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A NOVEL TYPE OF REGULATORY PROTEIN FOR THE GDP/GTP EXCHANGE REACTION OF *rho* p21, A *ras* p21-LIKE SMALL GTP-BINDING PROTEIN, IN RABBIT INTESTINE*

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INDEXING WORDS

rho p21; *ras* p21; the GDP/GTP exchange protein

SYNOPSIS

A novel regulatory protein for *rho* p21, a *ras* p21-like GTP-binding protein (G protein), was purified from the cytosol fraction of rabbit intestine. This protein, designated as *rho* GDP dissociation inhibitor (GDI), regulated the GDP/GTP exchange reaction of *rho* p21 by inhibiting the dissociation of GDP from and subsequent binding of GTP to it. *rho* GDI did not affect the GTPase activity of *rho* p21. *rho* GDI formed a complex with the GDP-bound form of *rho* p21 but not the GTP-bound form. *rho* GDI was inactive for other small G proteins including *ras* p21, *smg* p21 and *smg* p25A. These results indicate that *rho* GDI is a novel type of regulatory protein for the GDP/GTP exchange reaction of *rho* p21.

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**Abbreviation used are: G proteins, GTP-binding proteins; GAP, GTPase-activating protein; DTT, dithiothreitol; GTPγS, guanine 5'-(3-O-thio)triphosphate; EGTA, [ethylene-bis-(oxyethylenenitrilo)]tetraacetic acid.

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INTRODUCTION

rho p21 family belongs to a superfamily of *ras* p21/*ras* p21-like G proteins** of small molecular weights [2,34] and occurs widely in various species from yeast to man.[7,23,42] This family is composed of three highly homologous members, A, B and C.[7,23,42] All the members show Mr values of about 22,000 and have consensus amino acid sequences for GTP/GDP-binding and GTPase activities.[7,23,42] We have purified first *rho*B p21 and subsequently *rho*A p21 from bovine brain membranes.[13,41] The subcellular distribution analysis of *rho*B p21 in rat brain has shown that *rho*B p21 is mainly localized in synapses where it is found in synaptic plasma membranes but not in the cytosol.[21] *rho*A p21 has been purified from bovine adrenal gland cytosol and bovine aortic cytosol, and an unidentified *rho* p21 has been also purified from porcine brain cytosol.[5,15,26] These purified *rho* p21s indeed exhibit both GTP/GDP-binding and GTPase activities.[5,13,15,26,41] These results indicate that *rho* p21 is present in both membrane and cytosol fractions.

Although the definitive functions of *rho* p21 have not been clarified, several possible functions have been reported. Disruption of the yeast *RHO1* has been shown to be lethal for this microorganism.[24] Overexpression of the *rho*A gene in NIH/3T3 cells has been shown to reduce serum dependence for growth and to be tumorigenic when inoculated into nude mice.[1] *rho* p21 has been shown to be ADP-ribosylated by botulinum ADP-ribosyltransferase C3.[5,8,20,26] Botulinum ADP-ribosyltransferase C3 induces the morphological changes of several types of cells presumably through the changes of cytoskeletal components.[8,30] Microinjection of *rho*A p21 also affects the cytoskeletal system.[29]

By analogy with *ras* p21,[2] it is considered that *rho* p21 has the GDP-bound inactive and GTP-bound active forms and that there are a protein converting the inactive form to the active form and an effector protein whose function is modulated by the active form. A protein stimulating the GTPase activity of c-*ras* p21, designated as *ras* GAP, has recently been isolated

from several mammalian tissues and characterized.[10,35,36,39] A GAP for *rho* p21, designated as *rho* GAP, has been also isolated from human spleen, bovine brain and bovine adrenal gland.[9,25,40] Moreover, we have isolated a GAP for *smg* p21, designated as *smg* GAP, from human platelets and bovine brain.[20,37] *smg* p21 is another small G protein.[16,27]

In the present studies, we have found not only the *rho* GAP, *ras* GAP and *smg* GAP described in other tissues [9,10,20,25,35-37,39,40] but also another type of regulatory protein for *rho* p21 that inhibits the dissociation of GDP from and thereby the subsequent binding of GTP to this G protein in the cytosol fraction of rabbit intestine. We have tentatively designated this novel regulatory protein for *rho* p21 as *rho* GDP dissociation inhibitor (GDI). This paper describes the purification procedures and properties of *rho* GDI.

