

The post-translational modifications of Ral and Rac1 are important for the action of Ral-binding protein 1, a putative effector protein of Ral

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Abstract Ral-binding protein 1 (RalBP1) is a putative effector protein of Ral and possesses the GTPase-activating activity for Rac1 and CDC42. We examined the roles of the post-translational modifications of Ral and Rac1 for the action of RalBP1. In COS cells, Ral^{G23V}, a constitutively active form, was mainly detected in the membrane fraction while most of Ral^{G23V/C203S}, a Ral mutant which is not post-translationally modified, was found in the cytosol fraction. When RalBP1 was expressed alone in COS cells, it was found in the cytosol but not in the membrane fraction. When RalBP1 was coexpressed with Ral^{G23V}, a part of RalBP1 was found in the membrane fraction. However, when RalBP1 was coexpressed with Ral^{G23V/C203S}, all of RalBP1 was recovered in the cytosol fraction. Although Ral bound to RalBP1 at a molar ratio of 1:1, the interaction of Ral with RalBP1 did not affect the GTPase-activating activity of RalBP1 for Rac1. Furthermore, RalBP1 was more active on the post-translationally modified form of Rac1 and CDC42 than the unmodified form. These results suggest that the post-translational modification of Ral is important for the subcellular localization of RalBP1 and that the interaction of Ral with RalBP1 is not essential for the activity of RalBP1 but plays a role in recruiting RalBP1 to the membrane where its substrates, Rac1 and CDC42, reside.

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Key words: Ral; RalBP1; GAP; Subcellular localization; Rac1

1. Introduction

Ral is a member of small G-protein superfamily and consists of RalA and RalB [1]. As well as other small G proteins, Ral has the GDP-bound inactive and the GTP-bound active forms. The GDP-bound form of Ral is converted to the GTP-bound form by RalGDS [2], and inversely the GTP-bound form is changed to the GDP-bound form by RalGAP [3]. We and other groups have found that RalGDS is a putative effector protein of Ras [4–6]. Since RalGDS stimulates the

GDP/GTP exchange of Ral [2], it is possible that there is a signaling pathway from Ras to Ral through RalGDS. Indeed, it has been shown that RalGDS stimulates the GDP/GTP exchange of Ral in a Ras-dependent manner in COS cells and that a dominant negative form of Ral blocks a Ras-dependent transformation in NIH3T3 cells [7]. Furthermore, it has been demonstrated that RalGDS constitutes a Ras-signaling pathway distinct from Raf and that RalGDS and Raf synergistically regulate cell proliferation and gene expression [8,9]. Thus, evidence has been accumulated that Ral and RalGDS act downstream of Ras and that Ral is an important small G protein in the intracellular signal transduction system. However, the functions of Ral are not well known.

One possible clue to clarify the functions of Ral is RalBP1 which has been identified as a putative effector protein of Ral [10–12]. RalBP1 contains a Ral-binding domain in its C-terminal region and a RhoGAP homology domain in its central region. RalBP1 exhibits the GAP activity for Rac1 and CDC42 but not for RhoA. Therefore, RalBP1 may link between Ral and Rac1 or CDC42. However, the modes of activation and action of RalBP1 are not clear since the studies using full-length RalBP1 have not yet been done.

The post-translational modifications of small G proteins are critical for their activation and action [13,14]. In a Ras/RalGDS/Ral signaling pathway, we have shown that the post-translational modification of Ras is required for determining the subcellular localization of RalGDS and that the modification of Ral enhances the GDP/GTP exchange activity of RalGDS [15]. These results indicate that the post-translational modifications of Ras and Ral are important for the action of RalGDS. Therefore, we examined here the roles of the modifications of Ral and Rac1 in the action of RalBP1.

2. Materials and methods

2.1. Materials and chemicals

The RalB, and Rac1 and CDC42 cDNAs were provided by Drs. R. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA) and K. Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan), respectively. Baculoviruses expressing GST-RalB, GST-Rac1, and GST-CDC42 were supplied from Dr. Y. Matsuura (National Institute of Health, Tokyo, Japan). The cDNA of RalBP1 was isolated by reverse transcriptase PCR as described [15]. All procedures of passage, infection, and transfection of Sf9 cells and the isolation of baculoviruses were carried out as described [16]. MBP-RalBP1, MBP-RalBP1-(364–647), GST-RalB, GST-Rac1, and GST-CDC42 were produced in and purified from *E. coli* in accordance with the manufacturer's instructions as described [15,17,18]. Small G proteins purified from *E. coli* were used as the post-translationally unmodified form. The post-translationally modified form of GST-RalB was purified from the membrane fraction of Sf9 cell [15]. The post-translationally modified form of GST-Rac1 and GST-CDC42 were purified from Sf9 cells by the use of Triton X-114 as described [19,20]. Other materials and chemicals were from commercial sources.

