Characterization of Raf GDP Dissociation Stimulator-like (RGL) Activities to Regulate c-fos Promoter and the GDP/GTP Exchange of Raf*

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Raf GDP dissociation stimulator-like (RGL) has been identified to be a possible effector protein of Ras. RGL shares 50% amino acid identity with Raf GDP dissociation stimulator and contains the CDC25-like domain in the central region and the Ras-interacting domain in the C-terminal region. Since the modes of activation and action of RGL have not yet been clarified, in this paper we have analyzed the function of RGL. In COS cells, RGL interacted with RasG12V/E37G (a Ras mutant in which Gly-12 and Glu-37 were changed to Val and Gly, respectively) without binding to Raf, but with RasG12V/Y40C which bound to Raf. Raf did not inhibit the binding of RGL to RasG12V/E37G under the condition that Raf inhibited that of RGL to RasG12V. Expression of either Raf or Ral into NIH3T3 cells slightly activated c-fos promoter, while coexpression of both proteins greatly stimulated the c-fos promoter activity. RGL stimulated the GDP/GTP exchange of Raf and this action was enhanced by the post-translational modification of Raf. However, RGL was not active on Ras, Rac, CDC42, Rap, or Rh. Furthermore, this action of RGL to stimulate the GDP/GTP exchange of Raf was dependent on Ras in COS cells. These results suggest that RGL constitutes another Ras-signaling pathway which is distinct from the Raf pathway and indicate that the RGL pathway regulates the c-fos promoter activity and the GDP/GTP exchange of Raf.

Ras, a member of the small G protein1 superfamily, is an essential component in the transduction of extracellular signals that induce proliferation and differentiation (1, 2). Evidence shows that Ras exerts its functions through multiple effector proteins. These include Raf (3, 4), Raf-GDS (5–7), RGL (8), phosphatidylinositol 3-kinase (9), Cdc42Hs (10), stress-activated protein kinase (11), MAP kinase kinase kinase (12), MAP kinase kinase kinase (13), and AF-6 (14). Among these effector proteins, Raf has been extensively studied. Induction of Ras binds to Raf and promotes the activation of Raf, which in turn activates MAP kinase, which then triggers the activation of MAP kinase. Activated MAP kinase in turn translocates to the nucleus, where it phosphorylates and regulates the activities of nuclear transcription factors that cause changes in gene expression that control cell proliferation. Recent biochemical and biological evidence has implicated a second Ras-mediated signaling pathway which is distinct from the Raf/MAP kinase pathway and involves Rho family members, Rho, Rac, and CDC42 (15–19). Furthermore, the experiments using the effector loop mutants of Ras have suggested that Ras interacts with diverse effector proteins in the same cells (19–22). In vitro and yeast two-hybrid studies have shown that RasG12V/E37G (a Ras mutant in which Gly-12 and Thr-35 are changed to Val and Ser, respectively) binds to Raf, Raf-GDS, Ral, and AF6, that RasG12V/E37G interacts with Raf-GDS, Ral, yeast byr2, adenyl cyclase, and AF6, and that RasG12V/Y40C associates with AF6. Furthermore, RasG12V/E37G and RasG12V/Y40C activate Raf and Rac, respectively, and these Ras effector loop mutants cooperate to promote DNA synthesis in mammalian cells. Consistent with these results, coexpression of Raf and Rac induces transformation synergistically (15). Therefore, the Ras effector pathways could cooperate to exert the functions of Ras.

We have identified RGL as a Ras-interacting protein (5). RGL shares 50% amino acid identity with Raf-GDS (5, 23). RGL contains the Ras-interacting domain in the C-terminal region, named RID. RID binds to an active form of Raf, which is a member of the small G protein superfamily and possesses the same amino acid sequence as the effector loop of Raf (24). Expression of RID into Ras- but not Raf-transformed NIH3T3 cells reverses the malignant phenotypes such as growth in low serum condition and anchorage independence (25), and injection of RID into Xenopus oocytes inhibits Ras-dependent maturation and MAP kinase activation (26). Thus, RID has been well characterized and known to interact with the effector loop of an active form of Ras. Therefore, RGL could mediate some of Ras functions. The amino acid sequence of RGL shows that it contains the CDC25-like domain. Several GDP/GTP exchange proteins including CDC25, SOS, SCD25, Ste6, BUD5, LEE1, C3G, and Raf-GDS share the CDC25-like domain (23, 27). This domain of CDC25 and SOS stimulates the GDP/GTP exchange of Ral.

