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KINETIC ANALYSIS OF THE BINDING OF GUANINE NUCLEOTIDE TO BOVINE BRAIN smg p25A[1]

Ikuo Shoji, Akira Kikuchi, Shinya Kuroda and Yoshimi Takai[2]

Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan

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SUMMARY: Bovine brain smg p25A, a guanine nucleotide-binding protein with a Mr of about 25,000, bound specifically GTP, guanosine 5'-(3-O-thio)triphosphate (GTPγS) and GDP. The initial velocities of the binding of GTPγS to GDP-bound smg p25A and the dissociation of GDP from this protein increased by decreasing Mg^{2+} concentrations or increasing NaCl concentrations. The initial velocity of the binding of GTPγS to GDP-free smg p25A was not affected by changing Mg^{2+} concentrations. These results indicate that the dissociation of GDP from smg p25A limits the binding of GTP to this protein, and suggest that there is a protein stimulating the dissociation of GDP from smg p25A and thereby stimulating the binding of GTP to this protein in mammalian tissues. In fact, the protein stimulating the dissociation of GDP, but not of GTPγS, from smg p25A was detected in bovine brain cytosol. © 1989 Academic Press, Inc.

There are many G proteins with Mr values of about 20,000, designated here as small Mr G proteins, in mammalian tissues (for reviews, see Refs. 1,2). smg p25A has been isolated from bovine brain membranes as one of them (3). smg p25A has both guanine nucleotide-binding and GTPase activities (3). The smg p25A cDNA has been also isolated from a bovine brain cDNA library and its primary structure has been determined (4). smg p25A is composed of 220 amino acids with a calculated Mr of 24,954. The smg p25A

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[2]To whom requests for reprints should be addressed.

Abbreviations used are: G proteins, guanine nucleotide-binding proteins; smg p25A, the smg-25A protein; ras p21, the ras protein; GAP, GTPase-activating protein; GTPγS, guanosine 5'-(3-O-thio)triphosphate; DTT, dithiothreitol.

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cDNA is identical with the rab3 cDNA isolated from a rat brain cDNA library (5,6). Two other cDNAs encoding small Mr G proteins highly homologous to smg p25A are cloned from the same bovine brain cDNA library (4). The proteins encoded by these cDNAs are designated as smg p25B and smg p25C. Three smg p25s have the consensus amino acid sequences for guanine nucleotide-binding and GTPase domains as described for other small Mr G proteins (1,2).

Although the functions of smg p25s are not known at present, the smg p25A mRNA has been detected abundantly in brain and adrenal medulla (7,8). The smg p25A mRNA has been also detected in rat pheochromocytoma PC-12 cells, and its level increases after differentiation of these cells into sympathetic neuron-like cells in response to nerve growth factor or dibutyryl cyclic AMP (8). These results suggest that smg p25A is related at least to the regulation of neuronal functions, particularly synaptic functions.

The modes of activation and action of smg p25A are not known at present. Among many small Mr G proteins, the modes of activation and action of ras p21s have been most extensively investigated. ras p21s have the GDP-bound inactive and GTP-bound active forms (1). The reduction of GTPase activity by point mutation increases their biological activities such as those transforming and differentiating cells (1). Decreasing Mg^{2+} concentrations from 10^{-3} to 10^{-7} molar ranges or increasing $(NH_4)_2SO_4$ concentrations in the presence of a high concentration (5 mM) of Mg^{2+} causes a marked increase in the dissociation of GDP from N-ras p21 and Ha-ras p21 in a cell-free system (9,10). On the basis of these observations and by analogy with a group of G proteins serving as transducers for membrane receptors such as G_s, G_i and transducin (for reviews, see Refs. 11-13), it has been considered that there are a protein converting the inactive form to the active form of ras p21s and an effector protein of which function is modulated by the active form. Consistent with this assumption, a protein directly interacting with c-ras p21s, designated as GAP, has been identified first in Xenopus oocytes (14) and subsequently purified and characterized from bovine brain cytosol (15). c-ras p21 GAP stimulates the GTPase activity of c-ras p21s but not of the activated ras p21s (16,17). The cDNA of this GAP has been isolated and its primary structure has been determined (17,18). The proteins directly interacting with smg p25A have not been identified.

