SmgGDS stabilizes nucleotide-bound and -free forms of the Rac1 GTP-binding protein and stimulates GTP/GDP exchange through a substituted enzyme mechanism

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The Rac proteins, Rac1 and Rac2, are essential components of the NADPH oxidase system of phagocytes and regulate the actin assembly associated with membrane ruffling. These functions are controlled by the GTP-bound form of Rac. The biochemical interaction between Rac and its only known GDP-dissociation stimulator (termed smgGDS) was characterized. SmgGDS was able to stimulate the incorporation of guanosine 5′-[γ-thio]-triphosphate GTP[γS] into the RhoA, Rac2, Rac1, Rap1A and CDC42Hs GTP-binding proteins, but the activity was greatest toward RhoA and Rac2. Isoprenoid modification of these proteins was not absolutely required for the interaction with smgGDS. Interestingly, the activity of smgGDS toward Rac1 could not be observed in a [^{3}H]GDP/GTP exchange assay under conditions where it stimulated incorporation of GTP[γS] into Rac1. We determined that smgGDS prevented the loss of Rac1 activity during the [^{3}H]GDP/GTP exchange assay by demonstrating the ability of smgGDS to inhibit the loss of Rac1 GTP[γS]-binding during incubations at 30 °C. This stabilizing effect was exactly counterbalanced by the ability of smgGDS to stimulate the release of [^{3}H]GDP from Rac1, thereby producing no net observable effect in the exchange assay. SmgGDS was able to effectively stimulate the release of GDP but not GTP[γS] from Rac1. SmgGDS maintains Rac1 in a nucleotide-free form after release of GDP, indicating that the reaction between Rac1 and smgGDS involves a substituted enzyme mechanism.

INTRODUCTION

The Rac proteins, Rac1 and Rac2, are members of the Rho family of low molecular weight GTP-binding proteins (LMWGs). Rac1 has been shown to be involved in growth factor-stimulated actin filament assembly associated with the cellular processes of pinocytosis and membrane ruffling [1]. Rac2 and Rac1 have also been demonstrated to be crucial components of the phagocyte NADPH oxidase system, which is responsible for generating superoxide anion used in defending the human body from bacterial invasion [2–5].

As with all other GTP-binding proteins, the Rac proteins consist of two interconvertible guanine nucleotide binding forms, the active GTP-bound form and the inactive GDP-bound form. Switching between these two forms is regulated by three different types of regulatory proteins: GTPase-activating proteins which stimulate GTP hydrolysis, GDP dissociation inhibitors (GDIs) which inhibit GDP dissociation and may also inhibit GTP hydrolysis, and GDP dissociation stimulators (GDSs) which stimulate the exchange of GDP for GTP [6,7]. In resting phagocytes, the Rac proteins are maintained in an inactive cytosolic form by complexation with Rho-GDI [2,8–10]. Stimulation of cells with agonists (e.g. *N*-formylmethionyl-leucyl-phenylalanine, phorbol 12-myristate 13-acetate) which induce the production of superoxide anion activates the Rac proteins and induces their translocation, along with two other cytosolic oxidase components — $p47_{phox}$ and $p67_{phox}$, from cytoplasm to cell membrane to form the functioning NADPH oxidase [11]. The mechanism(s) of Rac protein activation in cells is not yet known. However, activation of superoxide formation stimulated by the Rac proteins in broken cell assays is dependent on the exchange of GTP for GDP on Rac catalysed by endogenous [12] or exogenously added exchange proteins [4]. Rac can also be induced to 'translocate' to the membrane *in vitro* by the addition of guanosine-5′-[γ-thio]triphosphate (GTP[γS]) ([13] G. M. Bokoch, B. P. Bohl and T.-H. Chuang, unpublished work). Currently, the only protein reported to be able to catalyse nucleotide exchange on Rac is the smgGDS originally described by Takai and co-workers [6,14,15].

SmgGDS is a 61 kD protein with 558 amino acid residues, originally isolated from bovine brain cytosol [14] and cloned by Kaibuchi et al. [15]. This protein and Dbl, a human oncogene product, are the only two proteins reported so far to have GDP/GTP exchange activity toward Rho family proteins [16,17]. Dbl, however, has substantially more activity toward CDC42Hs and RhoA than toward Rac proteins [17,18]. SmgGDS appears to be distinct from Dbl and other known exchange-factors which act solely on Ras, such as CDC25, SCD25, SOS, Ras-GRF and Vav [19], in that it has been shown to have GDP/GTP exchange activity toward a number of small GTP-binding proteins, including Rac1, Rac2, RhoA, k-Ras, and Rap1B [16]. An isoprenylated carboxyl terminus of these GTP-binding proteins has been reported to be required for functional and physical interactions with smgGDS [20,21].

