. RalGEF2 is able to activate Ral both in vivo and in vitro. Deletion of the PH domain results in an increased cytoplasmic localization of the protein and a corresponding reduction in activity in vivo, suggesting that the PH domain functions as a membrane anchor necessary for optimal activity in vivo.

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MATERIALS AND METHODS

Plasmids and Constructs—The KIAA0351 cDNA containing the complete coding sequence was kindly provided by the Kazusa DNA Research Institute. This cDNA was isolated in a random cloning strategy (39). A polymerase chain reaction fragment containing RalGEF2 flanked by a SalI site at the 5′ (primer A: 5′-GTGCACTATGTGAAACAGGAAATGTGTCG-3′) and a HpaI site at the 3′ (primer B: 3′-CGTGTTGGAATACGATTTAACATCGAC-5′) end, was subcloned into the pGEM-T vector (Promega). This clone was subsequently used to generate HA-RalGEF2, by introducing a SalI fragment containing RalGEF2 into SalI-digested pMT2-SM-HA. Cat-RalGEF2 (amino acids 1–289), flanked by a SalI site at the 5′ end (primer A) and a HpaI site at the 3′ end (primer C: 3′-CGATCTTCTAGCTGTTGTTAACGTCAAT-5′), and APH-RalGEF2 (amino acids 1–431), flanked by a SalI site at the 5′ end (primer A) and a HpaI site at the 3′ end (primer D: 3′-CTTGACGGTGCTCAGATGATCAAT-5′), were also subcloned in pGEM-T. These SalI-HpaI fragments were also cloned into SalI-HpaI-digested pBluescript in which SalI-NotI Rfl-RBD-CAAX had been cloned previously to obtain RalGEF2 proteins with the C-terminal polybasic sequence and CAAX box of Ki-Ras (5). Subsequently, SalI-NotI fragments from these clones were isolated and ligated into SalI-NotI-digested pMT2-SM-HA to generate HA-cat-RalGEF2-CAAX (HA-cat-CAAX) and
HA-APH-RalGEF2-CAAX (HA-APH-CAAX). In addition, cat-RalGEF2 and ΔPH-RalGEF2 were also isolated as SalI-Not1 fragments from the pGEM-T constructs and cloned into SalI-Not1-digested pMT2-SHA vector, generating HA-cat-RalGEF2 (HA-cat) and HA-APH-RalGEF2 (HA-ΔPH). From HA-cat-RalGEF2, the mutants HA-catRalGEF2 was generated, raising against a synthetic peptide that consists of the amino acids 543–557 (KSNRPQVPANLMSFE) of RalGEF2. Other antibodies used were anti-HA (12CA5) (45), anti-Ral, anti-Ras, and anti-Rap (all three are anti-HA (12CA5) (45), anti-Ral, anti-Ras, and anti-Rap (all three are anti-HA (12CA5) (45), anti-Ral, anti-Ras, and anti-Rap (all three are anti-HA (12CA5) (45), anti-Ral, anti-Ras, and anti-Rap (all three are anti-HA (12CA5) (45), anti-Ral, anti-Ras, and anti-Rap (all three are anti-HA (12CA5) (45). Alternatively, we used a reverse assay (49) in which 150 nM RalGDP was used to perform immunoprecipitations.

\[ \text{HA-APH-RalGEF2-CAAX (HA-APH-CAAX).} \]

\[ \text{In vitro activation of small GTPases—In vitro GEF activity was measured as described (48). Briefly, 250 nM purified GTPase, loaded with fluorescently labeled GTP, was added to a cuvette containing 100 nM eluted protein. The eluted protein was dialyzed for 20 h in the same buffer without glutathione. The purification procedure of the small GTPases used in the in vitro experiments is described elsewhere (42–44).} \]

\[ \text{Immunoprecipitation and Western Blotting—Western blotting of all protein samples was performed using polyvinylidene difluoride membranes. The antisera used for detecting endogenous RalGEF2 was raised against a synthetic peptide that consists of the amino acids 543–557 (KSNRPQVPANLMSFE) of RalGEF2. Other antibodies used were anti-HA (12CA5) (45), anti-Ral, anti-Ras, and anti-Rap (all three from Transduction Laboratories). Anti-RalGEF2 and 12CA5 were also used to perform immunoprecipitations.} \]