In the present studies, we have investigated the kinetics of the binding of guanine nucleotide to smg p25A and have found that

the rate limiting step for the binding of GTP to smg p25A is the dissociation of GDP from this protein.

MATERIALS AND METHODS

Material and Chemicals——smg p25A was purified from bovine brain membranes (3). Bovine brain cytosol was prepared as described (19). [^{3}H]GDP (specific activity, 11 Ci/mmol) and [^{35}S]GTPγS (specific activity, 1,350 Ci/mmol) were obtained from Amersham Corp. and Du Pont-New England Nuclear, respectively.

Assay for the Binding of [^{35}S]GTPγS to smg p25A——The binding of [^{35}S]GTPγS to smg p25A was determined by use of the nitrocellulose filter method (3). smg p25A (2 pmol of protein) was incubated with 400 nM [^{35}S]GTPγS (specific activity, 2,000-4,000 cpm/pmol) for the indicated periods of time at 30°C in the reaction mixture (100 μl) containing 20 mM Tris/HCl at pH 7.5, 1 mM L-α-dimyristoylphosphatidylcholine, 1 mM DTT, 5 mM $MgCl_2$ and 400 mM NaCl. The reaction was stopped as described previously (3).

Assay for the Dissociation of [^{3}H]GDP from smg p25A——To make a [^{3}H]GDP-smg p25A binary complex, smg p25A (2 pmol of protein) was preincubated for 1.5 h at 30°C in the same reaction mixture (50 μl) as used for the assay for the binding of [^{35}S]-GTPγS to smg p25A except that 600 nM [^{3}H]GDP (2,000-4,000 cpm/pmol) and 100 mM NaCl were used instead of 400 nM [^{35}S]GTPγS and 400 mM NaCl, respectively. Another mixture (50 μl), containing 20 mM Tris/HCl at pH 7.5, 1 mM L-α-dimyristoylphosphatidylcholine, 1 mM DTT, 5 mM $MgCl_2$, 60 μM non-radioactive GTPγS and 20 mM EDTA and/or 1.5 M NaCl, was added to this preincubation mixture and incubated for the indicated periods of time at 30°C to measure the dissociation of [^{3}H]GDP from smg p25A.

Preparation of GDP-free smg p25A——Since the purified smg p25A appeared to be complexed with endogenous GDP, GDP-free smg p25A was prepared by the same method as used for the preparation of GDP-free Ha-ras p21s (10). Endogenous GDP complexed with smg p25A was replaced by [^{3}H]GDP by incubating 50 pmol of the protein with 1 μM [^{3}H]GDP for 20 min at 30°C in the same reaction mixture (500 μl) as used for the assay for the binding of [^{35}S]GTPγS to smg p25A except that 10 mM EDTA was added. The sample was then applied to a Sephadex G-25 column (1 x 27 cm) equilibrated with 20 mM Tris/HCl at pH 8.0, 1 mM DTT, 10% glycerol, 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 200 mM $(NH_4)_2SO_4$ and 5 mM EDTA. The elution was performed with the same buffer. Fractions of 0.5 ml each were collected. Under these conditions, [^{3}H]GDP bound to smg p25A was completely dissociated from this protein. GDP-free smg p25A was detected by measuring the activity of the binding of [^{3}H]GDP to this protein. The active fractions were pooled and dialyzed overnight against a large volume of 20 mM Tris/HCl at pH 8.0 containing 100 mM NaCl, 1 mM DTT, 10% glycerol and 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The dialyzed sample was used as GDP-free smg p25A.

Determinations——Free Mg^{2+} concentrations were calculated as described (20). Protein concentrations were determined by the method of Bradford (21) with bovine serum albumin as a reference protein.