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Abbreviations: G protein, GTP-binding protein; RalGDS, Ral GDP dissociation stimulator; GAP, GTPase-activating protein; RalBP1, Ral-binding protein 1; PCR, polymerase chain reaction; MBP, maltose-binding protein; GST, glutathione-S-transferase; *E. coli*, *Escherichia coli*; HA, hemagglutinin; DTT, dithiothreitol; GTPγS, guanosine 5'-(3-O-thio)triphosphate; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid

2.2. Plasmid constructions

pMAL/RalBP1-(364–647) (amino acid number 364–647), pV-IKS/RalB, pV-IKS/Rac1, pV-IKS/CDC42, pGEX2T/RalB, pGEX2T/Rac1, and pGEX2T/CDC42 were constructed as described [15,17,18]. To construct pCGN/RalB^{G23V} (in which Gly-23 was changed to Val), pCGN/RalB^{S28N}, and pCGN/RalB^{G23V/C203S}, the 0.6-kb fragments encoding RalB^{G23V}, RalB^{S28N}, and RalB^{G23V/C203S} with *Xba*I and *Bam*HI sites were synthesized by PCR, and inserted into the *Xba*I and *Bam*HI cut pCGN. To construct pBJ-Myc/RalBP1, pUC19/RalBP1 [15] was digested with *Bam*HI and the 1.9-kb fragment encoding full-length RalBP1 was inserted into the *Bam*HI cut pBJ-Myc [18]. To construct pBJ-Myc/RalBP1-(1–415), pUC19/RalBP1 was digested with *Bam*HI and *Bgl*II and the 1.2-kb fragment encoding RalBP1-(1–415) was inserted into the *Bam*HI cut pBJ-Myc. To construct pBJ-Myc/RalBP1-(364–647), pBSKS/RalBP1-(364–647) [15] was digested with *Bam*HI and the 0.8-kb fragment encoding RalBP1-(364–647) was inserted into the *Bam*HI cut pBJ-Myc. To construct pMAL/RalBP1, pUC19/RalBP1 was digested with *Bam*HI and the 1.9-kb fragment encoding full-length RalBP1 was inserted into the *Bam*HI cut pMAL-c2.

2.3. Interaction of Ral with RalBP1 in COS cells

After COS cells (60–70% confluent on a 10-cm-diameter plate) were transfected with pCGN- and pBJ-derived constructs described above by the DEAE-dextran method [21], the cells were lysed as described [22]. The lysates expressing Ral with RalBP1 were prepared and the proteins of the lysates (0.6 mg) were immunoprecipitated with the anti-Myc antibody and probed with the anti-Myc and HA antibodies [22,23]. To determine the subcellular localization of Ral and RalBP1, COS cells were fractionated into the cytosol and membrane fractions [15]. Aliquots (20 µg of protein) of the cytosol and membrane fractions were probed with the anti-Myc and HA antibodies.

2.4. Assay for the Ral-binding activity of RalBP1

To make RalBP1 or RalBP1-(364–647) immobilized on amylose resin, *E. coli* lysates (100 mg of protein) expressing MBP-RalBP1 or MBP-RalBP1-(364–647) in lysis buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, and 10 µg/ml leupeptin) were incubated with 200 µl of amylose resin for 2 h at 4°C. The resin was precipitated by centrifugation and washed 3 times with 10 mM Tris-HCl (pH 7.5). One microlitre of the resin bound 0.6 pmol of MBP-RalBP1 and 4.8 pmol of MBP-RalBP1-(364–647). The [³⁵S]GTPγS- or [³H]GDP-bound form of GST-RalB was made as described [15] and incubated for 30 min at 4°C with immobilized RalBP1 or RalBP1-(364–647) (4 pmol of each) in 120 µl of reaction mixture (50 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 10 mM EDTA, 1 mM DTT, and 1 mg/ml BSA). After the immobilized RalBP1 and RalBP1-(364–647) were precipitated by centrifugation and washed, the remaining radioactivities were counted. During the procedures of this assay, neither [³⁵S]GTPγS nor [³H]GDP was dissociated from GST-RalB.

