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A stimulatory GDP/GTP exchange protein for smg p21 is active on the post-translationally processed form of c-Ki-ras p21 and rhoA p21

(ras p21/rho p21/prenylation)

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ABSTRACT **We have purified a stimulatory GDP/GTP exchange protein for smg p21A and -B, ras p21-like small GTP-binding proteins (G proteins), cloned its cDNA, and named it GDP dissociation stimulator (smg p21 GDS). We show here that smg p21 GDS is active not only on smg p21A and -B but also on c-Ki-ras p21 and rhoA p21, all of which are post-translationally processed. Furthermore, we show that smg p21 GDS is inactive on the post-translationally unprocessed form of these proteins and on the post-translationally processed form of c-Ha-ras p21 and smg p25A. All of the small G proteins recognized by smg p21 GDS have a cDNA-predicted C-terminal "CAAX" motif (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid) and a polybasic region upstream of this motif. These results suggest that smg p21 GDS is at least active on a group of small G proteins having these unique C-terminal structures. Moreover, they suggest that the C-terminal post-translational processing of these small G proteins, by farnesylation or geranylgeranylation of the C-terminal cysteine residue, removal of amino acids in positions denoted "AAX", and carboxyl methylation of the exposed cysteine residue, is important for the smg p21 GDS action.**

There is a superfamily of small GTP-binding proteins (G proteins) (for reviews, see refs. 1–3). Small G proteins have distinctive C-terminal structures, "CAAX", "CC", or "CXC" (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid), and can be post-translationally processed by (*i*) farnesylation or geranylgeranylation of the cysteine residue, (*ii*) removal of the amino acids in positions denoted "AAX", (*iii*) carboxyl methylation of the exposed cysteine residue, and (*iv*) palmitoylation of a cysteine residue upstream of the prenylated cysteine residue (for reviews, see refs. 4 and 5, and also see refs. 6–8). These post-translational processing steps affect the ability of small G proteins to bind to membranes and to exert their functions (3–5).

Small G proteins have GDP-bound inactive and GTP-bound active forms that are interconvertible by GDP/GTP exchange and GTPase reactions (2, 3). The GDP/GTP exchange and GTPase reactions are regulated by GDP/GTP exchange protein (GEP) and GTPase activating protein, respectively (2, 3). There are stimulatory and inhibitory GEPs, named GDP dissociation stimulator (GDS) and GDP dissociation inhibitor, respectively (9–20). GDSs have been identified for ras p21s, smg p21s, and rho p21s (14–20), whereas GDP dissociation inhibitors have been identified for rho p21s and smg p25A (9–13). We have proposed (21–23) that the post-translational processing of smg p21B, rhoA p21, and smg p25A may be important for the actions of their respective GEPs but not for the actions of their respective GTPase activating proteins. Our results indicate that these GEPs are inactive on the post-translationally unprocessed form of small G proteins produced in *Escherichia coli*, whereas GTPase activating proteins are still active on the bacterial proteins (22, 23). Furthermore, we have studied the specificity of smg p21 GDS toward ras p21s produced in *E. coli* and have reported (14) that smg p21 GDS is inactive on bacterial c-Ha-ras p21. However, other investigators have reported that ras p21 GDS is active on bacterial ras p21s (16–19). The reason for this discrepancy between our results and their results is unknown.

c-Ha- and c-Ki-ras p21s have been purified in post-translationally unprocessed and processed forms by use of a baculovirus/insect cell system (24, 25). We used these forms of ras p21s in the present study to reexamine the specificity of smg p21 GDS. We report here that smg p21 GDS is active on the post-translationally processed form of smg p21B, c-Ki-ras p21, and rhoA p21 but inactive on the unprocessed form of these proteins and on the post-translationally processed form of c-Ha-ras p21 and smg p25A. Therefore, we tentatively term this GEP smg GDS in this paper.

MATERIALS AND METHODS

Materials and Chemicals. The post-translationally processed forms of smg p21B, c-Ki(2B)-ras p21, rhoA p21, and smg p25A were purified from human platelet, bovine brain, bovine aortic smooth muscle, and bovine brain, respectively (26–29). The cDNAs of c-Ki(2B)-ras p21, c-Ha-ras p21, and rhoA p21 were kindly provided by R. A. Weinberg (Massachusetts Institute of Technology), F. Tamanoi (The University of Chicago), and P. Madaule (Centre National de la Recherche Scientifique Laboratoire, Gif sur Yvette, France), respectively. smg GDS was purified from smg GDS-overexpressing *E. coli* (20).

