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Purification and Characterization of *Rab* GDIβ from Rat Brain[1]

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Summary: A new *Rab* GDI was purified from the synaptic soluble fraction of rat brain by several column chromatographies as a protein that inhibited the dissociation of [^{3}H]GDP from *Rab*3A but was not recognized by an anti-*Rab* GDIα antibody. The partial amino acid sequence analysis revealed that it was identical with rat *Rab* GDIβ. Purified *Rab* GDIβ showed the kinetic properties similar to those of *Rab* GDIα, including the inhibitory effect on the dissociation of GDP from *Rab*3A, the substrate specificity, the requirement of the post-translational lipid modifications of *Rab*3A, the stoichiometric interaction with the GDP-bound form of *Rab*3A, the inhibitory effect on the binding of *Rab*3A to erythrocyte ghosts, and the stimulatory effect on the dissociation of *Rab*3A from the membrane.

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The abbreviations used are: G protein, GTP-binding protein; GDI, GDP dissociation inhibitor; SS, synaptic soluble fraction; API, *Achromobacter* protease I; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; GEP, GDP/GTP exchange protein.

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The *Rab* family small G proteins regulate intracellular vesicle transport such as exocytosis, endocytosis, and transcytosis (for reviews, see [1-6]). One proposed mode of action of *Rab* small G proteins is as follows: When the GDP-bound form of a *Rab* small G protein in the cytosol is converted to the GTP-bound form, it interacts with its specific vesicle and transports the vesicle to its specific acceptor membrane. After the fusion of the vesicle with the membrane, the GTP-bound form is converted to the GDP-bound form, which is translocated from the membrane to the cytosol. In this model, the conversion of the *Rab* small G protein between the GDP-bound and GTP-bound forms and its cyclical translocation between the vesicle/membrane and cytosol fractions are essential.

In our laboratory, we have isolated a regulatory protein having these two activities [7,8]. This protein, named *Rab* GDI, has originally been isolated from bovine brain as a cytosolic protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to *Rab*3A [7]. *Rab*3A terminates in Cys-Ala-Cys [9-11]. These two cysteines are geranylgeranylated and the C-terminal cysteine is furthermore carboxylmethylated [12]. *Rab* GDI forms a stable ternary complex with the GDP-bound form of lipid-modified *Rab*3A but neither with the GTP-bound form of lipid-modified *Rab*3A nor with the GDP-bound or GTP-bound form of lipid-unmodified *Rab*3A [13,14]. In addition, *Rab* GDI has both activities to inhibit the binding of the GDP-bound form of lipid-modified *Rab*3A to membranes, but not of the GTP-bound form, and to induce the dissociation of the GDP-bound form from the membranes [13]. Moreover, *Rab* GDI is active not only on *Rab*3A but also on all other *Rab* family members thus far studied [7,15-19]. On the basis of these biochemical properties, we have proposed that *Rab* GDI functions as a regulatory protein for the cyclical translocation of the *Rab* small G proteins.

Recently, two forms of the *Rab* GDI cDNA have been cloned from rat and mouse and named *Rab* GDIα and -β and *Rab* GDI-1 and -2, respectively [20,21]. Rat *Rab* GDIα and mouse *Rab* GDI-1 are the counterpart of bovine *Rab* GDI [8]. In contrast, rat *Rab* GDIβ and mouse *Rab* GDI-2 appear to belong to different isoforms, although rat *Rab* GDI-2 cDNA has not been cloned. In contrast to the well-characterized biochemical properties of bovine *Rab* GDIα, *Rab* GDIβ has neither been purified nor well characterized. In the present study, therefore, we have attempted to purify a new *Rab* GDI (*Rab* GDIβ) from the synaptic soluble fraction (SS) of rat brain to near homogeneity and to study the biochemical properties of *Rab* GDIβ in comparison with those of bovine *Rab* GDIα.

Materials and Methods

Materials and Chemicals——Lipid-modified *Rab*3A and *Rab*11 were purified from the membrane fraction of *Spodoptera frugiperda* cells infected with the baculovirus carrying the respective cDNAs [20,22]. Lipid-unmodified *Rab*3A was purified from *Rab*3A-overexpressing *Escherichia coli* [14]. An anti-bovine *Rab* GDIα antibody was prepared as described previously [23]. *Rab* GDIα was purified from bovine brain cytosol [7,24]. Other materials and chemicals were obtained from commercial sources.