RESULTS

Effect of Mg^{2+} on the Binding of [^{35}S]GTPγS to smg p25A——When smg p25A was incubated with 400 nM [^{35}S]GTPγS at 0.5 μM or 5 mM Mg^{2+}, smg p25A bound [^{35}S]GTPγS in a time-dependent manner

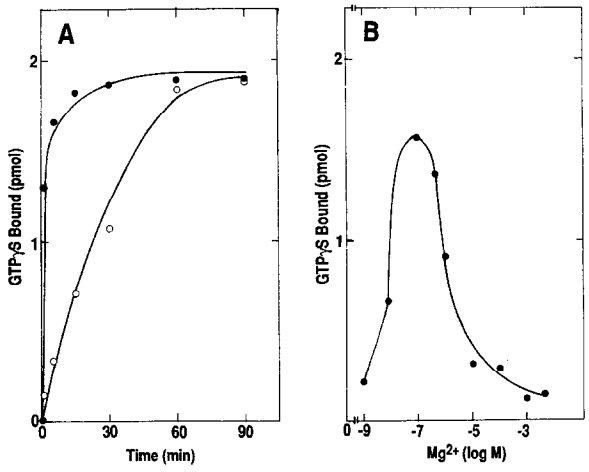

Fig. 1. Effect of Mg^{2+} on the binding of [^{35}S]GTPγS to smg p25A. A, time course. smg p25A (2 pmol of protein) was incubated with 400 nM [^{35}S]GTPγS in the presence of 400 mM NaCl either at 0.5 μM or 5 mM Mg^{2+} for various periods of time at 30°C. (●——●), at 0.5 μM Mg^{2+}; (○——○), at 5 mM Mg^{2+}. B, dose-dependent effect. smg p25A (2 pmol of protein) was incubated with 400 nM [^{35}S]GTPγS in the presence of 400 mM NaCl at various concentrations of Mg^{2+} for 2 min at 30°C. The results shown are representative of three independent experiments.

and one mol of the protein maximally bound about one mol of the nucleotide as shown Fig. 1A. However, the initial velocities between the two reactions were markedly different and that obtained at 0.5 μM Mg^{2+} was about thirty-fold greater than that obtained at 5 mM Mg^{2+}. Figure 1B shows the optimum concentrations of Mg^{2+} for this initial velocity. The maximal velocity was obtained in the range of 10^{-7} M Mg^{2+}, the velocity was decreased by increasing Mg^{2+} concentrations, and the minimum velocity was observed at over 10^{-5} M Mg^{2+}.

Effect of NaCl on the Binding of [^{35}S]GTPγS to smg p25A——In the presence of a high concentration (5 mM) of Mg^{2+}, a high concentration (2 M) of NaCl markedly stimulated the initial velocity of the binding of [^{35}S]GTPγS to smg p25A as shown in Fig. 2A. The velocity obtained at 2 M NaCl was about ten-fold greater than that obtained at 2 mM NaCl. This stimulatory effect of NaCl was dose-dependent as shown in Fig. 2B.

Effect of Mg^{2+} and NaCl on the Dissociation of [^{3}H]GDP from smg p25A——A [^{3}H]GDP-smg p25A binary complex was first prepared and the effect of Mg^{2+} and NaCl on the dissociation of [^{3}H]GDP from smg p25A was examined. As shown in Fig. 3, decreasing Mg^{2+} concentrations and increasing NaCl concentrations markedly stimulated the dissociation of [^{3}H]GDP from smg p25A. The half-life ($t_{1/2}$) for the dissociation of [^{3}H]GDP from smg p25A at 5 mM Mg^{2+}

