*rap*1 p21 regulates the interaction of *ras* p21 with RGL, a new effector protein of *ras* p21

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Abstract **We have recently found that ralGDS family members (RGL and ralGDS) are putative effector proteins of *ras* p21. *rap*1 p21 is a small GTP-binding protein which has the same amino acid sequence as the effector loop of *ras* p21. We examined the effect of *rap*1 p21 on the interaction of *ras* p21 with RGL. The GTP-bound form of *rap*1 p21 interacted with RGL as well as did *ras* p21. *rap*1 p21 inhibited the interaction of *ras* p21 with RGL. RGL was phosphorylated by cyclic AMP-dependent protein kinase (protein kinase A). Phosphorylation of RGL did not affect its binding to *ras* p21 and *rap*1 p21 under the conditions that phosphorylation of Raf-1 reduced its affinity for *ras* p21. These results demonstrate that *rap*1 p21 but not protein kinase A regulates the interaction of *ras* p21 with RGL and suggest that *rap*1 p21 and protein kinase A may cooperate to distinguish the signal of *ras* p21 to RGL from that to Raf-1.**

Key words: *rap*1 p21; *ras* p21; RGL; ralGDS; Protein kinase A; Raf-1

1. Introduction

ras p21 is a member of small G protein superfamily and is important for cell growth and differentiation [1–6]. Several mutations of *ras* p21 have been found in human cancers such as colon cancer and pancreatic cancer [1,3]. To identify effector protein(s) which directly interacts with the active form of *ras* p21 and exerts biological effects has been a major goal of research of *ras* p21 to clarify the mode of action of *ras* p21. The interaction of *ras* p21 with effector protein(s) requires the effector loop of *ras* p21 (amino acids 32–40) [1,3]. The effector protein(s) of *ras* p21 has some activity that is regulated by its interaction with the active form of *ras* p21, and this activity is responsible for some of biological effect of *ras* p21 in the cells.

Raf-1 is a serine/threonine kinase and is identified to be an effector protein which mediates a *ras* p21-dependent signal [5–13]. Raf-1 phosphorylates and activates MEK and in turn activates MAPK. This pathway mediates the signal into the nuclei, leading to activation of primary-response nuclear proto-oncogenes such as c-*fos* and c-*myc* by phosphorylation [5,6]. However, it is possible that *ras* p21 has effector proteins other than Raf-1, since *ras* p21 possesses multiple functions. p120-GAP, p110 subunit of PI3-kinase, and MEKK are possible effector proteins of *ras* p21 in that these proteins interact with the GTP-bound form of *ras* p21 and that these proteins have an influence downstream of *ras* p21 in various signaling pathway [14–17]. However, several evidence has suggested that p120-GAP and p110 subunit of PI3-kinase act as regulators of *ras* p21 [18,19]. Whether GAP or PI3-kinase is an effector protein of *ras* p21 might depend on cell types.

We have identified RGL (ralGDS like) as a new effector protein of *ras* p21 [20]. RGL has 70% amino acid homology with ralGDS [21]. The C-terminal domain of RGL and ralGDS has been found to associate with the GTP-bound form of *ras* p21 through the effector loop of *ras* p21 [20,22,23]. ralGDS stimulates GDP/GTP exchange of *ral* p24 [21]. *ral* p24 has been originally isolated by probing with an oligonucleotide corresponding to one of the GTP-binding domain of *ras* p21 [24]. Although the function of *ral* p24 is not yet understood, ralGDS and RGL may regulate the signal from *ras* p21 to *ral* p24.