MATERIALS AND METHODS

Materials and Chemicals

Japanese White rabbit intestine was washed in ice-cold 0.9% NaCl. *rho*B p21, *rho*A p21 and *smg* p25A were purified to near homogeneity from the bovine brain membranes as described.[13,18,41] *smg* p25A is another small G protein.[18] c-Ha-*ras* p21 was purified from c-Ha-*ras* p21-expressing *Escherichia coli* as described.[31] *smg* p21B was purified from human platelet membranes as described.[27] [^{3}H]GDP (0.99 TBq/mmol) and [^{35}S]GTPγS (49.7 TBq/mmol) were from Du Pont-New England Nuclear. BA-85 nitrocellulose filters (pore size 0.45 μm) were obtained from Schleicher & Schuell. Other materials and chemicals were obtained from commercial sources.

*Assay for Dissociation of [^{3}H]GDP from rho*B p21

The [^{3}H]GDP-bound form of *rho*B p21 was first made by incubating *rho*B p21 (50 ng of protein) for 20 min at 30°C in a mixture (25 μl) containing 20 mM Tris/HCl at pH 7.5, 1 mM L-α-

dimyristoylphosphatidylcholine, 1 mM DTT, 5 mM $MgCl_2$, 10 mM EDTA and 1 μM [^{3}H]GDP (0.9-1.2 x 10^4 cpm/pmol). After this first incubation, $MgCl_2$ (1 μl) was added to give a final concentration of 20 mM, and the mixture was immediately cooled on ice to prevent the dissociation of [^{3}H]GDP from *rho*B p21. Each radiolabeled sample was incubated with the assay sample of *rho* GDI in a mixture (75 μl) containing 20 mM Tris/HCl at pH 7.5, 1 mM DTT, 1.35 μM GTP, 10.1 mM EDTA or 10.1 mM $MgCl_2$, and the second incubation was performed for the indicated periods of time at 30°C. The free Mg^{2+} concentration in the second incubation mixture was 0.5 μM or 10 mM. After the second incubation, about 2 ml of ice-cold 20 mM Tris/HCl at pH 7.5 containing 25 mM $MgCl_2$ and 100 mM NaCl was added to the reaction mixture followed by rapid filtration on nitrocellulose filters and washed with the same solution four times. The radioactivity was counted as described.[20]

Assay for the Binding of [^{35}S]GTPγS to rhoB p21

The GDP-bound form of *rho*B p21 (50 ng of protein) was first made as described above except that nonradio-active GDP was used instead of [^{3}H]GDP. After this first incubation, $MgCl_2$ (1 μl) was added to give a final concentration of 20 mM, and the mixture was immediately cooled on ice. Each sample was incubated with the assay sample of *rho* GDI as described above except that [^{35}S]GTPγS (0.9-1.2 x 10^4 cpm/pmol) was used instead of non-radioactive GTP. The second incubation was performed for the indicated periods of time at 30°C. The reaction was stopped as described above.

Complex formation of rho GDI with the GDP-bound form of rhoB p21

*rho*B p21 (500 ng of protein) was incubated with 1 μM [^{3}H]GDP (2 x 10^4 cpm/pmol) and 1 μM GTPγS (2 x 10^4 cpm/pmol) for 20 min at 30°C to produce the [^{3}H]GDP-bound and [^{35}S]GTPγS-bound forms, respectively, as described above. Each radiolabeled sample (500 ng of protein) was mixed with or

without *rho* GDI (10 μg of protein) in a 300-μl mixture containing 20 mM Tris/HCl at pH 7.5, 250 mM GTP, 10 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM DTT, and subjected to 4.8-ml continuous sucrose density ultracentrifugation at 238,000 x g for 19 h. *rho* GDI alone was separately subjected to the same ultracentrifugation. After the centrifugation, fractions of 150 μl each were collected from the bottom. The amount of *rho*B p21 was measured by counting the radioactivity of a 50-μl aliquot from each fraction after filtration on nitrocellulose filters as described above. The activity of *rho* GDI was assayed by measuring the activity to inhibit the dissociation of [^{3}H]GDP from *rho*B p21.

