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A Mammalian Inhibitory GDP/GTP Exchange Protein (GDP Dissociation Inhibitor) for *smg* p25A Is Active on the Yeast *SEC4* Protein

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Evidence is accumulating that *smg* p25A, a small GTP-binding protein, may be involved in the regulated secretory processes of mammalian cells. The *SEC4* protein is known to be required for constitutive secretion in yeast cells. We show here that the mammalian GDP dissociation inhibitor (GDI), which was identified by its action on *smg* p25A, is active on the yeast *SEC4* protein in inhibiting the GDP/GTP exchange reaction and is capable of forming a complex with the GDP-bound form of the *SEC4* protein but not with the GTP-bound form. These results together with our previous findings that *smg* p25A GDI is found in mammalian cells with both regulated and constitutive secretion types suggest that *smg* p25A GDI plays a role in both regulated and constitutive secretory processes, although *smg* p25A itself may be involved only in regulated secretory processes. These results also suggest that a GDI for the *SEC4* protein is present in yeast cells.

The *SEC4* protein and *smg* p25A belong to a superfamily of *ras* p21/*ras* p21-like small GTP-binding proteins (G proteins) (7, 10, 18; for reviews, see references 2 and 22). The *SEC4* protein has been shown to play an essential role at the final stage of the constitutive secretory pathway of the yeast *Saccharomyces cerevisiae* (18). The *SEC4* protein is found in association with the cytoplasmic surface of secretory vesicles and the plasma membrane and may serve to control the exocytotic process (5). Although the definitive function of *smg* p25A has not yet been determined, it is likely that this protein plays an analogous role in secretory processes of mammalian cells. This supposition is based on the observations that this protein is found mainly on the cytoplasmic surface of secretory vesicles and granules and plasma membrane and partly in the cytosol (3, 4, 8, 11–13, 16, 19, 24), that a synthetic peptide designed from the putative effector region of the *rab3A* protein (identical to *smg* p25A) inhibits the fusion of Golgi vesicles in CHO cells as assayed in a cell-free system (17), and that *smg* p25A shares a high degree of amino acid sequence homology with the *SEC4* protein and the *YPT1* protein, which have been shown to be involved in intracellular protein transport in yeast cells (5, 18, 21, 25). Since *smg* p25A is found solely in cells possessing a regulated secretory pathway, its function is probably limited to regulated exocytotic events.

smg p25A has GDP-bound and GTP-bound forms which are interconvertible, as described for other small G proteins (2, 7, 10, 22). The conversion from the GDP-bound to GTP-bound form of *smg* p25A is regulated by a GDP/GTP exchange protein named GDP dissociation inhibitor (GDI) (9, 20). *smg* p25A GDI inhibits the dissociation of GDP from and thereby the subsequent binding of GTP to *smg* p25A. This protein was first purified from bovine brain cytosol (20); however, both immunoblot analysis with an anti-*smg* p25A GDI monoclonal antibody and Northern (RNA) blot analysis have revealed that *smg* p25A GDI is present in cells possessing only a constitutive secretion pathway as well as in cells that also have a regulated secretion pathway (9, 14). These results suggest that although *smg* p25A is involved only in regulated secretory processes, its GDI may interact with one or more related GTP-binding proteins involved in constitutive secretory processes as well. To address this issue, we have examined in this study whether *smg* p25A GDI can interact with the yeast *SEC4* protein.

smg p25A and its GDI were purified to near homogeneity from bovine brain membranes and cytosol, respectively (7, 20). The *SEC4* protein was purified to near homogeneity from an overproducing yeast strain (6). The *smg* p25A GDI activity which inhibited the dissociation of [^{3}H]GDP from and the binding of [^{35}S]GTPγS to *smg* p25A or the *SEC4*

