Purification and Characterization from Bovine Brain Cytosol of a Protein That Inhibits the Dissociation of GDP from and the Subsequent Binding of GTP to smg p25A, a ras p21-like GTP-binding Protein*

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A novel regulatory protein for smg p25A, a ras p21-like GTP-binding protein, was purified to near homogeneity from bovine brain cytosol. This regulatory protein, designated here as smg p25A GDP dissociation inhibitor (GDI), inhibited the dissociation of GDP, but not of guanosine 5'-[3-O-thio]triphosphate (GTP·S) from smg p25A. smg p25A GDI also inhibited the binding of GTP·S to the GDP-bound form of smg p25A but not of that to the guanine nucleotide-free form. GDI did not stimulate the GTPase activity of smg p25A and by itself showed neither GTP·S-binding nor GTPase activity. GDI was inactive for other ras p21/ras p21-like GTP-binding proteins including c-Ha-ras p21, rhoB p20, and smg p21. The Mₐ value of GDI was estimated to be about 54,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, about 65,000 from the S value (4.5 S), and about 82,000 by gel filtration. The isoelectric point of GDI was about pH 5.6. The activities of GDI were killed by trypic digestion or heat boiling. These results indicate that bovine brain cytosol contains a regulatory protein for smg p25A that inhibits the dissociation of GDP from and thereby the subsequent binding of GTP to this protein.

There is a superfamily of ras p21/ras p21-like G proteins with small molecular weights (for reviews, see Refs. 1 and 2). smg p25 family belongs to this superfamily and is composed of three highly homologous members, smg p25A, -B, and -C (5, 4). smg p25A was first isolated from bovine brain membranes and characterized (3). smg p25A is composed of 220 amino acids with a calculated Mₐ of 24,954 (4).

The functions of smg p25A are unknown at present, but the smg p25A mRNA is highly expressed in brain and adrenal medulla (6, 7). In brain, smg p25A is found partly in the soluble cytosol and mainly in synaptosomes where smg p25A is found in synaptic membranes, vesicles, and cytosol. This intrasynaptosomal distribution is different from that of c-ras p21s which are localized only in synaptic membranes (8). The smg p25A mRNA is also detected in rat pheochromocytoma PC-12 cells, and its level is increased after differentiation of these cells into neuron-like cells by nerve growth factor (7). Available evidence suggests that smg p25A is related to at least to neural functions.

The modes of activation and action of smg p25A have not been clarified. It is conceivable, however, by analogy with ras p21s (1) that smg p25A has the GDP-bound inactive and GTP-bound active forms and that there are proteins converting the inactive form to the active form and an effector protein whose function is modulated by the active form. None of these proteins directly interacting with smg p25A, however, have been identified. Recently, a protein that stimulates the GTPase activity of c-ras p21s, designated as a GTPase-activating protein for c-ras p21s, has been identified, purified, and characterized (9, 10). The primary structure of c-ras p21 GTPase-activating protein has been determined (11, 12). A different GTPase-activating protein for rhoA p21 has been also purified from human spleen (13).

In preceding papers (14, 15), we have partially purified two GTPase-activating proteins for smg p21 from bovine brain and human platelets. smg p21 is identical with the proteins encoded by the rap2A and Kreu-1 cDNAs (16, 17). We have also partially purified a GTPase-activating protein specific for rhoB p20 from bovine brain (3). We have not yet, however, succeeded in identifying a GTPase-activating protein for smg p25A. Instead, we have purified to near homogeneity another type of regulatory protein for smg p25A in the present studies. This protein lacks GTPase-activating protein activity but inhibits the dissociation of GDP from and thereby the subsequent binding of GTP to smg p25A. This paper describes the purification procedures and properties of this novel regul-
latory protein for smg p25A, which is tentatively designated as smg p25A GDI.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—Bovine brains were obtained as described (14), smg p25A and rbo P20 were purified from bovine brain membranes (5, 18), e. Ha-ras p21 was purified from human e Ha ras p21-expressing Escherichia coli, a gift from Drs. Y. Kaziro and T. Satoh (University of Tokyo, Tokyo, Japan), as described (15). smg p21 was purified from human platelet membranes (19). Advantec nitrocellulose filters for protein and nucleic acid study (A020A025A with 0.20-µm pore size and A045A025A with 0.45-µm pore size) were obtained from Toyoshi Co. (Tokyo, Japan). An anti-smg p25A monoclonal antibody, mAb SG-11-7, was prepared in collaboration with Dr. A. Mizoguchi (Kobe University).