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The abbreviations used are: G protein, GTP-binding protein; Ral-GDS, Raf GDP dissociation stimulator; RGL, RafGDS-like; MAP kinase, mitogen-activated protein kinase; RID, Ras-interacting domain; HA, hemagglutinin; GFP , green fluorescent protein; PCF, pyruvate carboxylase; GST, glutathione S-transferase; MBP, maltose-binding protein; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; GDI, GDP dissociation inhibitor; Rlf, RafGDS-like factor.
exchange of Ras and induces transformation in NIH3T3 cells (29–31). C3G and RaflGDS stimulate the GDP/GTP exchange of Rap and Raf, respectively (22, 32). However, the functions of RGL are not known since the studies using the full-length of RGL have not yet been done.

In this paper we have examined the roles of RGL in the Ras-signaling pathway. We show that RGL constitutes the Ras-mediated signaling pathway which is distinct from the Raf pathway and that it cooperates with Raf to activate c-fos promoter. Furthermore, we demonstrate that RGL stimulates the GDP/GTP exchange of Rap, that the post-translational modification of Rap is important for this action of RGL, and that RGL regulates the Ral activity. These results indicate that RGL mediates the Ras signals to stimulate the gene expression and activate Rap.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—pBJ-1, pcGNN, pfos-luc (a reporter vector in which the expression of luciferase is controlled by c-fos promoter) (33), the anti-HA and Myc antibodies, and wild type NIH3T3 cells were provided by Drs. R. Q. Hu, A. Klippel, and H. Cen (Chiron Corp., Emeryville, CA). pmt/RafCAAX and pmt/RafSAAX, pEXV/RacG12V, the ankyrin-Express km system (Takara Shuzo Co., Ltd., Ohtsu, Japan). To construct pEF-Bos/RasG12V/T35S, pEF-Bos, pcDNAIAmp/RasG12V were digested with XbaI and XhoI, and inserted into the BamHI cut pCGN. pGEX-2T/Rap1, pGEX-CDC42 were purified from the membrane and cytosol fractions of NIH3T3 cells as described (34). Briefly, the transformed E. coli were initially grown at 37 °C to an absorbance of 0.5 (optical density at 600 nm) and subsequently transferred to 20 °C, then isopropyl-1-p-D-galactoside was added to a final concentration of 0.1 or 0.3 mM and further incubation was carried out for 16 h at 20 °C. The expressed proteins were purified by affinity chromatography in accordance with the manufacturer’s instructions. GST-Rap1 and GST-RhoA were produced in E. coli and purified as described (24).

Interaction Assay of RGL and Ras in Intact Cells—Monolayers of NIH3T3 cells (2 × 10⁵ cells on a 15-cm diameter plate) were incubated with recombinant baculoviruses expressing RGL and Ras (35, 39). After 72 h, the cells were lysed as described (35) and the proteins of the lysates were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with the anti-Ras antibody. Y13–238 was used in the immunoprecipitation experiments (Fig. 1B lane 7), while Y13–238 was used as described previously (24).

Interaction Assay of RGL and Raf Effector Loop Mutants in Vitro—To make immobilized RGL (602–768) on amylase resin, 130 μg of MBP-RGL (602–768) was incubated with 200 μl of amylose resin in 500 μl of reaction mixture (20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 100 mM NaCl) for 2 h at 4 °C. The resin was precipitated by centrifugation and washed with 10 ml of Tris-HCl (pH 7.5) three times. To make the [35S]GTP[S]-bound form of Ras effector loop mutants, each of Ras mutants (25 pmol) was incubated with 10 μM [35S]GTP[S] (20,000 cpm/ pmol) for 15 min at 30 °C in 40 μl of reaction mixture (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM MgCl₂, and 1 mM DTT). After the incubation, the resin was washed with 10 ml of final centrifugation. The [35S]GTP[S]-bound form of Ras mutants were incubated for 30 min at 4 °C with immobilized RGL (602–768) (50 pmol) in 140 μl of reaction mixture (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 20 mM MgCl₂, 1 mM DTT, and 10 μM GTP[S]) in the presence or absence of GST-Raf (1–149). Immobilized RGL (602–768) were precipitated by centrifugation, the precipitated resin was washed, and the remaining radioactivities were...
Activation of c-fos Promoter and Ral by RGL