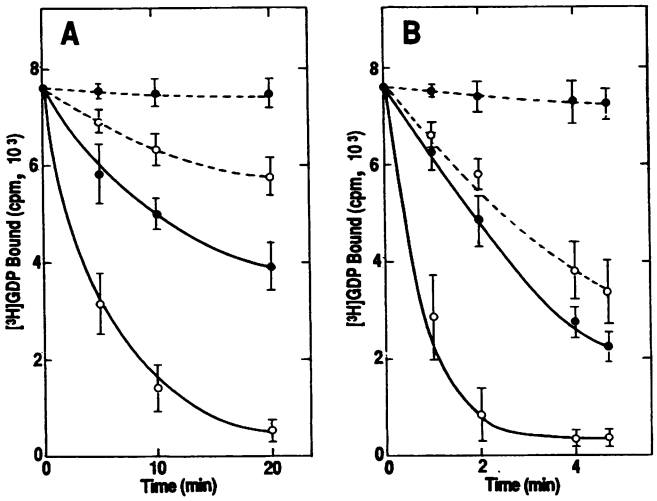

FIG. 1. Effect of *smg* p25A GDI on the dissociation of [^{3}H]GDP from *smg* p25A (A) and the *SEC4* protein (B). The activity of *smg* p25A GDI which inhibited the dissociation of [^{3}H]GDP from *smg* p25A and the *SEC4* protein was measured at 0.5 μM (——) and 10 mM (– – – –) Mg^{2+} in the presence (●) and absence (○) of GDI. Results are means ± standard errors of three independent experiments.

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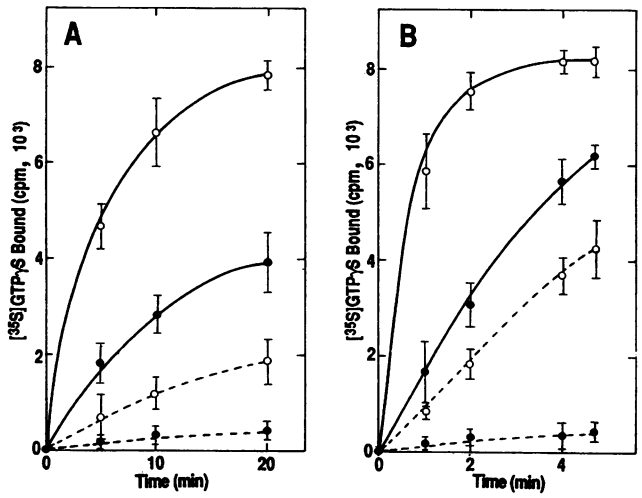

FIG. 2. Effect of *smg* p25A GDI on the binding of [^{35}S]GTPγS to *smg* p25A (A) and the *SEC4* protein (B). The *smg* p25A GDI activity which inhibited the binding of [^{35}S]GTPγS to *smg* p25A or the *SEC4* protein was measured at 0.5 μM (——) and 10 mM (- - - -) Mg^{2+} in the presence (●) and absence (○) of GDI. Results are means ± standard errors of three independent experiments.

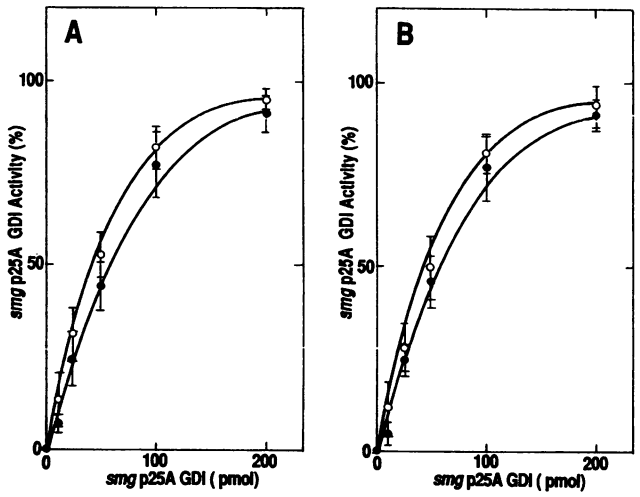

FIG. 3. Dose-dependent effect of *smg* p25A GDI on the dissociation of [^{3}H]GDP from and the binding of [^{35}S]GTPγS to *smg* p25A and the *SEC4* protein. The *smg* p25A GDI activity which inhibited the dissociation of [^{3}H]GDP from (A) and the binding of [^{35}S]GTPγS to (B) *smg* p25A (○) or the *SEC4* protein (●) (1 pmol each) was measured at 0.5 μM Mg^{2+} with various doses of *smg* p25A GDI. The second incubation time was 10 min for *smg* p25A and 2 min for the *SEC4* protein. *smg* p25A GDI activity was expressed as percent inhibition of the dissociation of [^{3}H]GDP from and the binding of [^{35}S]GTPγS to *smg* p25A or the *SEC4* protein. Results are means ± standard errors of three independent experiments.

