

The core domain of RGS16 retains G-protein binding and GAP activity in vitro, but is not functional in vivo

Canhe Chen, Sheng-Cai Lin*

Regulatory Biology Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, 30 Medical Drive, Singapore 117609, Singapore

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Abstract The regulators of G-protein signaling (RGS) family members contain a conserved region, the RGS domain, and are GTPase-activating proteins for many members of G-protein α -subunits. We report here that the core domain of RGS16 is sufficient for in vitro biochemical functions as assayed by its G-protein binding affinity and its ability to stimulate GTP hydrolysis by $G\alpha_o$ protein. RGS16 also requires, in addition to the RGS domain, the divergent N-terminus for its biological function in the attenuation of pheromone signaling in yeast, whereas its C-terminus region is dispensable. Together with other evidence, these data support the notion that RGS proteins interact with other cellular factors and may serve to link specific G-proteins to different downstream effectors in G-protein-mediated signaling pathways.

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Key words: RGS protein; RGS16; GTP-binding protein; GTPase-activating protein

1. Introduction

The regulators of G-protein signaling (RGS), defined by initial genetic studies as negative regulators of G-protein-mediated signaling, have been demonstrated to serve as GTPase-activating proteins (GAP) of a variety of G-protein α -subunits, thus terminating the signaling process by G-protein-coupled receptors [1–5]. The RGS proteins share an evolutionarily conserved domain of approximately 120 amino acid residues, referred to as the RGS domain. The RGS proteins bind to $G\alpha$ subunits with high affinity in the presence of both GDP and AlF_4^- , suggesting that they accelerate GTP hydrolysis by stabilizing the GTPase transition state [6]. This is supported by evidence obtained from crystallographic studies [7]. We have previously identified and characterized RGS16 (also known as RGS-r [8]) and shown that RGS16 binds $G\alpha$ subunits in the transition state [9]. However, RGS16 also binds G-proteins with significantly high affinity in the presence of GDP alone [9]. The significance of RGS binding to the inactive state of $G\alpha$ is unclear. It is possible that in addition to GAP activity, some of the RGS proteins may also modulate G-protein reactivation, or the RGS- $G\alpha$ (GDP) complex may have downstream effectors.

Alignment of RGS proteins reveals that they contain divergent sequences flanking the conserved RGS domain. Furthermore, they have a striking size variation ranging from 21 to

150 kDa [4,5,10]. In view of their diversity, RGS proteins may not only serve as GTPase-activating proteins, but could also be interacting with a diverse number of cellular proteins that may play crucial roles in G-protein-mediated signal transduction. Indeed, D-AKAP-2, a recently identified protein that binds to both type-I and type-II regulatory subunits of cAMP-dependent protein kinase (PKA), possesses an RGS domain, with the R-subunit-binding fragment at its divergent C-terminus [11]. RGS proteins may therefore play a direct role in the regulation of a signaling pathway that is commonly believed to be regulated by intracellular levels of the second messenger cAMP, primarily controlled by the G_s subunit. In addition, we have previously noticed that a short deletion of the N-terminus of RGS16 resulted in the loss of its biological activity although its G-protein binding capacity was intact. The size heterogeneity in the divergent sequences amongst the RGS protein family members further prompted us to study the structural requirements for the biochemical and biological functions of these proteins. In this report we show that the core RGS16 domain retains full GTPase activation activity and G-protein binding activity as assayed using several G subunits, including $G\alpha_o$, but the divergent N-terminus is required for its physiological function.