In this study, we determined the biochemical characteristics of the interaction between purified recombinant smgGDS and

Abbreviations used: GDS, GDP dissociation stimulator (smgGDS refers to the protein originally described by Takai, et al.); LMWG, low molecular weight GTP-binding protein; GTP[γS], guanosine 5′-[γ-thio]-triphosphate, DMPC, dimyristoylphosphatidylcholine; GDI, GTP-dissociation inhibitor; DTT, dithiothreitol.

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LMWG. Our data indicate that smgGDS stimulates the incorporation of GTP[γS] into a number of Ras-related GTP-binding proteins, and that this activity is observed with both post-translationally isoprenylated and non-prenylated forms of these proteins. We found that exchange activity was more potent towards RhoA and Rac2 than towards Rac1, Rap1A and CDC42Hs. SmgGDS releases GDP from Rac1 and has the previously unreported effect of stabilizing both nucleotide-bound and -free forms of Rac1 from denaturation *in vitro*. Our data indicate that a substituted enzyme kinetics mechanism governs the interaction of smgGDS with Rac1 and probably other LMWG substrates.

EXPERIMENTAL

Materials/proteins

Recombinant unprocessed Rac1, CDC42Hs and RhoA were purified from overexpressing *Escherichia coli* [8,12]. Processed (isoprenylated) Rac1 and Rap1A were isolated from cell membranes after protein expression in a baculovirus/Sf9 insect cell system [12,22]. GST–Rac1 fusion protein was purified from overexpressing *E. coli* (a gift from Dr. Alan Hall). [^{35}S]GTP[γS] (1400 Ci/mmol) and [^{3}H]GDP (32 Ci/mmol) were purchased from Dupont–NEN. All other reagents used were of the best grade commercially available.

Expression and purification of recombinant smgGDS

The smgGDS cDNA was generated by PCR amplification from a bovine brain cDNA library. A cDNA fragment encoding amino acid residues 1–240 of smgGDS was obtained by using a 5′-oligonucleotide primer (GGGGGATCCGGTACCATGGATAATCTCAGTGATACCTTGAAGAAGCTGAAGATAACAGC) which contains a *Bam*H1 site and a *Nco*1 site, and a 3′-oligonucleotide primer (GTTTTGAGCTCAGCGATATCC) containing an *Sst*1 site. A cDNA fragment encoding amino acid residues 241–558 was amplified by using a 5′-oligonucleotide primer (CGCTGAGCTCAAAACCGC) which contains an *Sst*1 site, and 3′-oligonucleotide primer (GGGGGATCCTCAGCTTTCCACAGCCAGTCTCTGCTCCTGTGAGAGAGGC) containing a *Bam*H1 site. Both PCR amplified products were subcloned into an M13mp18 vector. Single-stranded DNA was sequenced by using sequenase, essentially as described by the manufacturer (Upstate Biotechnology, Inc). The cDNA sequence was identical at the amino acid level to the previously published sequence for bovine smgGDS [15], except for an alanine for cysteine substitution at codon 335. This substitution was confirmed with cDNAs from three independent PCR amplifications. A similar substitution was reported in the human homologue [23], and is therefore not likely to perturb smgGDS function. The full length cDNA was subcloned into a bacterial expression vector, PET11d (Novagen), at the *Nco*1 and *Bam*H1 sites and transformed into *E. coli* strain BL21 (DE3) for protein expression. The transformed cells were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside to produce the recombinant smgGDS. For protein purification, the cells were harvested by centrifugation, suspended in buffer A [25 mM Tris pH = 8, 1 mM EDTA, 1 mM dithiothreitol (DTT)] plus 1 mM phenylmethanesulphonyl fluoride, 0.1% Triton X-100, 0.2 mg/ml lysozyme, incubated at 30 °C for 20 min, and then broken by sonication. After centrifugation, proteins in the supernatant were separated by a DEAE–Sephacel column equilibrated with buffer A. SmgGDS was resolved with a 0–1 M linear gradient of NaCl in buffer A and eluted at a concentration of around 0.4 M, as monitored by u.v. spectrophotometer and SDS/PAGE visualization. After dialysis against buffer A, further purification was performed with a Pharmacia f.p.l.c. 5/5 MonoQ column equilibrated with buffer A and eluted with a 0–0.6 M NaCl gradient in the same buffer. The isolated smgGDS was estimated by SDS/PAGE to be more than 95% pure.