Expression of Small G Proteins in *Spodoptera frugiperda* Cells (Sf9 Cells). The cDNA of smg p21B, c-Ki-ras p21, c-Ha-ras p21, or rhoA p21 with *Bam*HI sites upstream of the initiator methionine codon and downstream of the termination codon was synthesized by the polymerase chain reaction (30). These fragments were inserted into a pAcYM1 *Autographa californica* baculovirus transfer vector at the *Bam*HI cloning site to express each cDNA under the control of the polyhedrin promoter in Sf9 cells (31).

Purification of Small G Proteins from Sf9 Cells. c-Ki-ras p21-expressing Sf9 cells (1×10^8 cells) were suspended with

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Abbreviations: G proteins, GTP-binding proteins; GEP, GDP/GTP exchange protein; GDS, GDP dissociation stimulator; GTP[γ-^{35}S], guanosine 5′-[γ-[^{35}S]thio]triphosphate.

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10 ml of buffer A (10 mM Tris·HCl, pH 7.5/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol/10 μM (*p*-amidinophenyl) methanesulfonyl fluoride). This suspension was sonicated and centrifuged at 100,000 × *g* for 1 hr. About one-tenth of c-Ki-ras p21 was recovered in the membrane fraction. The post-translationally unprocessed form of c-Ki-ras p21 was purified from the soluble fraction. The supernatant (1.3 ml, 17 mg of protein) was diluted with 2.6 ml of buffer B (20 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol) containing 0.75% sodium cholate and applied to a Mono Q HR5/5 column equilibrated with buffer C (20 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/0.5% sodium cholate). After the column was washed with 26 ml of the same buffer, the material was eluted with a 60-ml linear gradient of NaCl (0–1.0 M) in buffer C. Fractions (1 ml) were collected. A single peak of guanosine 5′-[γ-[^{35}S]-thio]triphosphate (GTP[γ-^{35}S])-binding activity appeared in fractions 12–25.

The post-translationally processed form of c-Ki-ras p21 was purified from the membrane fraction. The 100,000 × *g* pellet was resuspended in buffer B containing 1% sodium cholate, stirred for 30 min, and then centrifuged at 100,000 × *g* for 1 hr. The supernatant (2 ml, 4 mg of protein) was diluted with 2 ml of buffer B and applied to a Mono Q HR5/5 column equilibrated with buffer C. After the column was washed with 26 ml of the same buffer, material was eluted with a 60-ml linear gradient of NaCl (0–1.0 M) in buffer C. Fractions (1 ml) were collected. A single peak of GTP[γ-^{35}S]-binding activity appeared in fractions 49 and 50.

The post-translationally unprocessed or processed forms of smg p21B, c-Ha-ras p21, and rhoA p21 were purified by the same method as described above except that 30 mM *n*-octyl glucoside was used for c-Ha-ras p21 and 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was used for rhoA p21.

We confirmed in three ways that smg p21B, c-Ki-ras p21, c-Ha-ras p21, and rhoA p21, which were purified from the soluble and membrane fractions, were post-translationally unprocessed and processed, respectively. (*i*) The proteins purified from the soluble fraction were completely digested with *Achromobacter* protease I. The C-terminal peptides were synthesized according to the amino acid sequences deduced from the respective cDNAs. The *Achromobacter* protease I digests and synthesized peptides were subjected to C_8 reverse-phase high performance liquid chromatography (28). The C-terminal peptides of the *Achromobacter* protease I digests were eluted at the same positions as those of the synthesized peptides (Ser-Ser-Cys-Gln-Leu-Leu for smg p21B, Cys-Val-Ile-Met for c-Ki-ras p21, and Ser-Gly-Cys-Leu-Val-Leu for rhoA p21). (*ii*) smg p21B and rhoA p21 purified from the soluble fraction were [^{3}H]geranylgeranylated, and c-Ki-ras p21 and c-Ha-ras p21 were [^{3}H]farnesylated in cell-free systems, whereas these proteins purified from the membrane fraction were not. (*iii*) When the baculovirus-infected Sf9 cells were labeled with [^{3}H]mevalonolactone, small G proteins in the membrane fraction were labeled, whereas those in the soluble fraction were not (see Fig. 5) (7, 24, 25).