2. Materials and methods

2.1. Construction of RGS16 deletion mutants

cDNA fragments for RGS16 deletion mutants were generated by polymerase chain reactions (PCR). The 5' sense oligonucleotide primers used to create three RGS16 N-terminal deletion mutants, $\Delta N13$, $\Delta N23$, and $\Delta N55$, were CCATGGAGAGAGCTAAAGAGTTCAAG, CCATGGGAATCTTTCTTCATAAATCA, and CCATGGAAGATGTACTGGGATGGAGA, respectively, each including an *NcoI* site. The 3' antisense oligonucleotide common for the three mutants was CGGAGGCTCAAGTGTGTGAAGGCT. The 5' oligonucleotide common for C-terminal deletion mutants $\Delta C16$, $\Delta C34$, and $\Delta C41$, was CTCGAGATGTGCCGCACCCTAGCCACCTTC. The corresponding antisense primers were: GATTAGGCCGAGGCTTG-GGCAGCC, CGTTAATAGGAGTCTTCTCCATC, and ATCTA-TGTGCGGGTCTTCCCCTGA, respectively. The sense primer of $\Delta N55$ and the antisense primer of $\Delta C16$ were used to generate the core RGS domain, $\Delta N55\Delta C16$. The PCR products were treated with Klenow DNA polymerase and polynucleotide kinase, and ligated to the *SmaI* site of pBluescript (Stratagene) for sequencing analysis.

2.2. GTPase-activating activity assays

The $G\alpha_o$ protein cDNA, generated by PCR as previously described [9], was ligated to the plasmid pQE30 (Qiagen). Wild type RGS16, $\Delta C16$, $\Delta C34$, and $\Delta C41$ cDNA fragments in pBluescript were released with *XhoI* and *BamHI*, blunt-ended with Klenow polymerase, and ligated to *SphI*/Klenow-treated pQE30 vector. The cDNA fragments for $\Delta N13$, $\Delta N23$, $\Delta N55$, and $\Delta N55\Delta C16$ cDNAs were released with *NcoI*/*BamHI*, blunt-ended with Klenow, and ligated to *BamHI*/Klenow-treated pQE30 plasmid. The ligation reactions were separately transformed into the bacterial strain M15 and transformants expressing individual protein were rapidly screened for from small-scale ex-

*Corresponding author. Fax: (65) 779-1117.
E-mail: mcblinsc@leonis.nus.sg

Abbreviations: RGS, regulator of G-protein signaling; GAP, GTPase-activating protein; G-protein, GTP-binding protein; GST, glutathione S-transferase; PKA, protein kinase A

pression cultures according to the manufacturer's instructions (Qia-gen). Eluted protein samples were dialyzed in the elution buffer without imidazole. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad).

The GAP activities of each RGS16 protein were determined as previously described [12]. Briefly, 0.25 μ M $G\alpha_o$ was incubated with [γ - 32 P]GTP (1 μ M) in a buffer containing 50 mM Na-HEPES (pH 8.0), 5 mM EDTA, 0.05% $C_{12}E_{10}$, and 2 mM DTT, at 30°C for 30 min; the temperature was then lowered to 10°C. A 5 μ l aliquot of mixture was added into 15 μ l reaction buffer containing 50 mM Na-HEPES (pH 8.0), 0.05% $C_{12}E_{10}$, 100 μ M unlabeled GTP and 2 mM DTT at 0°C. This was the zero timepoint. 40 μ l of GTP-preloaded $G\alpha_o$ mixture was then added into 120 μ l reaction buffer supplemented with $MgSO_4$ (12 μ M final concentration) and indicated RGS protein (2 μ M final concentration) or buffer to initiate GTP hydrolysis. 15 μ l aliquots were removed at the indicated timepoints and dotted onto BA85 filter discs kept on an ice-cold NUNC-Bio assay plate. After washing at 0°C in PBS buffer supplemented with 10 mM EDTA, filters containing residual unhydrolyzed GTP on $G\alpha_o$ were subjected to liquid scintillation spectrometry.

2.3. RGS-G-protein binding assays

The wild type RGS16 and three C-terminal deletion cDNAs were fused in-frame to the *Xho*I site of a derivative of the bacterial expression vector pGex2TK (Pharmacia). The cDNA fragments for the three N-terminal deletion mutants and the core RGS domain were released from pBluescript constructs with *Nco*I/*Bam*HI, and treated with Klenow. The blunt-ended fragments were then fused in-frame to the *Xho*I site (blunt-ended by Klenow) of the derivative vector. The fusion proteins were expressed in the *Escherichia coli* strain BL21(DE3)-pLysS and purified as GST-fusion proteins as described before [9]. The G-proteins ($G\alpha_{13}$, $G\alpha_{12}$, and $G\alpha_o$) were generated and 35 S-labeled by in vitro transcription and translation reactions using TNT coupled reticulocyte lysate systems (Promega) and [35 S]methionine. Deletion mutant proteins were assayed for their binding to G-proteins in the same way as previously described [9].