2.5. Assay for the GAP activity of RalBP1

After the post-translationally modified or unmodified form of GST-Rac1 and GST-CDC42 (4 pmol of each) were pre-incubated for 4 min at 30°C in 20 µl of the pre-incubation mixture (20 mM Tris-HCl (pH 7.5), 2 µM [^γ-³²P]GTP (4000–6000 cpm/pmol), 2 mM EDTA, 0.3% CHAPS, 1 mM L-α-dimyristoylphosphatidylcholine, 1 mM DTT, and 40 mg/ml BSA), 4 µl of mixture (100 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, and 10 mM DTT) was added. To this pre-incubation mixture, 16 µl of reaction mixture (17.5 mM Tris-HCl (pH 7.5), 2.5 mM GTP, and 1 mM DTT) containing MBP-RalBP1 was added, and the second incubation was performed with GST-Rac1 for 5 min at 25°C or with GST-CDC42 for 10 min at 25°C. Assays were quantified by rapid filtration on nitrocellulose filters. When the effect of the interaction of Ral with RalBP1 on the GAP activity of RalBP1 was examined, RalBP1 immobilized on amylose resin was incubated with 1 µM GTPγS-bound post-translationally modified or unmodified form of Ral to make immobilized RalBP1 interacting with Ral. The amylose resin was washed as described above and an aliquot was applied to SDS-polyacrylamide gel electrophoresis to estimate the amount of the immobilized proteins. The suspension of the amylose resin containing immobilized RalBP1 or RalBP1 interacting with Ral was used instead of RalBP1 solution in the GAP assay for the post-translationally modified form of Rac1.

2.6. Other methods

Protein concentrations were determined by the method of Lowry using BSA as a standard [24]. Triton X-114 phase separation assay of small G proteins was carried out as described [15,19].

3. Results

3.1. Interaction of Ral with RalBP1 in intact cells

RalBP1 has been identified as a putative effector protein of Ral, and in vitro and yeast two-hybrid experiments have shown that the C-terminal region of RalBP1 interacts with Ral [10–12]. However, the studies in mammalian cells have not yet been done. Therefore, we first examined whether full-length RalBP1 interacts with Ral in mammalian cells. RalBP1 was coexpressed with Ral^{G23V}, a constitutively active form, or Ral^{S28N}, a dominant negative form, in COS cells (Fig. 1A, lanes 1–4). RalBP1 was tagged with the Myc epitope at the N-terminus. Ral^{G23V} and Ral^{S28N} were tagged with the HA epitope at the N-terminus. When the lysates coexpressing Ral^{G23V} and RalBP1 were immunoprecipitated with the anti-Myc antibody, both Ral^{G23V} and RalBP1 were detected in the RalBP1 immune complex (Fig. 1A, lane 6). When the lysates expressing Ral^{G23V} alone were immunoprecipitated with the anti-Myc antibody, Ral^{G23V} was not detected (Fig. 1A, lane 5). Neither Ral^{G23V} nor RalBP1 was immunoprecipitated with non-immune immunoglobulin from the lysates expressing both proteins (data not shown). When RalBP1 was coexpressed with Ral^{S28N} (Fig. 1A, lane 3), RalBP1 did not make a complex with Ral^{S28N} (Fig. 1A, lane 7). These results indicate that RalBP1 interacts with the GTP-bound form of Ral in intact mammalian cells. To exclude the possibility that the interaction of Ral with RalBP1 in COS cells is non-specific due to overexpression of these proteins, we expressed Ral^{G23V} with RalBP1-(1–415) or RalBP1-(364–647). These RalBP1 deletion mutants were tagged with the Myc epitope at the N-terminus. The expression level of RalBP1-(1–415) was higher than that of RalBP1-(364–647) (Fig. 1B, lanes 1 and 2). The expression levels of Ral^{G23V} were similar in these cells. Ral^{G23V} interacted with RalBP1-(364–647) but not with RalBP1-(1–415) (Fig. 1B, lanes 3 and 4). These results indicate that the C-terminal region of RalBP1 is necessary and sufficient for its interaction with Ral in intact cells and that the interaction of Ral with RalBP1 observed in Fig. 1A is not non-specific.