**RESULTS**

**Interaction of RGL with Ras in Intact Cells**—RGL contains the CDC25-like domain which consists of 6 blocks in its central region and RID in the C-terminal region (5, 23) (Fig. 1A). Since experiments using the full-length of RGL have not yet been done, we coexpressed RGL with Ras in S9 cells to examine whether the full-length of RGL interacts with Ras in intact cells. RGL was tagged with the HA epitope at the N terminus. The expression level of transfected RGL in S9 cells expressing RGL alone was similar to that in the cells coexpressing RGL with RasG12V, an active form, or RasS17N, a dominant negative form, as assessed by immunoblotting (Fig. 1B, lanes 1–3). When the lysates coexpressing RGL with RasG12V were immunoprecipitated with the anti-Ras antibody, both RGL and Ras were detected in the Ras immune complex (Fig. 1B, lane 5). When the lysates expressing RGL alone were immunoprecipitated with the anti-Ras antibody, RGL was not detected (Fig. 1B, lane 4). When the lysates coexpressing RGL with RasS17N were immunoprecipitated with the anti-Ras antibody, RGL was not coprecipitated with RasS17N (Fig. 1B, lane 6). Neither RGL nor RasG12V was immunoprecipitated with non-immunoglobulin from the lysates expressing both proteins (data not shown). We used Y13–238 as the anti-Ras antibody to immunoprecipitate Ras for these experiments. Another anti-Ras antibody, Y13–259 which is known to be the neutralizing antibody (45), was tested for its ability to immunoprecipitate the Ras-RGL complex. In contrast to Y13–238, Y13–259 could not immunoprecipitate the Ras-RGL complex from the cell lysates coexpressing RGL with RasG12V under the same condition (Fig. 1B, lane 7), although Y13–259 and Y13–238 immunoprecipitated the similar amounts of Ras (Fig. 1B, lanes 5 and 6).
The association of RGL with Ras.

In two-hybrid experiments, it was shown that RGL binds to Ras through its effector loop in intact cells. RID of RGL is known to interact with the GTP-bound form of Ras through its effector loop (5, 25, 26). To examine whether RID is the only domain which is required for the association with Ras, two deletion mutants, RGL(1–505) and RGL(88–505), were generated (Fig. 1A). RGL(88–505) encodes only the CDC25-like domain. RasG12V was coexpressed with RGL, RGL-(1–505), or RGL-(88–505) in COS cells (Fig. 1C, lanes 1–3). RGL and its deletion mutants were tagged with the Myc epitope at the N terminus. Although RGL was coprecipitated with RasG12V, either RGL(1–505) or RGL(88–505) was not (Fig. 1C, lanes 4–6). Taken together with the previous observations (5, 25, 26), RID is sufficient and necessary for the association of RGL with Ras.