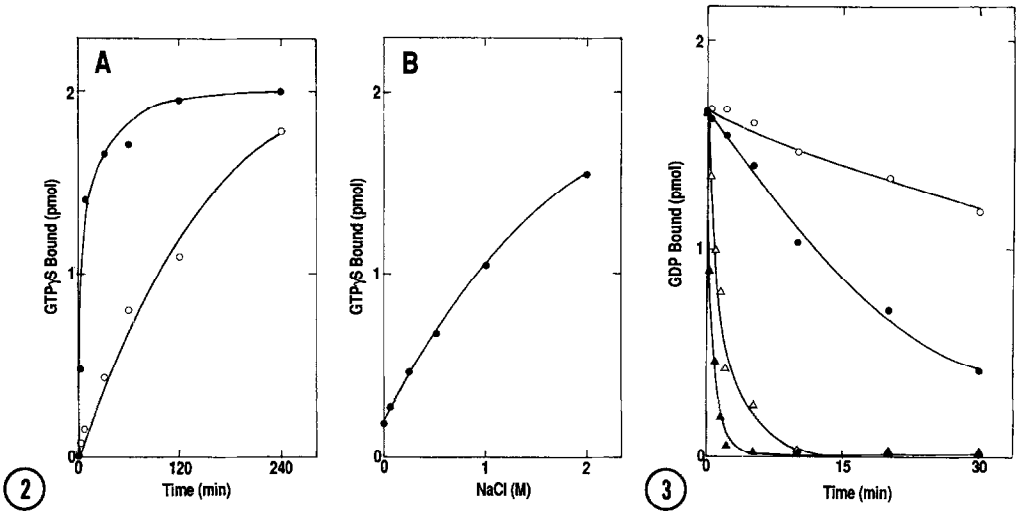

Fig. 2. Effect of NaCl on the binding of [^{35}S]GTPγS to smg p25A. A, time-course. smg p25A (2 pmol of protein) was incubated with 400 nM [^{35}S]GTPγS in the presence of 5 mM Mg^{2+} either at 2 mM or 2 M NaCl for various periods of time at 30°C. (○——○), at 2 mM NaCl; (●——●), at 2 M NaCl. B, dose-dependent effect. smg p25A (2 pmol of protein) was incubated with 400 nM [^{35}S]GTPγS in the presence of 5 mM Mg^{2+} at various concentrations of NaCl for 15 min at 30°C. The results shown are representative of three independent experiments.

Fig. 3. Effect of Mg^{2+} and NaCl on the dissociation of [^{3}H]GDP from smg p25A. A [^{3}H]GDP-smg p25A binary complex was incubated at the indicated final concentrations of Mg^{2+} and NaCl for various periods of time at 30°C. (○——○), at 5 mM Mg^{2+} and 50 mM NaCl; (●——●), at 5 mM Mg^{2+} and 800 mM NaCl; (△——△), at 0.5 μM Mg^{2+} and 50 mM NaCl; (▲——▲), at 0.5 μM Mg^{2+} and 800 mM NaCl. The results shown are representative of three independent experiments.

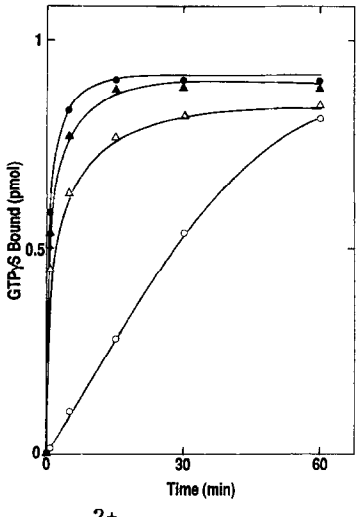

Fig. 4. Effect of Mg^{2+} on GDP-free smg p25A. smg p25A or GDP-free smg p25A (1 pmol of each protein) was incubated with 400 nM [^{35}S]GTPγS in the presence of 400 mM NaCl either at 0.5 μM Mg^{2+} or 5 mM Mg^{2+} for various periods of time at 30°C. (●——●), smg p25A at 0.5 μM Mg^{2+}; (○——○), smg p25A at 5 mM Mg^{2+}; (▲——▲), GDP-free smg p25A at 0.5 μM Mg^{2+}; (△——△), GDP-free smg p25A at 5 mM Mg^{2+}. The results shown are representative of three independent experiments.