[^{35}S]GTPγS incorporation assay

The ability of smgGDS to stimulate the incorporation of GTP[γS] into various LMWGs was determined by incubating 60 nM of each LMWG with 240 nM smgGDS in a solution containing 27.0 mM Tris/HCl (pH 8), 4.5 mM Hepes (pH 8), 1 mM DTT, 5.1 mM EDTA, 10.2 mM $MgCl_2$ (free Mg^{2+} = 5.1 mM) and 2.0 μM [^{35}S]GTP[γS] ($\sim 2.5 \times 10^4$ c.p.m./pmole) at 30 °C. At the indicated times, aliquots were removed and added to 2 ml of ice-cold stop solution (25 mM Tris/HCl (pH 8), 100 mM NaCl, 30 mM $MgCl_2$, 2 mM DTT, 0.1 mg/ml BSA) and incorporation of [^{35}S]GTP[γS] into the protein was determined by vacuum filtration on BA-85 nitrocellulose filters and liquid scintillation counting [8].

[H^3]GDP/GTP exchange assay

To determine the activity of smgGDS in the exchange of prebound [H^3]GDP from LMWGs in the presence of exogenous GTP, the LMWG (60 nM) was first loaded with [^{3}H]GDP in a solution containing 25 mM Tris/HCl (pH = 8), 1 mM DTT, 40 μg/ml BSA, 4.7 mM EDTA, 0.16 mM $MgCl_2$ (36 nM free Mg^{2+}) and 4.5 μM [^{3}H]GDP (10^4 c.p.m./pmole) at 30 °C for 7 min. In some experiments, dimyristoylphosphatidylcholine (DMPC) was added at a concentration of 1 mM. This reaction was stopped by adding $MgCl_2$ to a final concentration of 9.15 mM (final free Mg^{2+} = 5.5 mM). SmgGDS and unlabelled GTP were then added to a final concentration of 240 nM and 164 μM respectively to start the reaction. The amount of [^{3}H]GDP remaining on the LMWG was quantified as indicated in the [^{35}S]GTP[γS] incorporation assay.

[^{3}H]GDP dissociation assay

This assay was performed essentially the same way as the [^{3}H]GDP/GTP exchange assay described above except that the [^{3}H]GDP bound to the LMWG was released by smgGDS in the absence of any additional exogenous GTP at a free Mg^{2+} concentration of 7.0 mM.

[^{35}S]GTP[γS] dissociation assay

This assay was performed essentially the same way as the [H^3]GDP-dissociation assay except that the LMWGs were initially loaded with 2.58 μM [^{35}S]GTP[γS].

Stabilization effect of smgGDS toward Rac1

Rac1 protein (60 nM) was loaded with cold GDP and the concentration of free Mg^{2+} raised to 7.0 mM to stop the reaction, as in the [^{3}H]GDP-dissociation assay. After incubation of this reaction mixture in the presence or absence of 240 nM of smgGDS at 30 °C for the indicated times, aliquots were removed and added to an equal volume of 30 μM [^{35}S]GTP[γS] ($\sim 1 \times 10^4$ c.p.m./pmole) and 160 mM EDTA to adjust the final concentration of free Mg^{2+} to 165 nM. The reaction was incubated at 30 °C for 7 min, and [^{35}S]GTP[γS] bound to Rac1 was determined as in the [^{35}S]GTP[γS] incorporation assay.

Complex formation between Rac1 and smgGDS

Aliquots of 80 nM GST–Rac1 fusion protein were preloaded with or without guanine nucleotides in solution A [25 mM Tris/HCl (pH 8), 1 mM DTT, 40 μg/ml BSA, 4.7 mM EDTA, 0.16 nM $MgCl_2$ (36 nM free Mg^{2+})] containing either 40 μM GDP or GTP[γS] at 20 °C for 10 min. The reactions were stopped by the addition of $MgCl_2$ to a final concentration of 9.15 mM (free Mg^{2+} = 5.5 mM). SmgGDS was then added to a final concentration of 360 nM and incubated at 20 °C for 10 min. A 20 μl slurry of glutathione–sepharose beads was then added to absorb the protein complexes. After 10 min, the beads were washed five times with solution A containing either 40 μM GDP or GTP[γS], suspended in Laemmli sample buffer and the proteins were resolved with SDS/PAGE on 15% gels. Quantities of complexed smgGDS were determined by Western blotting using specific antibody raised against the purified recombinant smgGDS and quantitated by densitometry.