Other Assays and Procedures

The GAP activity for *rho*B p21, c-Ha-*ras* p21 or *smg* p21B was measured with the [γ-^{32}P]GTP-bound form of each G protein as a substrate using BA-85 nitrocellulose filters as described.[20,37] The Mr value of *rho* GDI was estimated by sucrose density gradient ultracentrifugation as described.[20] Protein concentrations were determined with bovine serum albumin as a reference protein as described.[4]

RESULTS

Purification of rho GDI

All the purification procedures were performed at 0-4°C. *rho* GDI was assayed by measuring the inhibition of the dissociation of [^{3}H]GDP from *rho*B p21 during the following purification procedures. The second incubation of the assay was performed for 10 min. Rabbit intestine (25 g wet weight) was homogenized using a Polytron homogenizer (PT10-35) with 125 ml of Buffer A (25 mM Tris/HCl at pH 7.5, 1 mM DTT, 1 mM $MgCl_2$ and 1 mM EGTA) containing 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride. The homogenate was centrifuged at 100,000 x g for 60 min. The supernatant (110

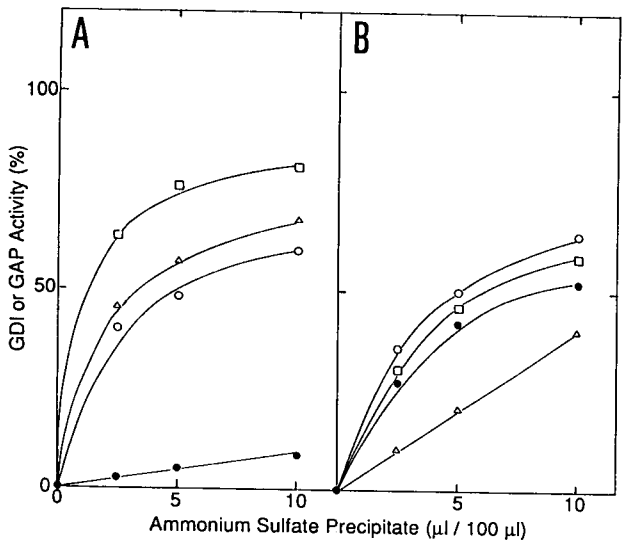

Fig. 1. The activities of *rho* GDI, *rho* GAP, *ras* GAP and *smg* GAP in the 0-40% and 40-60% ammonium sulfate precipitates. The 0-40% and 40-60% ammonium sulfate precipitates were dissolved and dialyzed as described in the text. The final volumes of both precipitates after the dialysis were adjusted to 6 ml by the addition of Buffer B. Various amounts of the 0-40% and 40-60% ammonium sulfate prepicitates were assayed for *rho* GDI, *rho* GAP, *ras* GAP and *smg* GAP. The assay for *ras* GAP was performed in 50 µl of the reaction mixture and the others were in 100 µl. The value of the radioactivity of [^{3}H]GDP bound to *rho*B p21 after the dissociation reaction in the absence of the assay sample (2,000 cpm) was subtracted from that before the dissociation reaction (20,000 cpm) and this value (18,000 cpm) was defined as 100%. The basal value (2,000 cpm) was subtracted from the value of the radioactivity of [^{3}H]GDP bound to *rho* p21 after the dissoiation reaction in the presence of the assay sample and the *rho* GDI activity of the assay sample was expressed as percent of these values to 18,000 cpm. The GAP activity was calculated from the decrease of the radioactivity of [γ-^{32}P]GTP compared to reactions performed in the absence of GAP and was expressed as percent decrease of the radioactivity. **A**, 0-40% ammonium sulfate precipitate; **B**, 40-60% ammonium sulfate precipitate. (●), *rho* GDI; (○), *rho* GAP; (□), *ras* GAP; (△), *smg* GAP. The results shown are representative of three independent experiments.

ml, 380 mg of protein) was applied to a DEAE-Sephacel column (5.2 x 7.5 cm) equilibrated with Buffer A. After the column was washed with 1.5 l of Buffer A and 75 ml of Buffer A containing 0.35 M NaCl, elution was performed with 300 ml of Buffer A containing 0.35 M NaCl in a stepwise manner. Most of *rho* GDI, *rho* GAP, *ras* GAP and *smg* GAP appeared in the fraction eluted by 0.35 M NaCl. Solid ammonium sulfate was added to this eluate (300 ml, 220 mg of protein) to give a final