2.4. Pheromone response assay

The yeast expression construct in the pMW29 vector encoding the wild type RGS16 was as previously described [9]. The fragments of individual RGS16 deletion mutant cDNAs were released with *Eco*RV/*Bam*HI from the pBluescript constructs and cloned into *Sma*I/*Bam*HI sites of pMW29, a yeast expression vector under the control of a galactose-inducible promoter [13]. The biological function of RGS16 deletion mutants was analyzed by a bioassay measuring the sensitivity of pheromone response in US356 yeast cells as previously described [9]. To monitor expression levels of each RGS16 construct, Western blotting analysis was performed as previously described [9], using affinity-purified specific antibody raised against full-length RGS16 protein.

3. Results

3.1. The core RGS16 domain retains full G-protein binding activity

To assess the minimum sequence requirement for the function of RGS16, we generated a series of deletion mutant con-

structs as schematically diagrammed in Fig. 1. These mutant proteins were expressed as GST-fusion proteins in *E. coli* and affinity-purified with glutathione-agarose. G-proteins, $G\alpha_o$, $G\alpha_{12}$, $G\alpha_{13}$, were generated and 35 S-labeled by in vitro transcription and translation. The GST-fusion RGS proteins were first analyzed for their binding efficiency to the G-proteins. As shown previously [8,9], the wild type RGS16 protein binds strongly to the G-proteins in the presence of both GDP and AlF_4^- . It also binds the G-proteins in the presence of GDP alone [9], albeit to a lesser extent (Fig. 2A–C). Whereas all the deletion mutants that included the core RGS domain (Δ N13, Δ N23, Δ N55, Δ N55 Δ C16, Δ C16) bound to the G-proteins with the same affinity as that of the wild type RGS16 (Fig. 2), a short deletion of the core RGS region (Δ C34) completely abolished its G-protein binding affinity (Fig. 2B).

3.2. The divergent regions are not required for GAP activity

We further examined the structural requirement of RGS16 function by measuring the GAP activity of the various deletion mutants on one of the G-proteins, $G\alpha_o$. The ability of the different RGS mutants to affect the GTP hydrolysis by $G\alpha_o$ was tested using a one-round hydrolysis assay with [γ - 32 P]GTP as substrate. The [32 P]_i released was calculated by subtracting remaining counts at an indicated time from 32 P counts on filters at zero timepoint. In the absence of RGS, the k_{cat} for $G\alpha_o$ to hydrolyze GTP was 0.014 s⁻¹ at 10°C (Fig. 3A,B). In the presence of wild type RGS16, the reaction was completed at the earliest timepoint (8 s); GTP hydrolysis was accelerated approximately seven-fold with a catalytic constant (k_{cat}) of 0.10 s⁻¹ (Fig. 3A,B). The N-terminal deletion mutants (Δ N13, Δ N23, Δ N55) and C-terminal deletion mutants outside the RGS domain (Δ C16) also stimulated the GTPase activity of $G\alpha_o$ at levels similar to that by wild type RGS16 (Fig. 3A,B). Similarly, the core RGS domain mutant, Δ N55 Δ C16, retained the GAP activity with a k_{cat} of 0.096 s⁻¹ (Fig. 3B). However, two C-terminal deletion mutants (Δ C34 and Δ C41) with some conserved amino acid residues in the RGS domain removed, failed to activate GTP hydrolysis and did not have obviously distinguishable kinetics from $G\alpha_o$ alone (Fig. 3B, and data not shown). These observations indicate that the divergent sequences flanking the core domain are not required for the GAP activity of RGS16, and that the GAP activity of RGS16 needs its full RGS domain.