\[ \text{In Vivo Activation of small GTPases—Cells were transiently transfected with HA-tagged versions of the small GTPases either alone or in combination with RalGEF2, and serum-starved for 20 h prior to lysis. GTP-bound forms of the different GTPases were isolated using activation-specific probes and subsequently quantified, as described (13, 46, 47). For RalGTP the Ral-binding domain of RIP2 was used, for Ras the RBD of RalF1, and for Rap the RBD of RalGDS.} \]

\[ \text{In Vitro Activation of small GTPases—In vitro GEF activity was measured as described (48). Briefly, 250 nM purified GTPase, loaded with fluorescently labeled GTP, was added to a cuvette containing 100 nM eluted protein. The eluted protein was dialyzed for 20 h in the same buffer without glutathione. The purification procedure of the small GTPases used in the in vitro experiments is described elsewhere (42–44).} \]
the presence of 50 μM purified GEF protein. Uptake of mantGDP results in an increase in fluorescence.

Subcellular Fractionation—Cells were harvested in lysis buffer (20 mM Hepes, pH 7.4, 4 mM EDTA, 1 mM sodium vanadate, 1 μM leupeptin, 0.1 mM aprotinin) and subsequently homogenized through a 23-gauge 1.25 M Microlance syringe. Intact cells and nuclear components were removed by a sequential two-step centrifugation at 6000 rpm for 1 min (Eppendorf table centrifuge). Subsequently, the samples were centrifuged at 100,000 × g at 4 °C for 90 min. The supernatant was collected as the soluble fraction, and the particulate fraction was dissolved in buffer containing 1% Triton X-100, 50 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM vanadate, 1 μM leupeptin, and 0.1 mM aprotinin. Soluble and particulate fractions were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

RESULTS

A Novel Ral Guanine Nucleotide Exchange Factor—In our ongoing search for regulators of Ras family members, we found a protein in the data base from the Kazusa DNA Research Institute (KIAA0351) with similarities to RasGEFs. A schematic representation of the non-coding and coding sequences of KIAA0351 is presented in Fig. 1A. As shown below, this protein is a GEF for the small GTPase Ral; hence, we named it RalGEF2, since this GEF forms a novel subclass, distinct from the RalGEF members, which are characterized by the presence of the RBD domain (Fig. 1B). RalGEF2 has the characteristic GEF domain present in all RasGEFs and shows considerable homology to previously described RasGEFs, especially in the structurally conserved regions scr1, scr2, and scr3 (data not shown). In addition, RaIGEF2 has a PH domain in its C terminus that shows highest homology to the PH domain in the Drosophila RhGEF Still Life (Sif) (50), and the N-terminal PH domains in the RacGEFs Tiam1 and Stef (51, 52) (Fig. 1C). Surprisingly, no Ras exchange motif (REM) is present in RaIGEF2, in contrast to all other Cdc25-like GEFs identified so far.

Expression of RaIGEF2—A polyclonal antibody (α-RaIGEF2) was raised against a C-terminal peptide of RaIGEF2. This antibody recognized the 60-kDa HA-RaIGEF2 protein immunoprecipitated from Cos7 cell lysate transiently transfected with HA-RaIGEF2 using an α-HA monoclonal antibody (Fig. 2). A similar-sized protein was identified in an α-RaIGEF2 immunoprecipitate from 293 cells. Both protein bands disappeared when the antibody was preincubated with the immunizing peptide. From this result, we concluded that α-RaIGEF2 recognizes RaIGEF2 both in immunoprecipitation and blotting experiments. Next, a Western blot containing protein samples from various human tissues was probed with either α-RaIGEF2 or α-RaIGEF2 blocked with peptide. As shown in Fig. 2B, RaIGEF2 expression is rather ubiquitous. It is high in brain, heart, kidney, adrenal gland, and colon; low in pancreas, skeletal muscle, thymus, and liver; and intermediate in lung and spleen. Ubiquitous expression of RaIGEF2 was also shown using reverse transcription-polymerase chain reaction by the Kazusa DNA Research Institute.

RaIGEF2 Activates Ral Both in Vivo and in Vitro—To investigate which Ras-like GTPase is activated by RaIGEF2, we incubated GST-cat (Fig. 3A) with various Ras-like GTPases loaded with fluorescent mantGDP. Release of guanine nucleotide bound to Ral was measured in real time as decrease in fluorescence. As shown in Fig. 3B, incubation of RaIGEF2 alone already resulted in a decrease in fluorescence, indicating that a fraction of the protein sample was unstable and degraded during the incubation. In the presence of GDP, intrinsic exchange activity is measured. We observed a clear increase in exchange when GST-cat was added as well, showing that GST-cat catalyzes Ral guanine nucleotide exchange. GST-cat did not affect the exchange rate of either Ras or Rap2 (Fig. 3C). As an alternative assay, we used a reverse procedure in which binding of mantGDP to Ral results in an increase in fluorescence.

