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SmgGDS-607 regulation of RhoA GTPase prenylation is nucleotide dependent.

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Abstract

Protein prenylation involves the attachment of a hydrophobic isoprenoid moiety to the C-terminus of proteins. Several small GTPases, including members of the Ras and Rho subfamilies, require prenylation for their normal and pathological functions. Recent work has suggested that SmgGDS proteins regulate the prenylation of small GTPases *in vivo*. Using RhoA as a representative small GTPase, we directly test this hypothesis using biochemical assays and present a mechanism describing the mode of prenylation regulation. SmgGDS-607 completely inhibits RhoA prenylation catalyzed by protein geranylgeranyltransferase I (GGTase-I) in an *in vitro* radiolabel incorporation assay. SmgGDS-607 inhibits prenylation by binding to and blocking access to the C-terminal tail of the small GTPase (substrate sequestration mechanism) rather than via inhibition of the prenyltransferase activity. The reactivity of GGTase-I with RhoA is unaffected by addition of nucleotides. In contrast, the affinity of SmgGDS-607 for RhoA varies with the nucleotide bound to RhoA; SmgGDS-607 has higher affinity for RhoA-GDP compared to RhoA-GTP. Consequently, the prenylation blocking function of SmgGDS-607 is regulated by the bound nucleotide. This work provides mechanistic insight into a novel pathway for the regulation of small GTPase protein prenylation by SmgGDS-607 and demonstrates that peptides are a good mimic for full-length proteins when measuring GGTase-I activity.

Graphical Abstract

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Author Contributions

BCJ conceived and coordinated the study and wrote the paper. BCJ, AJL, and ZR designed, performed, and analyzed experiments. CAF assisted with experimental design, data interpretation and revising the paper. All authors reviewed the results and approved the final version of the manuscript.

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Supporting Information. Protein construct sequences for SmgGDS-558, SmgGDS-607, and RhoA. Figure S1: Replot of data from Figure 2D fit to either substrate sequestration or competitive inhibition models. Equations S1 and S2 for fitting competitive inhibition model. Figure S2: Observed rate constants for SmgGDS-607 binding in Figures 4A and 4B. Table S1: Calculated rates and affinities for Figures 4 and S2 for SmgGDS-607 binding RhoA.

The authors declare no competing financial interest with the contents of this article.

Keywords

protein isoprenylation; protein farnesylation; small GTPase; Ras homolog gene family, member A (RhoA); geranylgeranylation; GGTase-I; SmgGDS; regulation; prenylation

INTRODUCTION

Protein prenylation is a post-translational modification that involves the addition of either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid to a cysteine residue four amino acids from the carboxy-terminus of a protein. The terminal residues within a -CaaX recognition motif help determine whether protein farnesyltransferase (FTase) or protein geranylgeranyltransferase-I (GGTase-I) catalyzes attachment of a farnesyl or geranylgeranyl group, respectively. Entry into and passage through the prenylation pathway has been viewed previously as an unregulated, constitutively active, housekeeping process.¹ Following prenylation, the majority of modified proteins undergo two additional processing steps: proteolytic removal of the -aaX residues and carboxymethylation of the terminal isoprenyl-cysteine residue. Recent work suggests that at least one CaaX-containing protein in yeast (Ydj1p) does not undergo these post-prenylation events and that these processing steps are deleterious to the normal function of this protein.²

Protein prenylation plays a crucial role in regulating the function, interaction, and subcellular localization of several proteins. Many small GTPases, including members of the Ras, Rho, Rap, and Rab subfamilies, are prenylated, and modification is often required for both normal and pathological functions. Therefore, developing compounds that target the protein prenylation pathway has been explored as a therapeutic approach to treating multiple diseases.³ A common approach has been to decrease prenylation through small-molecule inhibition of the prenyltransferases, FTase and GGTase-I.¹ Recent research suggest the existence of *in vivo* regulatory mechanisms for these pathways, which could provide new avenues for pharmacological intervention. Stimulating cells with glucose or insulin leads to increased phosphorylation of the common α -subunit of FTase and GGTase-I and this results in enhanced prenyltransferase activity for RhoA and Ras.⁴⁻⁷ Similarly, GGTase-I activity in mouse brain can be increased by neural depolarization, brain-derived neurotrophic factor (BDNF), or following exploration of a novel environment, all of which promote dendritic

arborization in neurons.⁸ Specifically, GGTase-I activity and membrane-bound Rac were elevated in mouse hippocampal lysates after exploring a novel environment, but total protein levels of GGTase-I and Rac were unchanged, which suggests a regulatory mechanism for GGTase-I activity. The prenylation of Rac1, a Rho family GTPase, was required for the GGTase-I-dependent effect on dendrite arborization.⁸ In a third example, stimulation of the adenosine A2B receptors reduces prenylation of Rap1B, leading to more cytosolic and nuclear nonprenylated Rap1B which reduces cell-cell adhesion and increases cell scattering.⁹ Together, these results add to the growing evidence that protein prenylation is regulated in cells, particularly prenylation of small GTPases.

SmgGDS proteins (small G-protein GDP dissociation stimulator, also known as RAP1GDS1) have been suggested as one regulator of the prenylation of Ras and Rho small GTPases.¹⁰ SmgGDS was initially characterized as a weak guanine nucleotide exchange factor (GEF) for multiple small GTPases; however, additional follow-up specificity studies suggest that the GEF activity may be limited to a few small GTPases.¹¹ SmgGDS is expressed as either a 558-amino acid splice variant with 12 armadillo repeats (SmgGDS-558) or a 607-amino acid variant with one additional armadillo repeat in the third position (SmgGDS-607).^{10,12} SmgGDS-607 co-immunoprecipitates from cells with the non-prenylated form of small GTPases that contain a polybasic region (PBR), including Rap1A, RhoA, Rac1, and K-Ras.¹⁰ In contrast, SmgGDS-558 co-immunoprecipitates with a form of GTPase that migrates faster on SDS-PAGE gels, likely representing a prenylated form of the GTPase.¹⁰ Furthermore, data suggest that SmgGDS-607 preferentially binds to GTPases that contain CaaX motifs ending in L (-CaaL), which are preferred substrates for GGTase-I.¹³ Cellular coexpression of SmgGDS-607 with small GTPases increases the amount of non-prenylated GTPase in the cell, which increases its partitioning to the aqueous fraction.¹⁰ Together, these data suggest that SmgGDS-607 may regulate the entry of certain small GTPases into the prenylation pathway.

