Identification of a Novel RalGDS-related Protein as a Candidate Effector for Ras and Rap1*

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Although Ras and Rap1 share interaction with common candidate effector proteins, Rap1 lacks the transforming activity exhibited by Ras proteins. It has been speculated that Rap antagonizes Ras transformation through the formation of nonproductive complexes with critical Ras effector targets. To understand further the distinct biological functions of these two closely related proteins, we searched for Rap1-binding proteins by yeast two-hybrid screening. We identified multiple clones that encode the COOH-terminal sequences of a protein that shares sequence identity with RaLGDS and RGL, which we have designated RGL2. A 158-amino acid COOH-terminal fragment of RGL2 (RGL2 C-158) bound to Ras superfamily proteins which shared identical effector domain sequences with Rap1 (Ha-Ras, R-Ras, and TC21). RGL2 C-158 binding was impaired by effector domain mutations in Rap1 and Ha-Ras. Furthermore, RGL2 C-158 bound exclusively to the GTP-, but not the GDP-bound form of Ha-Ras. Finally, coexpression of RGL2 C-158 impaired oncogenic Ras activation of transcription from a Ras-responsive promoter element and focus-forming activity in NIH 3T3 cells. We conclude that RGL2 may be an effector for Ras and/or Rap proteins.

Ras function is crucial for cell growth and differentiation (1). Ras, as a signaling molecule, is an allosteric switch that cycles between an active GTP-bound conformation and an inactive GDP-bound form. The nucleotide bound state of Ras is regulated by Ras guanine nucleotide exchange factors (GEFs;1 SOS and RasGRF), which activate Ras by promoting an exchange of GDP for GTP. Conversely, Ras GTPase-activating proteins (GAPs; p120- and nuclear factor-1 GAP) inactivate Ras by stimulating the hydrolysis of GTP to GDP (2). Activating mutations of Ras (positions 12, 13, and 61) render Ras insensitive to GAP-stimulated GTPase activity (3). Ras interacts with and participates in Raf-1 activation. Activation of Raf-1 kinase activity results in the phosphorylation of the mitogen-activated protein kinase (MAPK) kinase MEK, which in turn phosphorylates the p42 and p44 MAPKs ERK-1 and ERK-2 (4). Activated MAPKs translocate to the nucleus to regulate the activity of Elk-1 and other nuclear targets. Active mutants of Ras cause constitutive activation of the MAPK cascade and uncontrolled cell growth.

Although Raf-1 has been demonstrated to be a critical downstream effector of Ras function, there is increasing evidence that Ras may mediate its actions through activation of multiple downstream effector-mediated pathways (5). First, there is an increasing number of proteins that bind Ras and are candidate Ras effectors, suggesting that Ras activates both Raf-dependent and Raf-independent pathways. Like Raf-1, these functionally diverse proteins show preferential binding to the active GTP-bound form of Ras, and this interaction requires an intact Ras effector domain (amino acids 32–40). Included among these are the two Ras GAPs, phosphatidylinositol-3-OH kinase, GEFs for the Raf family of Ras-related proteins (RaLGDS and RGL), Rin-1, MEKK1, AF-6 and others (2, 6–13). Second, observations from genetic, biochemical, and biological studies suggest that Ras triggers the activation of members of the Rho family of Ras-related proteins and that Rho protein function is necessary for full Ras transformation (14–17). At present, the effector that connects Ras with Rho proteins is not known. Third, the recent observation that Raf-binding defective mutants of oncogenic Ras can still cause tumorigenic transformation supports the contribution of Raf-independent pathways to Ras transforming activity (18). Taken together, these observations support the importance of establishing the role of non-Raf effector targets in mediating Ras signaling and transformation.