*rap*1 p21 is a member of small G protein superfamily [25,26]. *rap*1 p21 shares 55% amino acid identity with *ras* p21 and *rap*1 p21 has the same amino acid sequence as the effector loop of *ras* p21 [25,26]. Therefore, it is conceivable that *rap*1 p21 exerts actions antagonistic or similar to those of *ras* p21. Indeed, *rap*1 p21 antagonizes *ras* p21 functions in several cells [25–30]. *rap*1 p21 has been shown to interact with Raf-1 by the yeast two hybrid system [8]. Recently the crystal structure of the complex between Raf-1 and *rap*1 p21 has been solved and it has been shown that the interaction of these proteins occurs between residues of the anti-parallel β-sheets formed from two β-sheets of the effector loop of *rap*1 p21 and two β-sheets of the *ras* p21-binding domain of Raf-1 [31]. These results strongly suggest that the biological effects of *rap*1 p21 are results of competition with *ras* p21 for the effector proteins of *ras* p21 and prompted us to examine whether *rap*1 p21 regulates the interaction of *ras* p21 with RGL. In this paper we show that *rap*1 p21 binds directly to RGL, a new effector protein of *ras* p21, and that *rap*1 p21 inhibits the binding of *ras* p21 to RGL.

2. Materials and methods

2.1. Materials and chemicals

The RGL cDNA was provided by Drs. Z. Ye and L.T. Willimas (Chiron Corp., USA). The c-H-*ras* p21, *rap*1A p21, *rac*1 p21, and *ral*B p24 cDNAs were provided by Drs. J. Downward (Imperial Cancer Research Institute, UK), C. Der (The University of North Carolina, USA), A. Hall (Imperial Cancer Research Institute, UK), and R. Weinberg (Whitehead Institute for Biomedical Research, USA), respectively. pMAL/Raf(1–257) was kindly supplied from Dr. T. Kataoka (Kobe University, Japan) [32]. The catalytic subunit of protein kinase A was from Dr. M. Inagaki (Tokyo Metropolitan Institute of Gerontology,

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Abbreviations: G protein, GTP-binding protein; MEK, MAPK and ERK kinase; MAPK, mitogen-activated protein kinase; MEKK, MEK kinase; GAP, GTPase activating protein; PI3-kinase, phosphatidylinositol 3-kinase; RGL, RalGDS like; protein kinase A, cyclic AMP-dependent protein kinase; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; RID, *ras* p21-interacting domain; PCR, polymerase chain reaction; GST, glutathione-*S*-transferase; MBP, maltose-binding protein; DTT, dithiothreitol.

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Japan). pMAL-c was purchased from New England Biolabs. [^{35}S]GTPγS and [^{3}H]GDP were from DuPont NEN Research Products.

2.2. Plasmid constructions

The amino acids 602–768 of RGL contained RID [20]. pMAL/RID (pMAL/RGL(602–768)) was constructed by the following procedures. The oligonucleotide corresponding to the Myc epitope was synthesized by PCR. This oligonucleotide was designed to have the *Eco*RI site at the 5′ end and the *Bam*HI site at the 3′ end. pBSKS/RID [20] was digested with *Xho*I and *Eco*RI. RID and the Myc epitope were ligated with pGEM7z which was digested with *Xho*I and *Bam*HI to generate pGEM7z/RID. pMAL-c was digested with *Pst*I and the protruding 3′ overhang was removed by T4 DNA polymerase. pGEM7z/RID was digested with *Shp*I and *Sac*I to obtain the RID-Myc fragment. This fragment was treated with T4 DNA polymerase. pMAL-c and the RID-Myc fragment were ligated to generate pMAL/RID. To construct pGEX-2T encoding H-*ras* p21, *rap*1A p21, *rac*1 p21, and *ral*B p24, 0.6 kb fragments containing these small G proteins with *Bam*HI and *Eco*RI sites were synthesized by PCR. These fragments were digested with *Bam*HI and *Eco*RI and inserted into the *Bam*HI and *Eco*RI cut pGEX-2T to generate pGEX/H-*ras* p21, pGEX/*rap*1A p21, pGEX/*rac*1 p21, and pGEX/*ral*B p24.