Interaction of RGL with Ras Effector Loop Mutants—Yeast two-hybrid experiments have shown that RGL binds to RasG12V/E37G and RasG12V/T35S but not to RasG12V/Y40C (19). To examine whether RGL interacts with these Ras effector loop mutants in mammalian cells, RGL was coexpressed with the Ras effector loop mutants in COS cells (Fig. 2A). RGL was coprecipitated with RasG12V and RasG12V/E37G but not with either RasG12V/T35S or RasG12V/Y40C (Fig. 2B, lanes 2–5). In COS cells without ectopically expressed Ras, RGL was not precipitated with the anti-Ras antibody (Fig. 2B, lane 1). These results were not partially consistent with the observations obtained from the yeast two-hybrid experiments. The specific binding of RGL to RasG12V/E37G was confirmed by in vitro experiments (Fig. 3). RGL(602–768), the Ras-interacting domain of RGL, was purified as MBP-fused to proteins from E. coli. Raf-(1–149), RasG12V, RasG12V/T35S, and RasG12V/Y40C were purified as GST-fused to proteins from E. coli. These Ras mutants showed the same GTP·S binding activity and the K_d for GTP·S for these Ras mutants were almost similar (data not shown). RGL(+602–768) bound to the GTP·S-bound form of RasG12V and RasG12V/E37G, although the binding activity of RGL(+602–768) to RasG12V was weaker than to RasG12V/E37G (Fig. 3A). However, RGL(602–768) interacted with neither the GTP·S-bound form of RasG12V/T35S nor RasG12V/Y40C (Fig. 3A). Raf-(1–149) which contains the Ras-binding domain inhibited the binding of RGL(602–768) to RasG12V in a dose-dependent manner, while Raf-(1–149) was unable to displace RGL(602–768) for binding to RasG12V/E37G (Fig. 3B).

Synergistic Activation of c-fos Promoter by RGL and Raf—It has been reported that the Raf/MAP kinase and Rac pathways synergize to induce focus formation in Rat1 cells and DNA synthesis in REF52 cells (15, 22). Since the Raf and Rac pathways are important for gene expression (46, 47), we examined whether the RGL pathway cooperates with other Ras effector pathways to activate c-fos promoter using c-fos luciferase. RafCAAX is an active Raf kinase targeted to the plasma membrane by virtue of the addition of a C-terminal membrane localization signal from Ki-Ras (48, 49). RafSAAX, in which the cysteine residue of CAAX box is changed to serine to prevent farnesylation, is a control protein (48). Transfection of RasG12V stimulated the c-fos luciferase expression. Transfection of either RGL or RafCAAX alone slightly stimulated the c-fos luciferase expression, while cotransfection of RafCAAX and RGL pathway.
greatly stimulated the expression (Fig. 4). RafSAAX did not stimulate the c-fos luciferase expression, and the expression level induced by cotransfection of RafSAAX and RGL was the same as that induced by transfection of RGL alone. Cotransfection of RGL and RacG12V, an active form of Rac, synergized to stimulate the c-fos luciferase expression, but the extent was weaker than that of RGL and RafCAAX (Fig. 4). These results indicate that the RGL pathway activates c-fos promoter synergistically with the Raf pathway.

**RGL Activity to Regulate the GDP/GTP Exchange of Small G Proteins**—Since RGL contains the domain highly homologous with CDC25, we examined whether RGL regulates the GDP/GTP exchange of small G proteins. Furthermore, we also investigated the effect of post-translational modification of small G proteins on the RGL activity to regulate the GDP/GTP exchange since it has been known that the GDP/GTP exchange regulatory proteins such as CDC25, SOS, smgGDS, RabGDI, and RhoGDI act more effectively on the post-translationally modified form of small G proteins than the unmodified form (30, 31, 39, 50). The post-translationally modified and unmodified forms of Ras, Ral, Rac, and CDC42 were purified from the membrane and cytosol fractions of Sf9 cells, respectively, as GST fused to proteins. RGL did not stimulate the binding of GTPγS to the post-translationally modified or unmodified form of Ras (Fig. 5). RGL was not active on the post-translationally modified or unmodified form of Rac or CDC42, either (Fig. 5). However, RGL stimulated the binding of GTPγS to both the post-translationally modified and unmodified forms of Ral (Fig. 5). RGL dissociated GDP from the modified form of Ral more effectively than the unmodified form (Fig. 6A). RGL stimulated the binding of GTPγS to Rac in a dose-dependent manner and the action was more effective on the modified form than the unmodified form (Fig. 6B). When the binding of GTPγS to Ral was assayed in the presence of RGL (40 nM) using various amounts of Ral, the apparent Kₘ value of RGL for the modified form was estimated to be 380 nM. That of RGL for the unmodified form was calculated to be more than 4 μM (data not shown). As shown in Fig. 6B, a large amount of RGL stimulated the GDP/GTP exchange of the post-translationally unmodified form of Ral. Under the same conditions, RGL was not active on the post-translationally unmodified form of either Rap or Rho (data not shown). These results indicate that RGL regulates the GDP/GTP exchange of Ral but not that of Ras, Rap, Rac, CDC42, or Rho. This characteristic of RGL is similar to that of RalGDS (23). To determine the region which is responsible for the GDP/GTP exchange activity, we examined the actions of RGL-(1–505) and RGL-(88–505) on Ral. RGL-(1–505) showed almost the same activity as RGL on Ral and the action of RGL-(88–505) on Ral was enhanced by the post-translational modification of Ral (Fig. 7).