3.2. Translocation of RalBP1 by Ral in COS cells

It has been reported that Ral is associated with the membrane fractions such as plasma membranes, clathrin-coated vesicles, and secretory vesicles [1]. By analogy with other small G proteins, the post-translational modification of Ral at its C-terminal cysteine could be important for its binding to the membrane [25]. Therefore, we examined the role of Ral in subcellular localization of RalBP1 in intact cells. When Ral^{G23V} was expressed in COS cells, Ral^{G23V} appeared in both the cytosol and membrane fractions (Fig. 2A, lanes 3 and 4). On the other hand, most of Ral^{G23V/C203S}, a Ral mutant which is not post-translationally modified, appeared in the cytosol fraction (Fig. 2A, lanes 5 and 6). When RalBP1 was expressed alone in COS cells, it was detected in the cytosol fraction (Fig. 2A, lanes 1 and 2). When RalBP1 was coexpressed with Ral^{G23V}, a part of RalBP1 was detected in the membrane fraction (Fig. 2A, lanes 3 and 4). Although the

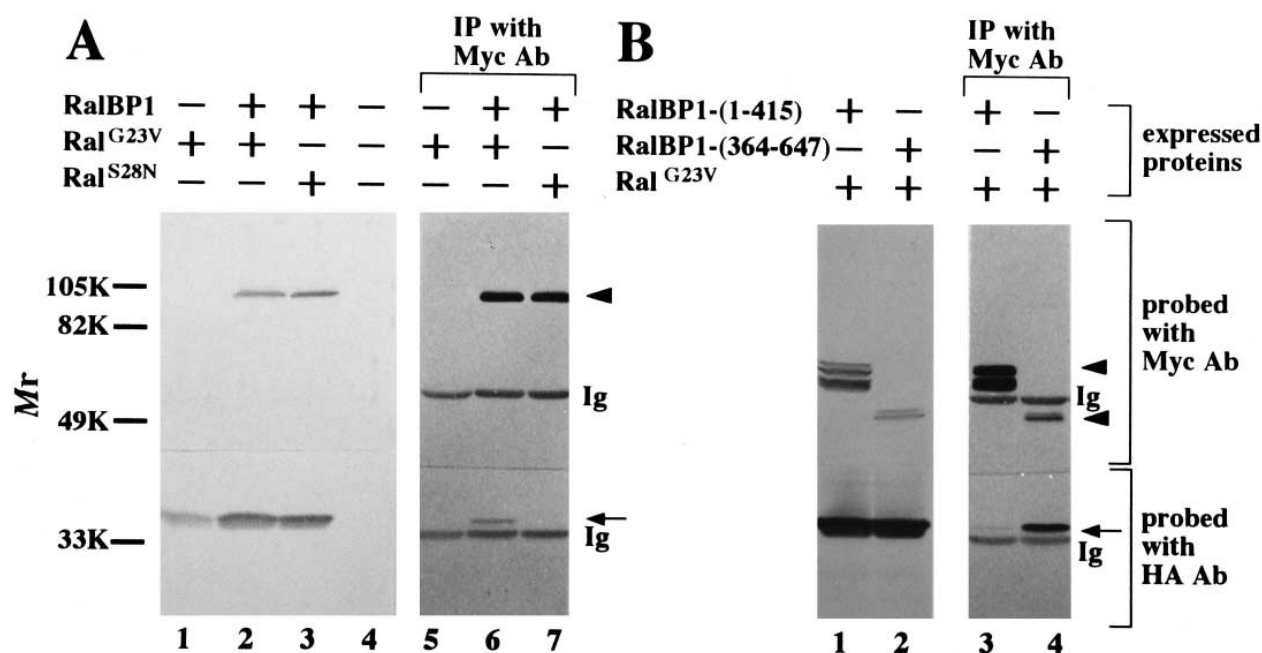


Fig. 1. Interaction of Ral with RalBP1 in mammalian cells. A: Interaction of Ral with RalBP1 in COS cells. Aliquots of the lysates of COS cells expressing Ral^{G23V} alone (lane 1), both Ral^{G23V} and RalBP1 (lane 2), or both Ral^{S28N} and RalBP1 (lane 3) were probed with the anti-HA and Myc antibodies. COS cells transfected with the empty vectors were control (lane 4). The lysates of COS cells expressing Ral^{G23V} alone (lane 5), both Ral^{G23V} and RalBP1 (lane 6), or both Ral^{S28N} and RalBP1 (lane 7) were immunoprecipitated with the anti-Myc antibody. The precipitates were probed with the anti-Myc and HA antibodies. B: Interaction of Ral with RalBP1-(364-647) in COS cells. The lysates of COS cells expressing both Ral^{G23V} and RalBP1-(1-415) (lanes 1 and 3) or both Ral^{G23V} and RalBP1-(364-647) (lanes 2 and 4) were directly probed with the anti-Myc and HA antibodies (lanes 1 and 2) or immunoprecipitated with the anti-Myc antibody, then probed with the anti-Myc and HA antibodies (lanes 3 and 4). Arrowheads indicate the positions of RalBP1, RalBP1-(1-415), and RalBP1-(364-647). Arrows indicate the positions of Ral^{G23V} and Ral^{S28N}. IP, immunoprecipitation; Ig, immunoglobulin; Ab, antibody. The results shown are representative of three independent experiments.