2.3. Expression and purification of GST-fused to small G proteins and MBP-fused to RID and Raf(1–257)

Transformed *Escherichia coli* were initially grown at 37°C to an absorbance of 0.8 (optical density at 600 nm) and subsequently transferred to 25°C. Then isopropyl-1-β-D-galactopyranoside was added at a final concentration of 0.1 mM and further incubation was carried out for 10 h at 25°C. The GST- and MBP-fused to proteins were purified by affinity chromatographies in accordance with the manufacturer's instructions. In the guanosine nucleotide-binding assay described below, about 0.3 mol of GTPγS or GDP bound to 1 mol of GST-fused to small G proteins. We do not know the exact reasons for the low stoichiometry of the guanosine nucleotide-binding to these small G proteins.

2.4. Binding assay of small G proteins and RID or Raf(1–257)

To make immobilized RID or Raf(1–257) on amylose resin, 500 μg of MBP-RID or 250 μg of MBP-Raf(1–257) was incubated with 200 μl of amylose resin in 500 μl of reaction mixture (20 mM Tris-HCl [pH7.5], 1 mM DTT, and 200 mM NaCl) for 2 h at 4°C. The resin was precipitated by centrifugation and washed with 10 mM Tris-HCl (pH 7.5) three times. One μl of aliquot of the resin bound to 20 pmol of MBP-RID or 2.2 pmol of MBP-Raf(1–257). To make the [^{35}S]GTPγS- or [^{3}H]GDP-bound form of small G proteins, each of small G proteins (10 pmol) was incubated with 2 μM [^{35}S]GTPγS (10,000–20,000 cpm/pmol) or 2 μM [^{3}H]GDP (40,000–60,000 cpm/pmol) for 10 min at 30°C in 40 μl of reaction mixture (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT). After the incubation, 1 M $MgCl_2$ was added at a final concentration of 20 mM. The [^{35}S]GTPγS- or [^{3}H]GDP-bound form of small G proteins were incubated for 30 min at 4°C with immobilized RID or Raf(1–257) (15 pmol) in 100 μl of reaction mixture (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 20 mM $MgCl_2$, 1 mM DTT, and 10 μM GTPγS or 10 μM GDP). Immobilized RID and Raf(1–257) were precipitated by centrifugation, the precipitated resin was washed, and the remaining radioactivities were counted. During the procedures of this assay, neither [^{35}S]GTPγS nor [^{3}H]GDP dissociated from [^{35}S]GTPγS- or [^{3}H]GDP-bound form of small G proteins.

2.5. Phosphorylation of RID and Raf-1 by protein kinase A

Immobilized RID and Raf(1–257) (15 pmol each) were incubated with the catalytic subunit of protein kinase A (500 ng of protein) for 30 min at 30°C in 30 μl of reaction mixture (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 25 μM ATP). Under these conditions 0.8 mol of phosphate was incorporated into 1 mol of RID and Raf(1–257).

3. Results

3.1. Specificity of the interaction of small G proteins with RGL

We have previously found that the amino acid 602–768 of RGL is a *ras* p21-interacting domain (RID) by the yeast two-hybrid system [20]. We first examined the direct binding of small G proteins such as *ras* p21, *rap*1 p21, *rac*1 p21 or *ral* p24 to RID using the purified proteins. Both the GTPγS-bound form of *ras* p21 and *rap*1 p21 associated with RID, but neither the GTPγS-bound form of *rac*1 p21 nor *ral* p24 bound to RID (Fig. 1). None of the GDP-bound form of *ras* p21, *rap*1 p21, *rac*1 p21 or *ral* p24 associated with RID (Fig. 1). These results show that *rap*1 p21 and *ras* p21 which have the same effector loop bind directly to RID of RGL in their GTP-bound active form.