protein was assayed as previously described (20). Briefly, to measure the dissociation of [^{3}H]GDP from *smg* p25A or the *SEC4* protein, 1 pmol of each small G protein was incubated at 30°C with 1 μM [^{3}H]GDP (8×10^3 cpm/pmol) for 20 or 2 min, respectively, in 25 μl of a mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM L-α-dimyristoylphosphatidylcholine, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 10 mM EDTA to produce the [^{3}H]GDP-bound form of each sample. The free Mg^{2+} concentration in the first incubation was 0.5 μM. After this first incubation, $MgCl_2$ (5 μl) was added to give a final concentration of 16.7 mM, and the mixture was immediately cooled on ice to prevent the dissociation of [^{3}H]GDP from *smg* p25A or the *SEC4* protein. Each radiolabeled sample was incubated with or without *smg* p25A GDI (50 pmol) in a mixture (70 μl) containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1.43 μM GTP, and 10.72 mM EDTA or 11.07 mM $MgCl_2$, and the second incubation was performed for the indicated periods of time at 30°C. Since the affinities of GDP for *smg* p25A and the *SEC4* protein are much lower than those of GTP, this concentration of cold GTP is sufficient to chase the reaction. The free Mg^{2+} concentration in the second incubation was 0.5 μM or 10 mM. About 2 ml of ice-cold 20 mM Tris-HCl (pH 7.5) containing 25 mM $MgCl_2$ and 100 mM NaCl was added to the reaction mixture, which was then rapidly filtered on nitrocellulose filters (BA-85; 0.45-μm pore size; Schleicher & Schuell). The filters were washed four times with this solution and the radioactivity was counted. To measure the binding of [^{35}S]GTPγS to *smg* p25A or the *SEC4* protein, the GDP-bound form of each small G protein (1 pmol) was first formed as described above except that nonradioactive GDP was used instead of [^{3}H]GDP. Each radiolabeled sample was incubated with or without *smg* p25A GDI (50 pmol) as described above, except that [^{35}S]GTPγS (8×10^3 cpm/pmol) was used instead of nonradioactive GTP. The second incubation was performed for the indicated times at 30°C.

[^{3}H]GDP dissociated from both *smg* p25A and the *SEC4* protein more rapidly at 0.5 μM Mg^{2+} than at 10 mM Mg^{2+} (Fig. 1). The initial velocities of this reaction at both low and high concentrations of Mg^{2+} were much faster for the *SEC4* protein than for *smg* p25A. *smg* p25A GDI slowed the rates of [^{3}H]GDP dissociation from the *SEC4* protein, as described for *smg* p25A (20). Similarly, [^{35}S]GTPγS bound to both *smg* p25A and the *SEC4* protein in a time-dependent manner (Fig. 2). The initial velocities of this reaction at both 0.5 μM and 10 mM Mg^{2+} were much faster for the *SEC4* protein than for *smg* p25A. *smg* p25A GDI slowed the rates of binding of [^{35}S]GTPγS to the *SEC4* protein, as described for *smg* p25A (20). The amounts of *smg* p25A GDI necessary for the inhibition of the dissociation of [^{3}H]GDP from and the subsequent binding of [^{35}S]GTPγS to *smg* p25A and to the *SEC4* protein were nearly the same (Fig. 3). In the experiments shown in Fig. 1 to 3, a large molar excess of *smg* p25A GDI over *smg* p25A and the *SEC4* protein was used, because the affinity of *smg* p25A GDI for *smg* p25A was not high (1) and the concentrations of *smg* p25A and the *SEC4* protein used here were low.

