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Regulation of RGS5 GAP activity by GPSM3

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Abstract Heterotrimeric G protein signaling is limited by intracellular proteins that impede the binding of or accelerate the hydrolysis of the activating nucleotide GTP, exemplified respectively by the G protein-signaling modifier (GPSM) and regulator of G protein-signaling (RGS) families of proteins. Little is known about how members of these groups of proteins might influence the impact of the other on G protein activity. In the present study, we have identified novel binding and functional interactions between GPSM3 (also known as activator of G protein-signaling 4 (AGS4) or G18) and RGS5, both of which were found to be expressed in primary rat aortic smooth muscle cell cultures. The binding of GPSM3 to RGS5 appears to be selective as no interactions were detected with other RGS proteins tested. In solution-based experiments, the addition of GPSM3 was found to enhance the ability of RGS5 to accelerate GTP hydrolysis by Gαi1 but not that of RGS4. In membrane-based assays utilizing M2 muscarinic receptor-activated Gαi1, GPSM3 decreased the rate of GTP hydrolysis in the presence of RGS4 but not RGS5, suggesting that the enhancement of RGS5 activity by GPSM3 is maintained under these conditions and/or that the binding of RGS5 to GPSM3 impedes its inhibitory effect on GTP turnover. Overall these findings show that it is possible for GPSM and RGS proteins to bind to one another to produce distinct regulatory effects on heterotrimeric G protein activity.

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Keywords GPSM proteins · RGS proteins · G protein activation · GTPase activity

Abbreviations

GPSM	G protein-signaling modifier
RGS	Regulator of G protein signaling
GPCR	G protein-coupled receptor
GAP	GTPase-accelerating protein
GEF	Guanine nucleotide exchange factor
VSMC	Vascular smooth muscle cell

Introduction

The magnitude, potency, localization, and duration of GPCR signals are governed by intracellular accessory proteins that coordinate and modulate interactions between the receptor, G protein, and effector. The ability of some Gαi/o proteins to attain or remain in the GTP-activated state can be limited by proteins containing G protein-signaling modifier (GPSM, also called GoLoco or GPR) motifs, which decrease nucleotide exchange rate in vitro [1], and also by regulators of G protein-signaling (RGS) proteins, which act as GTPase-accelerating proteins (GAPs) [2, 3].

Among the 20 genes that encode RGS proteins and the 10 that encode GPSM proteins, there are some that are ubiquitous, while others exhibit limited tissue distributions. RGS5 is highly expressed in the vascular system, and it is a key player in smooth muscle contraction and vascular remodeling [4]. Relatively little is known regarding the functional regulation of RGS5. Here, we identify GPSM3 (a.k.a. AGS4 or G18) as a novel binding partner for RGS5,

and also report GPSM3 to be expressed in VSMCs. Moreover, we have also investigated the possible cross-talk between RGS5 and GPSM3 with respect to the regulation of G Protein activity.

GPSM3 is a 160 amino acid protein that contains three tandem GPSM motifs at its C-terminus with a relatively short N-terminal domain that contains multiple prolines [5]. Full length GPSM3 binds to the inactive, GDP-bound form of Gαi1 and to fluoroaluminate-activated forms of both Gαi1 and Gαo. The GPSM motifs of GPSM3 can reduce the rate of GDP dissociation from Gαi, but not apparently from Gαo [6], while the N terminus of GPSM3 can serve as a guanine nucleotide exchange factor (GEF) for Gαi1. GPSM3 thus has the capacity to both impede and activate Gα signaling. As well, full length GPSM3 can inhibit the steady-state GTPase activities of both Gi1 and Go heterotrimers in the presence of RGS4 and an agonist-activated GPCR, although the underlying mechanisms are not completely clear [7].

Effects on G protein function of individual RGS and GPSM domains have been widely investigated; however, their combined effects generally have not. Increasing evidence suggests that these proteins may co-regulate G protein activities in cells. Indeed, RGS12 and RGS14 are two relatively large RGS proteins that also possess a GPSM domain [8]. Given that both RGS5 and GPSM3 are expressed in smooth muscle cells and are able to regulate Gi signaling, we investigated whether GPSM3 might functionally interact with RGS5 and further modulate G protein activity.

Materials and Methods

Generation of GPSM3 antibody

A 12 amino acid peptide was designed based on the N terminus of GPSM3 (amino acids 1–12). Peptides were synthesized by GeneScript Co., and rabbits were immunized through the GeneScript Co. antibody synthesis facility. Antiserum was characterized using both purified His-GPSM3 fusion protein and transiently transfected Chinese hamster ovary (CHO) and HEK293 cells overexpressing Flag-tagged GPSM3 to verify specificity and optimize conditions for immunoblotting and immunofluorescence experiments.