Assays. The smg GDS activities to stimulate the dissociation of [^{3}H]GDP from and the binding of GTP[γ-^{35}S] to the GDP-bound form of each small G protein were assayed (14). The GTP[γ-^{35}S]-binding activity was assayed by a filtration method using nitrocellulose filters (14).

Complex Formation of smg GDS with Small G Proteins. The GDP-bound form of the small G protein (30 pmol) was incubated with smg GDS (75 pmol) and subjected to 4.8-ml continuous sucrose density gradient ultracentrifugation (32). After the centrifugation, fractions (170 μl) were collected. The amounts of small G protein and smg GDS were determined by SDS/PAGE followed by protein staining with silver (32).

RESULTS

Effect of smg GDS on Small G Proteins. smg GDS stimulated the dissociation of [^{3}H]GDP from and the binding of GTP[γ-^{35}S] to the post-translationally processed form of smg p21B in a time-dependent manner (Fig. 1) (14). Under the same conditions, smg GDS stimulated the dissociation of [^{3}H]GDP from and the binding of GTP[γ-^{35}S] to c-Ki-ras p21 and rhoA p21 but was inactive on c-Ha-ras p21 and smg p25A, all of which were post-translationally processed (Fig. 1). The effect of smg GDS on smg p21B, c-Ki-ras p21, and rhoA p21 was dose-dependent, and the doses of smg GDS necessary for these proteins were similar (Fig. 2*A*). The doses of smg p21B, c-Ki-ras p21, and rhoA p21 for this smg GDS action were also similar (Fig. 2*B*). In these experiments, smg p21B, c-Ki-ras p21, rhoA p21, and smg p25A purified from mammalian tissues and recombinant c-Ha-ras p21 purified from the membrane fraction of the insect cells were used. When recombinant smg p21B, c-Ki-ras p21, and rhoA p21 purified from the membrane fraction of the insect cells were used, essentially the same results were obtained (Fig. 3). In contrast, smg GDS was inactive on the post-translationally unprocessed form of smg p21B purified from the soluble fraction of the insect cells (Fig. 3). smg GDS was also inactive on the post-translationally unprocessed form of c-Ki-ras p21 and rhoA p21 purified from the soluble fraction of the insect cells.

Complex Formation of smg GDS with Small G Proteins. When the post-translationally processed form of smg p21B was subjected to continuous sucrose density gradient ultracentrifugation, it mostly stuck to the centrifugation tube and was not recovered as a peak (Fig. 4*A*) (32). On the same ultracentrifugation, the post-translationally processed form of c-Ki-ras p21 and rhoA p21 appeared as a single peak at the positions corresponding to molecular weights of about 160,000 and 130,000, respectively (Fig. 4 *C* and *E*). Since the molecular weights of c-Ki-ras p21 and rhoA p21 estimated from SDS/PAGE and calculated from their primary structures are about 21,000, they might be aggregated (33, 34). In contrast, the post-translationally unprocessed forms of smg p21B, c-Ki-ras p21, and rhoA p21 appeared as a single peak