RESULTS

In order to evaluate the activity of smgGDS toward Rac and other LMWGs, bovine smgGDS was expressed in *E. coli* and purified to near homogeneity as described in the Experimental section. The ability of smgGDS to stimulate the incorporation of [^{35}S]GTP[γS] into various LMWGs was tested under conditions (i.e. at a high free Mg^{2+} concentration) in which the LMWGs have very low nucleotide-exchange rates in the absence of exchange factors [8]. Our initial experiments indicated that smgGDS was able to stimulate the exchange of [^{35}S]GTP[γS] for endogenous GDP, both on unprocessed and post-translationally processed LMWG, as illustrated by the data shown for Rac1 in Figure 1. In the presence of smgGDS, we could stimulate the binding of GTP[γS] to unprocessed RhoA up to a level equal to the amount of active RhoA protein added to the assay (i.e. 100% incorporation of [^{35}S]GTP[γS] after incubation for 50 min at 30 °C; Figure 1). SmgGDS was similarly effective on processed Rac2. In comparison, smgGDS only stimulated binding of GTP[γS] to levels of 53%, 42%, 32% and 17% of maximum for processed Rac1, unprocessed Rac1, unprocessed CDC42Hs, and processed Rap1A respectively by 50 min (Figure 1). Essentially no differences in the kinetics of guanine nucleotide exchange were observed for any of these proteins, in contrast to the report by Sasaki et al. [24] that the velocity of the exchange reaction for Rho was greater than for Rac1. Thus, smgGDS was more active toward RhoA and Rac2 than toward the other LMWGs, and isoprenyl modification of LMWGs is clearly not required for the interaction with smgGDS, although a direct comparison between processed and unprocessed Rac1 suggests that isoprenylation may facilitate the interaction.

The ability of smgGDS to stimulate the exchange of prebound [^{3}H]GDP with exogenous GTP at high free-Mg^{2+} concentration was also tested. As expected, smgGDS stimulated the release of [^{3}H]GDP from both RhoA and Rap1A (Figure 2). This effect of smgGDS on GDP dissociation was not observed with Rac1 however; a result which appeared to be inconsistent with the [^{35}S]GTP[γS] incorporation data of Figure 1. When we examined the effect of smgGDS on the release of prebound [^{3}H]GDP from Rac1 under low free-Mg^{2+} concentrations, at which Rac protein has a relatively high intrinsic GDP dissociation rate [8,25], we observed that [^{3}H]GDP/GTP exchange was stimulated by smgGDS (Figure 3). This observation suggested that smgGDS did indeed have the ability to stimulate the exchange of GTP for GDP on Rac1 under the appropriate assay conditions. Indeed, we subsequently determined that inclusion of 1 mM DMPC in

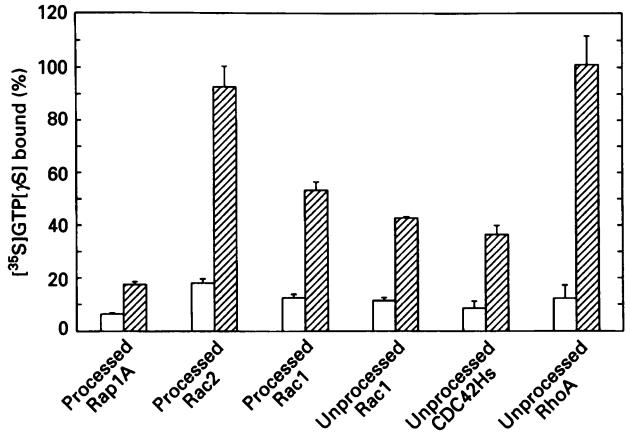

Figure 1 SmgGDS-stimulated incorporation of [^{35}S]GTP[γS] into various LMWGs

Incorporation rate of [^{35}S]GTP[γS] into 60 nM of various LMWGs, as indicated, was determined as described in the presence (▨) or absence (□) of 240 nM of smgGDS at 30 °C for 50 min. The results shown are representative of at least three independent experiments.

