Post-translational Modifications of Ras and Ral Are Important for the Action of Ral GDP Dissociation Stimulator*

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Takao Hinoi‡, Shosei Kishida‡, Shinya Koyama, Masahiro Ikeda, Yoshiharu Matsuura§, and Akira Kikuchi¶

From the Department of Biochemistry, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, J apan and the §Department of Virology II, National Institute of Health, 1-23-1 Toyama, Shinjyuku-ku, Tokyo 162, J apan

Ral GDP dissociation stimulator (RalGDS) is a GDP/GTP exchange protein of Ral and a new effector protein of Ras. Therefore, there may be a new signaling pathway from Ras to Ral. In this paper, we examined the roles of the post-translational modifications of Ras and Ral on this new signal transduction pathway. The post-translationally modified form of Ras bound to RalGDS more effectively than the unmodified form. The modification of Ras was required to regulate the distribution of RalGDS in the membranes. In the case of Ral, the post-translational modification of Ral enhanced the activities of RalGDS to stimulate the dissociation of GDP from and the binding of GTP to Ral. Furthermore, the modified form of Ral bound to Ral-binding protein 1 (RalBP1), a putative effector protein of Ral, more effectively than the unmodified form. Taken together with the observations that Ras and Ral are localized to the membranes, these results suggest that the post-translational modifications of Ras and Ral play a role for transmitting the signal effectively on the membranes in the signal transduction pathway of Ras/RaLGDS/Ral/RalBP1.

Ral is a member of small G protein superfamily and consists of RalA and RalB (1). Ral cDNA has been originally isolated by screening the simian B lymphocyte cDNA library using one of the consensus sequences of the GDP/GTP binding domains of Ras (2). Subsequently, Ral cDNA has been isolated from human placental, human pheochromocytoma, and marine ray electron lobe cDNA libraries (3–6). Northern blot analysis has demonstrated that RalA and RalB mRNAs are ubiquitously expressed with the high expression level in brain and testis (6, 7). Immunohistochemical and subcellular fractionation analyses have shown that Ral is localized to synaptic vesicles in synapses, and this small G protein has been purified from platele membranes (4, 5, 8). 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, and inversely the GTP-bound form is changed to the GDP-bound form by RalGAP (9, 10). The GTP-bound form of Ral could interact with the effector protein that can mediate Ral-dependent processes. However, the modes of activation and action of Ral have not been clear.

We and other groups have found that RaLGDS family members of RaLGDS and RGL are the putative effector proteins of Ras and proposed that there is a new signaling pathway from Ras to Ral through RaLGDS (11–13), since RaLGDS stimulates the GDP/GTP exchange of Ral (10). Furthermore, we have demonstrated that protein kinase A inhibits the Ral activation but not the interaction of Ras with RaLGDS induced by an extracellular signal and that Rap1 regulates the interaction of Ras with RaLGDS (14, 15). Therefore, protein kinase A and Rap1 selectively regulate the signal from Ras to either Ral or RaLGDS. Although the functions of Ral have long remained elusive, RalBP1 (also designated as RLIP (Ral-interacting protein) and RalGDS) has been found to be an effector protein of Ral (16–18). RalBP1 shares the domain homologous with RhoGAP and indeed exhibits the GAP activity for CDC42 and Rac (16–18). Rap1 also binds to PLD and is required for Ras-dependent PLD activity (19). These results suggest that the signaling pathway from Ras to Ral leads to the regulation of the actions of CDC42, Rac, and PLD. Thus, it is possible that RaLGDS is an important effector protein of Ras in the Ras-mediated signaling pathways as well as Ral.