**Assay for smg p25A GDI during the Purification Procedures (Method 1)—**The amount of smg p25A trapped after the rapid filtration assay (3) on nitrocellulose filters obtained from various commercial sources was quantified by use of an anti-smg p25A monoclonal antibody. Neither the [3H]GDP–nor [35S]GTPyS-bound form of smg p25A passed through nitrocellulose filters, such as Advantec A020A030D (0.20-µm pore size) and A045A030D (0.45-µm pore size) filters, but smg p25A GDI made the [3H]GDP-bound form, but not the [35S]GTPyS-bound form, pass through these filters in a dose-dependent manner. Since smg p25A GDI did not stimulate the dissociation of [3H]GDP from smg p25A as described below, smg p25A GDI could be assayed by measuring the decrease in the radioactivity of the [3H]GDP-bound form of smg p25A trapped on Advantec A020A030D or A045A030D nitrocellulose filters. Moreover, the sensitivity of this method was higher than that of other methods described below. Thus, this method (Method 1) was usually used during the purification procedures of smg p25A GDI. The [3H]GDP-bound form of smg p25A was first made by incubating smg p25A (50 pmol of protein, 25 nM in a mixture (25 μl) containing 20 mM Tris/HCl at pH 7.5, 1 mM EDTA, 5 mM MgCl2, 10 mM EGTA, and 1 μM [3H]GDP (0.9–1.2 × 104 cpm/pmol). After this first incubation, MgCl2 (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 [3H]GDP from smg p25A. The radioactivity was counted as described (3). Since over 0.2% sodium cholate interfered with this assay, this agent at less than 0.2% was used.

**Assay for the Dissociation of [3H]GDP from smg p25A (Method 2)—**The [3H]GDP-bound form of smg p25A was trapped on other nitrocellulose filters having 0.20- or 0.45-µm pore sizes, such as Advantec A020A030D (0.20-µm pore size) and A045A030D (0.45-µm pore size) filters, but smg p25A GDI made the [3H]GDP-bound form, but not the [35S]GTPyS-bound form, pass through these filters in a dose-dependent manner. Since smg p25A GDI did not stimulate the dissociation of [3H]GDP from smg p25A as described below, smg p25A GDI could be assayed by measuring the decrease in the radioactivity of the [3H]GDP-bound form of smg p25A trapped on Advantec A020A030D or A045A030D nitrocellulose filters. Moreover, the sensitivity of this method was higher than that of other methods described below. Thus, this method (Method 1) was usually used during the purification procedures of smg p25A GDI. The [3H]GDP-bound form of smg p25A was first made by incubating smg p25A (50 pmol of protein, 25 nM in a mixture (25 μl) containing 20 mM Tris/HCl at pH 7.5, 1 mM EDTA, 5 mM MgCl2, 10 mM EGTA, and 1 μM [3H]GDP (0.9–1.2 × 104 cpm/pmol). After this first incubation, MgCl2 (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 [3H]GDP from smg p25A. The radioactivity was counted as described (3). Since over 0.2% sodium cholate interfered with this assay, this agent at less than 0.2% was used.

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**RESULTS**

**Purification of smg p25A GDI**—During the purification procedures, the following buffers were used: Buffer A, 25 mM Tris/HCl at pH 7.5, 1 mM EDTA, 1 mM MgCl2, and 1 mM EGTA; Buffer B, 25 mM Tris/HCl at pH 7.5, 1 mM DTT, and 0.5 mM EDTA. All the purification procedures were performed at 0–4°C.