Among Ras-related proteins, the closest relatives of Ras proteins are R-Ras, TC21/R-Ras2, and Rap proteins (Rap1a and b and Rap2a and b). Interestingly, Ras proteins, TC21, and R-Ras have transforming capacity (19–22), whereas Rap proteins do not (23). In fact, Rap1a (Krev-1) was originally isolated based upon its ability to revert Ras-transformed NIH 3T3 cells to a normal flat phenotype (24). In addition to sharing over 50% sequence identity, Ras and Rap have identical sequences.

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The first two authors contributed equally to this work.

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The abbreviations used are: GEF(s), guanine nucleotide exchange factor(s); GRF, guanine nucleotide-releasing factor; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; GDS, guanine nucleotide dissociation stimulator; PCR, polymerase chain reaction; RBD, Ras/Rap binding domain; GST, glutathione S-transferase.

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within their respective effector domains (amino acids 32–40) (25, 26). A role for Rap1 as a Ras antagonist has been suggested in several systems (27–32). The sequence identity shared between the effector domains of Ras and Rap1 taken together with the ability of Rap1 to interact with Ras binding proteins, such as Ras GAP (33, 34) and Raf-1 kinase (35), has led to the speculation that Rap1 may antagonize Ras function through an interaction, in a nonfunctional manner, with Ras effector molecules. Direct support for this model has not yet been demonstrated.

As a step toward elucidating the functional basis for the divergent biological activities of Ras and Rap proteins, we used the yeast two-hybrid system to identify potential Rap1 effector molecules (36, 37). We identified clones encoding a new member of a growing family of candidate Ral GEFs which we have termed RGL2. We observed that the COOH-terminal fragment of RGL2 also bound to Ras and other Ras-related proteins which share identical effector domain sequences (R-Ras and TC21) and that binding to Rap and Ras was abolished by mutating the Sh3 domain. Hence, like Rap, RGL2 exhibits properties as an effector for Ras and Rap function. Finally, coexpression of the Ras-binding domain of RGL2 blocked oncogenic Ras signaling and transformation. We suggest that RGL2 may represent an important effector for Ras function and that Rap interaction with RGL2 may contribute to Rap antagonism of Ras function.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals—**pGBT9 (TRP1, amp’), containing the GAL4 DNA binding domain, pGAD10 (LEU2, amp’), containing a human placental oligo(dT)-primed cDNA library fused to the GAL4 transactivation activation domain, and the Saccharomyces cerevisiae strain HF7c were from Clontech (Palo Alto, CA). The vector pGEX9-carrying cDNA sequences encoding wild type and T35A and G12V;C186G mutant Ha-Ras proteins were provided by Linda van Aelst and Michael Wigler (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). [γ-32P]GTP (30 Ci/mmol) and [γ-32P]dCTP (3000 Ci/mmol) were from ICN Pharmaceuticals Inc. (Costa Mesa, CA). [3H]GDP and [3H]GTP (11.1 Ci/mmol) were from Amersham Corp. Sequenase was from U.S. Biochemical Corp. AmpliTaq was from Perkin-Elmer. 5-Bromo-4-chloro-3-indolyl β-D-galactoside was from the Biocolor Corp. Plasmid Constructions—Constrcuts encoding wild type Rap1b, Rhl, Rac1, Rabα, R-ras, and TC21 were made by PCR using primers that created an EcoRI site at the 5’ and a BamHI site at the 3’ end of the cDNA. cDNA sequences encoding G12V, G12V, C181G, S17N, E37G, and T35A mutations were made by PCR and cloned using the same restriction sites. This was amplified and cloned as a BamHI fragment. All of these fragments were cloned into the vector pGEX3. The COOH-terminal 158 amino acids of RGL2 was used to generate a GST fusion gene by digesting the vector pGAD10 carrying C-158 cDNA with EcoRI. The cDNA insert was subsequently ligated into EcoRI-digested pGEX-4T (Pharmacia Biotech Inc.). RGL2 RBD was constructed by PCR amplification of sequences encoding residues 1–98 (relative to C-158) with primers that created a 5’ BamHI and 3’ EcoRI site. PCR products were digested with BamHI and EcoRI and cloned into the BamHI site of the eukaryotic expression vector pCGN hygro (38). All clones created by PCR were sequenced to ensure that undesired mutations did not exist.