In this study, we directly test whether SmgGDS-607 inhibits the prenylation of RhoA catalyzed by GGTase-I using purified proteins and biochemical assays. RhoA was chosen as a representative small GTPase because multiple reports have observed RhoA associating with SmgGDS-607 in cells and it contains a CaaX motif ending in L (Figure 1A.; -CLVL).^{10,11,14} Indeed, we demonstrate that SmgGDS-607 inhibits GGTase-I-catalyzed prenylation of RhoA by binding to and sequestering the RhoA C-terminal CaaX motif rather than by inhibiting the catalytic activity of GGTase-I. Furthermore, the affinities of SmgGDS-607 for RhoA vary with the identity of the bound nucleotide. These results elucidate a novel cellular mechanism regulating the entry of RhoA, and likely other small GTPases, into the prenylation pathway.

MATERIAL AND METHODS

Molecular constructs.

Carol Williams (Medical College of Wisconsin) provided previously described human SmgGDS-558 (UniProt ID P52306–2) and SmgGDS-607 (P52306–1) constructs.¹³ She also provided human RhoA (P61586–1) cDNA¹⁰ that was subcloned into a bacterial His₆-tev expression construct. DNA sequencing verified final protein sequences (Supplemental data).

Protein expression and purification.

SmgGDS and RhoA were expressed in BL21(DE3) *E. coli* transformed with the His₆-tev expression constructs. The bacteria were incubated in 4 L shaker flasks containing 1 L of media (15 g tryptone, 7.5 g yeast extract, 7.5 g NaCl, 0.5% glucose, 50 µg/mL kanamycin) and grown at 34°C until OD₆₀₀ 0.6–0.8. Cultures were lowered to 21°C and SmgGDS proteins induced by addition of 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation for 18 hrs. Centrifuged cells were suspended in chilled lysis buffer (20 mM Tris, 300 mM NaCl, 5% glycerol, 5 mM imidazole, 1 mM TCEP, 1 mM PMSF, 10 µg/µL TAME, pH 8.0), aliquoted, flash frozen in liquid nitrogen, and stored at –80°C until purification. Cells representing 0.5 L of culture were rapidly thawed in warm water, diluted with additional lysis buffer, and passed three times through a chilled microfluidizer. Soluble fractions were obtained after centrifugation at 35,000 × *g* for 35 min and applied twice to a 4 mL nickel-NTA affinity column. The column was washed with 20 column volumes (CV) of lysis buffer with 500 mM NaCl and 20 mM imidazole. Protein was batch eluted six times with 1 CV elution buffer (20 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, 300 mM imidazole, 0.5 mM TCEP), and 1 mM ethylenediamine tetra-acetic acid (EDTA) final was added to the elution to inhibit metalloproteases. Fractions containing the desired protein were identified by SDS-PAGE and pooled, followed by dialysis (50K MWCO) against storage buffer (20 mM HEPES pH 7.8, 100 mM NaCl, 5% glycerol, 1 mM TCEP) overnight. Protein was concentrated by Amicon Ultra centrifugal filters, the final concentration determined by A₂₈₀ (15,470 cm^{–1}M^{–1} SmgGDS-607; 13,980 cm^{–1}M^{–1} SmgGDS-558), and aliquoted before flash freezing and storing at –80°C. SmgGDS-607 yielded 215 mg/L culture; SmgGDS-558 yielded 150 mg/L culture. RhoA was expressed and purified similarly with the following differences: RhoA was induced with 0.3 mM IPTG for 14 hrs at 25°C and lysis buffer contained additional protease inhibitors, 1 mM benzamidine and 1 µM pepstatin A. Column wash buffer contained 10 mM imidazole, and elutions were spiked with 5 mM EDTA (final concentration) to chelate Mg²⁺ ions, resulting in greatly reduced affinity for guanine nucleotide.¹⁵ Pooled elutions containing RhoA were then spiked with 1 mM TCEP and 2 mM benzamidine (final concentration) before addition of TEV protease at a 1:15 ratio (mg TEV:mg RhoA). The cleavage reaction was incubated overnight at 4°C with dialysis against storage buffer, which removed released guanine nucleotide, imidazole, and cleaved His₆tev tag. Cleaved RhoA was passed three times over nickel-NTA resin equilibrated with dialysis buffer and the flow through was collected. Resin was eluted with a step gradient of dialysis buffer with imidazole (0, 10, 20, 35, 250 mM imidazole). The flow through and fractions containing untagged RhoA were concentrated (10K MWCO), and protein concentration was determined using A₂₈₀ (18,450 cm^{–1}M^{–1}). Aliquoted protein was flash frozen and stored in –80°C. Untagged RhoA yielded 22 mg per L culture.

Radiolabel-prenylation assays.