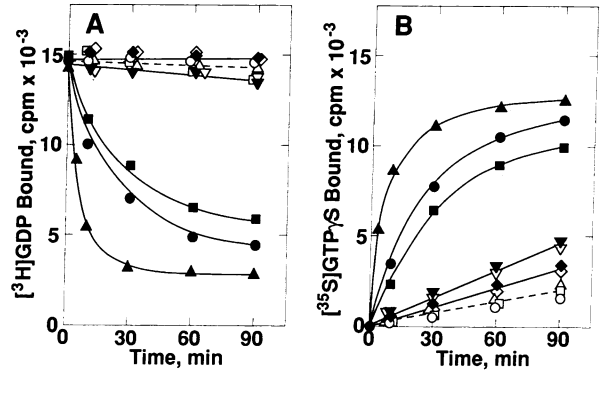

FIG. 1. Effect of smg GDS on the dissociation of [^{3}H]GDP from and the binding of GTP[γ-^{35}S] to small G proteins. The ability of smg GDS to stimulate the dissociation of [^{3}H]GDP from and the binding of GTP[γ-^{35}S] to each small G protein was assayed in the presence of smg GDS (6 pmol) and a small G protein (2 pmol). (*A*) Dissociation of [^{3}H]GDP. (*B*) Binding of GTP[γ-^{35}S]. Solid symbols, in the presence of smg GDS; open symbols, in the absence of smg GDS. ● and ○, Human platelet smg p21B; ■ and □, bovine brain c-Ki-ras p21; ▲ and △, bovine smooth muscle rhoA p21; ◆ and ◇, c-Ha-ras p21 purified from the membrane fraction of insect cells; ▼ and ▽, bovine brain smg p25A. The results are representative of three experiments.

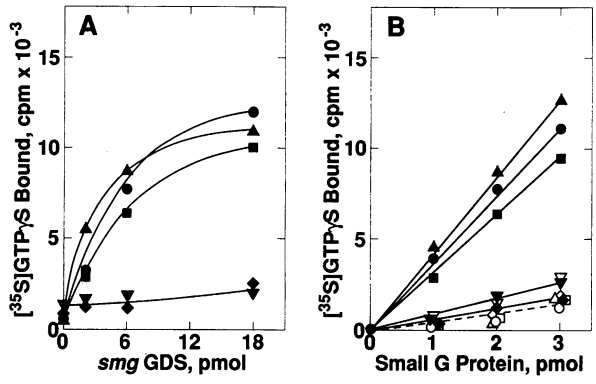

FIG. 2. Dose-dependent effect of smg GDS and small G proteins on the binding of GTP[γ-^{35}S]. The ability of smg GDS to stimulate the binding of GTP[γ-^{35}S] to various small G proteins was assayed for 10 min for rhoA p21 and for 30 min for other small G proteins. The same preparations of the various small G proteins were used as described in Fig. 1. (*A*) Effect of various doses of smg GDS. Various doses of smg GDS and a fixed amount of various small G proteins (2 pmol) were used. ●, smg p21B; ■, c-Ki-ras p21; ▲, rhoA p21; ◆, c-Ha-ras p21; ▼, smg p25A. (*B*) Effect of various doses of small G proteins. A fixed amount of smg GDS (6 pmol) and various doses of small G proteins were used. Solid symbols, in the presence of smg GDS; open symbols, in the absence of smg GDS. ● and ○, smg p21B; ■ and □, c-Ki-ras p21; ▲ and △, rhoA p21; ◆ and ◇, c-Ha-ras p21; ▼ and ▽, smg p25A. The results are representative of three experiments.

at the positions corresponding to their minimum molecular weights, suggesting that they were not aggregated (Fig. 4 *B*, *D*, and *F*). smg GDS also appeared at a position corresponding to the molecular weight of its monomeric form, that is,

