The Proline-rich N-terminal Domain of G18 Exhibits a Novel G Protein Regulatory Function*

Peishen Zhao, Chau H. Nguyen, and Peter Chidiac

From the Department of Physiology and Pharmacology, The University of Western Ontario, London, Ontario N6A 5C1, Canada

The classical model of G protein signaling includes three major components: G protein-coupled receptor (GPCR), heterotrimeric G protein, and effector. The inactive Ga subunit binds with high affinity to GPCR, Gβγ, and GDP. The binding of an agonist to the receptor promotes its guanine nucleotide exchange factor (GEF) activity toward the G protein that results in the exchange of GDP for GTP. This activates the G protein and is thought to cause the dissociation of Ga and Gβγ. Both GTP-bound Ga and free Gβγ are capable of initiating signals by interacting with downstream effectors such as adenylyl cyclase, phospholipase Cβ, and various ion channels and kinases (1). Signaling is terminated by the intrinsic GTPase activity of the Ga subunit, thereby returning the latter to its inactive form and regenerating the inactive Gaβγ complex. It is now recognized that heterotrimeric G protein signaling is more complex than originally proposed, with a number of factors having been identified that can modulate G protein activity. These include the regulator of G protein signaling (RGS) proteins that accelerate G protein deactivation and the receptor-independent activator of G protein signaling (AGS) proteins that modulate G protein signals through several distinct mechanisms. The Gαi/o-Loco (GoLoco)/G protein regulatory (GPR) motif of the Group II AGS proteins can alter the activities of both Ga and Gβγ (2).

The GoLoco/GPR motif was originally identified in the Drosophila RGS12 homologue, Loco (3–5). The Loco motif is a 19-amino acid sequence that can bind to the Gα subunit of Gi/o proteins in their inactive state (Gαi/o-GDP) to inhibit the exchange of GDP for GTP. This biochemical activity serves as its function as a guanine nucleotide dissociation inhibitor (GDI) (3, 6–11) to impede Gα activation. Several important contact points have been identified between the GoLoco motif and Ga subunits, the most notable being the extension of its highly conserved Asp/Glu-Gln-Arg triad into the guanine nucleotide binding pocket of Ga that interacts directly with the α- and β-phosphates of GDP (8, 12). This interaction between the GoLoco motif and Ga subunits has been shown to promote the dissociation of the Gβγ dimer from Ga in vitro (12). In this way the GoLoco motif may act as a receptor-independent activator of Gβγ signaling (13–16). The Ga-GoLoco complex may also affect physical coupling between Ga and the receptor (17, 18), and this may further impact the control of G protein function.

The modulation of G protein activities by GoLoco motif-containing proteins has been implicated in multiple physiological processes. In Caenorhabditis elegans and Drosophila, GPR1/2 and Pins GoLoco motifs, respectively, have been characterized to play essential roles in asymmetric cell division (5, 19, 20), and analogous mechanisms appear to exist in mammalian systems. For example, the Pins homologue LGN recently was shown to be critical for cell polarization during oocyte meiosis in mice (21). Emerging evidence also points to a role in GPCR signaling. Endogenously expressed LGN, for example, has been found to regulate G protein-dependent GIRK channel function in hippocampal neurons (22), whereas another man-
malian GoLoco protein, Pcp2, is able to modulate receptor regulation of Cav2.1 calcium channels expressed in *Xenopus laevis* oocytes (23).

The current study examines the effects of G18 (AGS4/GP3SM3) on G protein activity in a variety of experimental contexts. G18 is a 160-amino acid protein that is composed of three tandem GoLoco motifs interspersed through its middle and C-terminal area plus an uncharacterized N-terminal segment that is rich in proline (14 of 60 residues). Previous biochemical analyses have shown that at least two GoLoco motifs of G18 can interact with GDP-bound Goαi1 both *in vitro* and in overexpressed cell systems (3, 24). However, the overall physiological function of G18 still remains unknown. In the current study we further examine the interactions between G18 and heterotrimeric G proteins, and moreover, we identify its N terminus as a novel G protein-interacting domain.