Tritium labeled geranylgeranyl pyrophosphate (³H-GGPP) was purchased from Perkin Elmer and diluted 1:19 with unlabeled GGPP (Sigma) in assay buffer (50 mM HEPES, 5 mM TCEP, 2 mM MgCl₂, 0.02% triton X-100, pH 7.8). GGTase-I was purified as previously described.^{16,17} Purified nucleotide-free RhoA was incubated in assay buffer with GDP or GTP before addition of purified rat GGTase-I and, if present, SmgGDS-607. Addition of labeled GGPP initiated the reaction, which was incubated at 30°C for 4 min before stopping

with 5X SDS sample buffer. Unless indicated otherwise in figure legends, final concentrations were: 3 μ M RhoA, 10 μ M GDP or GTP, 10 nM GGTase-I, 0.02% Triton X-100, and 1.8–2.5 μ M GGPP. GGTase-I K_M for GGPP is 3 nM.¹⁸ Samples were heated for 2 min at 70°C before resolving on SDS-PAGE gels, followed by coomassie staining and destaining. RhoA bands were excised, placed in scintillation vials, chopped into ~1 mm² pieces, and dissolved in 500 μ L of 34% H₂O₂, 0.2 mM CuSO₄ by incubation at 37°C overnight. Samples were counted on a Beckman LS 6500 liquid scintillation counter after addition of 4.5 mL of BioSafe II scintillation cocktail (Research Products International). Specific activity (dpm/pmol) was used to convert dpm to pmoles of GGPP incorporated. For the substrate sequestration model (Scheme 1), the following equations were derived:

$$V = \frac{k_{cat}[E]_{total}[S]}{K_M + [S]} \quad (\text{Eq. 1})$$

$$[S] = \frac{-I_{total} + S_{total} - K_I \pm \sqrt{(-I_{total} + S_{total} - K_I)^2 + 4S_{total}K_I}}{2} \quad (\text{Eq. 2})$$

where [S] is free RhoA concentration; S_{total} , I_{total} , and E_{total} are respectively the total concentrations of RhoA, SmgGDS, and GGTase-I; and K_I is the affinity of SmgGDS and RhoA. Equations were fit using nonlinear regression in GraphPad Prism with the standard errors reported.

Peptide prenylation assay.

Michaelis-Menten plots for the reactivity of GGTase-I with the CaaX sequence from RhoA (CLVL) were carried out using the dansylated peptide (dansyl-GCLVL), which increases in fluorescence (λ_{ex} =340 nm, λ_{em} =520 nm) following prenylation with a previously published assay.¹⁹ GGTase-I (10 nM) and 10 μ M GGPP were incubated at 30°C with various concentrations of dansyl-GCLVL and fluorescence measured every 40 s for 2 hr. The initial fluorescence change was linearly fit and converted to velocity (μ M prenylated peptide per second) using equation 3.

$$\frac{\Delta \text{Fluorescence/sec}}{\text{total fluorescence change}} = \frac{\Delta \text{product } (\mu\text{M/sec})}{\text{max amount peptide } (\mu\text{M})} \quad (\text{Eq. 3})$$

The Michaelis-Menten equation was fit to the concentration dependence of the initial velocity (V/E) to determine values for k_{cat} and K_M .

$$\frac{\text{Velocity}}{[\text{Enzyme}]} = \frac{k_{cat} * [\text{dansyl-GCLVL}]}{K_M[\text{dansyl-GCLVL}]} \quad (\text{Eq. 4})$$

SmgGDS binding peptide assay.

The affinity of SmgGDS-607 and GGTase-I for a peptide corresponding to the last 13 amino acids of RhoA was measured using fluorescence anisotropy.¹⁷ A peptide representing the last 13 amino acids of RhoA was N-terminally labeled with 5-carboxyfluorescein (5-fam-ARRGKKKSGCLVL, RhoA 13mer). Peptide (100 nM) was incubated with various concentrations of SmgGDS or GGTase in buffer (50 mM HEPPSO, 5 mM TCEP, pH 7.8) before measuring fluorescence anisotropy on a Tecan Ultra (λ_{ex} =485 nm, λ_{em} =535 nm). Equation 5 was fit to the anisotropy data to determine K_d values.

$$millianisotropy(mA) = r_o + \frac{(r_{max} - r_o) \times [protein]}{K_d + [protein]} \quad (\text{Eq. 5})$$

SmgGDS binding full-length GTPase.

The binding affinity of SmgGDS to full-length RhoA was measured using biolayer interferometry (BLI) on an Octet RED96 system (ForteBio). SmgGDS was biotinylated at a 1:1 molar ratio with EZ-link NHS-PEG₄-biotin reagent (Thermo), dialyzed to remove unreacted biotin, concentrated, and stored at -80°C . The assay was performed at 25°C in 96-well plates in binding buffer (50 mM Hepes pH 7.8, 150 mM NaCl, 2 mM TCEP, 2 mM MgCl₂, 20 μM nucleotide (GDP or GPPNP), and 0.25 mg/mL BSA) with 1000 rpm plate shaking. Streptavidin probes were incubated in 200 μL of 50 ng/ μL biotinylated SmgGDS for 600 s and washed in binding buffer (300 s) before cycling through each concentration of RhoA, from low to high. Cycles consisted of a baseline in binding buffer (120 s), an association phase with RhoA (600 s), a dissociation phase in buffer (300 s), and a regeneration phase in binding buffer with 1 M NaCl (180 s). Controls included a probe without SmgGDS but incubated with each RhoA concentration and a probe with SmgGDS incubated only with buffer and not RhoA. OctetRed analysis software was used to subtract controls and align phases before exporting the data to GraphPad Prism and fitting with a two-phase exponential, likely representing specific and non-specific binding. Five times the half-life of the specific binding phase was fit to a one-phase exponential

$$Y = Y_{eq}(1 - e^{-kt}) \quad (\text{Eq. 6})$$

The responses at equilibrium (Y_{eq}) for each RhoA concentration were fit to the binding isotherm

$$Y_{eq} = Y_{max}[RhoA]/(K_d + [RhoA]) \quad (\text{Eq. 7})$$

where K_d is the binding affinity.