Most of small G proteins undergo the post-translational modifications at the C-terminal regions (20, 21). The formation of thioether bonds between cysteine residues and isoprenyl groups derived from pyrophosphate intermediates of the cholesterol metabolic pathway is important (20–22). Two kinds of isoprenyl groups have been known: 15-carbon farnesyl and 20-carbon geranylgeranyl. The post-translational modifications of Ras have been extensively studied (23–25). The C-terminal region of Ras has a CAAX motif (where C is cysteine, A is aliphatic amino acid, and X is methionine or serine). Ras is first farnesylated at the cysteine residue in the C-terminal region, next the three amino acids C-terminal to the cysteine are removed, and finally the exposed cysteine is carboxymethylated. In the case of Ha-Ras and N-Ras, there is another cysteine on the N-terminal side of the farnesylated cysteine.
and this second cysteine is palmitoylated. Ki-Ras does not have the second cysteine, but it has a polybasic region on the N-terminal side of the farnesylated cysteine instead of the second cysteine. Among these post-translational modifications, farnesylation of Ras is most important for its transformation and membrane binding activities. Furthermore, it has been shown that the post-translational modification of Ki-Ras is required for the activation of yeast adenylate cyclase and mitogen-activated protein kinase (26, 27) and that those of Ha-Ras and Ki-Ras enhance the activities of smgMDS, mCDC25, and hSOS, which are GDP/GTP exchange proteins of Ras (28–30). We also found that the post-translational modification of Ha-Ras is essential for the Raf activation (31). However, the role of the post-translational modification of Ras on the RalGDS action is not known. Ral has a C-terminal L (L is leucine) and has been found to be geranylgeranylated (32). The observation that Ral is localized to the membranes suggests that Ral binds to the membranes through its post-translational modification (1, 4, 5, 8). However, other roles of the post-translational modification of Ral are not clear.

As described above, the signal from Ras to Ral could regulate various cell functions. In this paper we examined the roles of the post-translational modifications of Ras and Ral on this new signaling pathway. We show that the post-translational modifications of Ras and Ral enhance their binding activities to the effector proteins, RalGDS and RalBP1, that the modification of Ras is necessary for the localization of RalGDS on the membranes in intact cells, and that the modification of Ral is important for the RalGDS action but not for the RalGAP action. Furthermore, we show the actions of RalGDS and RalGAP for various Ral mutants.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The RalGDSs and RalB DNAs were provided by Dr. R. Weinberg (Whitehead Institute for Biomedical Research) (10). The c-Ha-Ras and RalAG23V cDNAs were provided by Drs. J. Downward (Imperial Cancer Research Institute, London, United Kingdom) and P. Chardin (Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, France), respectively (33). Mammalian expression vectors, pB11-1 and pCGN, and the mouse anti-influenza virus HA monoclonal antibody 12CA5 were provided by Dr. Q. Hu (University of California San Francisco) (34). pCGN was designed to express a 16-amino acid epitope from influenza virus HA fused to protein, pIKS were provided by Dr. D. Mirza (University of California San Francisco) (31). pV-KS was designed to express GST and HA fused to proteins in SF9 cells. All procedures of passage, infection, and transfection of SF9 cells and the isolation of baculoviruses were carried out as described (35). RasG12V (in which valine was substituted for glutamic acid at position 12) and RasG12V (in which the terminal four amino acids of RasG12V were deleted) were synthesized as described (15, 31). RalG12D, RalB14D, and RalB14G were synthesized by PCR (36). The RalBP1 cDNA was isolated by reverse transcriptase PCR using rat brain mRNA as a template. pMALc-2 is purchased from New England Biolabs (Beverly, MA). COS cells were obtained from the American Type Culture Collection. The anti-Ras antibodies, Y13–238 for immunoprecipitation assay and F22S for immunoblot analysis, were purchased from Science Inc. (Uniondale, NY) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. [35S]GTPgS, [3H]GDP, and [γ-32P]GTP were from DuPont NEN.