We have previously demonstrated that *smg* p25A GDI stoichiometrically forms a complex with the GDP-bound form of *smg* p25A but not with the GTP-bound form of *smg* p25A or the GDP-bound form of other small GTP-binding proteins, including *smg* p21B and *rhoB* p21 (1). *smg* p25A GDI similarly formed a complex with the GDP-bound form of the *SEC4* protein but not with the GTP-bound form, as shown by sucrose density gradient ultracentrifugation (Fig. 4). On these sucrose density gradients, both the GDP-bound and GTP-bound forms of *smg* p25A banded at a position corresponding to an M_r value of about 140,000 in the absence of *smg* p25A GDI, as previously described (1). Since the M_r values of *smg* p25A estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its primary structure were about 24,000 and 25,000, respectively, this *smg* p25A

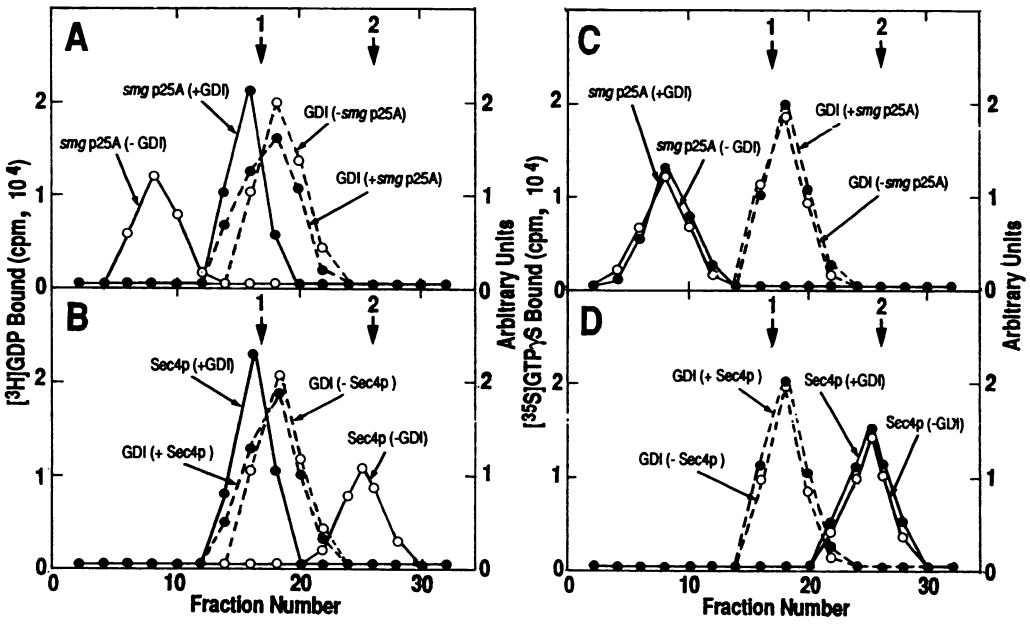

FIG. 4. Complex formation of *smg* p25A GDI with the [^{3}H]GDP-bound form of *smg* p25A and the *SEC4* protein. *smg* p25A (30 pmol) or the *SEC4* protein (15 pmol) was incubated at 30°C with [^{3}H]GDP and [^{35}S]GTPγS (2×10^4 cpm/pmol each) for 20 or 2 min, respectively, to produce the [^{3}H]GDP-bound or [^{35}S]GTPγS-bound form, respectively, of each protein as described previously (1). The [^{3}H]GDP-bound or [^{35}S]GTPγS-bound form of *smg* p25A (30 pmol) or the *SEC4* protein (15 pmol) was mixed with or without *smg* p25A GDI (670 pmol) and subjected to sucrose density gradient ultracentrifugation as described previously (1). *smg* p25A GDI alone was separately subjected to the same ultracentrifugation. After the centrifugation, fractions of 150 μl each were collected from the bottom. The amounts of *smg* p25A and the *SEC4* protein were measured by counting the radioactivity of a 40-μl aliquot for *smg* p25A and an 80-μl aliquot for the *SEC4* protein from each fraction after filtration on nitrocellulose filters as described previously (1). The amount of *smg* p25A GDI was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by protein staining with silver as described previously (1). (A) [^{3}H]GDP-bound form of *smg* p25A; (B) [^{3}H]GDP-bound form of the *SEC4* protein; (C) [^{35}S]GTPγS-bound form of *smg* p25A; (D) [^{35}S]GTPγS-bound form of the *SEC4* protein. GDI and Sec4p indicate *smg* p25A GDI and the *SEC4* protein, respectively. Arrows 1 and 2 indicate the positions of human hemoglobin (4.5S; M_r 64,000) and horse myoglobin (1.9S; M_r 17,000), respectively.