RESULTS

RhoA is a small GTPase that contains a C-terminal -CaaL motif and an upstream polybasic region (PBR, Figure 1A). GGTase-I has been demonstrated to prenylate RhoA in cells and *in vitro*.^{20,21} As a GTPase, RhoA cycles between three states: nucleotide free, GTP-, or GDP-bound; however, the effect of RhoA nucleotide status on GGTase-I reactivity has not yet been analyzed. Therefore, we measured the influence of RhoA nucleotide status on prenylation rates by assaying the incorporation of radiolabeled geranylgeranyl catalyzed by GGTase-I. No difference was observed between the initial rates of geranylgeranylation of nucleotide-free RhoA or RhoA that had been incubated with either 25 μ M GTP or GDP in the presence of magnesium (Figure 1B). Similarly, no significant difference was seen in the endpoint level of incorporated isoprenoid into the three species (Figure 1C).

To further characterize prenylation of RhoA, Michaelis-Menten constants were determined for GGTase-I-catalyzed modification of nucleotide-free RhoA. Measurement of the dependence of the initial rate of GGTase-I-catalyzed prenylation of RhoA at saturating ^3H -GGPP resulted in the following kinetic parameters: $k_{\text{cat}} = 0.06 \pm 0.02 \text{ s}^{-1}$ and $K_{\text{M}} = 2.3 \pm 1.4 \text{ }\mu\text{M}$ RhoA (Figure 1D). These values are similar to those previously reported for GGTase-I-catalyzed prenylation of the peptide substrate dansyl-GCVLL ($k_{\text{cat}} = 0.051 \text{ s}^{-1}$)¹⁶ and the protein substrate Ras-CVLL ($K_{\text{M}} = 1.3 \text{ }\mu\text{M}$).¹⁸

SmgGDS-607 directly regulates prenylation.

Previous data have suggested that SmgGDS-607 may regulate the entry of CaaX proteins into the prenylation pathway, particularly CaaX proteins that end in leucine residues such as RhoA (Figure 1A)¹³; however, SmgGDS-mediated regulation of prenylation has never been demonstrated *in vitro*. To examine this proposal, we added purified SmgGDS-607 to a prenylation reaction of RhoA catalyzed by GGTase-I; inclusion of 7.5 μ M SmgGDS-607 significantly decreased the incorporation of radiolabeled geranylgeranyl onto RhoA (Figure 2A, lane 1–3). Likewise, inclusion of increasing concentrations of SmgGDS-607 generated a dose-response curve (Figure 2B), indicating that under these conditions 5 μ M SmgGDS-607 inhibits greater than 90% of GGTase-I activity.

Two possible mechanisms to explain how SmgGDS-607 inhibits prenylation of RhoA are that: (1) SmgGDS-607 binds to GGTase-I and inhibits catalytic activity or (2) SmgGDS-607 binds to and sequesters RhoA in a conformation such that the prenylation site is inaccessible to GGTase-I. We tested each of these models. To examine whether SmgGDS-607 directly inhibits GGTase-I, geranylgeranylation of a dansylated peptide was measured from an increase in fluorescence after prenylation.¹⁹ The peptide sequence dansyl-GCLVL was chosen because it binds significantly tighter to GGTase-I than to either SmgGDS splice variant (Table 2). Under conditions of saturating GGPP, GGTase-I activity is not significantly altered by inclusion of either SmgGDS-607 or SmgGDS-558 (Figure 2C). Furthermore, SmgGDS proteins have little effect on the steady state kinetic parameters, K_{M} and k_{cat} (Table 1). These data demonstrate that SmgGDS-607 and SmgGDS-508 do not inhibit the reactivity of GGTase-I with a peptide substrate.

To test the second mechanism, that SmgGDS-607 binds and sequesters RhoA, we proposed a model in which SmgGDS-607 inhibits GGTase-I by binding substrate (Scheme 1). To test this model we carried out a series of radiolabeled prenylation reactions varying the concentrations of both RhoA and SmgGDS-607 (Figures 2D and S1). To analyze these data we derived equations describing the inhibitory effect of SmgGDS-607 on turnover using a simple kinetic scheme (Scheme 1) in terms of a Michaelis-Menten constant for RhoA (K_M) the affinity of SmgGDS-607 for RhoA (K_i) and the total concentrations of RhoA and SmgGDS-607 (Equations 1 and 2). Globally fitting equations 1 and 2 to these data, gave the following values, $k_{cat} = 0.05 \pm 0.01 \text{ s}^{-1}$, $K_M = 1.8 \pm 0.7 \text{ }\mu\text{M}$ and $K_i = 85 \pm 6 \text{ nM}$ reflecting the affinity of SmgGDS-607 for RhoA. As predicted for the model in Scheme 1, the k_{cat} value, even with SmgGDS-607 present (Figure S1A), is similar to the value observed in the absence of SmgGDS-607 ($k_{cat} = 0.06 \text{ s}^{-1}$, Figure 1D). Competitive inhibition is consistent with an unchanged k_{cat} , however, inhibition of GGTase-I was not observed with the peptide substrate (Figure 2C) and the data were poorly fit to a competitive inhibition model (Figure S1B). The data are more consistent with a substrate sequestration model for inhibition of GGTase-I activity rather than direct enzyme inhibition models.

SmgGDS binding affinity and specificity.