Cell culture and transfection

Primary rat vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) were generously provided by Dr Robert Gros and Bonan Liu, Robarts Research Institute. Briefly, male Wistar-Kyoto rats (WKY, 10–12 weeks of age; Charles River) were cared for in accordance with the Canadian Council on Animal Care guidelines. The animal protocol was approved by the Animal Use Subcommittee, University of Western Ontario. Isolation of rat aortic VSMCs was performed as described previously [9]. Isolated VSMCs and ECs were cultured until passage 7–9 for further experiments including RNA extraction, immunoblotting, and immunofluorescence.

CHO cells were seeded onto 10-cm (7×10^5 cells/plate) or 35-mm dishes (0.5×10^5 cells/plate) the day before transfection. Cells were transiently transfected with pcDNA3.1 Flag-tagged GPSM3, using lipofectamine 2000 (Invitrogen™). Control cells were transfected with empty pcDNA3.1 vector. At two-days post-transfection, cells were harvested for immunoblotting or fixed for immunofluorescence studies.

RNA Preparation and RT-PCR

Total RNA from isolated VSMCs was extracted using Trizol reagent (Invitrogen™) and further purified using RNeasy mini columns (Qiagen). 2 μg of total RNA was used for reverse transcription with the High Capacity Reverse Transcription kit (Applied Biosystems®). Primers specific for the open reading frames of GPSM3 and RGS5 were used in PCR reactions to examine transcript expression [7].

Immunofluorescence

Cells were seeded onto 35-mm glass-bottom microwell dishes at 30–50 % confluency. Cells were fixed using 2 % paraformaldehyde for 15 min, permeabilized in 100 % methanol for 10 min, blocked with 3 % BSA in PBST solution for 1 h to reduce nonspecific binding, after which anti-GPSM3 antibody was added (1:500), and fixed cells were incubated with the antibody in blocking solution at 4 °C overnight. After three washes with PBST, cells were probed with AlexaFluor 488 goat anti-rabbit secondary antibody (1:1000, Invitrogen) for 1 h at room temperature in the dark. DAPI was used to detect nuclei. As a negative control, cells were processed omitting the primary antibody. Finally, cells were visualized through a Zeiss LSM 410 confocal microscope equipped with a Krypton/Argon laser using a 63× oil immersion lens.

Constructs and protein purification

GPSM3 and RGS5 were subcloned into pET19b or pGEX4T2 vectors to make His-tagged or GST-tagged fusion proteins, respectively, which were expressed in *Escherichia coli* and purified by affinity chromatography followed by size exclusion FPLC as described [7]. Protein

concentrations were determined by Bradford assay, and purity was estimated by Coomassie staining.

Purified protein pull-down assay

GST-GPSM3 (300 nM) was incubated in binding buffer (50 mM Tris pH 7.5, 0.6 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.1 % TritonX-100, PMSF and protease inhibitor cocktail (P.I.)) with an equimolar amount of purified His-tagged RGS protein (RGS4, RGS5, or RGS16) in the presence or absence of increasing concentrations of Gαi1, which had been preincubated with 10 mM GDP. Glutathione 4B beads (10 μL bed volume) were added to the solution, and the incubation was continued overnight at 4 °C. Beads were pelleted by centrifugation and washed with binding buffer, and proteins were separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting.

Pre-steady-state GTPase assay

As described previously [7], purified His_6-Gαi1 (500 nM) was incubated with 10^6 cpm of [γ-^{32}P]-GTP for 15 min. The binding reaction was stopped by the addition of 500 μM unlabeled GTP, and a single round of GTP hydrolysis was initiated by adding 10 mM of Mg^{2+} in the presence or absence of RGS protein ± GPSM3. Aliquots were taken at indicated times and quenched with ice-cold activated charcoal, after which samples were centrifuged. The level of radioactive ^{32}Pi in the supernatant was measured by liquid scintillation counting.