and 50 mM NaCl was calculated to be about 80 min. At 5 mM Mg^{2+} and 800 mM NaCl, 0.5 μM Mg^{2+} and 50 mM NaCl, and 0.5 μM Mg^{2+} and 800 mM NaCl, $t_{1/2}$s were about 16 min, 1.9 min and 0.45 min, respectively. Dissociation rate constants (k), calculated by the equation: $k=0.693/t_{1/2}$, were about 0.0087 min^{-1}, 0.043 min^{-1}, 0.36 min^{-1}, and 1.5 min^{-1}, respectively.

Effect of Mg^{2+} on the Binding of [^{35}S]GTPγS to GDP-free smg p25A——Since the purified smg p25A appeared to be complexed with endogenous GDP, GDP-free smg p25A was prepared and the effect of Mg^{2+} on the initial velocity of the binding of [^{35}S]GTPγS to GDP-free smg p25A was examined. The initial velocity of the binding of [^{35}S]GTPγS to smg p25A, which might bind endogenous GDP, was more rapid at 0.5 μM Mg^{2+} than at 5 mM Mg^{2+} as shown in Fig. 4 (See also Fig. 1A). However, the initial velocities of the binding of [^{35}S]GTPγS to GDP-free smg p25A at 5 mM and 0.5 μM Mg^{2+} were almost the same.

Stimulation of the Dissociation of [^{3}H]GDP from smg p25A by Bovine Brain Cytosol——When a [^{3}H]GDP-smg p25A binary complex was incubated with bovine brain cytosol, the dissociation of [^{3}H]GDP from smg p25A was stimulated by the cytosol in time- and dose-dependent manners as shown in Fig. 5. This activity of the cytosol was killed by heat boiling or tryptic digestion. Under the same conditions, the cytosol did not stimulate the dissociation of [^{35}S]GTPγS from smg p25A. It remained to be clarified whether bovine brain cytosol stimulates the binding of [^{35}S]GTPγS to smg p25A, since it contains many G proteins as described (22).

DISCUSSION

It is established that many G proteins including those serving as transducers for membrane receptors and those involved in protein synthesis have two convertible forms, that is GDP-bound inactive and GTP-bound active forms (11-13, for reviews, see Refs. 23,24). In the case of G proteins serving as transducers for membrane receptors, the receptor stimulates the dissociation of GDP from the G protein and thereby stimulates the binding of GTP to the G protein when the receptor is occupied by its specific agonist (11-13). The GTP-bound active form then affects the function of its effector such as adenylate cyclase and cyclic GMP phosphodiesterase (11-13). Eukaryotic initiation factor-2 and prokaryotic elongation factor-Tu involved in protein synthesis have specific stimulatory proteins called GEF and EF-Ts, respectively, and the dissociation of GDP from and subsequent binding of GTP to each G protein are stimulated by these regulatory proteins