Cerebral tissue (450 g, wet weight) was homogenized for 1 min twice at 1-min intervals in a Waring blender with 450 ml of Buffer A containing 10 μM (p-amidinophenyl)methane-sulfonyl fluoride. The homogenate was further homogenized in a Potter-Elvehjem Teflon-glass homogenizer and centrifuged at 100,000 × g for 60 min. The supernatant (350 ml, 6.0 g of protein) was applied to a DEAE-Sephadex column (7.5 × 18 cm) equilibrated with Buffer A. After the column was washed with 8 liters of Buffer A and 400 ml of Buffer A containing 0.3 M NaCl, smg p25A GDI was eluted by 800 ml of Buffer A containing 0.3 M NaCl. Solid ammonium sulfate was added to this eluate (800 ml, 1.7 g of protein) to give a final concentration of 40% saturation. The sample was centrifuged at 20,000 × g for 20 min. All smg p25A GDI was precipitated. The GTPase-activating protein for rbo P20 was recovered in both the 0–40% and 40–60% precipitates. The 40–60% precipitate was dis-
solved in 26 ml of Buffer B and dialyzed overnight against Buffer B. The dialyzed sample was centrifuged at 100,000 × g for 60 min and the volume of the supernatant was adjusted to 32 ml by the addition of Buffer B.

One-fourth of the supernatant (8 ml, 147 mg of protein) was supplemented with 2.7 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 (1 × 10 cm) equilibrated with Buffer B containing 1% sodium cholate. After the column was washed with 190 ml of the same buffer, the elution was performed with a 180-ml linear gradient of NaCl (0–0.5 M) in Buffer B containing 1% sodium cholate and fractions of 4 ml each were collected. Three peaks of smg p25A GDI appeared (Fig. 1A). Fig. 1B shows SDS-PAGE analysis of the indicated fractions of the Mono Q column chromatography. Only a single protein band was observed in the first peak and smg p25A GDI activity coincided with this protein band. The first major peak was studied here, but two other minor peaks were not studied here. The active fractions of the first peak (Fractions 25–34) were collected and concentrated to 5 ml by an Amicon ultrafiltration cell equipped with a PM-10 filter. The concentrate (1.9 mg of protein) was dialyzed against Buffer B and used as a purified sample. The rest of the supernatant was subjected to the same Mono Q column chromatography in a similar manner. smg p25A GDI in the first peak of the Mono Q column chromatography was purified about 330-fold with about 30% yield. About 7.6 mg of pure GDI was obtained from 450 g of bovine brain.

smg p25A GDI was detected by Method 1 during these purification procedures, but essentially similar results were obtained when it was detected by Method 2 although its sensitivity was lower than that of Method 1.

**Kinetic Properties**—The initial velocity for the dissociation of [3H]GDP from smg p25A was faster at 0.5 μM Mg2+ than at 10 mM Mg2+ as estimated by Method 2 (Fig. 2A). This result was consistent with that described previously (23). smg p25A GDI inhibited this dissociation at both Mg2+ concentrations (Fig. 2A). This inhibitory action was dependent on the doses of GDI (Fig. 3). [35S]GTPγS was not dissociated from smg p25A irrespective of the presence of GDI at either Mg2+ concentration. These results were confirmed by gel filtration analysis.

The initial velocity for the binding of [35S]GTPγS to the GDP-bound form of smg p25A was faster at 0.5 μM Mg2+ than at 10 mM Mg2+ as estimated by Method 3 (23). In the case of the guanine nucleotide-free form, this velocity at 10 mM Mg2+ became faster but that at 0.5 μM Mg2+ did not markedly change (23).4 smg p25A GDI inhibited the binding of [35S]GTPγS to the GDP-bound form of smg p25A at both Mg2+ concentrations (Fig. 2B) but not of that to the guanine nucleotide-free form during its preparation.

4The lower amount of the binding of [35S]GTPγS to the guanine nucleotide-free form than that to the GDP-bound form shown in Fig. 2, B and C, was due to the inactivation of the guanine nucleotide-free form during its preparation.