3.3. An intact N-terminus is critical for RGS16 biological activity

As the deletion mutants containing the RGS domain retained both the G-protein binding affinity and GAP activity

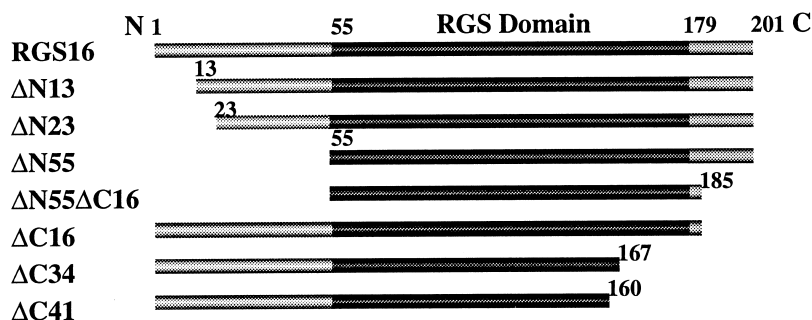


Fig. 1. Schematic diagram of the RGS16 full-length and deletion constructs. The names of different constructs are shown on the left; numbers at the top of each bar indicate the amino acid positions of the start and end of each construct, with respect to the wild type RGS16.

on $G\alpha_o$ in vitro, we tested their biological functions taking advantage of the fact that wild type RGS16 functionally resembles the yeast RGS protein Sst2p to attenuate pheromone signaling [2,9]. The biological function assay was performed by transforming different RGS16 pMW29 constructs into the yeast strain US356 that harbors a mutation in the *bar1* gene encoding a secreted protease capable of cleaving α -factor. In the absence of overexpressed RGS16, the yeast cells form a clear halo corresponding to the area of growth arrest. As previously shown [9], the RGS16 overexpressing yeast transformants are no longer sensitive to the pheromone factor (Fig. 4A,B). A short deletion of the N-terminus ($\Delta N13$) completely abolished the attenuating effect on pheromone signaling. Further deletion mutants of the N-terminus ($\Delta N23$, $\Delta N55$) behaved similarly (Fig. 4A). All the mutant cDNA constructs resulted in comparable levels of expression to those of the wild type RGS16, indicating the functional difference between the wild type RGS16 and its mutants is not due to impairment of expression. Relevance of the bioassay in yeast is supported by our recent finding that perturbation by fusion of a FLAG