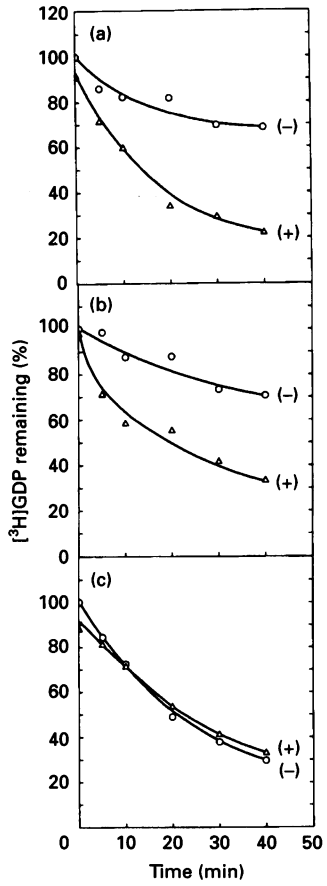

Figure 2 SmgGDS-stimulated [^{3}H]GDP/GTP exchange of RhoA (unprocessed), Rap1A (processed) and Rac1 (processed) under high free-Mg^{2+} concentration conditions

The exchange rate of prebound [^{3}H]GDP from 60 nM of RhoA (**a**), Rap1A (**b**) and Rac1 (**c**) with exogenous GTP in the presence (+) or absence (−) of 240 nM smgGDS was determined as described in the Experimental section. The results shown are representative of at least three independent experiments.

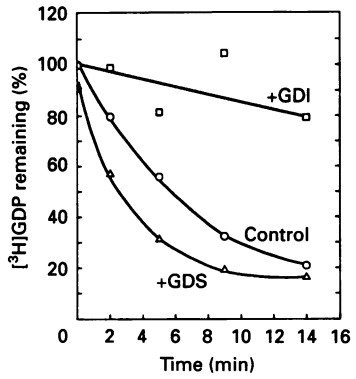

Figure 3 SmgGDS-stimulated [^{3}H]GDP/GTP exchange of processed Rac1 under low free-Mg^{2+} concentration conditions

The exchange-rate of prebound [^{3}H]GDP from 60 nM of processed Rac1 with exogenous GTP in the absence (○) or presence of 240 nm of smgGDS (△), or 180 nM of RhoGDI (□) was determined as described. RhoGDI is a GDP dissociation inhibitor that interacts only with isoprenylated Rac1.

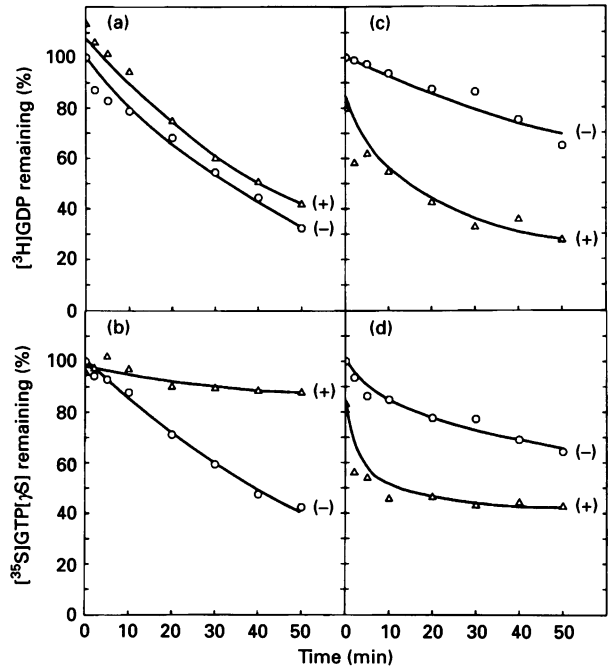

Figure 4 Nucleotide-releasing effect of smgGDS toward processed Rac1 and Rap1A

Rac1 (60 nM) (**a**, **b**) and Rap1A (**c**, **d**) were preloaded with [^{3}H]GDP (**a**, **b**) and [^{35}S]GTP[γS] (**b**, **d**) as described. The nucleotide-bound proteins were then incubated at 30 °C with (+) or without (−) 240 nM smgGDS in the absence of any exogenous unlabelled nucleotides. Under these conditions, in the presence of smgGDS only the nucleotide-release rate is detected, whereas in the absence of smgGDS the apparent nucleotide-release rate is equivalent to the decay rate of the nucleotide-binding protein.

the high Mg^{2+} assay allowed us to detect readily exchange activity of smgGDS toward Rac1 (not shown). This was due to the ability of this phospholipid to stabilize Rac1 from denaturation during the course of the assay (see following experiments). We took advantage of the lability of Rac1 in the absence of DMPC to characterize the interaction of smgGDS with an LMWG.