**EXPERIMENTAL PROCEDURES**

**RNA Preparation and Reverse Transcriptase PCR**—Tissues from 3-month-old C57BL/6 mice were collected and homogenized. Total RNA 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 frame of G18 were used in PCR reactions to examine the tissue distribution of G18. Furthermore, reverse transcriptase PCR reactions were performed to examine the tissue distribution of G18. The PCR reactions were performed with 100 ng of total RNA and 1.25 units of SuperScript II reverse transcriptase in a total volume of 20 μl of reaction buffer. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

**Preparation of Recombinant Epitope-tagged G18 Fusion Proteins**—GST-tagged G18wt (full-length G18), ΔNG18 (an N-terminal 60-amino acid truncation mutant containing only the three GoLoco motifs), and ΔNG18-mGL (lacking the N-terminal 60-amino acid and containing point mutations at the last amino acid of each GoLoco motif from Arg to Phe) were kindly provided by Dr. David P. Siderovski (The University of North Carolina, Chapel Hill, NC). G18-mGL (containing Arg to Phe mutations at the last amino acid of each GoLoco motif from Arg to Phe) was generated using the site-directed mutagenesis kit (Stratagene). G18AC (the N-terminal domain of G18), which contains only the first 60 amino acids of G18, was generated by inserting a stop codon at the appropriate position. The PCR product was subcloned into the pET-19b or pGEX4T2 vector to generate a GST-tagged or GST-tagged fusion protein, respectively. All other constructs of G18 were further subcloned into the pET-19b vector using primers listed in Table 1. Proteins were expressed and purified as described below.

**Protein Purification**—N-terminal His<sub>10</sub>-tagged G18 and mutants thereof were purified from *Escherichia coli* BL21 (DE3) strain as follows. Six liters of LB medium containing 100 μg/ml ampicillin was inoculated with previously transformed cells and grown with vigorous shaking at 37 °C to an *A*<sub>600</sub> ≥ 0.5. Expression of the proteins was induced by the addition of iso-

### TABLE 1

| Primers used for making His-tagged G18 and its mutants |
|--------------------------------------------------------|
| G18wt Sense primer                                      |
| ΔNG18 Sense primer                                      |
| ΔNG18 Antisense primer                                  |
| G18wt Antisense primer                                  |

Novel Regulatory Function of G18

**Experimental Procedures**

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propyl-β-D-thiogalactopyranoside to a final concentration of 500 μM and incubated for an additional 4 h before harvesting the bacteria by centrifugation. Bacteria were resuspended in 70 ml of buffer A (25 mM Tris (pH 8.0), 500 mM NaCl, 1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 5 mM imidazole) and incubated on ice with 0.2 mg/ml lysozyme for 30 min. After a further 20-min incubation with 25 μg/ml DNase I and 0.5 mM MgCl<sub>2</sub>, 3 ml of a 50% slurry of Ni-NTA affinity resin (Qiagen) pre-equilibrated in buffer A was added, and the mixture was gently rocked at 4 °C for 2 h. The resin was subsequently loaded onto a 30-ml column and washed with buffer B (25 mM Tris (pH 8.0), 500 mM NaCl, 1% Tween 20, 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 20 mM imidazole) followed by buffer C (25 mM Tris (pH 8.0), 500 mM NaCl, 0.2 mM PMSF, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 40 mM imidazole). Proteins were eluted from the Ni-NTA beads by adding 800 μl of buffer D (final concentrations: 25 mM Tris (pH 8.0), 500 mM NaCl, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 250 mM imidazole) after a 30-min incubation at 4 °C. This process was repeated a total of six times. This procedure yielded proteins that were ~60% pure as determined by Coomassie Blue staining. Samples were further purified by fast protein liquid chromatography using a Superdex 75 column (GE Healthcare) to yield proteins that were >90% pure. Peak fractions were pooled and stored in aliquots at −80 °C.

GST-tagged G18 proteins were purified using a previously established glutathione-Sepharose 4B affinity purification method (25). His-tagged Goαi1 and Goαs were grown in enriched medium (2% tryptone, 1% yeast extract, 0.2% glycogen, 0.5% NaCl, 50 mM KH<sub>2</sub>PO<sub>4</sub>), induced with 30 μM isoprropyl-β-D-thiogalactopyranoside, and purified as described previously (26).