Plasmid Constructions—pGEX/RalB, pCGN/RalGDS, and pB11-1RasG12V were constructed as described (15). To construct pMAL-RalGDS-(764–864), the 0.3-kb fragment encoding RalGDS-(764–864) with BamHI sites at 5’- and 3’-ends was synthesized by PCR. The fragment was digested with BamHI and inserted into the BamHI cut pMAL-c2 to generate pMAL/RalGDS-(764–864). To construct pMAL-RalBP1-(364–647), the full length of RalBP1 cDNA with BamHI sites synthesized by reverse transcriptase PCR was digested with BamHI and inserted into the BamHI cut pMAL-c2 to generate pMAL/RalBP1-(364–647). To construct pGEX-2T encoding RalGDS, pGAD/RalGDS (11) was digested with BamHI and EcoRI and then blunted with Klenow. The 2.7-kb fragment encoding RalGDS was inserted into pGEX-2T which was digested with EcoRI and then blunted with Klenow. To construct pV-KS/RalB, the 0.6-kb fragment encoding RalB with XbaI and PstI sites was synthesized by PCR. This fragment was digested with EcoRI and PstI and inserted into the EcoRI and PstI cut pV-KS to generate pV-KS/RalB. To construct pB11-1RalAG23V, pVL1393/RalAG23V was digested with XbaI and PstI and then blunted with T4 DNA polymerase. This fragment was inserted into pB11-1 which was digested with EcoRI and then blunted with Klenow to generate pB11-1RalG12V. To construct pGEX-2T encoding RalGDS-(764–864) with XbaI and BamHI sites was synthesized by PCR. This fragment was digested with XbaI and BamHI, blunted with mung bean nuclease, and then inserted into the Smal cut pGEX-2T to generate pGEX/RalB14D. To construct pGEX-2T encoding RalBP1-(364–647) with BamHI sites was synthesized by PCR. These fragments were digested with BamHI and inserted into the BamHI cut pGEX-2T to generate pGEX/RalB14G and pGEX/RalG12V.

Expression and Purification of GST Fused to Proteins and MBP Fused to Proteins from SF9 Cells and Escherichia coli—to Purify GST fused to RalB—RalGDS (GST-RalGDS) from SF9 cells, monolayers of SF9 cells (2 × 106 cells) were infected with high-titer recombinant baculoviruses (1 × 108 plaque-forming units/ml) at a multiplicity of infection of 5/cell. After 72-h post-infection, the cells were washed with cold phosphate-buffered saline and suspended in 1 ml of Buffer A (20 mM Tris/HCl (pH 7.5), 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 10 μg/ml leupeptin). The suspension was sonicated and incubated at 0–4°C for 5 min to remove unbroken cells and nuclei. The homogenate was centrifuged at 100,000 × g for 30 min. The supernatant was used as the cytosolic fraction. The pellet was resuspended in 1 ml of Buffer A containing 1% CHAPS, rocked for 1 h, and then centrifuged at 100,000 × g for 30 min. The supernatant was used as the membrane fraction. The post-translationally modified and unmodified forms of GST-Ras or GST-Ral were purified from the membrane and cytosol fractions, respectively, using glutathione-Sepharose 4B columns in accordance with the manufacturer’s instructions. To purify MBP fused to RalGDS-(764–864) (MBP-RalGDS-(764–864)) and MBP fused to RalBP1-(364–647) (MBP-RalBP1-(364–647)) from E. coli, transformed E. coli were initially grown at 37°C in LB medium to an absorbance of 0.8 (optical density at 600 nm) and subsequently transferred to 25°C. Ten isopyropyl-1-β-thiogalactopyranoside was added at a final concentration of 100 μM, and further incubation was carried out for 10 h at 25°C. To purify GST fused to RalGDS (GST-RalGDS) from E. coli, transformed E. coli were initially grown at 37°C in LB broth containing 1% glucose to an absorbance of 1.0 (optical density at 600 nm) and subsequently transferred to 25°C. Then isopyropyl-1-β-thiogalactopyranoside was added at a final concentration of 40 μM and further incubation was carried out for 4 h at 25°C. GST fusion to NF1 (GST-NF1) and RalB (GST-RalB) were produced in E. coli as described (11, 15). The GST and MBP fused to proteins were purified from E. coli by affinity chromatographies in accordance with manufacturer’s instructions.

To purify GST fused to proteins, Partial Purification of RalGAP—Bovine brain was obtained from the heads of freshly slaughtered cattle and frozen at −80°C until use. Cerebral tissue (approximately 140 g, wet weight) was homogenized in a Potter-Evemhen Teflon-glass homogenizer with 280 ml of Buffer B (25 mM Tris/HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 20,000 × g for 30 min, and the supernatant was centrifuged again at 100,000 × g for 1 h. The supernatant (200 ml, 1500 mg of protein) was applied to DE52 column (4.3 × 11 cm) equilibrated with Buffer B. After
the column was washed with 800 ml of Buffer B and 480 ml of Buffer B containing 100 mM NaCl, the elution was performed with Buffer B containing 200, 300, and 500 mM NaCl in a stepwise manner. All the Rap1 GAP activity appeared in the fractions eluted by 200 mM NaCl. Solid (NH₄)₂SO₄ was added to this eluate (24 ml, 144 mg of protein) at a final concentration of 40% saturation. The sample was centrifuged at 20,000 x g for 20 min. Most RapGAP activity was recovered in the precipitate. The precipitate was dissolved in 2.4 ml of Buffer B. After dialysis overnight against a large volume of Buffer B, the sample was centrifuged at 100,000 x g for 1 h. The supernatant (2 ml, 40 mg of protein) was applied to a Sephadryl S-300 column (1.5 x 86 cm) eluted with Buffer B and fractions of 2 ml each were collected. RapGAP activity appeared in Fractions 34–43. The partial purified RapGAP did not contain the GAP activity for Ras or Rho, but contained the weak GAP activity for Rap1.