amount of RalBP1 in the membrane fraction appeared to be small, this result was reproducible. However, when RalBP1 was coexpressed with Ral^{G23V/C203S}, RalBP1 was found in the cytosol fraction but not in the membrane fraction (Fig. 2A, lanes 5 and 6). These observations were confirmed using RalBP1-(364-647) (Fig. 2B). When RalBP1-(364-647) was expressed alone in COS cells, it was found in the cytosol fraction. When RalBP1-(364-647) was coexpressed with Ral^{G23V}, RalBP1-(364-647) was found in both the cytosol and membrane fractions, while when RalBP1-(364-647) was coexpressed with Ral^{G23V/C203S}, RalBP1-(364-647) was found in the cytosol fraction. These results suggest that Ral localized on the membrane recruits RalBP1 from the cytosol to the membrane.

3.3. Effect of the interaction of Ral with RalBP1 on the GAP activity of RalBP1

It has been demonstrated that the Ral-binding domain of RalBP1 is not necessary for the GAP activity [10]. Therefore, we examined whether the interaction of Ral with RalBP1 affected the GAP activity of RalBP1. Since the Ral-binding activity of RalBP1 using full-length RalBP1 had not yet been biochemically characterized, we measured its K_d and B_{max} . RalBP1 interacted with the GTP γ S-, but not with the GDP-, bound form of Ral in a dose-dependent manner (Fig. 3A). Scatchard plot analysis revealed that RalBP1 bound to Ral at a molar ratio of 1:0.9 with a K_d value of 230 nM. The Ral-binding activity of RalBP1-(364-647) was similar to that of full-length RalBP1, and this deletion mutant bound to Ral at a molar ratio of 1:0.8 with a K_d value of 200 nM (Fig.

3A). Previously we have shown that the post-translational modification of Ral increases its affinity for RalBP1 [15]. Under the conditions that the post-translationally modified or unmodified form of Ral bound to RalBP1 stoichiometrically, we measured the GAP activity of RalBP1 for Rac1. In this experiment we used RalBP1 immobilized on amylose resin as a source of GAP and the post-translationally modified form of Rac1 as a substrate. The GAP activity of immobilized RalBP1 was attenuated as compared with that of soluble RalBP1 (compare Fig. 3B with Fig. 4A). RalBP1 interacting with the modified or unmodified form of Ral exhibited the same GAP activity as RalBP1 itself (Fig. 3B). These results indicate that the interaction of Ral with RalBP1 does not affect the GAP activity of RalBP1.

3.4. Effect of the post-translational modifications of Rac1 and CDC42 on the GAP activity of RalBP1

Finally we examined the effect of the post-translational modifications of small G proteins on the GAP activity of RalBP1. We confirmed that GST-Rac1 and GST-CDC42 purified from Sf9 cells are post-translationally modified and those from *E. coli* are post-translationally unmodified by the Triton X-114 phase separation method (data not shown). GTP was hydrolyzed in both the modified and unmodified forms of these small G proteins with the similar efficiency (data not shown). Consistent with previous observations, RalBP1 stimulated the GTPase activity of the post-translationally unmodified form of Rac1 and CDC42 (Fig. 4A,B). RalBP1 was more active on the post-translationally modified form of Rac1 and CDC42 than the unmodified form (Fig.