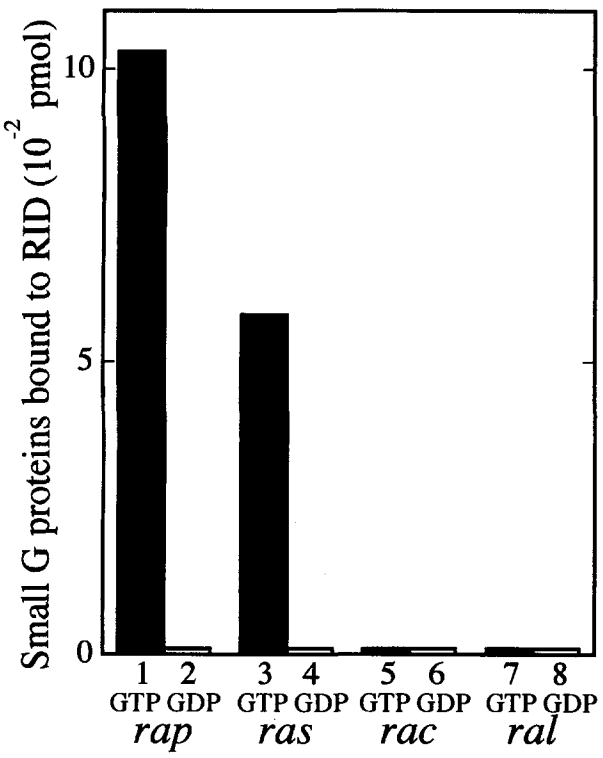

Fig. 1. Specificity of the interaction of small G proteins to RGL. The [^{35}S]GTPγS- (lanes 1, 3, 5, and 7) or [^{3}H]GDP- (lanes 2, 4, 6, and 8) bound form of *rap*1 p21 (lanes 1 and 2), *ras* p21 (lanes 3 and 4), *rac*1 p21 (lanes 5 and 6), and *ral* p24 (lanes 7 and 8) (10 pmol each) were incubated with immobilized RID (15 pmol). The radioactivities of the resin were counted. The results shown are representative of three independent experiments.

3.2. Affinity of rap1 *p21 for RGL*

Both the GTPγS-bound form of *rap*1 p21 and *ras* p21 interacted with RID in a dose-dependent manner (Fig. 2A). The maximal binding activity of *rap*1 p21 to RID was almost the same as that of *ras* p21 to RID. The affinity of *rap*1 p21 for RID was higher than that of *ras* p21 for RID. The K_d values of *rap*1 p21 and *ras* p21 for RID were estimated to be 10 nM and 80 nM, respectively, by Scatchard plot analysis.

3.3. Inhibition of the interaction of ras *p21 with RGL by* rap1 *p21*

*rap*1 p21 has the same amino acid sequence as the effector loop of *ras* p21. Since the effector proteins of *ras* p21 interact with *ras* p21 through its effector loop, ideally *rap*1 p21 should inhibit the interaction of *ras* p21 with its effector proteins. We

examined this possibility. As expected, *rap*1 p21 inhibited the binding of *ras* p21 to RID in a dose-dependent manner (Fig. 2B). The concentration of *rap*1 p21 required to inhibit 50% of the interaction of *ras* p21 with RID was 25 nM. The concentration of *ras* p21 used in this experiment was 200 nM. Therefore, these results indicate that the affinity of *rap*1 p21 for RGL is 8-fold higher than that of *ras* p21 for RGL and are consistent with the results in Fig. 2A.

3.4. *Effect of the phosphorylation of RID by protein kinase A on the interaction of* ras *p21 and* rap1 *p21 with RID*

It has been known that Raf-1 is phosphorylated by protein kinase A and that phosphorylation of Raf-1 reduces the binding of Raf-1 to *ras* p21 [33,34]. Therefore, we examined whether protein kinase A affects the interaction of RGL with *ras* p21 and *rap*1 p21. RID was phosphorylated by protein kinase A and 0.8 mol of phosphate was incorporated into 1 mol of RID (data not shown). The phosphorylation of RID did not affect its interaction with *rap*1 p21 or *ras* p21 (Fig. 3, lanes 1–4) although that of Raf(1–257) by protein kinase A reduced its binding to *ras* p21 (Fig. 3, lanes 5 and 6). These results suggest that protein kinase A does not regulate the interaction of *rap*1 p21 or *ras* p21 with RGL although protein kinase A inhibits the interaction of *ras* p21 with Raf-1.