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**Fig. 1. Mono Q column chromatography of smg p25A GDI.** An aliquot (10 μl) of the indicated fractions was assayed for smg p25A GDI by Method 1 using Advantec A045A304D nitrocellulose filters. Another aliquot (40 μl) of the indicated fractions was subjected to SDS-PAGE (12% polyacrylamide gel) followed by protein staining with Coomassie Brilliant Blue. Fractions 25–34 were collected, concentrated, and dialyzed as described in the text. An aliquot of the dialyzed sample (6 μg of protein) was subjected to SDS-PAGE followed by the same protein staining. A, elution profile of smg p25A GDI; B and C, protein staining. The radioactive of the [3H]GDP-bound form of smg p25A trapped on the filters; - - -, absorbance at 280 nm; ---, NaCl concentration. The protein markers used were bovine serum albumin (M₉ = 66,000), glyceraldehyde-3-phosphate dehydrogenase (M₉ = 36,000), carbonic anhydrase (M₉ = 29,000), and trypsin inhibitor (M₉ = 20,100). The results shown were representative of three independent experiments.

**Fig. 2. Effect of smg p25A GDI on the dissociation of [3H] GDP from and the binding of [35S]GTPγS to smg p25A.** A, effect of smg p25A GDI on the dissociation of [3H]GDP from smg p25A. The assay was performed in the presence or absence of smg p25A GDI (9.5 μg of protein) at 0.5 μM Mg2+ or 10 mM Mg2+ in the third incubation mixture by Method 2 using Advantec A020A025A nitrocellulose filters. B and C, effect of GDI on the binding of [35S]GTPγS to the GDP-bound and guanine nucleotide-free forms of smg p25A, respectively. The assay was performed in the presence or absence of GDI (9.5 μg of protein) at 0.5 μM Mg2+ or 10 mM Mg2+ in the third incubation mixture by Method 3 using Advantec A020A025A nitrocellulose filters. ---, at 0.5 μM Mg2+; ---, at 10 mM Mg2+; ●, in the presence of GDI; ○, in the absence of GDI. Essentially the same results were obtained in three independent experiments.
Figu res 2. Dose-dependent effect of smg p25A GDI on the dissociation of [3H]GDP from and the binding of [35S]GTPyS to smg p25A. The third incubations of Methods 2 and 3 were performed with the indicated doses of smg p25A for 10 min at 0.5 μM MgCl2. The smg p25A GDI activities measured by Methods 2 and 3 were calculated from the radioactivities of smg p25A trapped on the nitrocellulose filters in the presence and absence of smg p25A GDI. The smg p25A GDI activities measured by Methods 2 and 3 were expressed as percent inhibition of the dissociation of [3H]GDP from and the binding of [35S]GTPyS to smg p25A, respectively. \( A_0 \), smg p25A GDI activity measured by Method 2; \( A_1 \), smg p25A GDI activity measured by Method 3. Essentially the same results were obtained in three independent experiments.

Physical Properties—The purified sample of smg p25A GDI showed a single protein band as estimated by SDS-PAGE (Fig. 1C). This inhibitory action was dependent on the doses of GDI (Fig. 3). These results were confirmed by gel filtration analysis. The doses of GDI necessary for inhibiting the dissociation of [3H]GDP from and the binding of [35S]GTPyS to smg p25A were the same (Fig. 3).

GDI did not stimulate the GTPase activity of smg p25A and by itself showed neither [35S]GTPyS binding nor GTPase activity. GDI was inactive for c-Ha-ras p21, rhoB p20, and smg p21 in inhibiting both the dissociation of [3H]GDP and the binding of [35S]GTPyS and in stimulating GTPase activity.

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REFERENCES
7. We showed previously that bovine brain cytosol stimulated the dissociation of [3H]GDP from smg p25A (23). This dissociation was not, however, due to the real dissociation of [3H]GDP from smg p25A but to the action of smg p25A GDI in bovine brain cytosol making the [3H]GDP-bound form of smg p25A pass through Advantec A045A304D nitrocellulose filters used previously (23). We have not detected any activity that stimulates the dissociation of [3H]GDP from smg p25A in bovine brain cytosol.
A Regulatory Protein for smg p25


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