Assay for Rab GDI Activity——The *Rab* GDI activity was assayed by measuring the dissociation of [^{3}H]GDP from small G proteins (3 pmol each) by the filtration method using a nitrocellulose filter as described previously [24,25], except that the incubation with the small G proteins was performed at 0.5 μM Mg^{2+} for 10 min at 30°C.

Purification of a New Rab GDI (Rab GDIβ)——All the purification procedures were performed at 0-4°C. SS was prepared from forty-two rat brains as described previously [26]. SS (450 ml, 315 mg of protein) was directly applied to a Q-Sepharose FF column (2.6 x 24 cm) equilibrated with Buffer A (20 mM Tris/HCl at pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, and 1 mM DTT). After the column was washed with 600 ml of Buffer A, elution was performed with a 600-ml linear gradient of NaCl (0-0.5 M) in Buffer A, followed by 120 ml of 0.5 M NaCl in Buffer A at a flow rate of 5 ml/min. Fractions of 8 ml each were collected. Fractions 33-42 (80 ml, 24 mg of protein) representing the first peak of the *Rab* GDI activity were collected (see **Fig. 1A**) and diluted with 160 ml of Buffer B (20 mM potassium phosphate at pH 7.5, 0.5 mM EDTA, and 1 mM DTT). The sample was applied to a hydroxyapatite column (1 x 9 cm) equilibrated with Buffer B. After the column was washed with 70 ml of the same buffer, elution was performed with a 70-ml linear gradient of potassium phosphate (20-200 mM) in Buffer B at a flow rate of 0.25 ml/min. Fractions of 2 ml each were collected. One peak of the *Rab* GDI activity appeared in Fractions 11-14. These fractions (8 ml, 0.5 mg of protein) were collected and diluted with 16 ml of Buffer C (Buffer A with 10 % glycerol). The sample was applied to a Mono Q HR5/5 equilibrated with Buffer C. After the column was washed with 10 ml of the same buffer, elution was performed with a 30-ml linear gradient of NaCl (50-250 mM) in Buffer C at a flow rate of 0.5 ml/min. Fractions of 0.5 ml each were collected. One peak of the *Rab* GDI activity appeared in Fractions 27-29 (see **Fig. 2A**). These fractions (1.5 ml, 50 μg of protein) were pooled and stored at -80°C until use.

Analysis of Amino Acid Sequence——Purified new *Rab* GDI (20 μg of protein) was digested with API (0.4 μg of protein), and the digested peptides were separated by Bakerbond WP C8 reverse phase column chromatography as described [25,27]. The amino acid sequences of the peptides were determined with a peptide sequencer (Shimadzu PSQ-1-gas phase sequencer) [27].

Complex Formation of Rab3A with Rab GDI——The [^{3}H]GDP-bound or [^{35}S]GTPγS-bound form of lipid-modified *Rab*3A (75 pmol each) was incubated with *Rab* GDIβ or -α (300 pmol each), and subjected to 4.8-ml continuous sucrose density gradient ultracentrifugation [13,24]. After the centrifugation, fractions of 170 μl each were collected. The amount of the small G protein was determined by measuring the radioactivity bound to the small G protein by the filtration method, and the amount of *Rab* GDI was determined by SDS-PAGE followed by protein staining with silver [13,24].

Assays for Rab GDI to Regulate the Translocation of Lipid-Modified Rab3A——Assay for the binding of lipid-modified *Rab*3A to erythrocyte ghosts was done as described [28], except that

*Rho*A and *Rho* GDI were replaced with *Rab*3A and *Rab* GDI, respectively. Assay for the dissociation of lipid-modified *Rab*3A from the membrane was done as described [13,24], with slight modifications. Briefly, the [^{3}H]GDP-bound form of lipid-modified *Rab*3A (8 pmol) was mixed with erythrocyte ghosts (150 μg of protein) and centrifuged to separate the membrane and soluble fractions. The membrane fraction was collected and incubated with various doses of *Rab* GDIβ or -α. Each reaction mixture was centrifuged again to separate the membrane and soluble fractions. The amounts of *Rab*3A in both fractions were determined by measuring the radioactivity bound to the small G protein by the filtration method [13,24].

Other Procedures——Immunoblotting was performed as described [23]. Protein concentrations were determined with BSA as a reference protein [29].