**Two-hybrid Screening**—The yeast strain HF7c carrying the Rap1b GAL4 DNA binding domain fusion was transformed with pGAD10 containing a human placenta cDNA library. Transformsants capable of forming large colonies after 4 days in the presence of 5 mg 3-aminotriazole were tested for β-galactosidase activity. The vector pGAD10 carrying potential positive interacting cDNAs were rescued from yeast cells and used to transform Escherichia coli strain HB101. Those cDNAs that exhibited a Rap1-dependent β-galactosidase-positive phenotype upon retransformation were characterized further.

cDNA Cloning and Sequencing—cDNA libraries (Stratagene, La Jolla, CA) from human placenta (Uni-ZAP XR), skeletal muscle (λZAP II), heart (AZAP II), and testis (ZAP Express XR) were screened. In each case, 5.0 × 106 plaques were plated on E. coli strain XL-1 Blue MRF’. The largest cDNA from the two-hybrid screening was used to make a probe using the random priming procedure (Life Technologies, Inc.). Plasmids were generated from positive plaques using the helper phage Exassist (Stratagene). The 5’ most 500 nucleotides of the largest cDNA insert from the skeletal muscle library were used to resequence each of the libraries. The largest clones were approximately 2 kilometers in length and were sequenced using an ABI 373A automated sequencer (Applied Biosystems). Specific oligonucleotide primers were purchased from Oligo Therapeutics (Newtown, CT).

Two-hybrid Analysis of RGL2 C-158 Interaction with Small GTP-Binding Proteins—The yeast strain HF7c was cotransformed with RGL2 C-158 and wild type or mutated small GTP-binding proteins. Transformsants were selected on minimal medium lacking tryptophan and leucine. Colonies were then streaked on plates lacking tryptophan, leucine, and histidine to assess for growth and β-galactosidase activity.

**In Vitro Interaction of RGL2 C-158 with Rap1b and Ha-Ras**—Rap1b and Ha-Ras were loaded as follows. Approximately 10 pmol of Rap1b in 32 mm Tris-HCl, pH 7.5, 200 mM NH4SO4, 0.5 mM EDTA, 10 mM dithiothreitol, 100–200 mM GTP, and 60 μCi [γ-32P]GTP (30 Ci/mmol) were incubated for 3 h at room temperature. MgCl2 was then added to a final concentration of 5 mM. Similar amounts of Ha-Ras in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM dithiothreitol, 100–200 mM GTP, 60 μCi [γ-32P]GTP (30 Ci/mmol), and 5 mM EDTA were incubated for 5–10 min at room temperature. MgCl2 was then added to a final concentration of 10 mM. Reactions were run on a Sephadex G-50 column (equilibrated in 50 mM Tris-HCl, pH 7.5, 1 mg/m1 polyethylene glycol 20,000, 10 mM MgCl2) to separate unbound GTP from GTP-bound Rap1b or Ha-Ras. Typical loading efficiencies were 80% for Rap1b and 50% for Ha-Ras.

For the binding studies 8 pmol of RGL2 C-158 GST fusion or GST alone was bound to 50 μl of a 25% slurry of glutathione-Sepharose in 400 μl of binding buffer (1% Nonidet P-40, 20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mg/ml bovine serum albumin) at 4 °C for 20 min. After several washes with binding buffer, Rap1b or Ha-Ras loaded with [γ-32P]GTP was added and incubated with agitation for 60 min at 4 °C. Binding reactions were performed in a final volume of 250 μl. Reactions were washed eight times with 400 μl of binding buffer, and the bound nucleotide was quantified by liquid scintillation counting. For each quantity of Rap1b or Ha-Ras used, specific binding was determined by subtracting counts bound to GST alone from counts bound by RGL2 GST fusion protein.