4. Discussion

We have shown here that *rap*1 p21 directly binds to RGL with a high affinity as well as does *ras* p21 and that *rap*1 p21 inhibits the interaction of *ras* p21 with RGL. *rap*1 p21 is known to antagonize the signal of *ras* p21 to lead to gene induction and cell growth through the Raf/MAPK pathway in various cells [25–30]. Since *rap*1 p21 has the same amino acid sequence as the effector loop of *ras* p21 and interacts with Raf-1 [25,26,31], it is possible that *rap*1 p21 inhibits the binding of *ras* p21 to Raf-1 by competing with the effector loop of *ras* p21. By analogy with this inhibitory mechanism, *rap*1 p21 might inhibit the binding of *ras* p21 to RGL by competing with the effector loop of *ras* p21. Since RGL is a member of ralGDS family and has

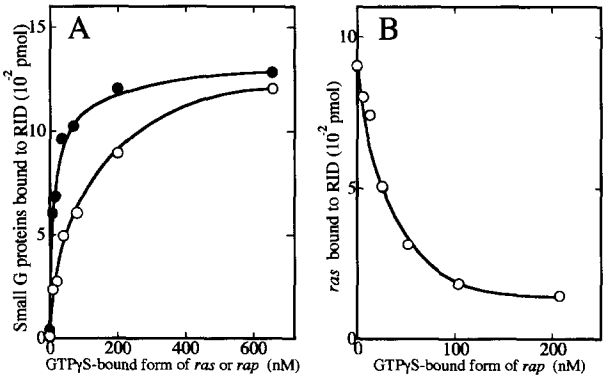

Fig. 2. (A) Affinity of *rap*1 p21 and *ras* p21 for RGL. The indicated concentrations of [^{35}S]GTPγS-bound form of *rap*1 p21 (●) and *ras* p21 (○) were incubated with immobilized RID (15 pmol). The radioactivities of the resin were counted. (B) Inhibition of the interaction of *ras* p21 with RGL by *rap*1 p21. The [^{35}S]GTPγS-bound form of *ras* p21 (20 pmol) was incubated with immobilized RID (15 pmol) in the presence of the indicated concentrations of the GTPγS-bound form of *rap*1 p21. The radioactivities of the resin were counted. The results shown are representative of three independent experiments.