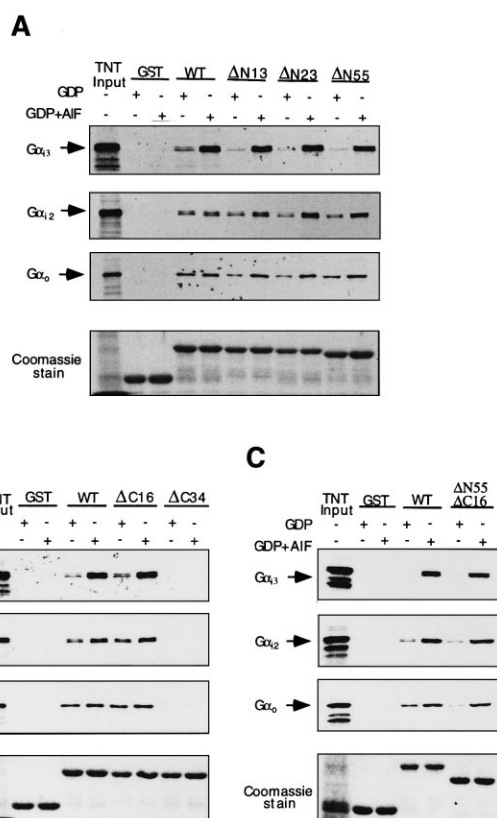


Fig. 2. Binding assays of RGS16 deletion mutants to $G\alpha$ proteins. The relative binding affinities of the N-terminal deletion mutants, C-terminal deletion mutants, and the core RGS domain of RGS16 to G-proteins are shown in panels A, B, and C, respectively. Recombinant RGS16 proteins and GST as a control (indicated at the top of each panel) were incubated separately with individual G-proteins (indicated on the left) pre-incubated with GDP or GDP plus AIF₄⁻. After washing, the beads were boiled with the SDS protein sample buffer and the eluted proteins were resolved on SDS-PAGE gels, along with the total input of each labeled G-protein (the first lane of each panel). The gels were stained with Coomassie blue, dried, and were exposed to X-ray films. Shown at the bottom of each panel is a representative SDS-PAGE gel stained with Coomassie blue, indicating relative GST-fusion RGS protein inputs.

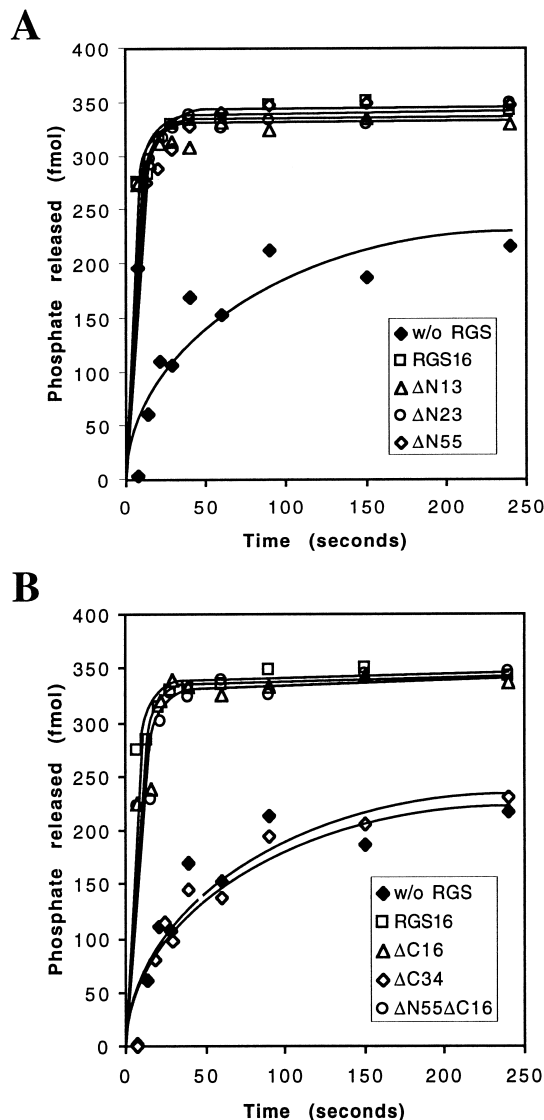


Fig. 3. GTPase-activating protein activity of various RGS16 proteins on $G\alpha_o$. Relative GAP activities on $G\alpha_o$ of the N-terminal deletion mutants ($\Delta N13$, $\Delta N23$, $\Delta N55$), and C-terminal mutants ($\Delta C16$, $\Delta C34$), the core RGS domain ($\Delta N55\Delta C16$), as well as the wild type RGS16, are shown in panels A and B, respectively.

tag to the N-terminus diminished RGS16 function in a cell growth assay in Chinese hamster ovary cells (Zhang and Lin, unpublished observation).

3.4. The $\Delta C16$ mutation retains both GAP activity and pheromone attenuation functions

In contrast to what was observed in the deletion mutants of the N-terminus, removal of the C-terminus ($\Delta C16$) did not affect the functions of RGS16. Compared to the wild type RGS16, $\Delta C16$ binds equally well to all the three G-proteins analyzed, $G\alpha_o$, $G\alpha_{i2}$, $G\alpha_{i3}$ (Fig. 2B), and as mentioned above, it retains full GAP activity on $G\alpha_o$ (Fig. 3B). Similarly, in the yeast bioassay, $\Delta C16$ suppressed the pheromone sensitivity of US356 cells, as does the wild type RGS16. Further deletion in the conserved region ($\Delta C34$, $\Delta 41$) destroyed both the G-protein binding and pheromone attenuation activity (Fig. 2B, Fig. 4B, and data not shown).