Fractions 34–43. The partial purified Rap1 GAP did not contain the GAP activity for Rho. The sample was centrifuged at 100,000 x g for 1 h. The supernatant (2 ml, 40 mg of protein) was applied to a Sephadryl S-300 column (1.5 x 86 cm) eluted with Buffer B and fractions of 2 ml each were collected. Rap1 GAP activity appeared in Fractions 34–43. The partial purified Rap1 GAP did not contain the GAP activity for Ras or Rho, but contained the weak GAP activity for Rap1. Localization of Rap1GDS Regulated by Ras in COS Cells—COS cells (60–70% confluent in a 10-cm dish) were transfected with pCGN/Ral-

Pellet was resuspended in 1 ml of Buffer A containing 1% Nonidet P-40, rocked for 1 h, and then centrifuged at 100,000 x g for 30 min. The supernatant was used as the membrane fraction. Aliquots (200 μg of protein) of the cytosol and membrane fractions were subjected to SDS-PAGE and probed with the anti-HA or Ras (F235) antibody. For immunoprecipitation assay, aliquots (200 μg of protein) of the cytosol and membrane fractions were immunoprecipitated with the anti-Ras antibody (Y13–238), subjected to SDS-PAGE, and probed with the anti-HA or Ras (F235) antibody as described (11, 31).

Binding Assays of Ras and Ral to Their Effector Proteins—The assay for the interaction of Ras and Ral with their effector proteins was carried out as described (14). To make immobilized RalGDS-(764–864) and RalBP1-(364–647) on amylose resin, MBP-RalGDS-(764–864) or MBP-RalBP1-(364–647). To make immobilized RalGDS-(764–864) with that of the unmodified form to MBP-

GST-Ral (5 pmol) was incubated for 10 min at 30°C in 20 mM Tris/HCl (pH 7.5), 2 mM MgCl₂, 10 mM EDTA, 0.3% CHAPS, 1 mM DTT, and 1 mg/ml BSA). After the incubation, 1 μl of 340 mM MgCl₂ was added. To this preincubation mixture, 30 μl of reaction mixture (50 μl Tris/HCl (pH 7.5), 1.3 mM GTP, 0.3 mM MgCl₂ and 27 mg/ml BSA) containing GST-NF1 (0.8 pmol) was added, and the second incubation was performed for 15 min at 24°C in the presence of the indicated concentrations of MBP-RalGDS-(764–864). Assays were quantified by rapid filtration on nitrocellulose filters. The GAP activity was calculated from the decrease of the radioactivity of [γ-32P]GTP compared with the reaction performed in the absence of GST-NF1, and the GAP inhibition activity was expressed as percent decrease of the GAP activity.

Rap1 GAP Assay—The Rap1 GAP activity was assayed by the method described above except that GST-Ral (3 pmol), RapGAP (7 μg of protein), and MBP-RalBP1 (364–647) were used instead of GST-Ras, GST-NF1, and MBP-RalGDS-(764–864). The second incubation was performed for the various periods of time at 30°C.

Other Assays—The K₅₀ for GDP or GTP γs of, dissociation rate of GDP from, and steady-state and actual catalytic rates of GTP hydrolysis of Ral mutants were determined as described (39–41).