**Effect of Ras on the RGL Activity to Stimulate the GDP/GTP Exchange of Ral**—Finally we examined whether the association of Ras with RGL regulates the RGL activity in intact cells.
Ral, which was tagged with the HA epitope at the N terminus, was coexpressed with RGL and Ras in COS cells. The expression level of Ral was not changed by the coexpression of RGL, RasG12V, or RasG12V/Y40C (Fig. 8A, lanes 1–4). The cells were labeled with 32Pi and Ral was immunoprecipitated with the anti-HA antibody. The proportion of GTP bound to Ral was quantified by the thin-layer chromatography method (42, 43) (Fig. 8B). When Ral was expressed alone, the percentage of the GTP-bound form of Ral was about 4%. Coexpression of RGL increased the proportion of GTP bound to Ral from 4 to 12%. Furthermore, RasG12V enhanced the RGL-dependent increase of the proportion of GTP bound to Ral to 18%, but not did RasG12V/Y40C which failed to bind to RGL. To confirm that the association of Ras with RGL regulates the RGL activity, RGL-(1–505) which lacks the Ras-interacting domain was studied. Ral and RGL-(1–505) were expressed with or without RasG12V (Fig. 8A, lanes 5 and 6). RGL-(1–505) increased the percentage of the GTP-bound form of Ral, but its activity did not raise by coexpression with RasG12V. These results indicate that the association of Ras with RGL enhanced the GDP/GTP exchange activity of RGL in intact cells.

DISCUSSION

Previously we isolated cDNA of RGL and found that the C-terminal region of RGL interacts with Ras (5, 24–26). However, the functions of RGL have not yet been clear. Using the full-length of RGL, we have clarified that RGL binds to a Ras effector loop mutant which activates neither the Raf nor Rac pathway, that it cooperates with Raf to activate c-fos promoter, and that it stimulates the GDP/GTP exchange of Ral in a Ras-dependent manner. These results are the first demonstration showing that RGL constitutes another Ras-signaling pathway which is distinct from the Raf pathway and that the signal from RGL leads to the gene expression and Ral activation. These findings provide new insight into the functional significance of Ras and RGL interaction and support the importance of RGL as a real effector protein of Ras.

Evidence has been accumulated that Ras transmits the signal to at least two pathways, the Raf and Rac pathways (15–19, 22). Our results have clearly shown that RGL specifically binds to RasG12V/T35S, but not to either RasG12V/T35S or RasG12V/Y40C. It has been reported that RGL interacts with both RasG12V/T35S and RasG12V/Y40C in yeast two-hybrid experiments (19). Although we do not know the reasons for the discrepancy between our and other’s results, it might be due to the difference of the assay methods. Furthermore, we have demonstrated that RGL activates c-fos promoter synergistically with Raf. These results suggest that the RGL pathway is a Ras-signaling pathway which is different from the...
Raf and Rac pathways. The Raf and Rac pathways have been shown to increase c-fos promoter activity through ternary complex factor and serum response factor, respectively (47). Furthermore, it has been demonstrated that the signal through serum response factor acts synergistically with the signal through ternary complex factor which is activated by MAP kinase (46, 47). Therefore, RGL could activate transcription factors other than ternary complex factor. It has been reported that RalGDS induces focus formation in NIH3T3 cells synergistically with Raf (51). Therefore, RalGDS family members could act downstream of Ras and regulate cell functions cooperatively with the Raf pathway.