**In vitro interaction of RGL2 RBD with Ras-GDP and Rap-GTP**—was determined by binding experiments as described above. RGL2 GST fusion protein (8 pmol) or an equivalent amount of GST alone was combined with various amounts of Ha-Ras loaded with either [3H]GDP or [3H]GTP (11 Ci/mmol).

NIH 3T3 Transcription Activation and Transformation Assays—NIH 3T3 cells were grown and transfected as described previously (39). The pZIP-Raf-N4-Luc reporter plasmid contains the cDNA for Raf-1 driven by a minimal fos promoter containing two tandem copies of the ets/AP-1 Ras-responsive promoter element from the polyomavirus enhancer (40). Cells were cotransfected with the pZIP-ras/H12 pCIS plasmid DNA encoding Ha-Ras (H12) (100 ng/60-mm dish) and 5 μg of either empty pCIS-hygro vector, pCIS-ras-N4 (encoding c-Raf-1 residues 23–284) (38), or pCIS-RGL2 RBD alone with 1 μg of the (Py52)/Lac reporter. Forty-eight h after transfection, total cell lysates were prepared, and luciferase activity was determined as described (41). A similar cotransfection was performed to assess the abilities of Raf-N4 and RGL2 RBD (5 μg/dish) to block Ras (Q61L) (10 ng/60-mm dish) transforming/focus-forming activity. Transfections were performed in duplicate, and transformed foci were quantitated after 14–16 days.

**RESULTS**

**Identification of RapGDS-related Protein as a Rap1b-interacting Protein**—Although Rap and Rap share identical effector domain sequences and interact with common candidate effector proteins, they exhibit distinct signaling and biological activities. To understand further the relationship between Ras and Rap1 signaling, we searched for Rap1b effectors using yeast two-hybrid library screening. The yeast strain HF7c was cotransformed with a plasmid containing the GAL4 DNA binding domain fused to Rap1b (pGBT9-Rap) and a plasmid containing the GAL4 transcriptional activation domain fused to cDNAs from human placental and testis cDNA libraries (pGAD10). From a final collection of 4.8 × 106 transformsants, we identified 238 His+ colonies. Ninety-six of the 238 His+ colonies were positive for β-galactosidase activity, and 48 possessed Rap1b-dependent β-galacto-
sidase activity. We sequenced the 5’ end of 23 of these clones and found that they contained different size fragments of the same gene. The smallest cDNA encoded the COOH-terminal 151 amino acids of the protein and defined this region as sufficient for Rap1b-specific binding. The largest clone obtained from the two hybrid screen was approximately 1.1 kilobases in length. Although the largest clones obtained after screening four libraries were approximately 2 kilobases in length, Northern blot analysis indicated that the transcript is approximately 3 kilobases in length (data not shown) indicating that these are not full-length clones. Further attempts to obtain full-length cDNA have been unsuccessful. The sequence of the partial cDNA was determined (Fig. 1A).

A comparison of the partial cDNA sequence with those in the GenEMBL data base revealed a high degree of amino acid sequence identity/similarity to RalGDS (42%/61%) and RGL (40%/61%). Based on this similarity we have named this protein RGL2 (Ral GDS like 2). Like RalGDS and RGL, the NH2 terminus of RGL2 contains a sequence with significant similarity to the catalytic domain of the yeast protein CDC25, which functions as a Ras GEF (8–10, 42). Among the proteins bearing CDC25 domains, the highest degree of similarity (62%) exists with RalGDS and RGL, whereas RGL2 possessed only 50% similarity when compared with other CDC25-related domains present in the mammalian Ras exchange factors Ras-GRF, SOS1, or the Rap1 exchange factor C3G (43–45). High levels of sequence identity were also seen in the COOH-terminal portion of RGL2 when compared with RalGDS and RGL (49 and 47%, respectively) (Fig. 1B). This sequence represents the RBD of RalGDS and RGL. RGL2 did not show any obvious sequence identity with any other known RBDs. Thus, like RalGDS and RGL, RGL2 is composed of an NH2-terminal CDC25 homology and a COOH-terminal RBD.