might be a polymerized form. In contrast, both the GDP-bound and GTP-bound forms of the *SEC4* protein appeared at a position corresponding to an M_r value of about 25,000. This M_r value of the *SEC4* protein was similar to that estimated by column chromatography and to that calculated from its primary structure (6, 18) and thus represents a monomeric form of the protein. In the presence of *smg* p25A GDI, only the GDP-bound form of both *smg* p25A and the *SEC4* protein altered migration and appeared at a position corresponding to an M_r value of about 85,000. Since the M_r values of *smg* p25A GDI estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the S value, and its primary structure were about 54,000, 59,000, and 51,000, respectively (9, 20), these results suggest that *smg* p25A GDI formed a complex with the GDP-bound form of both *smg* p25A and the *SEC4* protein at a molar ratio of 1:1 but not with the GTP-bound form. Under comparable conditions, neither the GDP-bound form of *smg* p25A nor the *SEC4* protein formed a complex with the stimulatory GDP/GTP exchange protein for *smg* p21s or with other *ras* p21-like small GTP-binding proteins (data not shown) (23).

We have shown here that mammalian *smg* p25A GDI is active on the yeast *SEC4* protein in inhibiting the GDP/GTP exchange reaction and is capable of forming a complex with the GDP-bound form of the yeast *SEC4* protein but not with the GTP-bound form. These interactions occur at GDI concentrations similar to those seen with *smg* p25A. Bovine *smg* p25A shares about 43% amino acid sequence homology with the yeast *SEC4* protein (10, 18, 25). The four domains of *ras* p21 that have been implicated in GTP binding and GTP hydrolysis, residues 10 to 17, 57 to 63, 113 to 120, and 143 to 149, are highly conserved in both *smg* p25A and the *SEC4* protein (2, 10, 18, 22, 25). Two additional domains, residues 51 to 59 and 83 to 97 of *smg* p25A, are highly homologous to the corresponding regions in the *SEC4* protein. Since *smg* p25A GDI interacts with *smg* p25A and the *SEC4* protein but not with *ras* p21s, it is possible that one or both of these two additional conserved domains are responsible for the specificity of the interaction of *smg* p25A GDI. The first of these two domains, residues 51 to 59 in *smg* p25A, corresponds to the region from 32 to 40 in *ras* p21 known as the effector domain.

Through the use of an anti-*smg* p25A monoclonal antibody, *smg* p25A has been found in secretory cells of the regulated secretion type, such as neurons, endocrine cells, and exocrine cells, but not in cells such as hepatocytes and lymphocytes which secrete only constitutively (11, 12). In contrast, we have found by immunoblot analysis with an anti-*smg* p25A GDI monoclonal antibody and by Northern blot analysis that *smg* p25A GDI is present in cells of both types (9, 14). These earlier findings together with the present results strongly suggest that *smg* p25A GDI is involved in both regulated and constitutive secretory processes and may interact with additional related GTP-binding proteins. Consistent with this hypothesis, we have recently purified a novel small GTP-binding protein from rat hepatocytes that is also recognized by *smg* p25A GDI (15). This liver small

GTP-binding protein is similar to but not identical to *smg* p25A (15).

While a GDI for the *SEC4* protein has not yet been identified in yeast cells, its existence was suggested by the kinetic properties of the purified protein (6). Since the affinity of GTP for the *SEC4* protein is 20-fold higher than that of GDP, the GDP/GTP exchange reaction could be regulated through the stabilization of the GDP-bound state of the protein by a GDI activity as follows. By analogy with *ras* p21, the *SEC4* protein is supposed to be predominantly the GDP-bound form in the resting state (2). The GDI for the *SEC4* protein forms a complex with the GDP-bound form of the *SEC4* protein and keeps it the GDP-bound form. When an unidentified signal comes to either the *SEC4* protein or its GDI, the GDI for the *SEC4* protein dissociates from the *SEC4* protein and consequently stimulates its GDP/GTP exchange reaction. Initial attempts to identify a yeast counterpart of *smg* p25A GDI through the use of its cDNA as a probe under low-stringency conditions have not been successful. Moreover, expression of this cDNA in a *sec4-8* mutant did not suppress the temperature-sensitive growth resulting from this mutation. Further investigation is necessary to understand the functions and modes of action of *smg* p25A GDI and *smg* p25A in mammalian secretory cells and the role of GDI in the regulation of the *SEC4* protein in yeast cells.

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