We have also found that RGL stimulates the GDP/GTP exchange of Ral as well as RalGDS. This activity of RGL is specific for Ral as far as we have examined. Although RGL is homologous with RalGDS, about 100 amino acids in the region between blocks 1 and 2 of the CDC25-like domain are lacking (5). Therefore, these 100 amino acids are not essential for the RGL activity to stimulate the GDP/GTP exchange of Ral. CDC25 and SOS share the CDC25-like domain containing blocks 1 to 6 (23). The region consisting of blocks 1 to 6 of CDC25 and SOS is sufficient for stimulating the GDP/GTP exchange of Ras (30, 31). However, we have found that RGL-(1–505) but not RGL-(88–505) has the activity to stimulate the GDP/GTP exchange of Ral although RGL-(88–505) covers the CDC25-like domain. These results indicate that the CDC25-like domain of RGL is not sufficient for its activity to stimulate the GDP/GTP exchange of Ral and that the region of RGL-(1–87) may be necessary to keep RGL in a structure to regulate the GDP/GTP exchange of Ral. The mode of regulation in the GDP/GTP exchange of Ral by RGL could be different from that of Ras by CDC25 and SOS. We have shown that RGL is not sufficient and necessary for the association with Ras but that it is not directly required for the action to stimulate the GDP/GTP exchange of Ral in vitro. We have also demonstrated that RasG12V but not RasG12V/N40C stimulates RGL-mediated GDP/GTP exchange of Ral in COS cells and that the activity of RGL-(1–505) is not enhanced by RasG12V. These results indicate that Ras-induced RGL activation is dependent upon the two proteins binding and that RGL mediates the redistribution of RGL to Ral which is present on the membranes. This characteristic of RGL is similar to that of RalGDS (43). Indeed RalGDS translocates from the cytosol to the membranes to bind to Ral which is present on the membranes (36). Therefore, it is possible that RGL is not important for the catalytic activity itself but essential for the determination of subcellular localization. However, we cannot exclude the possibility of the existence of a factor on the membranes which activates RGL.

Small G proteins undergo a series of post-translational modifications which regulate their localization to the membrane fractions such as plasma membrane, endoplasmic reticulum, and Golgi apparatus, and which is essential for their biological actions (2, 50, 52). It has been demonstrated that the post-translational modifications of small G proteins are important for the actions of their GDP/GTP exchange proteins (30, 31, 39, 50). Consistent with previous observations, RGL stimulates the GDP/GTP exchange of the modified form of Ral more effectively than that of the unmodified form. Since the K_m value of Ral for the modified form of Ral is smaller than that for the unmodified form, the post-translational modification of Ral could be important for not only its binding to the membranes but also its affinity for RGL. We have demonstrated that RalGDS is more active on the post-translationally modified form of Ral than the unmodified form (36). Therefore, the post-translational modification of Ral is important for the action of RalGDS family (RalGDS and RGL) to stimulate the GDP/GTP exchange of Ral.

Although it is clear that Ral is activated by RalGDS and RGL, the functions of Ral have long remained elusive. It has been shown that Ral is required for Sre- and Ras-dependent activation of phospholipase D (53). Furthermore, it has been reported that Ral is involved in Ras-dependent transformation in NIH3T3 cells and that this small G protein regulates the initiation of border cell migration in Drosophila oogenesis (43, 54). Thus, evidence has been accumulated that Ral is an important small G protein in the signal transduction system. Rlf has been recently identified as a novel Ras- and Rap1-associating protein (55). Rlf contains the CDC25-like domain and RID and it shares 34 and 24% amino acid identity with RalGDS and RGL, respectively. Although the functions of Rlf are not determined, it could have a GDP/GTP exchange activity of Ral from the structural similarity with RalGDS and RGL. The reasons of the existence of three GDP/GTP exchange proteins for Ral are not known at present. The difference of the modes of action and activation of RalGDS family remains to be clarified. Further studies are necessary to understand the whole picture of the Ras-signaling pathway through RalGDS family.

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Activation of c-fos Promoter and Ral by RGL

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