![Fig. 2. Expression of RaIGEF2 in human tissue. A, HA-RaIGEF2 was precipitated from lysate of Cos7 cells transiently transfected with HA-RaIGEF2 using anti-HA (12CA5) monoclonal antibody (lane 1), and endogenous RaIGEF2 was precipitated from lysate of 293 cells using a polyclonal peptide antibody of RaIGEF2 (α-RaIGEF2; lane 2). Blots were probed with either α-RaIGEF2 (left panel) or with α-RaIGEF2 preincubated with the peptide used for immunization (right panel). H-chain, immunoglobulin heavy chain. B, newborn human tissue samples were lysed and equal amounts of protein were separated by gel electrophoresis. Gels were blotted and probed with either α-RaIGEF2 (upper panel) or with α-RaIGEF2 preincubated with the peptide used for immunization (lower panel). The upper panel, arrows indicate RaIGEF2; in the lower panel, arrows indicate where RaIGEF2 should appear but is blocked. Lanes represent pancreas (p), skeletal muscle (sk), brain (b), skin (s), thymus (t), heart (h), kidney (k), liver (l), spleen (sp), lung (lu), adrenal gland (ag), and colon (c).](image-url)
Regulatory Function of the PH Domain—PH domains commonly interact with membrane lipids, in particular phospho-
rylated phosphatidylinositol lipids. As such, PH domains function either as membrane anchor or as a regulatory domain that
responds to the products of PI3K, phosphatidylinositol 3,4-
bisphosphate, or phosphatidylinositol 3,4,5-trisphosphate. To
determine whether RalGEF2 responds to PI3K signaling, we
introduced RalGEF2 in A14 cells and stimulated the cells with
insulin, a potent inducer of PI3K activity (45). However, we
did not observe any increase in RalGEF2 activity by insulin treat-
ment (data not shown). Additionally, other stimuli tested, in-
cluding epidermal growth factor, endothelin, forskolin, ionomycin,
lysophosphatidic acid, and serum, failed to activate
RalGEF2. We therefore investigated whether the PH domain is
involved in membrane localization of RalGEF2. Cells were
transfected with HA-RalGEF2 or mutants lacking the PH do-
main (HA-ΔPH and HA-cat), and the presence of these proteins
in the cytosol and the membrane-enriched particulate fraction
was determined. Deletion of the PH domain resulted in a clear
reduction of the level of RalGEF2 in the membrane fraction
(Fig. 5A), indicating that the PH domain is involved in mem-
brane localization of RalGEF2. This reduced association to
membranes could be restored by adding the C-terminal poly-
basic domain and CAAX sequence of Ki-Ras to HA-ΔPH and
HA-cat (HA-ΔPH-CAAX and HA-cat-CAAX). This region di-
rects the addition of a C-terminal isoprenyl group and as a con-
sequence membrane attachment (Fig. 5A).

Next, we investigated whether deletion of the PH domain
also affects GEF activity in vitro. Cells were cotransfected with
the various RalGEF2 constructs and HA-Ral, and the level of
RalGTP was determined. Even at lower levels of expression
(Fig. 5B, upper panel), full-length RalGEF2 is much more ef-
cient in activating Ral than the mutant lacking the PH domain
(Fig. 5B), showing that the PH domain is required for efficient
RalGEF2 activity. The reduced GEF activity of HA-ΔPH can be
restored by the addition of a membrane anchor (HA-ΔPH-
PH and HA-cat), and the presence of these proteins
in the cytosol and the membrane-enriched particulate fraction
was determined. Deletion of the PH domain resulted in a clear
reduction of the level of RalGEF2 in the membrane fraction
(Fig. 5A), indicating that the PH domain is involved in mem-
brane localization of RalGEF2. This reduced association to
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basic domain and CAAX sequence of Ki-Ras to HA-ΔPH and
HA-cat (HA-ΔPH-CAAX and HA-cat-CAAX). This region di-
rects the addition of a C-terminal isoprenyl group and as a con-
sequence membrane attachment (Fig. 5A).