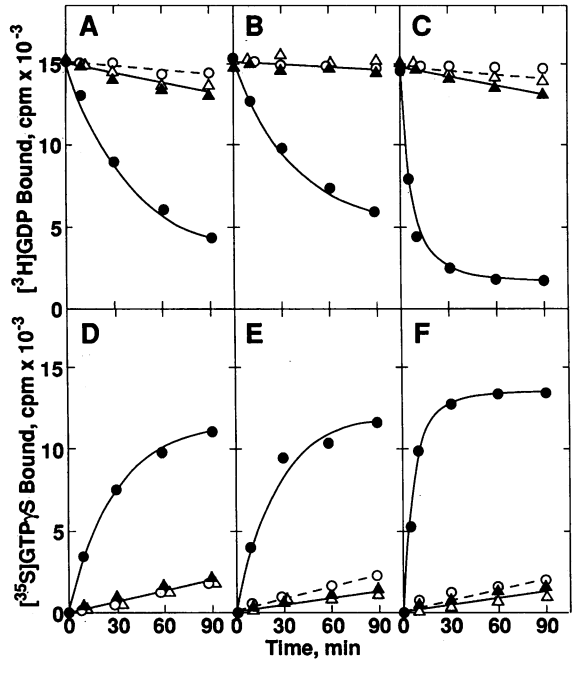

FIG. 3. Effect of smg GDS on the dissociation of [^{3}H]GDP from and the binding of GTP[γ-^{35}S] to small G proteins purified from the insect cells. The stimulatory activity of smg GDS on the dissociation of [^{3}H]GDP from and the binding of GTP[γ-^{35}S] to various small G proteins were assayed in the presence of smg GDS (6 pmol) and various small G proteins (2 pmol). (*A–C*) Dissociation of [^{3}H]GDP. (*D–F*) Binding of GTP[γ-^{35}S]. (*A* and *D*) smg p21B. (*B* and *E*) c-Ki-ras p21. (*C* and *F*) rhoA p21. ● and ○, Small G proteins purified from the membrane fraction of insect cells; ▲ and △, small G proteins purified from the soluble fraction of insect cells; ● and ▲, in the presence of smg GDS; ○ and △, in the absence of smg GDS. The results are representative of three experiments.

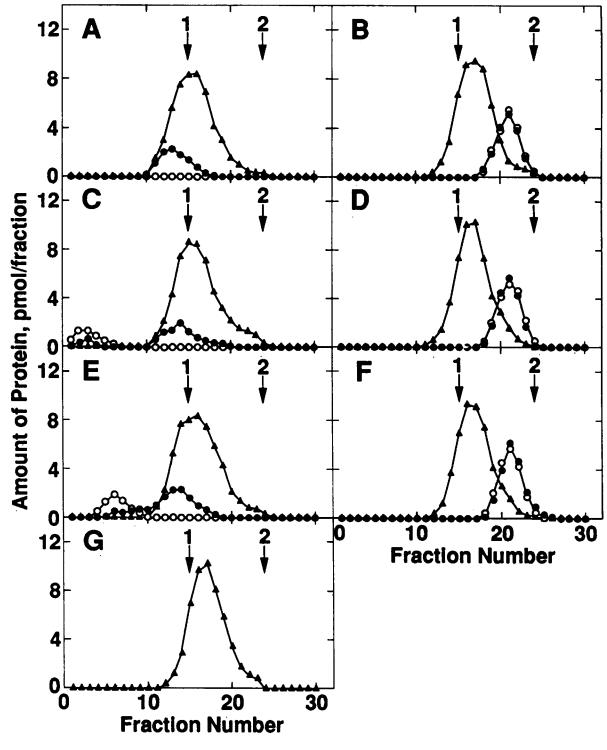

FIG. 4. Complex formation of smg GDS with the post-translationally processed form of various small G proteins. The GDP-bound form of various small G proteins (30 pmol) was incubated with or without smg GDS (75 pmol) for 10 min for rhoA p21 and for 20 min for other small G proteins and subjected to sucrose density gradient ultracentrifugation. (*A* and *B*) smg p21B. (*C* and *D*) c-Ki-ras p21. (*E* and *F*) rhoA p21. (*G*) smg GDS alone. (*A*, *C*, and *E*) Small G proteins purified from mammalian tissues. (*B*, *D*, and *F*) Small G proteins purified from the soluble fraction of insect cells. ●, Small G proteins in the presence of smg GDS; ○, small G proteins in the absence of smg GDS; ▲, smg GDS. Arrows 1 and 2 indicate the positions of human hemoglobin (4.5 S; M_r, 64,000) and horse myoglobin (1.9 S; M_r, 17,000), respectively. The results are representative of three experiments.

about 53,000 (Fig. 4*G*). When smg GDS was mixed with the post-translationally processed form of smg p21B, c-Ki-ras p21, or rhoA p21 and each mixture was subjected to the same ultracentrifugation, each small G protein appeared at the same position with a molecular weight of about 74,000 (Fig. 4 *A*, *C*, and *E*). Concomitantly, smg GDS partly shifted to a position with a higher molecular weight. The partial shift was due to the addition of an excess amount of smg GDS against each small G protein. In these experiments, the post-translationally processed form of small G proteins purified from mammalian tissues were used, but essentially the same results were obtained with the post-translationally processed form of small G proteins purified from the membrane fraction of the insect cells (data not shown). In contrast, when smg GDS was mixed with the post-translationally unprocessed form of smg p21B, c-Ki-ras p21, or rhoA p21 and each mixture was subjected to the same ultracentrifugation, smg GDS and each small G protein appeared separately at their original positions (Fig. 4 *B*, *D*, and *F*). Under comparable conditions, smg GDS did not form a complex with the post-translationally processed form of c-Ha-ras p21 or smg p25A (data not shown).