RESULTS

Purification of the Post-translationally Modified and Unmodified Forms of Ras and Ral—To examine the roles of the post-translational modifications of Ras and Ral on the signal pathway from Ras to Ral, we produced GST-Ras and GST-Ral in Sf9 cells and purified them from both the cytosol and membrane fractions of Sf9 cells. These proteins were tagged with an HA epitope. By the Triton X-114 phase-partition method, the proteins purified from the cytosol fraction of Sf9 cells were recovered in the aqueous phase, while the proteins purified from the membrane fraction were recovered in the detergent-enriched phase (Fig. 1). These results indicate that GST-Ras and GST-Ral purified from the cytosol fraction of Sf9 cells are post-translationally unmodified and those from the membrane fraction are post-translationally modified.

Effect of the Post-translational Modification of Ras on Its Binding to RapGDS—We originally found that RGL was an effector protein of Ras and that the C-terminal region of RGL (RGL–(632–734)) bound to the post-translationally unmodified GTP-bound form of Ras (11, 42). The C-terminal region of RapGDS (RapGDS-(764–864)) shared high homology with RGL-(632–734) and was found to bind to the post-translationally unmodified GTP-bound form of Ras (12). However, the effect of the post-translational modification of Ras to its binding to RapGDS was not yet studied. We compared the binding activity of the post-translationally modified forms of Ras to MBP-RalGDS-(764–864) with that of the unmodified form to MBP-RalGDS-(764–864). The post-translationally modified form of...
Ras bound to RalGDS-(764–864) two to three times more than the unmodified form (Fig. 2A). When 25 pmol of the modified and unmodified forms of Ras were used in this assay, 1.5 pmol of the modified form and 0.8 pmol of the unmodified form bound to 20 pmol of MBP-RalGDS-(764–864). As consistent with the previous observations (11, 13), RalGDS-(764–864) inhibited the GAP activity of NF1 for the post-translationally modified form of Ras (Fig. 2B). Furthermore, RalGDS-(764–864) inhibited the GAP activity of NF1 for the post-translationally modified form more effectively (Fig. 2B). The IC_{50} values of the GAP inhibitory activity of RalGDS-(764–864) for the modified and unmodified forms are 170 and 350 nM, respectively. These results suggest that the post-translational modification of Ras may render it effectively bind to RalGDS.

Localization of RalGDS Regulated by Ras in COS Cells—Next, we examined the effect of the post-translational modification of Ras on its interaction with RalGDS in intact cells. When Ras^{G12V} was expressed in COS cells, the unmodified and modified forms of Ras appeared in the cytosol and membrane fractions, respectively (Fig. 3A, lanes 3 and 4). The modified form migrated faster than the unmodified form on SDS-PAGE. On the other hand, Ras^{G12V1A} appeared in the cytosol fraction but not in the membrane fraction (Fig. 3A, lanes 5 and 6). When RasGDS alone was transfected into COS cells, most of RalGDS, which was tagged with an HA epitope at the N terminus, was detected in the cytosol fraction of COS cells (Fig. 3A, lanes 1 and 2). When RasGDS was transfected with Ras^{G12V}, RalGDS was detected in both the cytosol and membrane fractions, while when RasGDS was transfected with Ras^{G12V1A}, most of RalGDS was still recovered in the cytosol fraction (Fig. 3A, lanes 3–6). We also examined whether RalGDS interacted with Ras on the membranes. When the lysates expressing RasGDS alone were immunoprecipitated with the anti-Ras antibody, RalGDS was not detected in the precipitates (data not shown). As expected, RasGDS made a complex with Ras in both the cytosol and membrane fractions of COS cells expressing RalGDS and Ras^{G12V} (Fig. 3B, lanes 1 and 2), while RasGDS made a complex with Ras in the cytosol fraction but not in the membrane fraction of COS cells expressing RalGDS and Ras^{G12V1A} (Fig. 3B, lanes 3 and 4). As consistent with the previous observations (11, 15), nonimmune immunoglobulin did not immunoprecipitate a RalGDS-Ras complex (data not shown). These results suggest that the modified form of Ras localized to the membranes may recruit RalGDS from the cytosol to the membranes, resulting in placing RalGDS in the vicinity of Ras, which is also present on the membranes through its post-translational modification.