GTPγS binding assay

As previously [7], purified His_6–Gαi1 (100 nM) was preincubated for 1 h at 4 °C in the absence or presence of purified GPSM3 ± RGS5. Binding assays were initiated by adding 0.5 μM [^{35}S]-GTPγS (1.25×10^5 cpm/pmol). The combined proteins were further incubated at 30 °C for 30 min. The assay was terminated by the addition of cold stop buffer (Tris (pH 8.0) 20 mM, $MgCl_2$ 10 mM, NaCl 100 mM, Lubrol 0.1 %, GTP 1 mM, DTT 0.1 mM), and samples were filtered through nitrocellulose membranes followed by washing with ice-cold wash buffer. The level of radioactive ^{35}S binding to G protein was measured by liquid scintillation counting. Nonspecific binding was measured in the presence of 100 μM unlabeled GTPγS, and subtracted to yield specific binding.

Receptor- and agonist-stimulated steady-state GTPase assay

Sf9 membranes (from cells expressing N-terminal c-myc-tagged M2 muscarinic receptor, Gαi1, Gβ1, and Gγ2), as previously, were incubated with [γ-^{32}P]-GTP in the presence of purified RGS proteins (RGS4 or RGS5) at indicated concentrations with or without GPSM3 (1 μM) [7]. Non-specific membrane GTPase signal was estimated by adding 1 mM of unlabeled GTP to the above assay mix, and this value was subtracted from the total counts per minute. Reactions were stopped by the addition of activated charcoal followed by centrifugation, and the level of ^{32}Pi in the resulting supernatant was determined by liquid scintillation counting. Agonist-dependent GTPase activity was determined by subtracting the signal measured in the presence of the inverse agonist tropicamide.

Immunoblotting

SDS-PAGE was transferred to PVDF membranes, and then membranes were incubated with blocking buffer (Tris-buffered saline Tween 20 (TBST) with 5 % skim milk) for 1 h at room temperature and then probed with primary antibodies (1:1000) diluted in blocking buffer overnight on a rotating platform at 4 °C. Blots were subsequently washed 3 times with TBST and then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) (Promega) diluted in TBST for 1 h at room temperature. After another three washes with TBST, the blot was visualized by LumiGLO Reserve chemiluminescence substrate (KPL, Inc.) using a FluorChem 8000 imaging system.

Statistical analysis

All data are expressed as mean ± S.E.M., and statistical significance was determined with Student's *t* test or one-way ANOVA, followed by a Tukey's Multiple Comparison Test. Values of $p < 0.05$ were considered significant.

Results

Expression of GPSM3 and RGS5 mRNA in smooth muscle cells

GPSM3 is expressed at relatively high levels in spleen and heart [5, 7], suggesting that it might be involved in regulating immune function as well as cardiovascular function. It has been suggested that expression of GPSM3 in mature monocytes might be involved in Collagen Antibody-induced Arthritis [10]. However, the cellular expression and function of GPSM3 in the cardiovascular system has not been well investigated. Primary aortic smooth muscle cells were isolated from 3-month-old rats, and RT-PCR was used to identify genes expressed in these cells. Consistent with previous studies, RGS5 was detected

at the mRNA level (Fig. 1a), and as well, we have also identified the expression of GPSM3 mRNA in these cells (Fig. 1a).

GPSM3 protein expression

To confirm the expression of GPSM3 in smooth muscle cells, we developed a polyclonal antibody against the N-terminal region of the protein (Genscript Co.). To determine the specificity of our antibody, CHO cells were transiently transfected with Flag-tagged GPSM3. 48 h after transfection, cells were either subjected to immunofluorescence or lysed and processed for immunoblot analysis. As indicated in Fig. 1b, and consistent with a previous report [5], no endogenous GPSM3 was detected in non-transfected CHO cells. When GPSM3 was overexpressed, an immune reaction band occurred at the expected molecular weight (~ 20 kDa). Immunofluorescence experiments showed that only cells overexpressing GPSM3 were detected by the GPSM3-antibody, which revealed primarily cytosolic expression (Fig. 1c).

After validating the antibody, we used it to investigate the expression of endogenous GPSM3 at the protein level in both VSMCs and ECs, as shown in Fig. 1d, e. Consistent with its mRNA expression pattern, GPSM3 was found in both cell types, with relatively greater expression in VSMC