Purity of and [^{3}H]Mevalonate Labeling of Small G Proteins. The samples of smg p21B, c-Ki-ras p21, rhoA p21, and c-Ha-ras p21 purified from the insect cells were more than 95% pure on SDS/PAGE (Fig. 5). Small G proteins from the membrane fraction migrated as slightly faster bands on gels than those from the soluble fraction (24, 25). When the insect

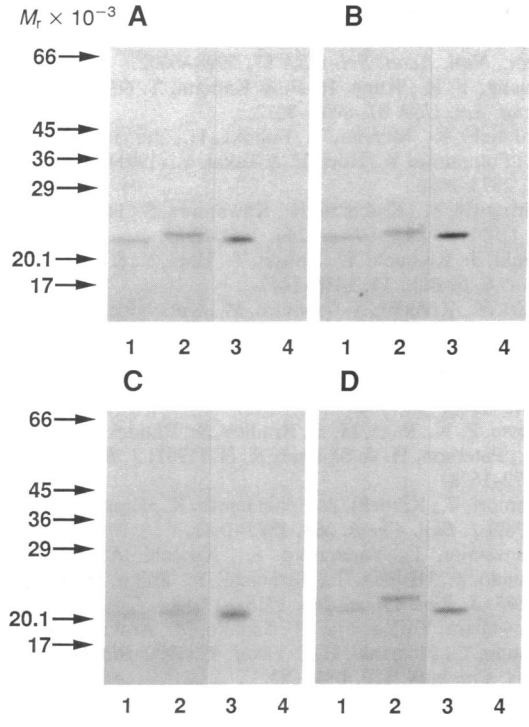

FIG. 5. Purity of and [^{3}H]mevalonate labeling of small G proteins. Sf9 cells were infected with baculovirus carrying the cDNA of each small G protein. Labeling with [^{3}H]mevalonate was performed as described (7, 24, 25). Small G proteins purified from the insect cells and the [^{3}H]mevalonate-labeled membrane and soluble fractions were analyzed by SDS/PAGE and by fluorography of the dried gel. (*A*) smg p21B. (*B*) c-Ki-ras p21. (*C*) rhoA p21. (*D*) c-Ha-ras p21. Lanes: 1, small G proteins purified from the membrane fraction; 2, small G proteins purified from the soluble fraction; 3, membrane fraction; 4, soluble fraction; 1 and 2, Coomassie brilliant blue staining; 3 and 4, fluorography. The protein markers used were bovine serum albumin (M_r, 66,000), chicken ovalbumin (M_r, 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r, 36,000), carbonic anhydrase (M_r, 29,000), trypsin inhibitor (M_r, 20,100), and horse myoglobin (M_r, 17,000).

cells were labeled with [^{3}H]mevalonate, small G proteins in the membrane fraction were labeled with [^{3}H]mevalonate, whereas those in the soluble fraction were not (Fig. 5).

DISCUSSION

We have shown that partial tryptic digestion of smg p21B separates it into the N-terminal GDP/GTP-binding fragment and the C-terminal prenylated membrane-binding tail (Lys-Lys-Ser-Ser-geranylgeranyl-Cys methyl ester) (21). We have found by use of this N-terminal fragment that smg GDS neither interacts with the N-terminal fragment nor affects its GDP/GTP exchange reaction (21). On the basis of this finding, we have proposed that either or both of the polybasic region and isoprenoid moiety in the C-terminal region of smg p21B may be important for the smg GDS action (21). Consistent with this proposal, we have shown here that smg GDS is inactive on the post-translationally unprocessed form of smg p21B purified from the soluble fraction of insect cells.

We have reexamined the effect of smg GDS on the post-translationally processed form of c-Ha- and c-Ki-ras p21s and have shown here that smg GDS is inactive even on the post-translationally processed form of c-Ha-ras p21 but interacts with the post-translationally processed form of c-Ki-ras p21 and stimulates its GDP/GTP exchange reaction. Consistent with the result of smg p21B, smg GDS is inactive on the post-translationally unprocessed form of c-Ki-ras p21.