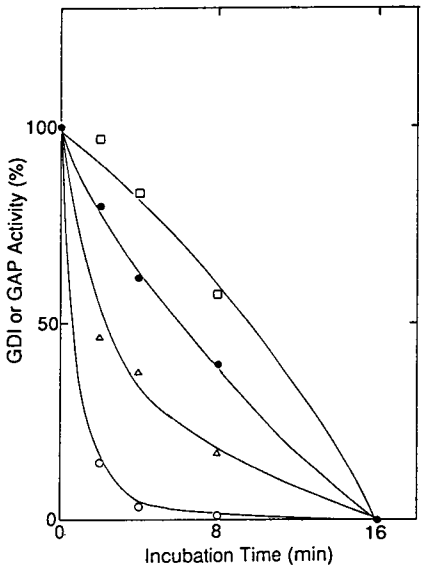

Fig. 2. Sensitivity of *rho* GDI, *rho* GAP, *ras* GAP and *smg* GAP to heat treatment. The 40-60% ammonium sulfate precipitate (200 µl) was incubated at 50°C for various periods of time. After the incubation, each sample was quickly cooled in ice-water and assayed for *rho* GDI, *rho* GAP, *ras* GAP and *smg* GAP. The GDI and GAP activities were calculated as described in the legend to Fig. 1. Each point was expressed as percent to the activity of the samples without heat treatment. The symbols were the same as those used in the legend to Fig. 1. The results shown are representative of three independent experiments.

concentration of 40% saturation. The sample was centrifuged at 20,000 x g for 20 min. Most of *rho* GDI was recovered in the supernatant while 50-80% of *rho* GAP, *ras* GAP and *smg* GAP were precipitated (Fig. 1). Solid ammonium was added to the supernatant to give a final concentration of 60% saturation. All *rho* GDI and three GAPs were precipitated by centrifugation at 20,000 x g for 20 min. The precipitate was dissolved in 4 ml of Buffer B (25 mM Tris/HCl at pH 7.5, 1 mM DTT and 0.5 mM EDTA). After dialysis overnight against the same buffer, the sample was centrifuged at 100,000 x g for 60 min. The volume of the supernatant (60 mg of protein) was adjusted to 6 ml by the addition of Buffer B.

Since the 40-60% precipitate of the ammonium sulfate fractionation contained *rho* GDI, *rho* GAP, *ras* GAP and *smg* GAP, we attempted to separate *rho* GDI from these GAPs by several column chromatographies and separated it from *ras* GAP and *smg* GAP but did not succeed in separating it from *rho* GAP. When the sample, however, was incubated at 50°C for various periods

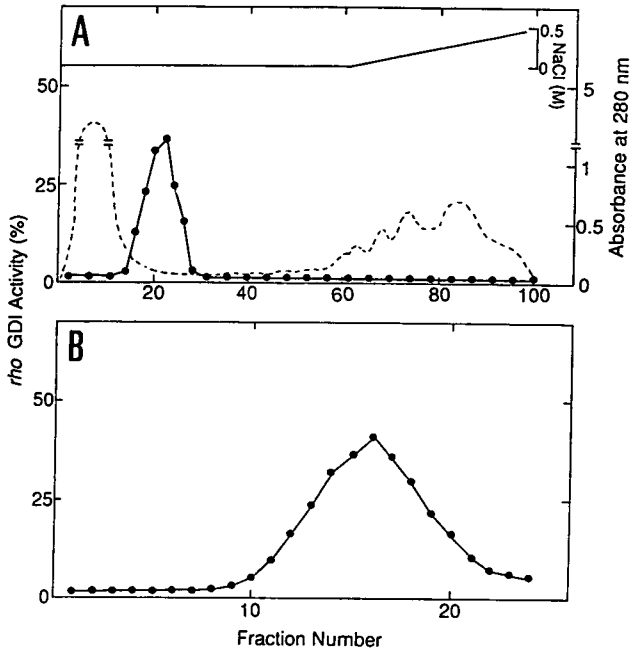

Fig. 3. Mono Q column chromatography and sucrose density gradient ultracentrifugation of *rho* GDI. Aliquots (10 µl) of each fraction of Mono Q column chromatography and sucrose density gradient ultracentrifugation were assayed for *rho* GDI. The *rho* GDI activity of the assay sample was calculated and expressed as described in the legend to Fig. 1. **A**, Mono Q column chromatography; **B**, sucrose density gradient ultracentrifugation. (●), *rho* GDI; (----), absorbance at 280 nm; (——), NaCl concentration. The results shown are representative of three independent experiments.