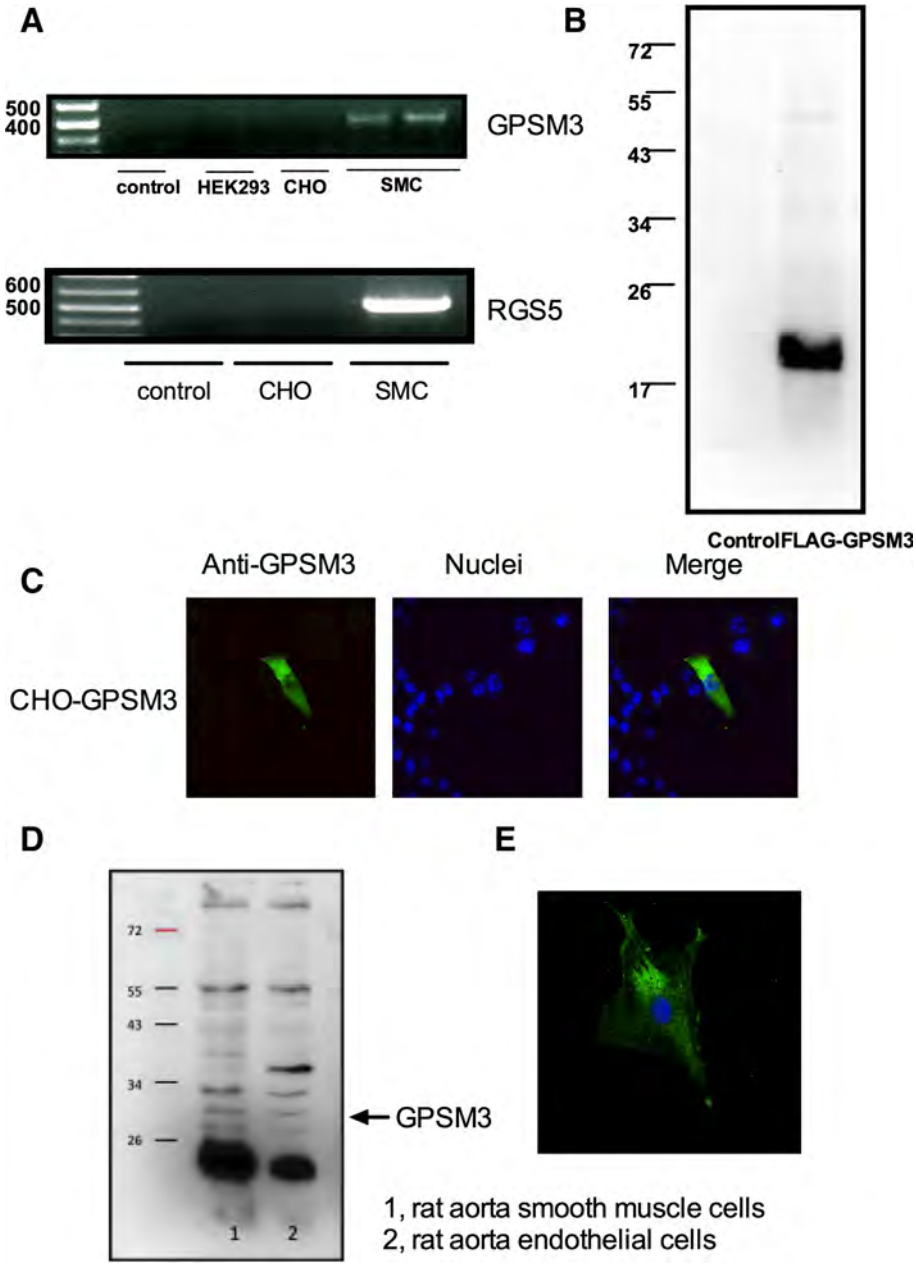

Fig. 1 Validation of GPSM3 antibody and expression of GPSM3 and RGS5 rat VSMC. **a** Total RNA was isolated from established cell lines and primary cells and reverse transcribed to cDNA, after which PCR was performed using primers designed to specifically probe for GPSM3 and RGS5. CHO cells were seeded in 10-cm plates and transiently transfected with pcDNA3.1 vector (control) or a plasmid encoding Flag-GPSM3. 48 h after transfection, cells were either (**b**) lysed and the lysate separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting or (**c**) fixed and subjected to immunofluorescence. Blots or fixed cells were probed with anti-GPSM3 antibody (1:1000) and anti-rabbit secondary antibody (1:1000 (immunoblotting), or 1:500 (immunofluorescence). **d** Lysates from cultured primary aortic smooth muscle cells (lane 1) and endothelial cells (lane 2) were separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting. **e** Primary aortic smooth muscle cells were fixed and subjected to immunofluorescence. Blots or fixed cells were probed with anti-GPSM3 antibody (1:1000). Data are representative of three independent cell isolations from different animals

compared to ECs (Fig. 1d). Immunocytochemistry-based assays showed that, consistent with a previous report and our own earlier results using an overexpression system, endogenous GPSM3 was localized mainly in the cytosolic fraction (Fig. 1e).