To characterize determinants of the affinity of SmgGDS for RhoA, we compared the binding affinities of SmgGDS-607, SmgGDS-558, and GGTase-I for a RhoA peptide. Fluorescence anisotropy provides a sensitive, quantitative approach for measuring binding constants by detecting the change in rotational diffusion of a molecule following binding of another molecule.²² A peptide containing the C-terminal 13 amino acids of RhoA, including the polybasic region and CaaX motif, was synthesized with a 5-carboxyfluorescein label at the N-terminus (5-fam-ARRGKKKSGCLVL, RhoA 13mer). Varying concentrations of SmgGDS were added to a fixed concentration of the RhoA peptide and the resulting increase in fluorescence anisotropy was measured (Figure 3A and 3B). A binding isotherm (Equation 5) was fit to these data to calculate the values of dissociation constants describing the affinity of SmgGDS-607 or SmgGDS-558 for the RhoA 13mer ($K_d = 1.9 \text{ }\mu\text{M}$ and $8.1 \text{ }\mu\text{M}$, respectively, Table 2). Finally, as a basis for comparison, the affinity of GGTase-I for the RhoA 13mer was determined ($K_d = 56 \text{ nM}$, Figure 3C), indicating that GGTase-I has significantly higher affinity for the peptide than either splice variant of SmgGDS. Unexpectedly, a decrease in anisotropy was observed for the binding of GGTase-I to the RhoA 13mer suggesting that the 5-FAM fluorophore is rotating more rapidly in the GGTase-I•peptide complex. One possible explanation is in the RhoA 13mer, the 5-FAM fluorophore, largely made up of aromatic rings, interacts with the hydrophobic LVL residues of the CaaX motif to decrease fluorophore rotation. However, in the GGTase-I•peptide complex, the fluorophore may extend away from the protein complex and experience greater diffusional rotation.

Work by others suggests that the polybasic residues within a truncated form of RhoA (1–190, C190S) are important for binding to SmgGDS-607.¹¹ To test this proposal, we measured the affect on affinity of removing the polybasic region from the RhoA peptide (5-fam-GCLVL). Indeed, the polybasic region enhances the peptide affinity for SmgGDS-607, SmgGDS-558 and GGTase-I by 32-fold, >5-fold and 39-fold, respectively (Table 2). These

data demonstrate that the polybasic region contributes to the binding affinity of both SmgGDS and GGTase-I for RhoA.

Both the inhibition of activity and affinity data support a model where SmgGDS-607 recognizes nonprenylated RhoA, consistent with data from cells suggesting that SmgGDS-607 prefers binding to nonprenylated GTPases.¹⁰ However, it was unclear whether SmgGDS-607 could bind to the prenylated form of RhoA. To begin to answer this question, the RhoA 13mer peptide was incubated with GGTase-I and GGPP for increasing lengths of time to generate samples with an increasing fraction of geranylgeranylated peptide. The anisotropy of each sample was then measured before and after addition of SmgGDS-607 (Figure 3D). In this experiment, an increase in anisotropy is observed upon addition of SmgGDS-607 to the peptide incubated with GGTase-I for short times, indicative of complex formation. However, at longer times little complex is formed, as indicated by the small increase in anisotropy. These data indicate that the affinity of SmgGDS-607 for the prenylated form of RhoA is decreased significantly (8-fold).

Nucleotide status regulates SmgGDS binding affinity.

A comparison of the binding affinities of SmgGDS-607 for RhoA 13mer (1.9 μ M) versus full-length protein (85 nM) suggests that SmgGDS-607 makes additional contacts with RhoA outside the polybasic and CaaX regions. As a GTPase, RhoA undergoes conformational changes depending on whether it is in the GTP- or GDP-bound state and these conformational changes can affect the affinity for GEFs, GAPs, and effectors.^{23,24} Thus, we hypothesized that the nucleotide status of RhoA could regulate the affinity with SmgGDS-607 and this in turn, would regulate prenylation by GGTase-I. To begin to test this model, we first measured the binding affinities of SmgGDS-607 for RhoA-GDP and RhoA-GTP using a bio-layer interferometry (BLI) assay. Biotinylated SmgGDS-607 was bound to streptavidin probes, and the time-dependent signal change was measured for binding various concentrations of RhoA with either bound GDP or GppNp, a non-hydrolyzable analog of GTP. Signal changes were best fit with a two-phase association equation; the two phases are consistent with a rapid, specific-binding phase and a slower second step likely representing non-specific interactions. A single exponential was fit to five half-lives of the rapid binding phase and the observed rate constant (supplemental data Figure S2) and the endpoint value were determined (Figures 4A and 4B). A single binding isotherm was fit to the dependence of the endpoint on the concentration of RhoA to calculate binding affinities. The binding affinity of SmgGDS-607 for RhoA-GDP measured using the BLI assay ($K_d = 77 \pm 7$ nM) is comparable to the value measured from inhibition of GGTase-I catalyzed prenylation ($K_i = 85$ nM, Figure 2D). Additionally these data demonstrate that SmgGDS-607 has decreased affinity for RhoA-GppNp ($K_d = 249 \pm 13$ nM, Figure 4C). This result indicates that the affinity of SmgGDS-607 for RhoA depends on the bound nucleotide.

Nucleotide status can regulate GTPase prenylation.