of time, all the activities of *rho* GDI, *rho* GAP, *ras* GAP and *smg* GAP gradually decreased, but the activity of *rho* GAP decreased most rapidly while the activity of *rho* GDI decreased more slowly (Fig. 2). Therefore, we partially purified *rho* GDI from the sample in which the activity of *rho* GDI was intact but *rho* GAP was completely destroyed.

The 40-60% precipitate of the ammonium sulfate fractionation (6 ml, 60 mg of protein) was divided into 30 tubes and treated at 50°C for 2 min. After the incubation, each sample was quickly cooled in ice-water and collected. Half of the heat-treated sample was supplemented with 1 ml of Buffer B containing 4% sodium cholate. The sample was adjusted to pH 8.0 by 1.5 M Tris and applied to a Mono Q column (0.5 x 5 cm)

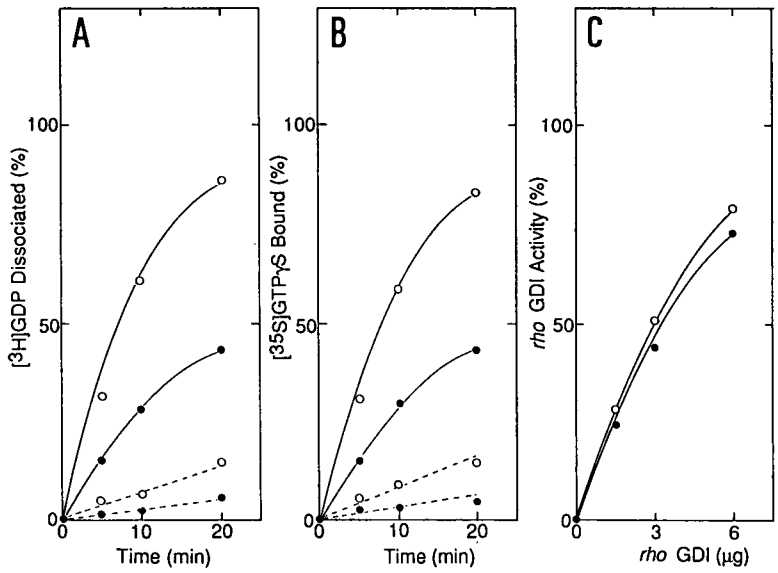

Fig. 4. Effect of *rho* GDI on the dissociation of [^{3}H]GDP from and the binding of [^{35}S]GTPγS to *rho*B p21. **A**, effect of *rho* GDI on the dissociation of [^{3}H]GDP from *rho*B p21. **B**, effect of *rho* GDI on the binding of [^{35}S]GTPγS to *rho*B p21. (——), at 0.5 μM Mg^{2+}; (----), at 10 mM Mg^{2+}. (●), in the presence of GDI; (○), in the absence of GDI. **C**, dose-dependent effect of *rho* GDI on the dissociation of [^{3}H]GDP from and the binding of [^{35}S]GTPγS to *rho*B p21. The *rho* GDI activities were measured at 0.5 μM Mg^{2+} with various doses of GDI. The *rho* GDI activity for inhibiting the dissociation of [^{3}H]GDP from *rho*B p21 was calculated and expressed as described in the legend to Fig. 1. The value of the radioactivity of [^{35}S]GTPγS bound to *rho*B p21 in the presence of *rho* GDI was subtracted from that in the absence of *rho* GDI (37,000 cpm), and the *rho* GDI activity for inhibition of the binding [^{35}S]GTPγS to *rho*B p21 was expressed as percent of the former values to 37,000 cpm. (●), *rho* GDI activity to inhibit the dissociation of [^{3}H]GDP from *rho*B p21; (○), *rho* GDI activity to inhibit the binding of [^{35}S]GTPγS to *rho*B p21. The results shown are representative of three independent experiments.