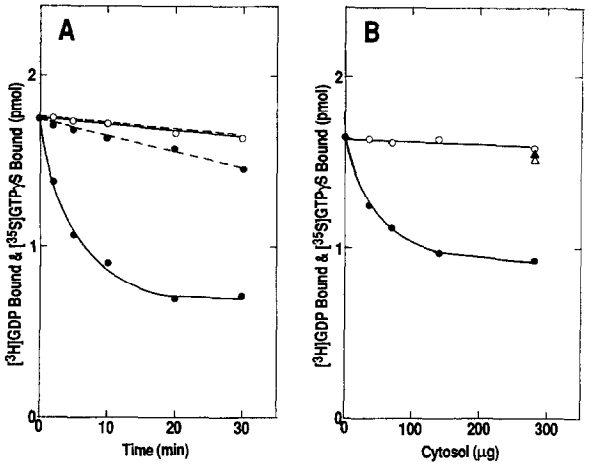

Fig. 5. Stimulation of the dissociation of [^{3}H]GDP from smg p25A by bovine brain cytosol. A, time course. smg p25A (2 pmol of protein) was preincubated with 600 nM [^{3}H]GDP or [^{35}S]GTPγS (2,000-4,000 cpm/pmol) for 1.5 h at 30°C to make a [^{3}H]GDP-smg p25A or [^{35}S]GTPγS-smg p25A binary complex. Another mixture (50 μl), containing 20 mM Tris/HCl at pH 7.5, 1 mM L-α-dimyristoylphosphatidylcholine, 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, 60 μM nonradioactive GTPγS and bovine brain cytosol (280 μg of protein), was added to this preincubation mixture and incubated for various periods of time at 4°C. (●), [^{3}H]GDP-smg p25A binary complex; (○), [^{35}S]GTPγS-smg p25A binary complex. (———), in the presence of the cytosol; (- - - -), in the absence of the cytosol. B, dose-dependent effect. A [^{3}H]GDP-smg p25A or [^{35}S]GTPγS-smg p25A binary complex was incubated for 10 min at 4°C in the same reaction mixture described above except that various doses of bovine brain cytosol were used. Tryptic digestion was performed by incubating the cytosol (280 μg of protein) with trypsin (17.5 μg of protein) for 1 h at 30°C. The reaction was stopped by the addition of trypsin inhibitor (52.5 μg of protein). Heat boiling of the cytosol was performed by boiling the cytosol for 3 min at 100°C. The boiled sample was cooled on ice and used. The [^{3}H]-GDP-smg p25A binary complex was incubated with these samples. (●), [^{3}H]GDP-smg p25A binary complex; (○), [^{35}S]GTPγS-smg p25A binary complex. (▲), the cytosol after tryptic digestion; (△), the cytosol after heat boiling. The results shown are representative of three independent experiments.

(23,24). In all of these cases, the rate limiting step for the binding of GTP to each G protein is the dissociation of GDP from the respective G proteins.

Similarly, ras p21s have been shown to have GDP-bound inactive and GTP-bound active forms and have been suggested to possess its stimulatory regulatory protein which probably stimulates the dissociation of GDP from ras p21s and thereby stimulates the binding of GTP to ras p21s. In yeast, the protein encoded by the CDC25 gene has been shown to stimulate guanine nucleotide exchange of RAS (25,26). However, such a stimulatory regulatory protein for mammalian ras p21s has not been identified.

This paper has shown that the initial velocities of the binding of GTPγS to GDP-bound smg p25A and the dissociation of GDP

from this protein increase by decreasing Mg^{2+} concentrations or increasing NaCl concentrations. Moreover, evidence is presented that the initial velocity of the binding of GTPγS to GDP-free smg p25A is not affected by changing Mg^{2+} concentrations. These results are consistent with earlier observations that decreasing Mg^{2+} concentrations from 10^{-3} to 10^{-7} molar range stimulates the dissociation of GDP from G proteins involved in protein synthesis and ras p21s (9,10,27). The present results together with these earlier observations suggest that the rate limiting step for the binding of GTP to smg p25A is the dissociation of GDP from smg p25A as described for other many G proteins, and that there is a protein stimulating the dissociation of GDP from smg p25A and thereby stimulating the binding of GTP to smg p25A in mammalian tissues. Consistent with this assumption, bovine brain cytosol stimulates the dissociation of GDP from smg p25A. Since bovine brain cytosol does not stimulate the dissociation of GTPγS from smg p25A, the stimulation of the dissociation of GDP from smg p25A by the cytosol is not merely due to the non-specific degradation of smg p25A. This activity of the cytosol is killed by tryptic digestion or heat boiling, suggesting that this activity is derived from a protein(s). It is conceivable that this protein also stimulates the binding of GTP to smg p25A, but this possibility remains to be clarified since bovine brain cytosol contains many G proteins (22). We are currently isolating this regulatory protein for smg p25A.

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