Effect of Mutations of Ral on the Actions of RalGDS and RalGAP.—To analyze the role of the signaling pathway from Ras to Ral, Ras mutants are useful tools. However, the characterization of these mutants has not yet been systematically done. We constructed wild type Ral and four Ral mutants (Ral^{G23V}, Ral^{G86E}, RalT46A, and Ral^{G86E}) in bacterial expression vector. These Ral mutants correspond to Ras mutants, Ras^{G23V}, Ras^{G12V1A}, Ras^{G12V}, and Ras^{G86E}. The characterization of these Ral mutants were summarized in Table I. The K_{cat} values of wild type Ral for GDP and GTP·Y·S were similar and about 20 nM. Ral^{G23V} and RalT46A also showed the similar K_{cat} values for both GDP and GTP·Y·S. The affinity of Ral^{G23V} for GDP was 10-fold higher than for GTP·Y·S, while Ral^{G86E} exhibited the higher affinity for GTP·Y·S than GDP. The GDP dissociation constants (K_{d}) of Ral mutants were variable. The K_{d} values of wild type Ral for GDP and GTP·Y·S were 0.0043, 0.0011, 0.13, 0.0067, and 0.017, respectively. RalGDS stimulated the dissociation of GDP from Ral 10-fold and showed the similar effect on Ral^{G23V}.

RalGDS stimulated the dissociation of GDP from Ral^{G23V} 4-fold, but affected that from Ral^{G28N} or Ral^{G86E} very little. The steady-state rates (K_{cat}) of GTPase activity of Ral, Ral^{G23V}, Ral^{G86E}, and Ral^{G86E} were 0.0043, 0.0011, 0.13, 0.0067, and 0.0017, respectively. RalGDS stimulated the dissociation of GDP from Ral 10-fold and showed the similar effect on Ral^{G23V}.

### Figure 2

**Effect of the post-translational modification of Ras on its interaction with RalGDS.**

*Panel A:* Interaction of Ras with RalGDS. The modified (●) and unmodified (□) [γ-32P]GTP-bound forms of Ras (125 or 250 nM) were incubated with immobilized RalGDS-(764–864) (200 nM). Radioactivities bound to the resin were counted. *Panel B:* Inhibition of NF1 by RalGDS. The modified (●) and unmodified (□) [γ-32P]GTP-bound forms of Ras (75 nM) was incubated for 15 min with the indicated concentrations of MBP-RalGDS-(764–864) in the presence or absence of GST-NF1 (20 nM). The results shown are representative of three independent experiments.

### Figure 3

**Localization of RalGDS regulated by Ras in COS cells.**

*Panel A:* Translocation of RalGDS by Ras^{G12V} but not by Ras^{G12V1A}. COS cells expressing RalGDS alone (lanes 1 and 2), both RalGDS and Ras^{G12V} (lanes 3 and 4), or both RalGDS and Ras^{G12V1A} (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 subjected to SDS-PAGE and probed with the anti-HA and Ras (F235) antibodies. *Panel B:* Interaction of Ras with RalGDS on the membranes. The proteins of the cytosol (lanes 1 and 3) and membrane (lanes 2 and 4) fractions of COS cells expressing both RalGDS and Ras^{G12V} (lanes 3 and 4) were immunoprecipitated with the anti-Ras antibody (Y13–238). The precipitates were subjected to SDS-PAGE and probed with the anti-HA and Ras (F235) antibodies. Ig, immunoglobulin. The Arrowhead and arrow indicate the positions of RalGDS and Ras, respectively. The results shown are representative of three independent experiments.
GTP-γS with the similar efficiency (data not shown). GDP was dissociated from both the modified and unmodified forms of Ral with the similar efficiency in a time-dependent manner (Fig. 4A). The action of RalGDS to stimulate the dissociation of GDP from Ral was more effective on the modified form of Ral than on the unmodified form (Fig. 4A). RalGDS stimulated the binding of GTP-γS to the modified form of Ral more effectively than to the unmodified form (Fig. 4B). When the binding of GTP-γS to Ral was assayed in the presence of RalGDS (50 nm) using various amounts of Ral, the apparent Kd values of RalGDS for the modified and unmodified forms of Ral were estimated to be 380 nM and 3.2 μM, respectively. The Vmax values of RalGDS for the modified and unmodified forms were 1.5 and 1.6 nmol/min/nmol, respectively. These results taken together with the results of Table I suggest that the post-translational modification of Ral is not essential for the action of RalGDS but that the modification is important for increasing the affinity of RalGDS for Ral. GTP was hydrolyzed in both the modified and unmodified forms of Ral with the similar efficiency in a time-dependent manner (Fig. 5). In contrast to the case of RalGDS, the post-translational modification of Ral did not affect the RalGAP activity (Fig. 5).