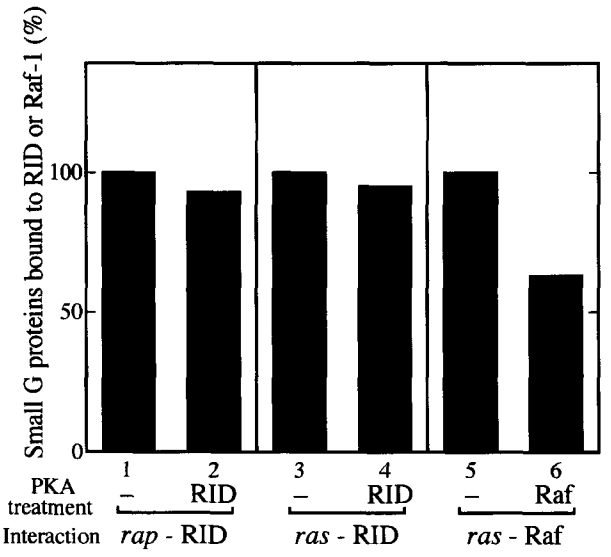

Fig. 3. Effect of the phosphorylation of RGL by protein kinase A on the interaction of RGL with *rap*1 p21 and *ras* p21. The [^{35}S]GTPγS-bound form of *rap*1 p21 was incubated with the unphosphorylated or phosphorylated form of immobilized RID (lanes 1 and 2). The [^{35}S]GTPγS-bound form of *ras* p21 was incubated with the unphosphorylated or phosphorylated form of immobilized RID (lanes 3 and 4). The [^{35}S]GTPγS-bound form of *ras* p21 was incubated with the unphosphorylated or phosphorylated form of immobilized Raf(1–257) (lanes 5 and 6). The radioactivities of the resin were counted. The binding activity of *rap*1 p21 or *ras* p21 to RID or Raf(1–257) was expressed as percentage of that without protein kinase A treatment (lanes 1, 3, and 5). PKA, protein kinase A. The results shown are representative of three independent experiments.

a GDP/GTP exchange activity of *ral* p24[1], RGL may mediate the signal from *ras* p21 to *ral* p24. Our results suggest that *rap*1 p21 regulates the signal from *ras* p21 to *ral* p24 through RGL (RalGDS) in addition to the action antagonistic to *ras* p21/Raf/MAPK pathway.

We have also found that phosphorylation of RID by protein kinase A does not affect the interaction of *rap*1 p21 or *ras* p21 with RID. In RID, RKKNS^{735} is a consensus sequence of the phosphorylation by protein kinase A. Although S^{735} could be phosphorylated by protein kinase A, the physiological significance of the phosphorylation of RID is not known. In contrast, the phosphorylation of Raf-1 by protein kinase A reduced its binding to *ras* p21 [33,34]. The decrease of the affinity of Raf-1 for *ras* p21 by protein kinase A might be one of the mechanism by which the signals through cyclic AMP inhibit the proliferation of the cells [35]. Taken together with the previous observations, *rap*1 p21 and protein kinase A inhibit the signal from *ras* p21 to Raf-1, while *rap*1 p21 but not protein kinase A inhibits the signal from *ras* p21 to RGL. Therefore, it is conceivable that *rap*1 p21 and protein kinase A are important for *ras* p21 to distinguish two different effector proteins.

We have previously found that *rap*1 p21 stimulates DNA synthesis as well as *ras* p21 in Swiss 3T3 cells [36]. These results indicate that *rap*1 p21 exerts its own action depending on cell types. Although both *rap*1 p21 and *ras* p21 are ubiquitously detected in various tissues, *rap*1 p21 shows tissue and subcellu-

[1] Akira Kikuchi and Lewis T. Williams, unpublished observations.

lar distributions partly distinct from those of *ras* p21 [25,26]. The amount of *rap*1 p21 is at least ten times higher than that of *ras* p21 in bovine aortic smooth muscle, rat heart, and human platelets. In the synapse of rat brain, *rap*1 p21 is found in the synaptic plasma membrane, the synaptic vesicle, and the synaptic mitochondria, while *ras* p21 is primarily located to the synaptic plasma membrane. Since ralGDS is ubiquitously expressed in various tissues [21], *rap*1 p21 may exert its own specific actions through RGL (ralGDS) in places where *rap*1 p21 alone is expressed. Taken together with the observations that *ral* p24 is localized to the synaptic vesicle [37], it is intriguing to speculate that the signal from *rap*1 p21 to *ral* p24 through RGL (ralGDS) is important for the function of the synaptic vesicle.

Recently, ralBP1 has been found to be a putative effector protein of *ral* p24 [38]. ralBP1 has a GTPase stimulating activity for CDC42 and *rac* p21. CDC42 and *rac* p21 are known to regulate the assembly of multimolecular focal complexes at the plasma membrane [39]. Therefore, RGL(ralGDS) might be a key protein to mediate cross talk between *ras* p21, *rap*1 p21, *ral* p24, CDC42, and *rac* p21. Further studies are necessary for understanding this new signal transduction pathway of *ras* p21 which regulates the action of *ral* p24.

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