Working under high Mg^{2+} conditions in the absence of DMPC, we noted (see Figure 2) that the apparent rate of [^{3}H]GDP dissociation from Rac1 (~ 70% of prebound [^{3}H]GDP lost during the 50 min assay) was much greater than that from either RhoA or Rap1A (~ 30% loss of [^{3}H]GDP over the 50 min assay). Two possibilities might account for this rapid loss of GDP from Rac1. The first is that Rac1 has a higher intrinsic GDP exchange rate than does RhoA or Rap1A. The second is that Rac1 is not totally stable during the course of the release assay at 30 °C at high Mg^{2+}, and that part of the rapid loss of [^{3}H]GDP is actually due to denaturation of the Rac1 protein. If the latter possibility were true, then we reasoned that the binding of smgGDS to Rac1 might stabilize Rac1 and prevent degradation, with the stabilization effect exactly counterbalancing the stimulated exchange of [^{3}H]GDP for GTP induced by smgGDS, thereby effectively 'masking' our ability to detect the stimulated release of [^{3}H]GDP in these assays.

We designed experiments to attempt to differentiate between these two possibilities. The release of [^{3}H]GDP from Rac1 was determined as in Figure 2, but in the absence of excess unlabelled GTP. In the absence of competing nucleotides, any [^{3}H]GDP released should rebind to the Rac1, effectively making this a measure of the rate of loss of Rac1 nucleotide-binding activity. We found that the rate of loss of [^{3}H]GDP under these conditions was essentially the same as that observed in the presence of excess unlabelled nucleotide (see Figure 4a and compare Figure 2c). The loss of bound [^{3}H]GDP in this experiment thus appears to be due to denaturation; we estimate the decay rate for Rac1 to have a half-time of ~ 30 min at 30 °C. By contrast, Rap1A denatured relatively slowly, with only 20–30% loss of active protein over a 50 min timespan (Figure 4c and d). Since the overall rate of [^{3}H]GDP dissociation from Rac1 in Figure 2c had a half-time of ~ 20 min at 30 °C, the actual intrinsic rate of GDP dissociation must be quite low, with most of the loss accounted for by denaturation of Rac1 binding-activity during the assay. The decay rate of [^{3}H]GDP-bound Rac1 was about the same as the decay rate for [^{35}S]GTP[γS]-bound Rac1 (Figure 4b).

Can the binding of smgGDS to Rac1 prevent the denaturation of the protein that occurs under these assay conditions? Direct evidence of the stabilizing effect of the smgGDS towards GDP-bound Rac1 is shown in Figure 5. We incubated GDP-preloaded Rac1 at 30 °C in the presence of high Mg^{2+}, and measured the amount of active protein remaining at various times in the presence or absence of smgGDS by rapidly lowering the free Mg^{2+} concentration with EDTA and determining the total amount of [^{35}S]GTP[γS]-binding activity remaining. Figure 5 shows that there was substantial loss of active Rac1 during the incubation, and that this decay could be prevented largely by incubation in the presence of smgGDS. In contrast, addition of smgGDS (±EDTA) subsequent to the incubation at 30 °C did not restore binding activity. We therefore conclude that smgGDS stabilizes the GDP-bound form of Rac1 *in vitro*.

It is obvious from Figure 4b that we could detect a stabilizing effect of smgGDS on the degradation of [^{35}S]GTP[γS]-bound Rac1, while we could not see the stabilizing effect on the [^{3}H]GDP-bound Rac1, even though we knew it occurred based on the data of Figure 5. SmgGDS appears to stabilize the GTP[γS]-bound form of Rac1 but does not stimulate the release of GTP[γS] from Rac1 under these assay conditions. Observation of the stabilizing effect of smgGDS on [^{3}H]GDP-bound Rac1 appears to be exactly compensated for by the smgGDS-catalysed stimulation of the release of [^{3}H]GDP from Rac1 in the absence of excess unlabelled GTP. That the ability of smgGDS to

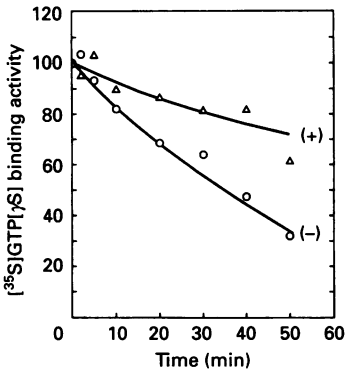

Figure 5 Stabilization effect of smgGDS towards processed Rac1

GDP bound Rac1 (60 nM) was incubated in the presence (+) or absence (−) of 240 nM smgGDS at 30 °C. Aliquots were taken at the indicated time-points and the remaining [^{35}S]GTP[γS] binding activity of Rac1 was determined as described in the Experimental section.