Effect of the Post-translational Modification of Ral on the Binding to RalBP1—RalBP1 has been identified as an effector protein of Ral by yeast two-hybrid system (16–18). The Ral-binding domain of RalBP1 has been found to be localized in its C-terminal half. We purified Ral-binding domain of RalBP1 as MBP-RalBP1(364–647). As consistent with the previous observations (16–18), the post-translationally unmodified GDP-bound form of Ral interacted with RalBP1(364–647) (Fig. 6A). The post-translationally modified form of Ral bound to RalBP1(364–647) two to three times more than the unmodified form (Fig. 6A). When 25 pmol of the modified and unmodified forms of Ral were used in this assay, 2.5 pmol of the modified form and 1.2 pmol of the unmodified form bound to 20 pmol of MBP-RalBP1(364–647). RalBP1 has been shown not to bind to Ral(349), which is a mutant in the putative effector loop (16). As shown in Table I, RalGAP did not act on Ral(346), which is also a mutant in the putative effector loop. These results indicate that RalGAP and RalBP1 share a common binding domain on Ral. We examined the effect of RalBP1 on the RalGAP activity for the post-translationally modified and unmodified forms of Ral. RalBP1(364–647) slightly inhibited the endogenous GTPase activities of both the modified and unmodified forms of Ral with the similar efficiency (data not shown). RalBP1(364–647) inhibited the RalGAP activity in a dose-dependent manner (Fig. 6B). This inhibitory action of RalBP1(364–647) was stronger for the modified form of Ral than for

| Kd (nm) | GDP dissociation constant | GTPase sensitivity | GDPase activity | GAP sensitivity |
|--------|--------------------------|-------------------|-----------------|----------------|
|        | GDP-γS                   | −GDS              | +GDS            | −GAP           | +GAP           |
|        |                         | Kd (min⁻¹)        |                 |                |                |
| Ral(346)          | 20 ± 2                   | 22 ± 1            | 4.3 ± 0.1       | 44 ± 2         | 10 ± 2         |
| Ral(348)          | 50 ± 3                   | 50 ± 2            | 1.1 ± 0.1     | 4.5 ± 0.3     | 4.0 ± 2        |
| Ral(349)          | 64 ± 4                   | 667 ± 8           | 130 ± 10       | 250 ± 30      | 1.9 ± 0.4      |
| Ral(350)          | 17 ± 1                   | 17 ± 2            | 6.7 ± 0.5      | 67 ± 5        | 10 ± 2         |
| Ral(351)          | 647 ± 7                  | 116 ± 5           | 1.7 ± 0.2      | 2.8 ± 0.2     | 1.6 ± 0.3      |

*ND = not determined.*

Table I Characterization of Ral mutants

The actual catalytic rate (Kcat) of GTPase activity was determined in the presence or absence of RalGAP (7 μg of protein) as described (39). GTPase activity of Ral(348) was not determined because most of [γ-32P]GTP was dissociated during these assays. The results shown are means ± standard deviations of three independent experiments.
form. Since the fied form of RalGDS stimulates the GDP/GTP exchange of the modified form more effectively than that of the unmodified form (43, 44). RalGDS and RalBP1, two to three times more than the unmodified forms. We have also shown that RalGDS and RalBP1 inhibit the activities of NF1 and RalGAP, respectively. The inhibitory actions of RalGDS and RalBP1 are more effective on the modified form than on the unmodified form. These results suggest that the post-translational modifications of Ras and Ral may increase their affinities for the effector proteins.