GPSM3 selectively interacts with RGS5

Based on their known biochemical selectivity, GPSM3 and RGS5 would be expected to target an overlapping set of heterotrimeric G proteins in VSMCs. There is an increasing evidence demonstrating that the various proteins that modulate G protein nucleotide binding and hydrolytic activities can interact with one another functionally and in some cases physically as well [11]. Thus, further experiments were designed to examine the combined effects of GPSM3 and RGS5 on G protein activity in the absence and presence of an activated receptor, and also to determine whether GPSM3 and RGS5 might be able to form a complex with one another.

As shown in Fig. 2a, purified His-RGS5 directly interacts with GST-GPSM3, with no noticeable interaction with the GST tag or the Glutathione 4B beads. In an effort to identify the RGS5 binding site, we tested GPSM3 constructs lacking either the N-terminal region (aa1–60) or the C-terminal region (aa61–160) for their ability to bind to RGS5. Neither one of these constructs exhibited any appreciable binding to RGS5 (data not shown), suggesting that elements of both truncated regions or are needed to maintain the RGS5 binding surface of GPSM3. The fact that we observed a direct interaction between RGS5 and GPSM3 suggests that there might be cross-talk between how these two proteins to govern signaling processes within the cell. In contrast to its interaction with RGS5, GPSM3 does not appear to interact with RGS2, RGS4 (data not shown), or RGS16 (Fig. 2a).

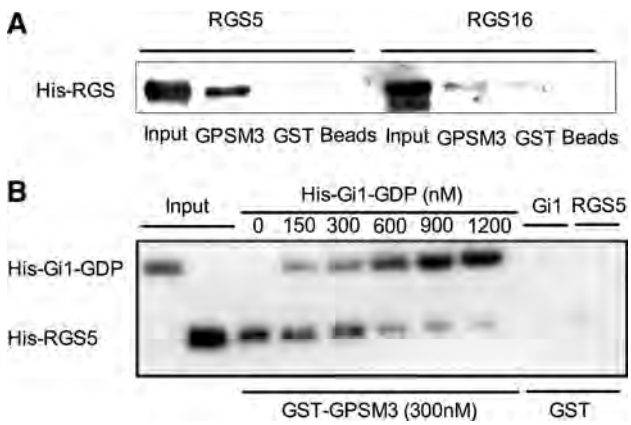

Fig. 2 Interactions between GPSM3 and RGS5. In vitro pull-down assays were performed as indicated in Materials and Methods. (**a**) Purified His-tagged RGS proteins (300 nM) were mixed with wild type GPSM3 (300 nM), purified GST protein (300 nM), or buffer and then incubated overnight with glutathione-sepharose beads in a total volume of 200 μL. Beads and associated proteins were precipitated by centrifugation and washed three times with buffer, and the final pellet was and resuspended in 2× protein loading dye and heated to 99 °C for 5 min. The denatured proteins were separated using 12 % SDS-PAGE, transferred to a PDVF membrane and probed for His-tagged proteins as described in the legend to Fig. 1. The input lanes were generated using 50 ng of purified RGS proteins. (**b**) His-tagged RGS5 (300 nM) was incubated with GST-tagged wild-type GPSM3 (300 nM), plus increasing concentrations of purified His-Gαi1 as indicated. In vitro pull-down assays were performed as noted for **a**. The input lanes were generated using 50 ng of purified RGS5 and purified Gαi1 protein respectively. The blots shown in **a** and **b** are representative of 3 independent experiments

Gαi1 and RGS5 inhibit each other's binding to GPSM3

The binding of RGS5 and GPSM3 to one another could potentially alter each of their abilities to bind to and/or modulate the function of Gα. For example, the binding of RGS proteins to various receptors, effectors, and scaffolding proteins can variously promote or inhibit their interactions with G proteins [12]. One common target of GPSM3 and RGS5 is Gαi1, and to determine whether any of these three proteins might either increase or decrease binding between the other two, we carried out a GST-pull-down assay combining purified polyhistidine-tagged RGS5, polyhistidine-tagged Gαi1, and a GST-GPSM3 fusion protein (Fig. 2b). As expected, we found that complexes between GPSM3 and either RGS5 or Gαi1-GDP and GPSM3 could be isolated from mixtures of these respective proteins using glutathione beads. When increasing amounts of Gαi1-GDP (from 150 nM to 1.2 μM) were added to a mixture of RGS5 and GPSM3 (300 nM respectively), we observed a gradual diminution of binding between GPSM3 and RGS5. It follows that RGS5 might inhibit the binding of GPSM3 to the inactive state of G protein (Gαi1-GDP), although the incomplete inhibitory effect of Gαi1 on RGS5–GPSM3 binding suggests the possible existence of a ternary complex between the G protein and its two binding partners. Regardless, the effect of one of these proteins on G protein activity might be influenced by the other, and thus, further experiments were designed to test the possible functional consequences of the RGS5–GPSM3 interaction.