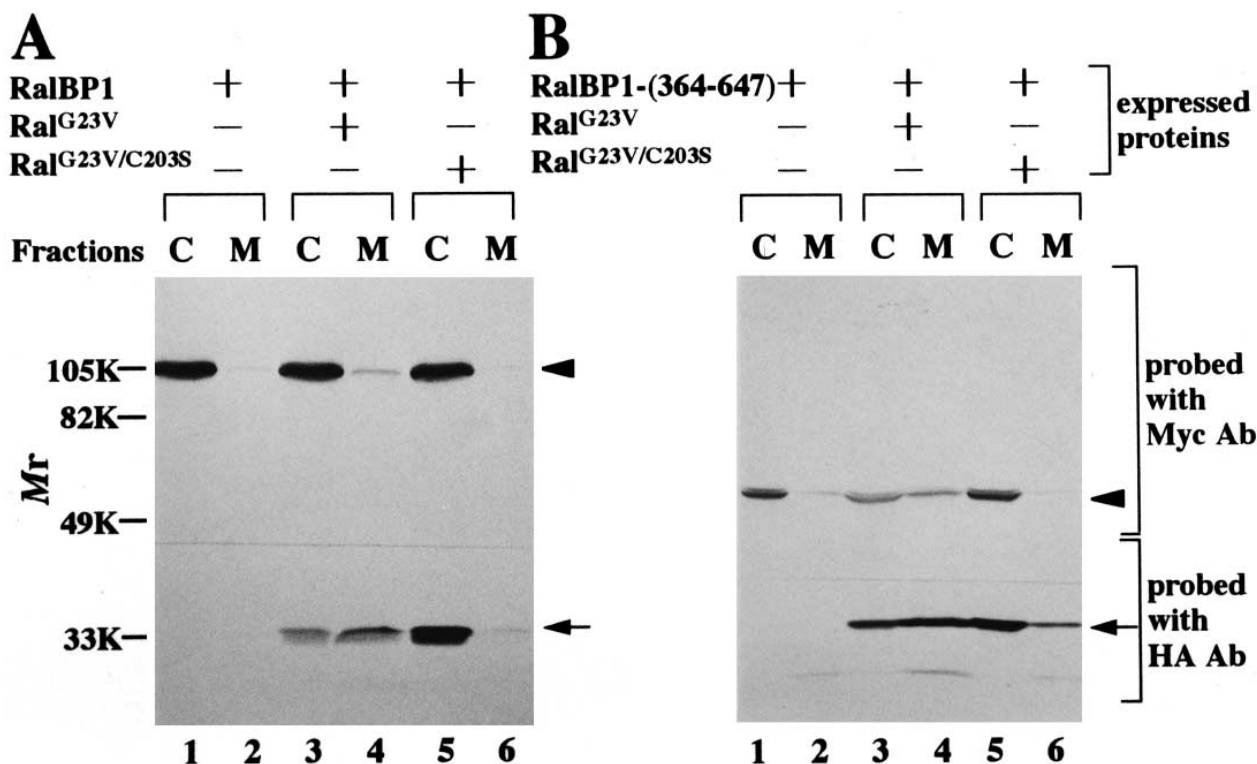


Fig. 2. Translocation of RalBP1 by Ral. A: Translocation of RalBP1 by Ral^{G23V} but not by Ral^{G23V/C203S}. COS cells expressing RalBP1 alone (lanes 1 and 2), both Ral^{G23V} and RalBP1 (lanes 3 and 4), or both Ral^{G23V/C203S} and RalBP1 (lanes 5 and 6) were disrupted and separated into the cytosol (lanes 1, 3, and 5) and membrane (lanes 2, 4, and 6) fractions. Aliquots of each sample were probed with the anti-Myc and HA antibodies. B: Translocation of RalBP1-(364-647) by Ral^{G23V}. COS cells expressing RalBP1-(364-647) alone (lanes 1 and 2), both Ral^{G23V} and RalBP1-(364-647) (lanes 3 and 4), or both Ral^{G23V/C203S} and RalBP1-(364-647) (lanes 5 and 6) were disrupted and separated into the cytosol (lanes 1, 3, and 5) and membrane (lanes 2, 4, and 6) fractions. Aliquots of each sample were probed with the anti-Myc and HA antibodies. C, cytosol fraction; M, membrane fraction; Ab, antibody. Arrowheads indicate the positions of RalBP1 and RalBP1-(364-647). Arrows indicate the positions of Ral^{G23V} and Ral^{G23V/C203S}. The results shown are representative of three independent experiments.