We have not yet demonstrated whether the bindings of Ras to RalGDS and Ral to RalBP1 regulate the activities of RalGDS and RalBP1. The binding of Ras to Ral is not sufficient for the activation of Ral (31). The role of the post-translational modification of Ras to activate Raf could be to bring Raf to the membranes where some activator may be present (52, 53). Therefore, the post-translational modifications of Ras and Ral might be important for the subcellular localization of RalGDS and RalBP1, resulting in transmitting the signals to downstream molecules. Indeed, our results have demonstrated that the post-translational modification of Ras is necessary for the localization of RalGDS on the membranes. Since Ral is localized to the membranes through the post-translational modification, it is possible that RalGDS on the membranes acts on Ral. RalBP1 has been found to show the GAP activities for CDC42 and Rac (16–18). CDC42 and Rac regulate the cytoskeleton and the stress-activated protein kinase activity (54–57). Since CDC42 and Rac are also localized to the membranes, Ral might regulate the subcellular localization of RalBP1 to act on CDC42 and Rac on the membranes. It has been reported that RalGDS-mediated GDP/GTP exchange of Raf is activated by Ras in COS cells and that a RalGDS mutant in which Ras-interacting domain is deleted fails to respond to Ras (58). These results indicate that Ras-induced RalGDS activation is dependent upon the two protein binding and that Ras mediates the redistribution of RalGDS to Raf. This is analogous to the mechanism to receptor activation of SOS, a GDP/GTP exchange protein of Ras (59, 60). SOS is in a complex with Grb2 in the cytosol in the absence of growth factors. After a growth factor induces autophosphorylation of its receptor, the Grb2-SOS complex translocates from the cytosol and associates with the receptor on the membranes thereby placing it in the vicinity of Ras. Therefore, our results suggest that the post-translational modifications of Ras and Ral are also important for regulating the localization of their effector proteins in the cells, resulting in bringing them into contact with their substrates on the membranes. However, since our experiments have been done by overexpression of RalGDS and/or Ras in COS cells, we cannot exclude the possibility that endogenous RalGDS is localized on the membranes, that overexpressed RalGDS is in the cytosol simply because RalGDS is already saturated on the membranes, and that addition of RasG12V takes this excess RalGDS to the membranes. Therefore, it is necessary to determine subcellular localization of endogenous RalGDS.

We have also characterized several Ras mutants. RasS17N, RasT24N, RasG12A, and RasG12V are analogous to RasG12V, RasG12N, and RasG12E respectively. RasG12N exhibits a more effective than on the unmodified form (29, 30). RabGDI and RhoGDI inhibit the GDP/GTP exchange of the modified forms of the members of Rab subfamily and Rho subfamily, respectively, but not that of the unmodified forms (43, 44). Consistent with these observations, we have demonstrated that RalGDS 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 RalGDS for the modified form of Ral is smaller than that for the unmodified form, the post-translational modification of Ral could increase the affinity of RalGDS for Ral. In contrast to RalGDS, RalGAP activity is not affected by the post-translational modification of Ral. These results are also consistent with the previous observations that the post-translational modifications of small G proteins do not affect the activities of RasGAP, RapGAP, and RhoGAP (44–46).

It has been known that the post-translational modifications of small G proteins are critical for their functions (21, 23). Deletion or mutation of the C-terminal region of Ras abolishes the transforming activity (21, 23, 47). The modified form of Ras stimulates adenylate cyclase more effectively than the unmodified form in vitro (26). The modified form of Ras activates B-Raf in the presence of 14-3-3 protein, but the unmodified form does not in vitro (48). We have also found that the post-translational modification of Ras is essential for the Raf activation in intact cells (31). Therefore, the post-translational modifications of Ras and Ral may increase their affinities for the effector proteins.
higher affinity for GDP than GTP and markedly increases the intrinsic GDP dissociation activity thereby suppressing the effect of RalGDS. RalGDS shows a higher affinity for GTP than GDP and is not sensitive to RalGDS. These properties of RalGDS and RalGDS are similar to those of Ras and Ras, indicating that Gly is important for the interaction with hSOS or mCDC25. By analogy with Ras, it is likely that Ser of Ral is required for RalGDS stimulation and that Gly of Ral is important for RalGDS binding. However, RalGDS acts on Ral as well as on wild-type Ras. This result is different from the case ofcdc25 and Ras, sincecdc25 does not stimulate the GDP/GTP exchange of Ras (64, 65). Although the functions of Ral are not clear, it is possible that Ser of Ral is required for RalGDS stimulation and that Gly75 of Ras is important for RalGDS binding. However, RalGDS acts on Ral as well as on wild-type Ras. This result is different from the case ofcdc25 and Ras, sincecdc25 does not stimulate the GDP/GTP exchange of Ras (64, 65).

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