Protein-Protein Interaction Assay—Purified His<sub>6</sub>-Goαi1 or His<sub>6</sub>-Goαs (500 nm) was preincubated for 1 h in binding buffer (50 mM Tris (pH 7.5), 0.6 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 2 μg/ml leupeptin, and 20 μg/ml aprotinin) at 30 °C in the presence of either 10 μM GDP or GDP + AMF (10 mM NaF, 10 mM MgCl<sub>2</sub>, 20 μM AlCl<sub>3</sub>). An equimolar amount (500 nm) of GST-tagged G18wt, G18-mGL, ΔNG18, ΔNG18-mGL, or G18AC was added to the Goα mixture and incubated on a rotating platform at 4 °C for 2 h. Glutathione-Sepharose 4B beads or Ni-NTA-agarose beads (20 μl bed volume) were then added into the protein mixture and incubated overnight. The protein mixture was washed three times with binding buffer in the presence of GDP with/without AMF, and the beads were resuspended in 2× Laemmli buffer. Eluted proteins were separated on a 12% SDS gel and transferred to a polyvinylidene fluoride transfer membrane (Pall Corp.) for immunoblotting.
Novel Regulatory Function of G18

immunoblotting—Membranes were incubated with blocking buffer (Tris-buffered saline Tween 20 (TBST) with 5% skim milk) for 1 h and then probed with anti-His or anti-GST antibody (1:1000) (Santa Cruz biotechnology) 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.

**Presteady State GTPase Assay**—Presteady state GTPase activity of purified G proteins was measured as described earlier (26). Purified His₆-Gα₁₁ or His₆-Gα₆ (1 μM) was incubated with [γ-³²P]GTP (1 × 10⁶ cpm/assay) plus 1 μM nonradioactive GTP for 15 min at 30 or 20 °C in GTP binding buffer (50 mM HEPES (pH 7.5), 0.05% Lubrol, 1 mM EDTA, 5 μg/ml bovine serum albumin) and then kept on ice. The GTP binding reaction was stopped by the addition of 0.25 volumes of ice buffer (50 mM HEPES (pH 7.5), 10 mM MgCl₂, 500 μM GTP), and a single round of GTP hydrolysis was initiated by adding 10 mM Mg²⁺ in the presence or absence of 1 μM G18, one of its mutants, RGS4 (300 nM in Fig. 5, 100 nM in Fig. 8), or both RGS4 and G18. Aliquots were taken at the indicated time points and quenched with ice-cold 5% (w/v) Norit in 0.05 M NaH₂PO₄. The level of radioactive ³²P in the supernatant was detected by liquid scintillation counting on a Packard Tri-Carb 2900TR liquid scintillation counter (PerkinElmer Life Sciences).

**Solution-based Steady State GTPase Assay**—Purified His₆-Gα₁₁ (100 nM) or His₆-Gα₆ (100 nM) was incubated for 1 h at 4 °C in binding buffer (20 mM HEPES (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 1 mM DTT, 2 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 0.1% Lubrol, PMSF, 1 μg/ml leupeptin, and 10 μg/ml aprotinin) in the presence or absence of 1–2 μM G18 or its mutants. Binding assays were initiated by adding 0.5 μM [³⁵S]GTPγS (1.25 × 10⁵ cpm/pmol). The incubation continued for 30 min at 30 °C (Gα₁₁) or 60 min at 20 °C (Gα₆). The assay was terminated by adding ice-cold stop buffer (20 mM Tris (pH 8.0), 10 mM MgCl₂, 100 mM NaCl, 0.1% Lubrol, 1 mM GTP, and 0.1 mM DTT). Samples were filtered through nitrocellulose membranes (Millipore) followed by washing four times with 2 ml of ice-cold wash buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 10 mM MgCl₂) and radioactivity was measured using liquid scintillation counting. The nonspecific binding was determined in the presence of 100 μM unlabeled GTPγS, and these values were subtracted to yield specific binding.

**Solution-based Steady State GTPase Assay**—Purified His₆-Gα₁₁ or His₆-Gα₆ (250 nM) was incubated with 3 μM G18 or one of its mutants for 30 min at 4 °C in assay buffer (50 mM Na-Hepes (pH 8.0), 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 5 mM MgCl₂), and a single round of GTP hydrolysis was initiated by adding 10 mM Mg²⁺ in the presence or absence of 1 μM G18, one of its mutants, RGS4 (300 nM in Fig. 5, 100 nM in Fig. 8), or both RGS4 and G18. Aliquots were taken at the indicated time points and quenched with ice-cold 5% (w/v) Norit in 0.05 M NaH₂PO₄. The level of radioactive ³²P in the supernatant was determined by liquid scintillation counting.
Receptor- and Agonist-stimulated GTPase Assay—Sf9 membranes overexpressing M2 muscarinic receptor and heterotrimeric G proteins were prepared as indicated previously (27). These Sf9 cell membranes (8 μg/tube) were assayed for 100 μM carbachol-stimulated GTP hydrolysis at 30 °C for 5 min in the absence or presence of the indicated purified proteins in the reaction buffer (20 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 mM NaCl, 2 mM MgCl₂ (7.5 mM free Mg²⁺), 1 μM GTP, 1 mM ATP, [γ-32P]GTP (1 × 10⁶ cpm per assay), and protease inhibitors) in a total reaction volume of 50 μl. The assay was stopped by adding 950 μl of ice-cold 5% (w/v) Norit in 0.05 M NaH₂PO₄. The reaction mixture was centrifuged, and the level of 32P in the resulting supernatant was determined by liquid-scintillation counting. The nonspecific GTPase activity was defined as that in the presence of the inverse agonist tropicamide (10 μM), and these values were subtracted from the total counts per minute to yield the agonist- and receptor-dependent GTP hydrolysis.