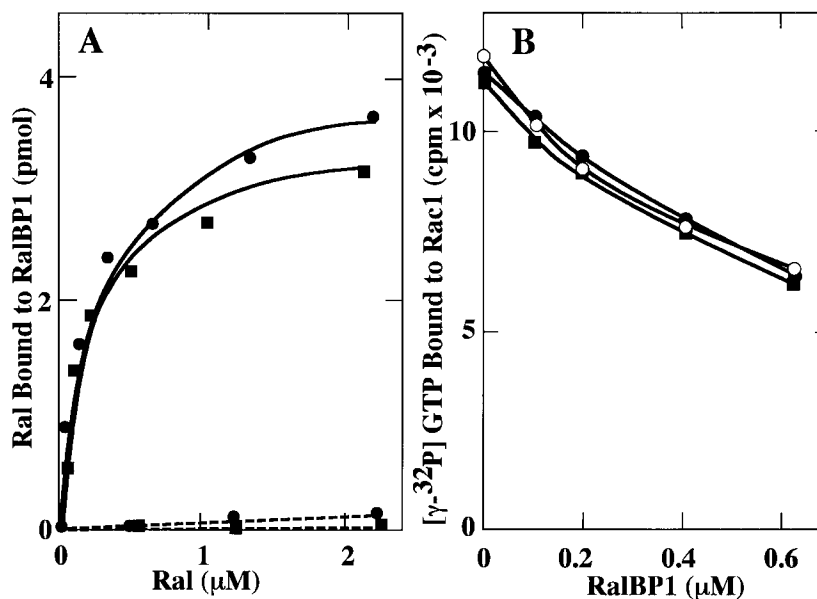


Fig. 3. Effect of the interaction of Ral with RalBP1 on the GAP activity of RalBP1. A: Interaction of Ral with RalBP1 in vitro. RalBP1(●) or RalBP1-(364-647) (■) immobilized on amylose resin (4 pmol of each) was incubated with the indicated concentrations of the [³⁵S]GTPγS- (—) or [³H]GDP- (-----) bound post-translationally unmodified form of GST-RalB. After the samples were precipitated by centrifugation, the remaining radioactivities were counted. B: The GAP activity of RalBP1. The [γ-³²P]GTP-bound post-translationally modified form of GST-Rac1 (4 pmol) was incubated for 5 min at 25°C with the indicated concentrations of immobilized RalBP1 (■) or immobilized RalBP1 stoichiometrically interacting with the GTPγS-bound modified (●) or unmodified (○) form of GST-RalB. The results shown are representative of four independent experiments.

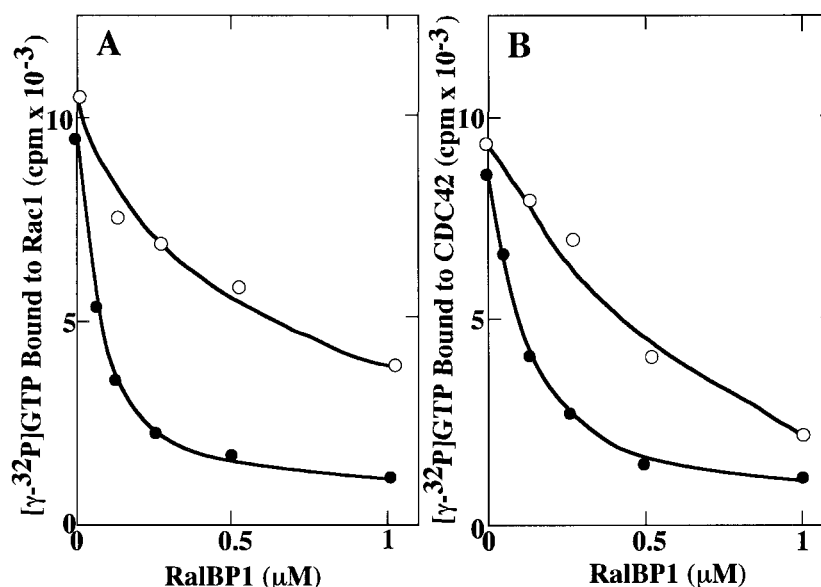


Fig. 4. Effect of the post-translational modifications of small G proteins on the GAP activity of RalBP1. The GAP activities of the indicated concentrations of RalBP1 for the post-translationally modified (●) and unmodified (○) forms of GST-Rac1 or GST-CDC42 (4 pmol of each) were assayed. A: Rac1. B: CDC42. The results shown are representative of three independent experiments.

4A,B). These results indicate that the post-translational modifications of Rac1 and CDC42 enhance the GAP activity of RalBP1.