We have shown here that smg GDS is active on the post-translationally processed form of rhoA p21 and is inactive on the post-translationally unprocessed form. We have previously partially purified rho p21 GDS, which is active on mammalian rhoA and -B p21s but inactive on mammalian smg p21B and bacterial c-Ha-ras p21 (15). rho p21 GDS is active on the post-translationally processed form of rhoA p21 but inactive on the post-translationally unprocessed form (23). The relationship between rho p21 GDS and smg GDS is not clear at present, since rho p21 GDS has not yet been purified to homogeneity.

smg GDS is inactive on smg p25A, which is also geranylgeranylated but has a C-terminal CXC motif and lacks a polybasic upstream region (8). Among small G proteins thus far studied, smg GDS is active on the proteins having both the C-terminal prenylated cysteine residue and the upstream polybasic region. smg p21A and -B, c-Ki-ras p21, and rhoA p21 have this structure (3–7). In contrast, smg GDS is inactive on c-Ha-ras p21. c-Ha-ras p21 has a C-terminal prenylated cysteine residue and a second cysteine residue that may be palmitoylated but lacks a nearby polybasic region (3–5). These results suggest that smg GDS is active on a group of small G proteins that have a polybasic region upstream of the C-terminal prenylated cysteine residue and that the post-translational processing of these small G proteins may be important for their interaction with smg GDS. If the isoprenoid moiety is important for the smg GDS action, smg GDS may recognize both geranylgeranyl and farnesyl moieties since smg GDS is active on both the geranylgeranylated and farnesylated proteins. It is unknown, however, at present which of the C-terminal post-translational modifications is responsible for the interaction of small G proteins with smg GDS. Furthermore, we can not rule out the possibility that other post-translational modifications such as protein phosphorylation may be responsible for this interaction. It may be noted that the previously reported ras p21 GDS (rGEF) is also active on several small G proteins, including c-Ha-ras p21, rap1A p21 (smg p21A), and rho p21, but that this GEP is active on the post-translationally unprocessed form of these proteins (19). Since the primary structure of rGEF has not yet been determined, the relationship between smg GDS and rGEF is not known.

We have found previously that smg GDS is inactive on rhoB p21 purified from bovine brain membranes but have found in this study that smg GDS is also active on this protein (data not shown) (14). rhoB p21 lacks a polybasic region but has a second cysteine residue that could be palmitoylated (3–5). The reason for the previous failure of smg GDS to affect the rhoB p21 activity (14) might be due to the presence of a high concentration of CHAPS in the rhoB p21 sample, because this detergent at >0.1% inhibits completely the smg GDS action (unpublished observation). Since we have not yet determined the post-translationally processed C-terminal structure of rhoB p21, we will conclude the effect of smg GDS on rhoB p21 after the determination of this structure.

smg p21A and -B are phosphorylated by protein kinase A, and this phosphorylation makes them sensitive to the stimulatory effect of smg GDS on GDP/GTP exchange (35–37). It is likely that the protein kinase A-catalyzed phosphorylation of smg p21 induces its activation by smg GDS. c-Ki-ras p21 is also phosphorylated by protein kinases A and C (38). It is possible that this phosphorylation affects the interaction of c-Ki-ras p21 with smg GDS as is the case with smg p21. A protein kinase C-activating phorbol ester stimulates the conversion of an unidentified type of ras p21s from the GDP-bound to GTP-bound form in Jurkat cells (39). It could be speculated that the protein kinase C-catalyzed phosphorylation of c-Ki-ras p21 induces the activation of c-Ki-ras p21 by smg GDS. rhoA p21 could be also phosphorylated by protein kinase A, since rhoA p21 has a potential phosphorylation site

by protein kinase A in a region comparable to those of smg p21s and c-Ki-ras p21 (3). Generally, several small G proteins are present in a single type of cell. Therefore, it is possible that smg p21s, c-Ki-ras p21, and rhoA p21 are regularly activated by smg GDS through their phosphorylation and exert their specific functions in intact cells.

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