To further test the model that nucleotide status of RhoA can regulate prenylation through altering SmgGDS-607 affinity, nucleotide-free RhoA was incubated with GTP prior to reaction with GGTase-I and ³H-GGPP. Inclusion of GTP causes no detectable change in either the initial rate of geranylgeranylation of RhoA (Figure 1B) or in the observed end-

point of labeling (Figure 1C). However, inclusion of GTP reverses the SmgGDS-607-dependent inhibition of RhoA geranylgeranylation seen in Figure 2A (lanes 3 and 4 vs. lane 6). To further examine the nucleotide-dependent affect on prenylation we measured the initial rate for geranylgeranylation of RhoA catalyzed by GGTase-I in the presence of 5 μ M SmgGDS-607 and the dependence on the concentration of GTP or GDP. These data demonstrate that pre-incubation of RhoA with GTP enhances prenylation while pre-incubation with GDP has little effect on prenylation (Figure 5). GDP, even at high concentrations, does not enhance geranylgeranylation. In contrast, increasing the concentration of RhoA-GTP at higher GTP concentrations leads to dissociation of the RhoA-SmgGDS-607 complex to form unbound RhoA-GTP, thereby increasing the initial rate for prenylation. The concentration of GTP required to reach saturating RhoA geranylgeranylation in the presence of SmgGDS-607 is ~ 3 μ M, similar to the concentration of RhoA in the reaction (3 μ M) and consistent with the nanomolar affinity reported for RhoA and GTP in the presence of magnesium (K_d of RhoA for GTP γ S with Mg^{2+} is 0.16 μ M).¹⁵ Interestingly, in the presence of SmgGDS-607, even saturating concentrations of GTP do not restore RhoA geranylgeranylation to the level observed in the absence of SmgGDS-607 (Figure 5, triangle). This is consistent with data from Figure 4 that SmgGDS-607 can still bind RhoA-GTP, albeit with weaker affinity than RhoA-GDP. These data confirm the proposal that SmgGDS-607 binds RhoAGTP more weakly than RhoA-GDP and that varying the bound nucleotide can regulate the initial rate of prenylation.

DISCUSSION

Prenylation is regulated by SmgGDS and nucleotides.

Recent work suggests that SmgGDS proteins can regulate small GTPase prenylation.^{10,13} Here we used biochemical assays to directly test this hypothesis and present a mechanism for regulation of prenylation. SmgGDS-607 reduces RhoA geranylgeranylation catalyzed by GGTase-I and this occurs through a substrate sequestration mechanism rather than direct inhibition of the prenyltransferase (Scheme I). This inhibition depends on the relative affinities and cellular concentrations of GGTase-I and SmgGDS-607. Furthermore, although the reactivity of GGTase-I is unaffected by nucleotides (Figure 2C) or the nucleotide status of RhoA (Figure 1B), the affinity of SmgGDS-607 for RhoA depends on the bound nucleotide. Consequently, the prenylation blocking function of SmgGDS-607 is also regulated by the nucleotide bound to RhoA.

The nucleotide dependent affinity of SmgGDS-607 for RhoA parallels observations seen from cell-based work. A dominant negative mutant of RhoA, RhoA T19N, which is predominantly nucleotide-free in cells, co-immunoprecipitates greater amounts of SmgGDS-607 than wildtype RhoA.¹⁴ In contrast, a constitutively active mutant, RhoA G14V, predominantly bound to GTP, did not co-immunoprecipitate detectable amounts of SmgGDS.²⁵ Furthermore, dominant negative RhoA transfected into cells partitions more into the aqueous phase than the detergent phase following fractionation, whereas wildtype RhoA partitions more into the detergent phase, consistent with prenylation. Co-expression of SmgGDS-607 further increased the amount of dominant negative RhoA in the aqueous fraction.¹⁰ This is consistent with our model indicating that GDP-bound RhoA (as mimicked

by the dominant negative mutant) interacts more strongly with SmgGDS-607, reducing its level of geranylgeranylation and resulting in greater nonprenylated RhoA in the cytosol. In the absence of bound nucleotide, recent data indicate that the affinity of SmgGDS-558 and -607 for farnesylated and non-farnesylated RhoA is high.¹² We measured similar values for the affinity of SmgGDS for nucleotide-free, non-modified RhoA (data not shown), demonstrating tighter binding than with the GDP or GppNp forms of RhoA.

In the absence of SmgGDS, *in vitro* prenylation of RhoA was unaffected by nucleotide status (Figure 1B, 1C). This is consistent with earlier work showing that FTase activity for farnesylation of the small GTPase H-Ras was unaffected by addition of GDP or GTP γ S.²⁶ Likewise, structural studies suggest FTase and GGTase-I mainly interact with the C-terminal CaaX sequence of potential substrates.²⁷ In contrast, Rab GGTase (or GGTase-II) activity is highly dependent on the nucleotide status of Rab GTPases.²⁸ The preference for GDP-bound Rab results from a second protein, Rab escort protein (REP), which preferentially binds GDP-bound, nonprenylated Rabs and increases the affinity of the association with RabGGTase by >50-fold.²⁹ Similar to REP, SmgGDS-607 may escort newly synthesized Rho and Ras GTPases to the prenyltransferases for modification. However, unlike REP, SmgGDS-607 would likely need to release the GTPase before prenylation since GGTase-I and SmgGDS-607 compete for interaction with the CaaX motif. Disassociation could occur through the mechanism of nucleotide exchange described here or via an unknown release factor present in cells, similar to Arl2/3-mediated release of lipidated cargo from PDE δ and Unc119.^{30,31}

SmgGDS interacts with the body of the small GTPase.

Previous data¹¹ and our peptide affinity data (Table 2) demonstrate that SmgGDS-607 interacts with the upstream polybasic region (PBR) and the CaaX motif. However, the enhanced affinity of SmgGDS-607 for RhoA (77–249 nM, Figure 4) compared to the RhoA peptides (1.9 μ M, Table 2) and the nucleotide-dependence of SmgGDS-607 affinity for RhoA argue that SmgGDS-607 also interacts with other regions of RhoA. A recently published crystal structure of SmgGDS-558, which only differs from SmgGDS-607 by the lack of the third armadillo-repeat, provides structural insight into this interaction. The structure reveals two charged regions: one negatively charged that interacts with the PBR and CaaX motif and one positively charged region, which when mutated reduced nucleotide exchange activity.¹² This second region in the structure is consistent with our model that SmgGDS-607 makes additional contacts with RhoA outside the polybasic and CaaX regions. Additionally, consistent with our data that SmgGDS-607 prefers non-geranylgeranylated RhoA (Figure 3), this recent study indicated that non-farnesylated RhoA had higher affinity for SmgGDS-607 than farnesylated RhoA.¹²

Peptides are good substrate mimics for GGTase-I.