Effects of GPSM3 and RGS5 on free Gαi1

Solution-based single-turnover GTPase assays were used to examine whether there might be an effect of GPSM3 on RGS5 GAP activity. This assay measures only a single cycle of GTP hydrolysis and therefore allows us to examine changes in GTP hydrolysis absent any effects on nucleotide exchange. We have previously shown that GPSM3 itself has

no effect on GTP hydrolysis [7]. As shown in Fig. 3a, RGS5 as expected greatly enhanced GTP hydrolysis by Gαi1 GTPase activity. Interestingly, in the presence of an equimolar amount of GPSM3, a further increase in the rate of GTP hydrolysis was observed with an additional threefold increase in the rate of GTP hydrolysis relative to the effect of RGS5 alone ($t_{1/2}$ of ~24 vs. ~87 s). The addition of GPSM3 showed no measurable change in the maximal GTP hydrolysis level promoted by RGS5 (Fig. 3a), indicating a negligible effect on nucleotide exchange over this relatively short (<2 min) period. RGS4 was also tested for comparison since it does not interact with GPSM3, and consistent with this, GPSM3 showed no appreciable effect on RGS4 activity (Fig. 3b). These results suggest that GPSM3 can specifically enhance RGS5 GAP activity, and that this effect may due to a direct interaction between the two proteins.

In other solution-based experiments, we examined the binding of [^{35}S]GTPγS to Gαi1 in the presence of GPSM3 with and without RGS5. We previously showed that full length GPSM3 has a negligible net effect on GTPγS binding to Gαi1, which is attributable to countervailing GDI and GEF effects of the central/C-terminal and N-terminal parts of the protein, respectively [7]. We found that RGS5 alone had no effect on the binding of [^{35}S]GTPγS to Gαi1 nor did it alter binding in the presence of full length GPSM3 (data not shown). These results suggest that the binding of RGS5 to GPSM3 does not alter either of its GDI or GEF effects on Gαi1, although it cannot be ruled out that both of those may be altered albeit equivalently and in opposite ways.

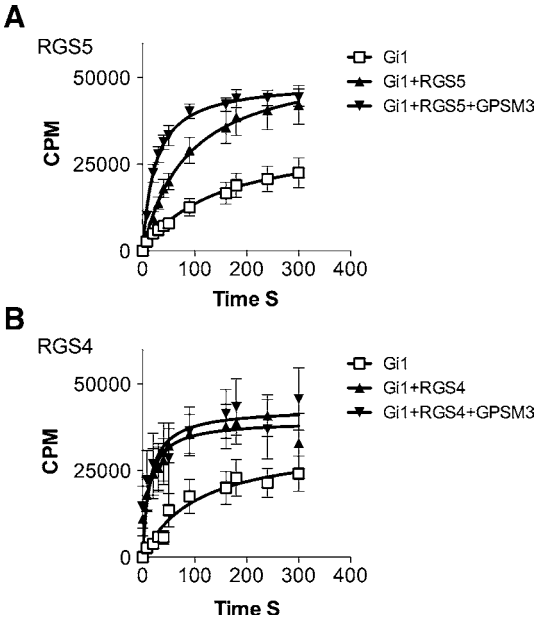

Fig. 3 The effect of GPSM3 on RGS5 GAP activity. Purified His-Gαi1 (250 nM) was incubated with [γ-^{32}P]-GTP (1 × 10^6 cpm/assay) plus 1 μM nonradioactive GTP for 15 min at 30 °C in a volume of 200 μL. The binding reaction was quenched by the addition of 50 μL of 500 μM unlabeled GTP. A single round of GTP hydrolysis was initiated by adding 10 mM of Mg^{2+} in the presence RGS5 (**a**), or RGS4 (**b**) (100 nM) with or without GPSM3 (1 μM), and aliquots were withdrawn and quenched at the times indicated. The points shown are mean ± S.E.M. of 3 independent experiments