RGL2 Exhibits Properties of an Effector Target for Rap1 and Ras—RalGDS shows preferential interaction with Ras-GTP and requires an intact effector domain (8–10). Thus, RalGDS exhibits properties of a candidate Ras effector target. To determine if RGL2 also possessed properties of an effector protein, RGL2 C-158 was tested with various wild type and mutated GTPases. Wild type and constitutively activated (G12V) mutants of Rap1b and Ras interacted with very similar affinities in the semiquantitative β-galactosidase filter assay (Table I). The lack of an endogenous yeast GAP activity for Ras and Rap may explain why activating mutations do not result in higher interaction with RGL2 C-158.

As shown in Table I, effector domain mutants Rap1b T35A and Ras T35A were unable to interact with RGL2 C-158. These results suggest that, like Raf-1, RGL2 binding requires an intact effector domain. Interestingly, a second effector domain mutation, Rap1b E57G, did interact with RGL2 C-158 with nearly wild type affinities. Since the E57G mutation in Ras inhibited its ability to interact with Rap1b, but not RalGDS, Raf-1 and RGL2 are predicted to have overlapping but distinct structural requirements for binding (18). Rap1b S17N, which by analogy to the Ras S17N dominant negative mutation is thought to have reduced affinity for GTP, showed no detectable interaction with RGL2 C-158, suggesting that RGL2 C-158 showed preferential binding to the active GTP-bound form of Rap1b. Mutant versions of Rap1b and Ras, with mutations in the cysteine residues of their COOH-terminal CAAX prenylation signal sequence (Rap1b G12V;C181G and Ras G12V;C186G) retained strong binding. Thus, CAAX-dependent, post-translational modifications (e.g. prenylation) are not required for RGL2 binding.

The fact that RGL2 interacted with both Ras and Rap1 and that the interaction required the effector domain prompted us to establish the specificity of the interaction between RGL2 and other Ras-related GTPases. Although both Ras, Rap1, R-Ras, and TC21/R-Ras2 share complete sequence identity within their respective effector domains, R-Ras and TC21 interacted with RGL2 C-158 to a lesser extent than did wild type and activated forms of Ha-Ras and Rap1b. RGL2 C-158 displayed some affinity for RalA and Rac1, although these two proteins diverge significantly within their effector domain sequence compared with Ras. Finally, we were unable to detect any interaction of RGL2 C-158 with RhoA, Rab3a, or Rheb under the same assay conditions (Table I). Thus, RGL2 C-158 showed strong preferential binding to only Ras and Rap1b.
RGL2 interacts with Ras and focuses transformation in NIH3T3 assays (80 and 50%, respectively) (Fig. 3). These observations suggest that coexpression of the RGL2 RBD with Ras causes a significant reduction in oncogenic Ras-induced activation of transcription from the ets/AP-1 Ras-responsive promoter element and focus formation in NIH 3T3 transformation assays (80 and 50%, respectively) (Fig. 3). These observations suggest that RGL2 may interact with Ras in vivo.

**DISCUSSION**

It is becoming increasingly apparent that Ras-dependent cellular transformation is mediated by interactions with multiple effector proteins (5–13). Although Ras and Rap share interaction with common candidate effector targets (e.g. Raf-1, RalGDS), they exhibit divergent signaling and biological activities. Whereas constitutively activated mutants of Ras cause cellular transformation, analogous mutants of Rap1a antagonize Ras signaling and transformation (23, 24, 27–34). Therefore, identifying common and distinct effectors that interact with these two closely related proteins may provide a critical clue to establish the mechanism by which Rap antagonizes Ras and to define the distinct signaling pathways controlled by Rap proteins. In the present study, we used yeast two-hybrid library screening analysis and identified RGL2 as a candidate effector for Ras and Rap. RGL2 constitutes the third member of a family of proteins that are candidate GEFs for the Raf family of Ras-related GTPases. Like Raf-1, the interaction of the Ras/Rap binding domain of RGL2 (RGL2 C-158) with Ras and Rap requires an intact effector domain. Also like Raf-1, RGL2 (RGL2 C-158) was able to block oncogenic Ras signaling and transformation. However, whereas Raf-1 showed preferential binding to the active, GTP-bound forms of Ras and Rap, RGL2 C-158 showed exclusive binding to GTP-Ras. These findings support the possibility that RGL2 interacts with Ras in mammalian cells. Thus RGL2 interaction with Rap may represent a mechanism for Rap inhibition of Ras.