Several analogs of FPP and GGPP have been developed to characterize the protein prenylome and evaluate the effectiveness of prenyltransferase inhibitors.^{32–35} These studies often use fluorescent peptides to measure prenyltransferase activity; however, uncertainty remains about how well peptides mimic full-length protein substrates for GGTase-I. FTase binds full-length H-Ras (K_d = 0.5 μ M) approximately ten-fold weaker than its corresponding

CaaX peptide (GCVLS, $K_d = 0.058 \mu\text{M}$).³⁶ Here, we demonstrate that the rate for GGTase-I catalysis of full-length RhoA (0.06 s^{-1}), determined using the radiolabel incorporation assay (Figure 1D, or 0.05 s^{-1} Figure 2D), is comparable to k_{cat} for prenylation of the RhoA CaaX motif peptide (dansyl-GCLVL, 0.046 s^{-1}) measured using the fluorescent assay. These values agree with previously reported rates of GGTase-I-catalyzed prenylation of protein substrates (*Sf9* produced GGTase-I with GGPP on Ras-CVLL, $k_{\text{cat}} = 0.02 \text{ s}^{-1}$ ¹⁸; GGTase-I with NBD-FPP on GST-RhoA, $k_{\text{cat}} = 0.08 \text{ s}^{-1}$).³⁷ Likewise, the Michaelis-Menten constant, K_M , for RhoA protein ($2.3 \mu\text{M}$, Figure 1D) and peptide ($1.9 \mu\text{M}$, Table 1) agree with each other and previously reported values ($1.2 \mu\text{M}$ Ras-CVLL with GGTase-I).¹⁸ Additionally, RhoA contains a polybasic region upstream of its CaaX motif (Figure 1A, underlined). For peptide substrates, the polybasic region was shown to decrease catalytic efficiency for prenylation by FTase, but not GGTase-I.¹⁷ Consistent with this result, the reactivity of GGTase-I with the RhoA peptide without the PBR was similar to full-length RhoA. Together these data demonstrate that peptides can be used in lieu of full-length proteins for measuring GGTase-I activity and that the radiolabel incorporation and dansyl fluorescence assays yield similar rates.

Summary.

The data presented support a model, initially hypothesized from cell-based work, where SmgGDS-607 regulates the prenylation of small GTPases by a substrate sequestration model. Although only geranylgeranylation of RhoA was discussed here, SmgGDS-607 likely regulates the prenylation of other small GTPases in a similar fashion. Indeed, SmgGDS-607 also blocked the farnesylation of a mutant RhoA construct containing a farnesylation CAAX motif (ending in -CLVM, data not show), which suggests that SmgGDS-607 also inhibits FTase activity. Given the role small GTPases play in many diseases, exploiting the mechanism of SmgGDS-607 to regulate prenylation may present a new avenue for therapeutic treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Nucleotide status of RhoA does not affect prenylation catalyzed by geranylgeranyltransferase-I (GGTase-I) in the absence of SmgGDS.

A. Human RhoA amino acid sequence (UniProt P61586–1) with prenylated cysteine (C190) in bold and 13 amino acid peptide with polybasic region and CaaX motif underlined.

B. Initial velocity of RhoA geranylgeranylation catalyzed by GGTase-I is unaffected by nucleotide status. Nucleotide-free RhoA (1.3 μM final) was incubated with buffer (black circles), 25 μM GTP (orange triangles), or 25 μM GDP (blue squares) before addition of GGTase-I (5 nM final) and ³H-GGPP (9 μM final). Aliquots were removed at the indicated times, resolved by SDS-PAGE, and ³H-geranylgeranyl incorporation into RhoA measured by scintillation counting.

C. The endpoint of RhoA geranylgeranylation is unaffected by nucleotide status. Reaction was identical to **B** except that the incubation time was 2 hr.

D. Concentration dependence of prenylation of nucleotide-free RhoA catalyzed by GGTase-I. Varied concentrations of nucleotide-free RhoA were incubated for 3 min with GGTase-I (5 nM) and ³H-GGPP (17.5 μM) before processing, as described in **B**. A fit of the Michaelis-Menten equation (Equation 1) to the data using nonlinear regression gave values of $k_{cat} = 0.06 \text{ s}^{-1}$ and $K_M = 2.3 \text{ μM RhoA}$.

Figure 2. SmgGDS-607 blocks RhoA geranylgeranylation through a substrate sequestration mechanism rather than direct inhibition of GGTase-I.

A. SmgGDS-607 inhibits the prenylation of nucleotide-free or GDP-RhoA more than GTP-RhoA. Nucleotide-free RhoA (4 μ M) was pre-incubated with the indicated components and then labeled with 3 H-GGPP (2 μ M) for 4 min.

B. Prenylation of RhoA catalyzed by GGTase-I is inhibited by SmgGDS-607 in a dose-dependent manner. Nucleotide-free RhoA (2.5 μ M) was pre-incubated with the indicated concentration of SmgGDS-607 and then labeled for 6 min by addition of GGTase-I (50 nM) and 3 H GGPP (2 μ M).

C Addition of SmgGDS (5 μ M final) or nucleotide (30 μ M final) has no effect on the kinetics of GGTase-I catalyzed prenylation of a RhoA CaaX peptide. GGTase-I (10 nM) and 10 μ M GGPP were incubated with various concentrations of dansyl-GCLVL and an increase in fluorescence reflecting prenylation was measured (every 40 s for 2 hr). The Michaelis-Menten equation (eq. 4) was fit to initial reaction velocities to calculate values of k_{cat} and K_M (reported in Table 1).