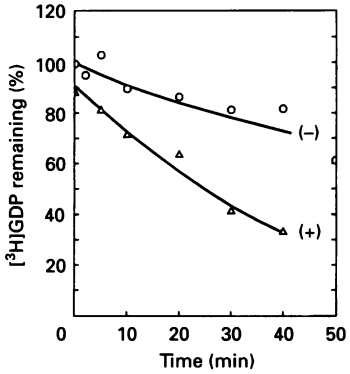

Figure 6 The [^{3}H]GDP/GTP exchange effect of smgGDS towards processed Rac1

The [^{3}H]GDP/GTP exchange effect of smgGDS toward Rac1 can be estimated by comparing the result in Figure 2c with the stabilization effect of smgGDS shown in Figure 5. ○, Data from Figure 5 which represents the true amount of active Rac1 in the presence of smgGDS under the reaction condition. △, Data from Figure 2c which represents the nucleotide exchange rate of Rac1 in the presence of smgGDS under the reaction conditions used.

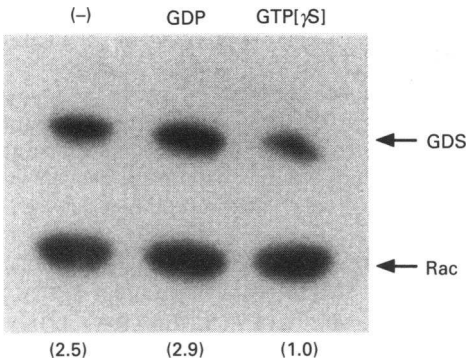

Figure 7 Complex formation between smgGDS and Rac1 in various nucleotide-bound states

The amount of smgGDS bound to forms of Rac1 is shown by Western blotting with a specific smgGDS polyclonal antibody; the level of Rac1 in each set of bead precipitates is shown for comparison as well. The numbers in parentheses at the bottom of each lane represent the relative amount of smgGDS bound to that particular form of Rac1 after normalization for the amount of bound Rac1 and subtraction of the glutathione bead blank, which was ~ 10% of the total smgGDS bound.

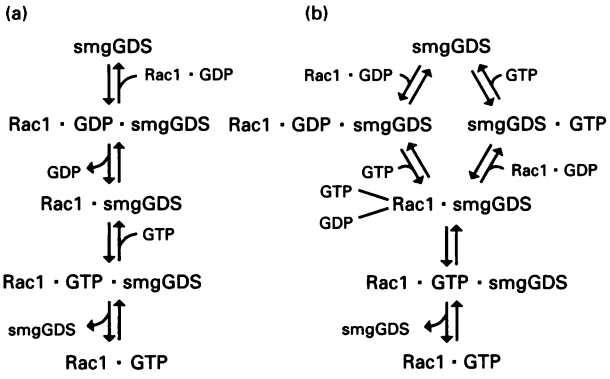

Figure 8 Possible mechanisms for the interaction between Rac1 and smgGDS

(**a**) Substituted enzyme mechanism; and (**b**) ternary complex mechanism.

stimulate the release of GDP is independent of the presence of excess GTP is demonstrated in Figures 2b and 4c, where the release of prebound [^{3}H]GDP from Rap1A stimulated by smgGDS is the same in the presence or absence of exogenous GTP. In addition, unlike the Rac1 protein, release of prebound [^{35}S]GTP[γS] from Rap1A stimulated by smgGDS could be detected in the absence of any exogenous nucleotides (Figure 4d).

Using the data of Figure 5 as a measure of the actual amount of active Rac1 present during the assay in the presence of smgGDS, we can 'correct' the data of Figure 2c to compensate for the actual level of active Rac1 in the presence of smgGDS; the [^{3}H]GDP-releasing effect of smgGDS on Rac1 is thereby revealed (Figure 6). This data is in good agreement with the ability of smgGDS to stimulate [^{3}H]GDP release from Rac1 in the presence of DMPC (not shown).

We examined the ability of smgGDS to form physical complexes with various guanine-nucleotide-bound forms of Rac1 (Figure 7). Effective complex formation between Rac1–GDP and nucleotide-free Rac1 and smgGDS was readily detectable, while complexation with Rac1–GTP was substantially less at the same protein ratios. This weaker affinity for Rac in the GTP-form would be consistent with the need for the two proteins to dissociate once GTP/GDP exchange has taken place (see Figure 8).

DISCUSSION

The stabilizing effect of smgGDS-binding has not been noted previously. The data of Figures 4, 5, and 7 show that smgGDS binds to and stabilizes both nucleotide-bound forms of Rac1, as well as a nucleotide-free form of the protein. The formation of a stable complex of smgGDS with nucleotide-free Rac1 is indicated by the ability of smgGDS to release prebound [^{3}H]GDP from Rac1 in the absence of exogenous GTP (Figure 4a). Under that circumstance, the nucleotide-depleted Rac1 protein would be expected to be unstable unless maintained in an active, nucleotide-free form by smgGDS. The latter was shown to be true because the nucleotide-depleted Rac1 retains its GTP-binding activity

(Figure 5) only in the presence of smgGDS. Finally, direct physical complexes of nucleotide-free Rac with smgGDS could be detected (Figure 7).