equilibrated with Buffer B containing 1% sodium cholate. Elution was performed with 25 ml of the same buffer followed by a 22.5-ml linear gradient of NaCl (0-0.5 M) in Buffer B containing 1% sodium cholate. Fractions of 0.5 ml each were collected. *rho* GDI appeared as a single peak in fractions 16-26 (Fig. 3**A**). This peak did not contain *ras* GAP or *smg* GAP. The active fractions were collected and concentrated to 200 μl by Centricon 10. The concentrate (880 μg of protein) was

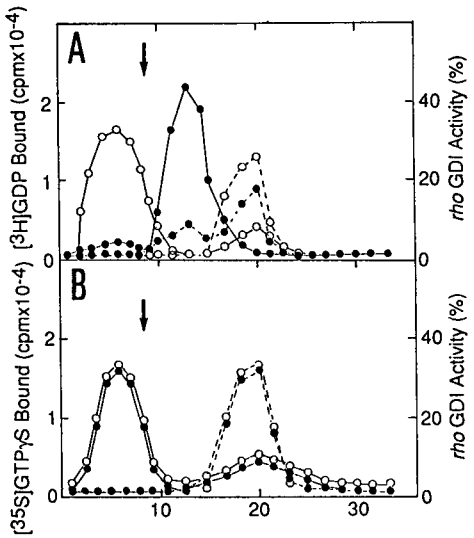

Fig. 5. Complex formation of *rho* GDI with the GDP-bound form of *rho*B p21. **A**. the [^{3}H]GDP-bound form of *rho*B p21. **B**. the [^{35}S]GTPγS-bound form of *rho*B p21. (●——●), *rho*B p21 in the presence of *rho* GDI; (○——○), *rho*B p21 in the absence of *rho* GDI; (●---●), *rho* GDI in the presence of *rho*B p21; (○---○), *rho* GDI in the absence of *rho*B p21. An arrow indicates a position of hemoglobin (4.5 S, Mr=64,500). The results shown are representative of three independent experiments.

subjected to ultracentrifugation using 4.8-ml sucrose density gradient (5-15% sucrose in Buffer B). Centrifugation was performed at 238,000 x g for 19 h. Fractions of 0.2 ml each were collected. *rho* GDI appeared as a single peak in fractions 14-18 (Fig. 3**B**). The active fractions (1 ml, 250 μg of protein) were collected and used as a partial purified sample of *rho* GDI. The other half of the heat-treated sample was subjected to Mono Q column chromatography and sucrose density gradient ultracentrifugation in a similar manner.

Properties of rho GDI

[^{3}H]GDP was dissociated from *rho*B p21 in a time-dependent manner (Fig. 4**A**). The initial velocity for the dissociation of [^{3}H]GDP from *rho*B p21 was much faster at 0.5 μM Mg^{2+} than at 10 mM Mg^{2+} as described for other G proteins.[12,14,33] *rho* GDI inhibited this dissociation at both Mg^{2+} concentrations. This inhibitory action was dependent on the doses of *rho* GDI (Fig. 4**C**). [^{35}S]GTPγS was not dissociated from *rho*B p21 irrespective of the presence of *rho* GDI at either Mg^{2+} concentration. [^{35}S]GTPγS bound to rhoB p21 more rapidly at 0.5 μM Mg^{2+} than at 10 mM Mg^{2+} (Fig. 4**B**). *rho* GDI inhibited this binding at both Mg^{2+} concentrations. This inhibitory action was also dependent on the doses of *rho* GDI (Fig. 4**C**). The doses of *rho* GDI necessary for inhibiting the dissociation of [^{3}H]GDP from and the binding of [^{35}S]GTPγS to *rho*B p21 were the same (Fig. 4**C**).

rho GDI did not stimulate the dissociation of [^{35}S]GTPγS from *rho*B p21 or the GTPase activity of *rho*B p21. *rho* GDI by itself did not show [^{35}S]GTPγS-binding activity. *rho* GDI was active on *rho*A p21 in inhibiting both the dissociation of [^{3}H]GDP and the binding of [^{35}S]GTPγS, but not on c-Ha-*ras* p21, *smg* p21B or *smg* p25A (data not shown).