Results

SS was first subjected to Q-Sepharose FF column chromatography. When each fraction was assayed for the *Rab* GDI activity, two peaks appeared (**Fig. 1A**). When each fraction was immunoblotted with an anti-*Rab* GDIα antibody, a single peak appeared, corresponding to the second peak of the *Rab* GDI activity (**Fig. 1B**). The fractions representing the first peak of the *Rab* GDI activity were collected and subjected to hydroxyapatite column chromatography. The *Rab* GDI activity appeared as a single peak (data not shown). The active fractions were collected and subjected to Mono Q HR5/5 column chromatography. The *Rab* GDI activity appeared as a single peak (**Fig. 2A**). **Fig. 2B** shows the protein staining of the active fractions of the Mono Q HR5/5 column chromatography on an SDS-polyacrylamide gel. This protein staining revealed only a single protein with an *Mr* value of about 47,000. This value was slightly smaller than the *Mr* value of *Rab* GDIα that was about 54,000. This protein band was not recognized by the anti-*Rab* GDIα antibody (**Fig. 2C**). This new *Rab* GDI was purified about 300-fold with about 3 % yield from SS. About 50 μg of pure new *Rab* GDI was obtained from forty-two rat brains.

When the purified *Rab* GDI was completely digested with API and subjected to Bakerbond WP C8 column chromatography, more than 20 peptides were separated (data not shown). Two peaks were sequenced by an automated gas-phase sequencer. The amino acid sequences of the peaks were LPGQPPASMGRGRDWNV and VLHMDQNPYYGGESASITPLEDLYK, which were identical with those of the residues 58-74 and 30-54 of rat *Rab* GDIβ, respectively [20]. These results indicate that the purified *Rab* GDI is rat *Rab* GDIβ.

Rab GDIβ as well as -α inhibited the dissociation of GDP from lipid-modified *Rab*3A and *Rab*11 (**Fig. 3A**). These activities were