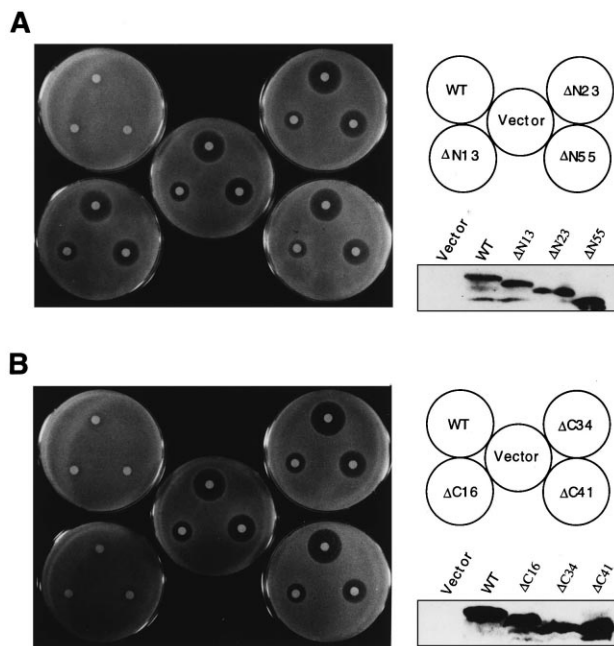


Fig. 4. Yeast pheromone response assays. Halo assays of the N-terminal deletion mutants and C-terminal deletion mutants are shown in panels A and B, respectively. The arrangement of each dish is diagrammed at the top right; the expression levels of each RGS16 protein, accessed by Western analysis using anti-RGS16 antibody, are indicated at the bottom right.

4. Discussion

We have described in this report that the core domain of RGS16 retains GTPase-activating protein activity on $G\alpha_o$ in vitro that is virtually indistinguishable from that of the full-length protein. Our results obtained with RGS16 are consistent with the crystallographic studies showing that only the RGS domain residues are in contact with G-proteins [7], and that the core domains of other RGS proteins such as the retina-specific RET-RGS1, RGS4, retain full GAP activities on G-proteins [14,15]. We further show that the core RGS16 domain binds equally well to the G-proteins, $G\alpha_o$, $G\alpha_{i2}$, $G\alpha_{i3}$, providing an explanation for the full GAP activity of the core domains of RGS proteins.

However, bioassays based on the ability to attenuate pheromone signaling in yeast show that RGS16 needs an intact N-terminus to function. In contrast, removal of 16 amino acid residues from the C-terminus did not affect either the in vitro biochemical or in vivo functions. The biological relevance of the bioassay is further supported by other evidence showing that fusion of a FLAG tag to the N-terminus of full-length RGS16 diminished its in vivo function in modulating cell growth (Zhang and Lin, unpublished results). Similar to what we observed in the bioassay using the C-terminal deletion mutants, the tagging to the C-terminus did not affect RGS16 activity in the cell growth assays. Furthermore, it

should be noted that the deletions of the N-terminus or the C-terminus did not cause protein misfolding or alteration of protein stability as no obvious change in protein expression levels or solubility of deletion mutant proteins was observed in either bacterial cells, yeast cells, or CHO cells. How the divergent N-terminal and C-terminal regions modulate the function of RGS proteins remains to be further studied. One study with the GAIP ($G\alpha$ interacting protein) RGS protein suggests that palmitoylation may be involved in the subcellular localization and function in membrane trafficking of GAIP [16,17]. However, RGS16 does not have the consensus sequence for such a modification. The recent identification of D-AKAP-2 that binds to the regulatory subunits of PKA suggests an exciting possibility that there exist a variety of cellular factors that bind to the divergent regions of RGS proteins [11]. These proteins may link different G-proteins to specific downstream effectors. Identification and characterization of putative cellular factors that bind to the N-terminus of RGS16 is under way.

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