Regulation of receptor-stimulated G protein activity by RGS5 and GPSM3

The GAP activity of RGS proteins can be measured under steady-state conditions in membrane-based assays that include Gα, Gβγ, and an agonist-stimulated receptor. We previously showed that GPSM3 decreases steady-state Gαi1 GTPase activity in the presence of Gβγ, agonist-activated M2 muscarinic receptor, and RGS4 (which, respectively, promote GTP binding and hydrolysis) [7]. Although the exact mechanism is unclear, such an inhibitory effect is presumed to result from a decrease in receptor-promoted nucleotide exchange. We hypothesized that the ability of GPSM3 to increase RGS5 GAP activity might counterbalance its inhibitory effect on steady-state GTPase activity. To examine this possibility, we compared the effect of purified GPSM3 on GPCR-dependent steady-state Gi GTPase activity in the presence of either RGS4 or RGS5.

As shown in Fig. 4(c, d), consistent with previous findings, GPSM3 inhibited RGS4-stimulated GTP hydrolysis in a concentration-dependent manner [7]. In the presence of GPSM3, the magnitude of the RGS4 effect was reduced, but RGS4 potency was essentially unchanged, which implies that GPSM3 does not compete with RGS4 for binding to Gαi in this context. Since the rate limiting step in the presence of RGS protein is nucleotide exchange, this decrease in steady-state GTP hydrolysis most likely reflects an inhibition in nucleotide exchange caused by the GDI activity of GPSM3. Notably, there was no observable effect of GPSM3 when we used RGS5 instead of RGS4 (Fig. 4a, b). This observation seems to be formally consistent with the aforementioned hypothesis, as an increase in RGS5 GTPase-accelerating activity concomitant with a reduced rate of GTP binding could result in a net effect of zero. Alternatively, it may be that the binding of RGS5 to GPSM3 somehow interferes with its ability to act on Gαi1 under these conditions, resulting in a failure to inhibit steady-state GTPase activity. As discussed below, we consider the second explanation to be more likely.

Discussion

The activation state of heterotrimeric G protein Gα subunits is controlled by multiple binding partners, which variously affect the binding and hydrolysis of GTP. These