4. Discussion

RalBP1 has been identified as a putative effector protein of Ral and shown to possess the GAP activity for Rac1 and CDC42 [10–12]. However, it has not yet been shown that RalBP1 interacts with Ral in intact cells. We have demonstrated for the first time that full-length RalBP1 binds to an active form of Ral but not to a dominant negative form of Ral in COS cells. Furthermore, we have shown that RalBP1 stoichiometrically binds to the GTPγS-bound form of Ral with a K_d value of 230 nM. These results strongly support the possibility that RalBP1 is an effector protein of Ral. We have also demonstrated that the interaction of Ral with RalBP1 does not affect the GAP activity of RalBP1 for Rac1 under the condition that RalBP1 binds to Ral stoichiometrically. Taken together with the previous observation that the Ral-binding domain of RalBP1 is not necessary for its GAP activity [10], these results suggest that Ral does not serve as an allosteric regulator of RalBP1 and that there is some mechanism by which Ral influences the functions of Rac1 and CDC42 in cells.

Many signal transduction molecules translocate to the membrane. Ras resides on the plasma membrane through its post-translational modification [13,14,26]. Raf is an important Ras effector protein and usually present in the cytosol. When Ras is activated, Raf translocates from the cytosol to the membrane by binding to Ras and is activated to transmit the signal to mitogen activated protein kinase cascade [27]. Raf which has a membrane-localizing motif is constitutively activated without the Ras activation [28,29]. Thus, a role of Ras in the activation of Raf could be to recruit Raf to the membrane. Consistent with these observations, our results have shown that Ral localized on the membrane induces the translocation of RalBP1 from the cytosol to the membrane.

Ral is present not only in the plasma membrane along with Ras but also in the intracellular vesicles [1]. Rac and CDC42 interact with the membrane and cytoskeleton [30,31] and regulate cell shape and motility [32–34]. Recently Rac has been shown to regulate receptor-mediated endocytosis, especially clathrin-coated vesicle formation [35]. These results suggest that Rac is also present in the intracellular vesicles. Therefore, RalBP1 may act as a negative regulator for Rac1 and CDC42 when these small G proteins are colocalized with Ral on the same sites. Inversely, when RalBP1 translocates to the membrane where Rac1 and CDC42 are not present, Ral may potentiate the activities of Rac1 and CDC42 by removing RalBP1 from a compartment in which it serves as a GAP for Rac1 and CDC42. However, since our experiments have been done by overexpression of RalBP1 and/or Ral in COS cells, we cannot exclude the possibility that endogenous RalBP1 is localized on the membrane, that overexpressed RalBP1 is in the cytosol simply because RalBP1 is already saturated on the membrane, and that addition of Ral^{G23V} takes this excess of RalBP1 to the membrane. Therefore, it is necessary to determine subcellular localization of endogenous Ral and RalBP1 by their specific antibodies.

It has been known that the post-translational modifications of small G proteins are critical for their functions and the actions of their GDP/GTP exchange proteins [14,26]. In contrast to GDP/GTP exchange proteins, the post-translational modifications of small G proteins do not affect the activity of RasGAP, RapGAP, or RalGAP [15,36,37]. However, we have shown that the post-translational modifications of Rac1 and CDC42 enhance the GAP activity of RalBP1. Recently Rab3-GAP has been purified from rat brain and found to be active on the post-translationally modified form of Rab3A but not on the unmodified form [38]. Thus, the roles of the post-translational modifications of small G proteins in the GAP action vary depending on GAP types. Taken together with our previous observations that the post-translational modification of Ras is necessary for the translocation of RalGDS and that the modification of Ral enhances the action of RalGDS [15], it is

conceivable that the modifications of Ras, Ral, and Rac1 play a role not only for their subcellular localization but also for transmitting the signals effectively on the membranes in the signaling pathway of Ras/RalGDS/Ral/RalBP1/Rac1.

The GAP activity of RalBP1 for Rac1 and CDC42 is rather weak as compared with those of other RhoGAP family members. It is known that Raf, Rabphilin-3A, and phospholipase C- β are the effector proteins of Ras, Rab3A, and Gq, respectively, and that they have the weak GAP activities for these G proteins [39–41]. Furthermore, it has been shown that RalBP1 interacts with Rac1 in yeast two-hybrid experiment [11]. These results suggest that RalBP1 may act as an effector protein rather than serve as a negative regulator of Rac1 and CDC42. In addition to the RhoGAP homology domain, RalBP1 has two α -helix regions [10,11]. These regions may associate with other molecules which induce downstream events. Further studies are necessary to understand the whole picture of the physiological roles of the Ral–RalBP1 pathway in the intracellular signal transduction system and cellular functions.

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