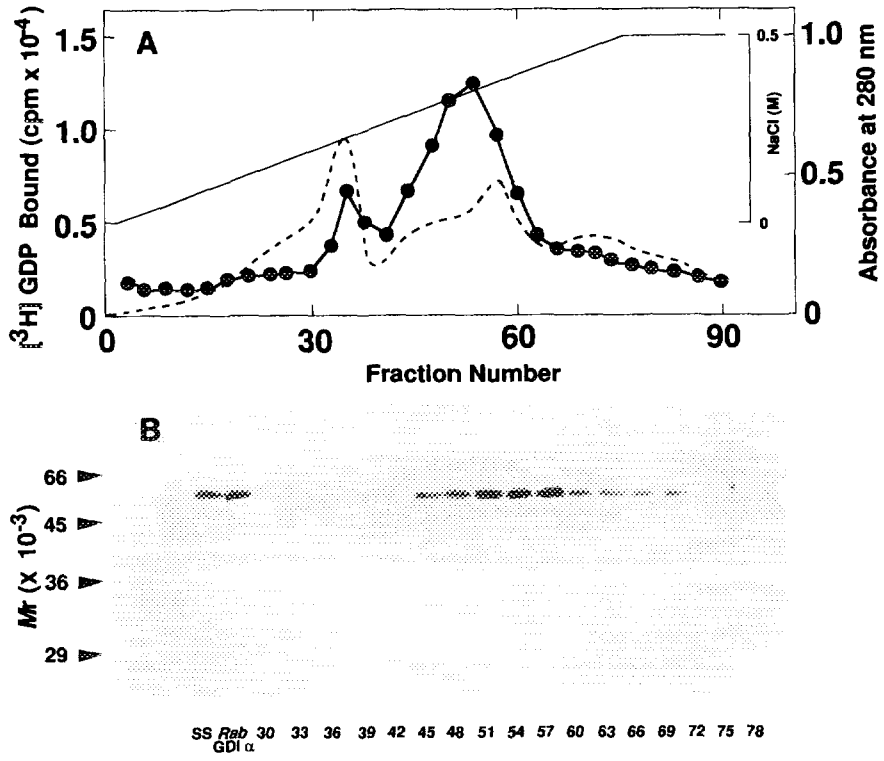


Fig. 1. Q-Sepharose FF column chromatography. A, Elution profile of *Rab* GDI. An aliquot (50 μl) of each fraction was assayed for the *Rab* GDI activity with the [^{3}H]GDP-bound form of lipid-modified *Rab*3A. (●), [^{3}H]GDP bound; (---), absorbance at 280 nm; (——), NaCl concentration. **B,** Immunoblot analysis with an anti-*Rab* GDIα antibody. An aliquot (10 μl) of each fraction was subjected to SDS-PAGE (10 % polyacrylamide gel), followed by immunoblot analysis. The protein markers used were BSA (*Mr*, 66,000), ovalbumin (*Mr*, 45,000), glyceraldehyde-3-phosphate dehydrogenase (*Mr*, 36,000), and carbonic anhydrase (*Mr*, 29,000). The results shown are representative of three independent experiments.

dose-dependent, and the doses of *Rab* GDIβ necessary for these activities were similar to those of *Rab* GDIα. **Fig. 3B** shows the requirement of the post-translational lipid modifications of *Rab*3A for the *Rab* GDIβ and -α activities. *Rab* GDIβ was also active on lipid-modified *Rab*3A and inactive on lipid-unmodified *Rab*3A.

Rab GDIβ as well as -α formed a complex with the [^{3}H]GDP-bound form of lipid-modified *Rab*3A, but not with the [^{35}S]GTPγS-bound form, as estimated by sucrose density gradient ultracentrifugation (**Fig. 4**). Both the [^{3}H]GDP-bound and [^{35}S]GTPγS-bound forms appeared at a position corresponding to an *Mr* value of about 320,000 in the absence of *Rab* GDI. Since the *Mr* values of *Rab*3A

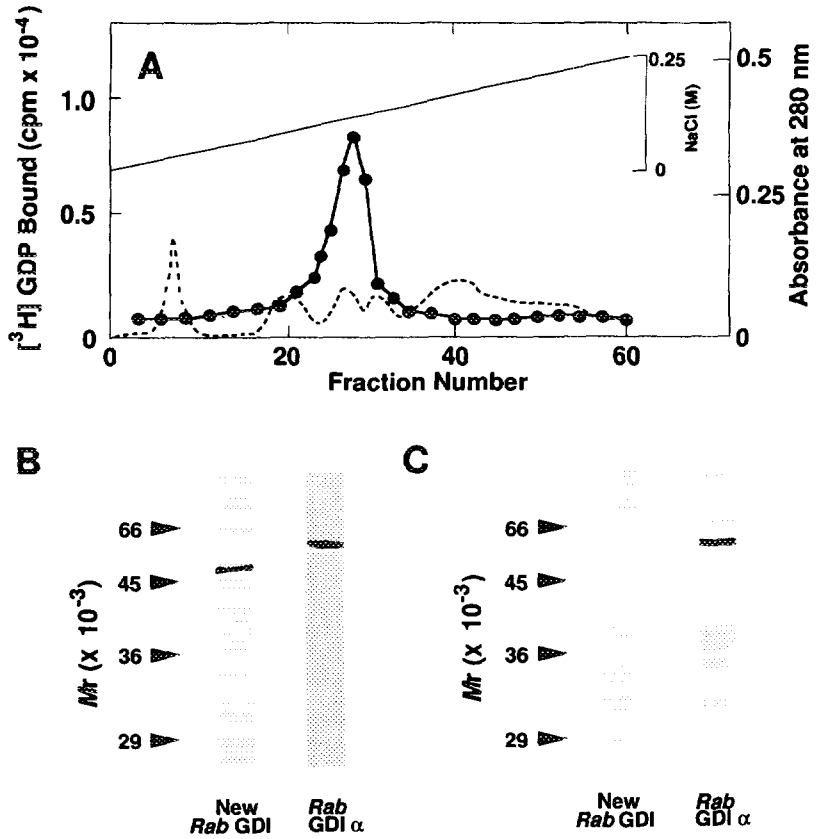
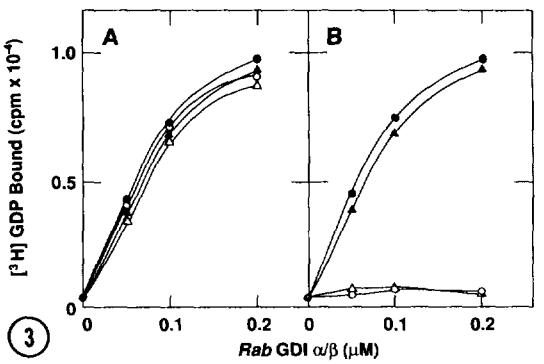