Two basic kinetic schemes can be proposed to explain the mechanism by which nucleotide exchange is stimulated by exchange factors such as smgGDS: an enzyme-substituted (ping-pong) mechanism and a ternary complex mechanism, as illustrated in Figure 8. A stable transition complex between smgGDS and the nucleotide-free form of Rac1 supports the concept that the reaction between smgGDS and Rac1 follows a substituted enzyme mechanism. This mechanism is similar to that proposed for SDC25, a *Saccharomyces cerevesiae* gene product which stimulates nucleotide exchange on Ras [26,27], as well as for the guanine nucleotide exchange reaction catalysed by eukaryotic initiation factor 2 [28], and the *E. coli* elongation factor Ts [29]. These results predict a lower affinity of smgGDS for GTP-bound LMWG proteins, and this was observed when complex formation was assessed directly (Figure 7).

Contrary to previous reports of Takai et al. [6], we observed that smgGDS was active towards both post-translationally processed (isoprenylated) and unprocessed Rac1. The isoprenyl modification of LMWGs appeared not to be absolutely required for interaction with smgGDS, and this was further confirmed by the high activity of smgGDS towards unprocessed RhoA. SmgGDS has been reported to have activity only toward processed Rap1, c-K-Ras, Rho and Rac [16,20,21], and has been shown to induce the release of Rap1 and c-K-Ras from cell membranes to cytoplasm [30,31], probably by binding to the isoprenylated C-terminus and preventing membrane association. The isoprenylated C-terminus region of Rap1 was shown to bind to smgGDS by a cross-linking method [32]. In light of our current data, it is likely that smgGDS interacts with more than one site on the LMWGs and that the isoprenyl portion is not absolutely required for catalysed nucleotide exchange to occur. Isoprenylation might enhance the smgGDS binding, as we observed that smgGDS has slightly higher activity toward processed Rac1 than to unprocessed Rac1 (Figure 1).

Our results also indicate that smgGDS has greater capability to stimulate nucleotide exchange on RhoA and Rac2 than on Rac1, CDC42Hs or Rap1A (both processed and unprocessed), implying that if smgGDS is indeed the sole stimulatory exchange-regulator for both Rac1 and Rac2, then Rac2 might be activated by smgGDS more readily than Rac1. Sasaki et al. [24] have suggested that, based upon the differing kinetics of Rho and Rac1 activation by smgGDS they observed, Rho would be a primary target of smgGDS. However, we see very effective stimulation of GTP/GDP exchange on Rac2, comparable to the activity of smgGDS toward Rho, and we could not detect more rapid exchange-kinetics when Rho was compared with Rac1 or Rac2. Since in the signal transduction pathways linking growth factors to assembly of the actin cytoskeleton it appears that Rac is an upstream regulator of Rho [1], it is likely that there exist other, unidentified stimulatory GTP/GDP exchange regulators which may be selectively active toward the Rac proteins.

We demonstrated that smgGDS could stimulate both the incorporation of GTP[γS] and the release of GDP from Rac1 and Rac2, although the latter activity was obscured under high Mg^{2+} assay conditions in the absence of DMPC by the ability of smgGDS to stabilize Rac from denaturation which occurred during the assay. Thus, smgGDS can serve as a positive regulator for Rac proteins as originally reported by Hiraoka et al. [16], and Rac2 is an effective substrate. Our observations are of note for determination of the GTP/GDP exchange capability of other proteins reported to be potential stimulatory exchange factors based upon their sequence homology with the nucleotide exchange domain of the *Dbl* proto-oncogene [19]. [^{3}H]GDP dissociation is the most commonly used assay to detect such activity, and our results indicate that under certain assay conditions and with certain LMWG, the ability to detect exchange may be compromised.

In summary, we have described the ability of smgGDS to stimulate GTP/GDP exchange on various LMWG, showing it to be most effective toward RhoA and Rac2, and have demonstrated that smgGDS binding stabilizes the nucleotide-free form of Rac1. The latter supports a substituted enzyme mechanism for stimulated guanine nucleotide exchange by this regulatory factor. SmgGDS appears to be capable of acting on both unprocessed and post-translationally processed forms of LMWG, suggesting sites of binding that do not absolutely require an isoprenylated C-terminus.

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