Fig. 3. RalGEF2 specifically activates Ral in vitro. A, Coomassie
Blue-stained gel, which demonstrates the purification result of a fusion
protein containing GST and the catalytic domain of RalGEF2 (GST-cat;
residues 1–289). M stands for marker. B, 250 nM human RalB loaded
with fluorescent mantGDP was incubated either alone to measure
stability of the protein (1, open circles), with 5 mM GDP to measure the
intrinsic release (2, closed circles) or with 5 mM GDP and 50 mM GST-cat
(3, open squares). The right panel is a magnification of the initial part
of the reaction in the left panel, to clearly show the rate differences.
C, 250 nM Ha-Ras.mantGDP (left panel) or 250 nM Rap2.mantGDP (right
panel) was incubated with either 5 mM GDP (1, open circles) or 5 mM
GDP and 100 nM GST-cat (2, closed circles). D, 100 nM mantGDP was
incubated either alone (1, open circles), in the presence of 50 (2, closed
circles) and 100 (3, open squares) nM GST-cat, or Rif (4, closed squares).
At the time indicated (arrow), 150 nM simian Ral loaded with GDP was
added. Two different RalA proteins were used, one containing residues
1–206 (left panel) and one containing residues 1–178 (right panel). E,
150 nM simian RalGDP (residues 1–178) was incubated with 100 nM
mantGDP either alone (1, open circles), or in the presence of 50 nM
GST-cat (2, closed circles) or 100 nM GST-cat (3, open squares) for a pro-
longed period of time. Subsequently, 50 nM Rif was added (arrow)
and incubation continued.
domain is most similar to PH domains present in the Rho/RacGEFs Tiam1, SIF, and Drosophila Still life. PH domains may bind to phosphatidylinositol lipids, which are either constitutively present or which are induced by certain stimuli. For instance, the product of PI3K, phosphatidylinositol 3,4-bisphosphate, recruits target proteins to the membrane by binding to their PH domains (53, 54) Via this relocalization, a protein is for instance either brought into the vicinity of its target or of its activators. However, the fact that insulin, a very strong inducer of PI3K, fails to activate RalGEF2 suggests that RalGEF2 activity is not induced by these lipids. Thus, the PH domain may serve to constitutively anchor RalGEF2 to membranes. Indeed, deletion of the PH domain results in an increased cytoplasmic localization of the protein and a corresponding reduction in the efficiency of Ral activation in vivo. Both membrane localization and efficient GEF activity could be restored by the addition of the C-terminal polybasic region and CAAX motif of Ki-Ras, which targets proteins to the membranes.
From these results, we conclude that the PH domain is predominantly responsible for membrane localization of RalGEF2.

Our failure to induce the activity of RalGEF2 by external stimuli may indicate that RalGEF2 is a constitutively active GEF, or that we have not yet identified the proper stimulus. Whereas Ral clearly serves as a downstream target of Ras signaling, through the direct binding of members of RalGDS, Rgl and/or Rif to activated Ras, it is clear from several studies that other pathways are also mediating Ral activation. For instance, calcium can activate Ral independently of Ras (13, 18), and in neurophilts as yet unidentified pathways exist (15). RalGEF2 may mediate one of these pathways.

RalGEF2 is distinct from the other RalGEFs in that it does not have a REM domain. This REM domain is thought to play a role in stabilizing the catalytic domain of Ras-like GEFs. Perhaps the PH domain or another region serves a similar function. An additional striking difference between RalGEF2 and other RalGEFs, in particular Rif, is that RalGEF2 does not exchange nucleotide in vitro from a truncated version of Ral. Apparently the C-terminal region of Ral contains amino acids essential for the proper binding of the catalytic domain of RalGEF2. Whether RalGEF2 directly interacts with this region, or if the absence of the REM domain is responsible for this difference awaits further investigation. However, this result clearly indicates that RalGEF2 uses a different molecular mechanism to release GDP from Ral than Rif does.

Ral has been implicated in a variety of cellular processes. Most notable is the role of Ral in coupling signals from Ras to the induction of transcription, such as transcription from serum response elements (5, 55) and the inhibition of the transcription factor AFX (56). These effects may, at least in part, explain the effects of Ral-mediated signaling on cell proliferation (1–7) and differentiation (8–10). However, the mechanism by which Ral regulates transcription is unclear. Another function of Ral is the regulation of the cytoskeleton. This is indicated by the association of the active form of Ral with RalBP, a protein involved in the cross-linking of actin filaments. Indeed, Ral activates the RalBP transcript factor AFX (56). These effects may, at least in part, explain the effects of Ral-mediated signaling on cell proliferation.