Fig. 2. Mono Q HR5/5 column chromatography and analyses of the purified new *Rab* GDI. ***A,*** Elution profile of the new *Rab* GDI. An aliquot (25 μl) of each fraction was assayed for the *Rab* GDI activity with the [^{3}H]GDP-bound form of lipid-modified *Rab*3A. (●), [^{3}H]GDP bound; (---), absorbance at 280 nm; (——), NaCl concentration. ***B,*** Protein staining with Coomassie Brilliant Blue. The new *Rab* GDI and *Rab* GDIα (1 μg of protein each) were subjected to SDS-PAGE (10 % polyacrylamide gel). ***C,*** Immunoblot analysis. The new *Rab* GDI and *Rab* GDIα (500 and 50 ng of proteins, respectively) were subjected to SDS-PAGE followed by immunoblot analysis. The protein markers used were the same as those used in **Fig. 1B**. The results shown are representative of three independent experiments.

estimated from SDS-PAGE and its primary structure were about 24,000 and 25,000, respectively [9,30], this *Rab*3A might be polymerized. In the presence of *Rab* GDIβ, only the [^{3}H]GDP-bound form of *Rab*3A altered migration and appeared at a position corresponding to an *Mr* value of about 76,000. Since the *Mr* values of *Rab* GDIβ estimated from SDS-PAGE and its primary structure were about 47,000 and 51,000, respectively [20], these results indicate that *Rab* GDIβ formed a complex with the GDP-bound form of lipid-modified *Rab*3A at a molar ratio of 1:1, but not with the GTP-bound form.



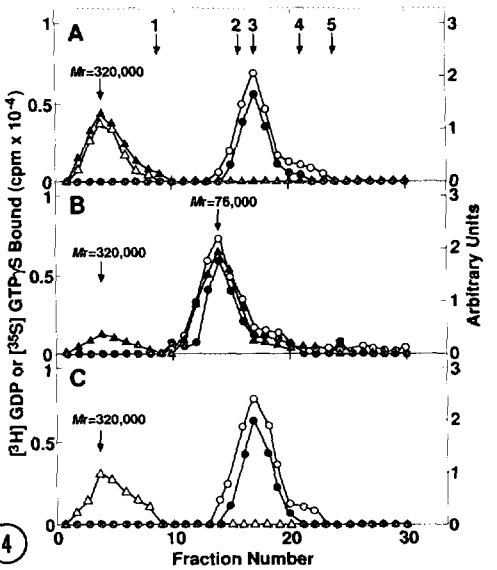


Fig. 3. Substrate specificity and requirement of the lipid modifications for *Rab* GDIβ and -α. ***A***, Substrate specificity. The dissociation of [^{3}H]GDP from lipid-modified *Rab*3A or *Rab*11 was assayed in the presence of various doses of *Rab* GDIβ or -α. (▲, △), with *Rab* GDIβ; (●, ○), with *Rab* GDIα; (●, ▲), with *Rab*3A; (○, △), with *Rab*11. ***B***, Requirement of the lipid modifications of *Rab*3A. The dissociation of [^{3}H]GDP from lipid-modified or -unmodified *Rab*3A was assayed in the presence of various doses of *Rab* GDIβ or -α. (▲, △), with *Rab* GDIβ; (●, ○), with *Rab* GDIα; (●, ▲), with lipid-modified *Rab*3A; (○, △), with lipid-unmodified *Rab*3A. The results shown are representative of three independent experiments.

Fig. 4. Complex formation of *Rab* GDIβ or -α with lipid-modified *Rab*3A. ***A***, The [^{3}H]GDP-bound or [^{35}S]GTPγS-bound form of lipid-modified *Rab*3A alone was subjected to 4.8-ml sucrose density gradient ultracentrifugation. *Rab* GDIβ or -α alone was also subjected to the same ultracentrifugation. ***B***, The [^{3}H]GDP-bound form of *Rab*3A was incubated with *Rab* GDIβ or -α, and subjected to the same ultracentrifugation. ***C***, The [^{35}S]GTPγS-bound form of *Rab*3A was incubated with *Rab* GDIβ or -α and subjected to the same ultracentrifugation. (▲), the [^{3}H]GDP-bound form; (△), the [^{35}S]GTPγS-bound form; (●), *Rab* GDIβ; (○), *Rab* GDIα. Arrows indicate the positions of the protein markers: **1**, γ-globulin (*Mr*, 160,000); **2**, BSA (*Mr*, 66,000); **3**, ovalbumin (*Mr*, 45,000); **4**, carbonic anhydrase (*Mr*, 29,000); **5**, myoglobin (*Mr*, 24,000). The results shown are representative of three independent experiments.