Recent evidence indicates that Ras interaction with non-Raf

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**A New Effector for Rap1 or Ras**

**TABLE I**

| Clone         | β-Galactosidase activitya |
|---------------|--------------------------|
| Rap1b         | ++                       |
| Rap1b G12V    | ++                       |
| Rap1b G12V;C181G | ++                  |
| Rap1b S17N   | –                        |
| Rap1b T35A   | –                        |
| Rap1b E37G   | +                        |
| Ha-Ras       | +                        |
| Ha-Ras T35A  | +                        |
| Ha-Ras G12V;C186G | ++                |
| Rac1         | +                        |
| R-Ras        | +                        |
| TC21         | +                        |
| RalA         | +                        |
| Rhos         | –                        |
| Rab3a        | –                        |
| Rheb         | –                        |

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a The yeast strain HF7c was cotransformed with C-158 and Rap1b or other GTPases. After 3 days of growth, colonies were assayed for β-galactosidase activity. ++ indicates strong activity; + indicates weak, but detectable, activity; – indicates no activity. The results were obtained at least two times in independent experiments.
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Effector targets contribute significantly to Ras transforming activity. For example, analysis of two Ras effector domain mutants that fail to bind to and activate Raf showed that these mutants retained strong tumorigenic transforming activity (18). In the present study, we show that one of these mutants, Ras G12V,E37G, retained the capacity to bind to RGL2, indicating that RGL2 could serve as a critical effector for mediating the transforming action of this mutant Ras protein. Consistent with this, we have observed that coexpression of RalGDS synergistically enhanced the transforming activity of an effector domain mutant that had lost RafGDS binding (Ras G12V,T35S).2

Another possible role for RGL2 might be as an effector mediating signaling from R-Ras and/or TC21. Like Ras, constitutively activated mutants of TC21 and R-Ras cause potent tumorigenic transformation of NIH 3T3 cells (19–22). However, neither TC21 nor R-Ras causes the same up-regulation of Raf-1 or B-Raf kinase activity seen with Ras transformation of NIH 3T3 cells.3 Thus, these two Ras-related proteins utilize Ras-independent signaling pathways to cause transformation. We found that RGL2 C158 also bound to TC21 and R-Ras, although this interaction was weaker than that seen with Ras or Rap. Similarly, it has been reported that RafGDS also interacts with TC21 and R-Ras (9, 48). Thus, these candidate Raf GEFs may represent the key effectors for mediating the transforming actions of TC21 and R-Ras.

Full morphological transformation induced by Ras is dependent upon the activities of the Ras-related GTPases Rac1, RhoA and Rap (16, 17, 49). Recent data support a role for RafGDS as a functional Ras effector (49, 50). However, the effectors coupling Ras to Rac1/RhoA have not yet been identified. The two-domain structure of the members of the RafGDS family makes them ideal candidates for coupling Ras or Rap activity with that of other GTPase-mediated signaling cascades. Since the RGL2 CDC25-related domain shares the strongest sequence similarity with RafGDS, it is possible that RGL2 also functions as a GEF for Raf or Rap-related proteins. Therefore, Ras or Rap binding to RGL2 may stimulate this activity, which in turn may result in the subsequent activation of other low molecular weight GTPases. Future studies will be aimed at determining whether RGL2 functions in an overlapping but distinct manner as RafGDS or if instead it couples active Ras to other Rap-related GTPases.

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