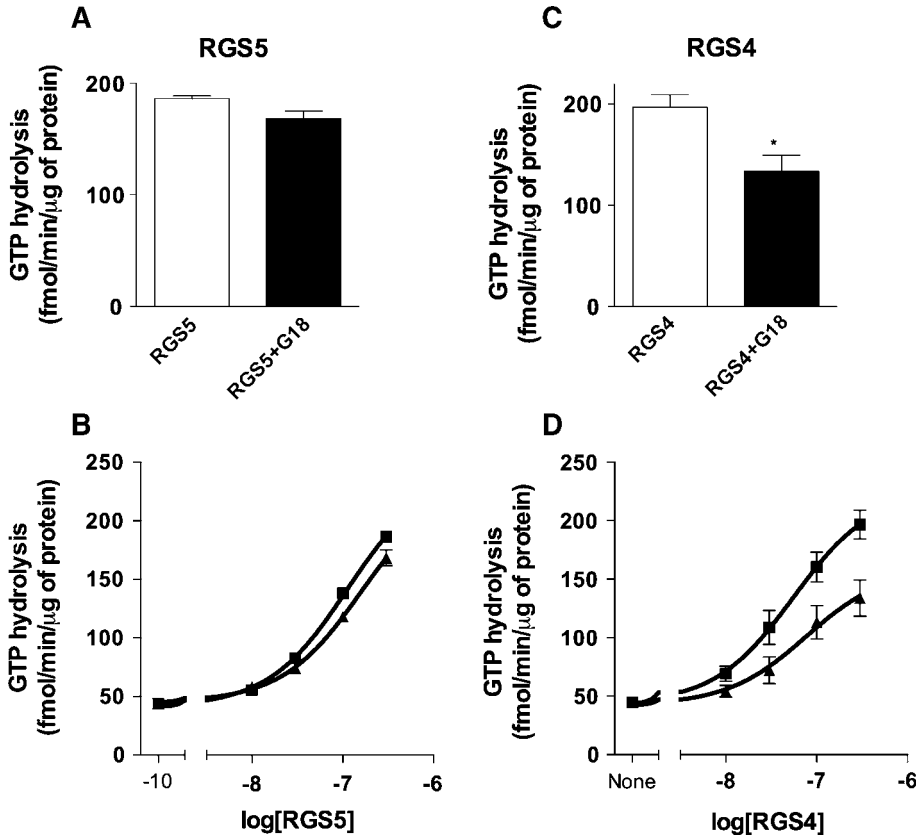

Fig. 4 Regulation of G protein activity by RGS5 and GPSM3. Membranes derived from Sf9 cells co-expressing M2 muscarinic acetylcholine receptor plus heterotrimeric Gi1 were assayed for GTPase activity with the agonist carbachol (100 μM) or the inverse agonist tropicamide (10 μM), in the presence of indicated concentrations of RGS5 (**a**) or RGS4 (**b**) without or with GPSM3 (1 μM). Data shown represent mean ± S.E.M. taken from 3 independent experiments carried out in triplicate. *$p < 0.05$ (Student's *t*-test)

include GPCRs and non-receptor GEFs, which promote the binding of the activating nucleotide GTP by facilitating GDP dissociation, Gβγ dimers, and GPSM motif-containing proteins, which bind to overlapping sites on Gα and delay GDP dissociation, and RGS proteins and certain effectors, which promote the hydrolysis of Gα-bound GTP [11]. Frequently, these modulatory proteins are found to work with one another, but the combined results are not always straightforward to predict from their known individual effects on Gα proteins [11, 12].

In the present study, RGS5 and GPSM3 were found to bind to one another with functional consequences. Multiple previous studies have shown that the binding of RGS proteins to various receptors, effectors, and scaffolding proteins can variously promote or inhibit their interactions with G proteins [12]. The binding of GPCRs to RGS proteins for example promotes their targeting to activated Gα proteins [12], and may additionally increase their ability to promote GTP hydrolysis [13]. However, little is known regarding potential RGS-GPSM interactions either functionally or physically.

We found the binding of RGS5 to GPSM3 to be reduced by an excess of purified Gαi1 (Fig. 2b). One possible explanation of this is that the binding of GPSM3 and Gαi1 to RGS5 is mutually exclusive; however, based on our other observations, a more likely explanation may be that their affinities for the G protein are higher than for one another and/or that there are allosteric effects wherein the affinity between two of these proteins is decreased by the binding of the third. These latter explanations would allow for the existence of a complex containing GPSM3, RGS5, and Gαi1, wherein the effects on G protein function of either RGS5 or GPSM3 might be altered. Indeed, we found that the GAP activity of RGS5 in solution was increased by GPSM3. Such a change may have occurred in GPCR-driven membrane-based assays as well. GPSM3 as expected reduced the rate of RGS4-promoted steady-state GTPase activity [7], but in contrast, no decrease was observed with RGS5 when GPSM3 was included in the assay. This could reflect a compensatory increase in the RGS5 GAP effect due to GPSM3 to offset the expected negative effect of GPSM3. Alternatively, as nucleotide exchange is likely to be rate limiting in these experiments, a more probable explanation may be that the binding of RGS5 to GPSM3 impeded its inhibitory effect under these circumstances. It follows that the respective effects of RGS5 and GPSM3 on G protein activity, and the impact of their mutual binding on these effects, may be context dependent, i.e., these could differ depending upon whether or not additional components such as Gβγ, a GPCR, and membrane phospholipids are present.

As a point of comparison to the present study, both RGS12 and RGS14 along with their RGS domains

additionally contain functional GPSM motifs [6, 10, 14]. In the presence of free Gαi1 and the non-receptor GEF Ric-8A, the GDI function of RGS14 appears to predominate [15], whereas the GAP function of its RGS domain predominates in the presence of an agonist-activated receptor [16]. Notably, the central region of RGS14 that lies between its RGS and GPSM domains, under certain conditions, appears to impede GPSM activity and also increase the effect of the RGS domain on receptor-activated G proteins, and moreover, this domain when isolated can similarly enhance the activities of B/R4 subfamily RGS proteins [16]. The present findings show that an analogous relationship exists between RGS5 and GPSM3, although the underlying mechanism may differ. As well, the physiological significance of interactions between RGS and GPSM proteins remains to be determined; however, such cross-talk could conceivably play a role in modulating GPCR signaling as well as in G protein-dependent intracellular processes such as asymmetric cell division. Roles in cell division have been identified with most GPSM family members, including RGS14, and as well the RGS domains of RGS14 [17] and the *Caenorhabditis elegans* protein RGS7 [18] have been implicated in such processes. Comparable roles for RGS5 and GPSM3 have yet to be reported, although the present results suggest the possibility that these two proteins could act in tandem in receptor-independent cellular functions where G proteins are involved.

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