Rab GDIβ as well as -α inhibited the binding of lipid-modified *Rab*3A to the membrane and induced the dissociation of lipid-modified *Rab*3A from the membrane (**Fig. 5**). The doses of *Rab* GDIβ necessary for these activities were similar to those of *Rab* GDIα.

Discussion

We have purified a new *Rab* GDI, which inhibits the GDP/GTP exchange reaction of *Rab*3A but is not recognized by an anti-*Rab*

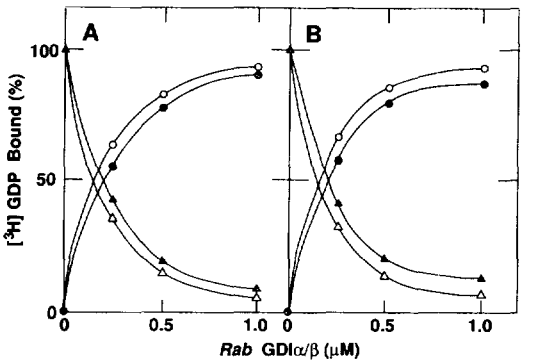


Fig. 5. Effects of *Rab* GDIβ and -α on the binding of lipid-modified *Rab*3A to erythrocyte ghosts and on the dissociation of lipid-modified *Rab*3A from the membrane. ***A***, Effect on the binding of lipid-modified *Rab*3A to erythrocyte ghosts. The [^{3}H]GDP-bound form of lipid-modified *Rab*3A was first incubated with various doses of *Rab* GDIβ or -α and then incubated with erythrocyte ghosts. ***B***, Effect on the dissociation of lipid-modified *Rab*3A from erythrocyte ghosts. The [^{3}H]GDP-bound form of lipid-modified *Rab*3A which was prebound to erythrocyte ghosts was incubated with various doses of *Rab* GDIβ or -α. (●, ▲), with *Rab* GDIβ; (○, △), with *Rab* GDIα; (●, ○), the soluble fraction; (▲, △), the membrane fraction. The results shown are representative of three independent experiments.

GDIα antibody, and identified it as *Rab* GDIβ. The doses of *Rab* GDIβ necessary for the inhibition of the GDP/GTP exchange reaction are similar to those of *Rab* GDIα. On the assumption that the two isoforms are separated on the Q-Sepharose FF column chromatography with the same yield, the amount of *Rab* GDIβ in SS is about five-times lower than that of *Rab* GDIα. The yield of the purification of *Rab* GDIβ is calculated to be about 18 %. The biochemical properties of *Rab* GDIβ are moreover indistinguishable from those of *Rab* GDIα, including the inhibitory effect on the dissociation of GDP from *Rab*3A, the substrate specificity, the requirement of the post-translational lipid modifications of *Rab*3A, the stoichiometric interaction with the GDP-bound form of *Rab*3A, the inhibitory effect on the binding of *Rab*3A to the membrane, and the stimulatory effect on the dissociation of *Rab*3A from the membrane.

An important question is why different isoforms exist. It has been shown that these isoforms show the different tissue distribution and intracellular localization [20,21,31,32]. *Rab* GDIα is highly expressed in brain and much lower in other tissues, whereas *Rab* GDIβ and *Rab* GDI-2 are ubiquitously expressed [20,21, 31]. *Rab* GDIα is totally cytosolic [23,31], whereas a higher level of *Rab* GDI-2 is associated with membranes [31]. On the basis of these observations, it has been proposed that each isoform may

have a different function [31]. Recently, *Rab* GDIα has been shown to deliver the *Rab* small G proteins to their specific acceptor membranes, accompanied with their GDP/GTP exchange reaction [33,34]. Therefore, it is possible that the interactions of each isoform with other proteins such as a GDI-displacement factor [33,34] or a stimulatory GEP which stimulates the GDP/GTP exchange reaction, may be different. Further studies are necessary to clarify the significance for the presence of the different isoforms.

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