The activities of *rho* GDI in inhibiting the dissociation of [^{3}H]GDP from and the binding of [^{35}S]GTPγS to *rho*B p21 were destroyed by tryptic digestion or heat boiling (data not shown). The S value of *rho* GDI was estimated to be about 2.5 S which corresponded to a Mr value of about 27,000.

Complex Formation of rho GDI with the GDP-bound Form of rhoB p21

Both the GDP-bound and GTPγS-bound form of *rho*B p21 appeared in one major and one minor peaks with Mr value of about 77,000 and 25,000, respectively, on sucrose density gradient ultracentrifugation (Fig. 5, **A** and **B**). The large Mr peak appeared to be an aggregated form of *rho*B p21. When *rho* GDI was first mixed with the GDP-bound form of *rho*B p21 and the mixture was then subjected to the same centrifugation, *rho*B p21 at the position with Mr values of about 77,000 and

25,000 mostly disappeared and that at the position with a Mr value of about 48,000 appeared (Fig. 5**A**). In contrast, when the mixture of *rho* GDI with the GTPγS-bound form of *rho*B p21 was subjected to the same centrifugation, *rho*B p21 appeared at the original position with Mr values of about 77,000 and 25,000 (Fig. 5**B**). These results indicate that *rho* GDI formed a complex with only the GDP-bound form of *rho* p21.

DISCUSSION

In the present studies, we have partially purified a novel type of regulatory protein for *rho*A p21 and *rho*B p21 from the cytosol fraction of rabbit intestine that is distinct from the *rho* GAP, *ras* GAP and *smg* GAP described earlier.[9,10,20,25,35-37,39,40] This protein, designated here as *rho* GDI, lacks GAP activity but inhibits the dissociation of GDP from *rho* p21. *rho* GDI also inhibits the binding of GTPγS to the GDP-bound form of *rho* p21. *rho* GDI is active for *rho* p21 and is inactive for other *ras* p21/*ras* p21-like G proteins including c-Ha-*ras* p21, *smg* p21B and *smg* p25A. Moreover, we have presented the evidence that rho GDI forms a complex only with the GDP-bound form of *rho* p21 but not with the GTP-bound form. Our previous kinetic studies have revealed that the rate-limiting step for the GDP/GTP exchange reaction of *rho* p21 is the dissociation of GDP from this G protein[22] as described for other many G proteins.[3,11,12,14,17,28,32, 33,38] The present results together with these previous observations indicate that *rho* GDI interacts with the GDP/GTP exchange reaction of *rho* p21 by inhibiting the dissociation of GDP from and the subsequent binding of GTP to this G protein.

rho p21 has another type of regulatory protein designated as a GAP that stimulates the GTPase activity of *rho*A p21 and *rho*B p21.[9,40] The Mr value of *rho* GDI is similar to that of *rho* GAP that is estimated to be about 25,000-30,000. *rho* GDI lacks, however, GAP activity, whereas *rho* GAP lacks the activity to affect the GDP/GTP exchange reaction of *rho*B

p21.[40] Therefore, it is evident from these properties that *rho* p21 has two different types of regulatory proteins, GAP and GDI.

It has been shown that the agonist-bound membrane receptors linked to oligomeric G proteins and guanine nucleotide-exchange proteins for initiation and elongation factors for protein synthesis stimulate the dissociation of GDP from and the subsequent binding of GTP to the respective G proteins.[3,11,17,28,33,38] The *CDC25* protein has been also suggested to serve as a stimulatory regulatory protein for the *RAS* protein in yeast.[6] Since *rho* GDI inhibits the dissociation of GDP from and the subsequent binding of GTP to *rho* p21, this protein appears to serve as an inhibitory regulatory protein for *rho* p21 and is apparently different from membrane receptors and the *CDC25* protein. It is, however, possible that the inhibitory action of *rho* GDI in the dissociation of GDP from *rho* p21 is reversed by covalent protein modification or by another factor which is regulated by intracellular messengers such as Ca^{2+}-calmodulin, cyclic AMP-dependent protein kinase, protein kinase C, and tyrosine kinases. It is also possible that *rho* GDI exhibits the stimulatory action in the dissociation of GDP by these modifications. If these are the cases, *rho* GDI could regulate *rho* p21 activity in the same manner as membrane receptors and the *CDC25* protein. Thus, the activity of *rho* p21 appears to be regulated in a dual manner by both *rho* GDI and GAP.

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