D. A substrate sequestration model (Scheme 1) best describes SmgGDS-607 mediated inhibition of RhoA geranylgeranylation. Varying concentrations of SmgGDS-607 were pre-incubated with the indicated concentration of RhoA and then labeled for 4 min by addition of GGTase-I (10 nM) and 2 μ M 3 H-GGPP. Equations 1 and 2 describing Scheme 1 were fit to the data (solid lines) resulting in the following values: $k_{\text{cat}} = 0.05 \text{ s}^{-1}$, $K_M = 1.8 \mu\text{M}$, and $K_i = 85 \text{ nM}$. Replotted in Figure S1.

Figure 3. RhoA polybasic tail and CaaX motif peptide has higher affinity for GGTase-I than for SmgGDS-607 or SmgGDS-558.

A peptide representing the polybasic region and CaaX motif of RhoA was fluorescently labeled (5-fam-ARRGKKKSGCLVL, RhoA 13mer) and binding measured using fluorescence anisotropy. *A*, *B*, and *C*, Indicated concentration of SmgGDS-607 (*A*), SmgGDS-558 (*B*), or GGTase-I (*C*) was added to peptide (100 nM final *A* and *B*; 10 nM *C*) and anisotropy measured. Equation 5 was fit to these data using nonlinear regression. *D*, SmgGDS-607 has decreased affinity for geranylgeranylated RhoA 13mer peptide. GGTase-I (20 nM) and GGPP (2 μM) were added to RhoA 13mer (10 nM) and incubated for the indicated amount of time leading to an increased concentration of prenylated peptide. Anisotropy was measured for two controls that show little change over time: before (squares, fit to horizontal line) and after (circles) addition of GGPP/GGTase for the indicated time. For each time point, anisotropy was also measured after a 5 min incubation with SmgGDS-607 (750 nM, triangles, exponential fit). Binding of the nonprenylated peptide to SmgGDS-607 leads to an increase in anisotropy ($t=0$). A decrease in anisotropy is observed as prenylation of the peptide occurs. After 250 min of reaction, the observed anisotropy approaches the unbound value, suggesting that the K_d of SmgGDS-607 for the geranylgeranylated RhoA 13mer peptide is $>16 \mu\text{M}$.

Figure 4. SmgGDS-607 affinity for RhoA is nucleotide-dependent.

A biolayer interferometry assay was used to measure the binding affinity of SmgGDS-607 and RhoA with either GDP (*A*) or GppNp, a non-hydrolyzable analog of GTP (*B*). Streptavidin-coated probes were incubated with biotinylated SmgGDS-607 (50 ng/ μ L) in a 96-well format, followed by washing and recording a baseline in wells with buffer. Probes were then transferred to wells containing RhoA and nucleotide, and the time-dependent association of RhoA recorded. A two-phase exponential was fit to these data, representing fast specific and slow non-specific binding. A single-phase exponential (eq. 4) was fit to five halftimes of the fast phase (shown) to calculate the observed rate constant and endpoint. *C*, A single binding isotherm (equation 5) as fit to the concentration dependence of the endpoint by non-linear regression to determine binding affinities.

Figure 5. Addition of GTP, but not GDP, alleviates SmgGDS-607-dependent inhibition of RhoA geranylgeranylation.

Radiolabel prenylation assays were performed as in Figure 1. RhoA (3 μM) prenylation was catalyzed by GGTase-I (10 nM) and ³H-GGPP (1.8 μM) for 4 min in the absence of SmgGDS-607 (triangle). This reaction was repeated in the presence of 5 μM SmgGDS-607 and increasing concentrations of either GDP (filled squares) or GTP (circles). A control reaction containing 20 μM GDP without SmgGDS-607 (open square) indicates that GDP does not affect GGTase-I activity for RhoA.

Scheme 1.

Reaction mechanism where SmgGDS (I) sequesters RhoA (S) from GGTase-I (E).
Described by equations 1 and 2.

Table 1.
GGTase-I is not inhibited by either nucleotide or SmgGDS proteins.

A non-linear regression fit of the Michealis-Menten equation (Equation 4) to GGTase-I catalyzed prenylation of dansyl GCLVL (data in Figure 2C) allows calculation of steady state kinetic parameters and demonstrates that SmgGDS proteins do not directly inhibit GGTase-I.

	K_M (μM)	k_{cat} (s^{-1})
Buffer	1.9 ± 0.8	0.046 ± 0.009
+ GTP	2.1 ± 0.9	0.052 ± 0.011
+ GDP	2.1 ± 1.1	0.052 ± 0.013
+ SmgGDS-607	3.1 ± 1.7	0.057 ± 0.017
+ SmgGDS-558	2.0 ± 1.2	0.039 ± 0.012
+ SmgGDS-607 & GTP	2.5 ± 1.6	0.049 ± 0.016

Table 2.
The polybasic region (PBR) of RhoA peptide enhances affinity for SmgGDS and GGTase-I.

Fluorescence anisotropy was used to measure the binding affinities (K_d) of SmgGDS splice variants or GGTase-I for RhoA tail peptides. Equation 5 was fit to the data in Figure 3 by non-linear regression. ^aNo complex formation was observed at 40 μ M SmgGDS-558.

	K_d RhoA PBR and CaaX (5-fam-ARRGKKKSGCLVL)	K_d RhoA CaaX only (5fam-GCLVL)
SmgGDS-607	$1.9 \pm 0.3 \mu\text{M}$	$61 \pm 20 \mu\text{M}$
SmgGDS-558	$8.1 \pm 1.5 \mu\text{M}$	$> 40 \mu\text{M}^a$
GGTase-I (with 1 μ M GGPP)	$0.056 \pm 0.008 \mu\text{M}$	$2.2 \pm 1.6 \mu\text{M}$