RESULTS

Tissue Distribution of G18 in Mice—G18 is a 160-amino acid protein encoded by a 1472-bp mRNA with 88% similarity between human and mice at the amino acid level (3). To determine the tissue distribution of G18 in mice, total RNA was extracted from different tissues of 3-month-old C57BL/6 mice, and primers specific for the open reading frame of G18 were used to probe for G18. As shown in Fig. 1, full-length G18 was detected at about 500 bp, corresponding well to the open reading frame of 480 bp. We found that G18 was highly expressed in spleen and lung and moderately expressed in heart, kidney, liver, brain, and adipose tissue. These results are consistent with a previous report by Cao et al. (3) using a human RNA blot, thus indicating a similar tissue distribution between human and mouse.

Purified G18 Can Interact with Both Inactive and Fluoroaluminate-activated G Proteins—Previous studies have shown that the GoLoco motifs of G18 have higher binding affinity toward Gαᵢ-GDP compared with Gαᵦ-GDP (3, 24). To extend these findings, we tested the binding between purified G18 and purified Gαᵢ or Gαᵦ in both their inactive GDP-bound and fluoroaluminate-activated states. Three G18 mutants were examined (Fig. 2) that contain an N-terminal truncation (NG18), inactivating point substitutions within each GoLoco motif (G18-mGL), or a combination of both modifications (NG18-mGL). Consistent with previous studies (3, 24), G18 wt and NG18 interacted with Gαᵢ or Gαᵦ in both their inactive GDP-bound and fluoroaluminate-activated states. Three G18 mutants were examined (Fig. 2) that contain an N-terminal truncation (ΔNG18), inactivating point substitutions within each GoLoco motif (G18-mGL), or a combination of both modifications (ΔNG18-mGL). Consistent with previous studies (3, 24), G18 wt and ΔNG18 interacted with Gαᵢ-GDP, whereas G18-mGL and ΔNG18-mGL did not (Fig. 3). None of the purified G18 proteins displayed any detectable binding to the GDP-bound form of Gαᵦ under the conditions employed in our studies (Fig. 3).

To determine whether the observed G18 interactions were specific for inactive G protein, we performed parallel in vitro pulldown
assays in the presence of AlF₄⁻ to mimic the transition state of G protein. Surprisingly, in the presence of AlF₄⁻, both Gαᵢ₁ and Gαₒ interacted with G₁₈wt (Fig. 3). Moreover, removal of the G₁₈ N-terminal domain diminished its binding to G proteins (Fig. 3). These results suggest that whereas the GoLoco motifs of G₁₈ are responsible for its interaction with inactive Gαᵢ₁, the N-terminal segment of G₁₈ may serve to bind the fluoroaluminate-activated Gαᵢ/o.

The N-terminal Domain of G₁₈ Can Interact with Fluoroaluminate-activated Gα Proteins—We also generated and tested an additional truncation mutant of G₁₈ containing only the first 60 residues (G₁₈C) to confirm the binding between the N terminus of G₁₈ and the transition state of G proteins. Indeed, this segment of G₁₈ was sufficient to bind to fluoroaluminate-activated forms of both Gαᵢ₁ and Gαₒ (Fig. 4).

G₁₈ Has No Effect on G Protein GTPase Activity in Presteady State GTPase Assays—We examined the effects of G₁₈wt, ΔNG18, and G₁₈C on the various stages of the G protein guanine nucleotide binding cycle to determine the biochemical significance of the interaction between the G₁₈ N terminus and Gα subunits. Because GTPase-activating proteins (GAP) tend to have high affinity for fluoroaluminate-activated G proteins (28, 29), we first investigated the possibility that the G₁₈ N terminus might have GAP activity toward Gαᵢᵢ or Gαₒ (Fig. 5). RGS4, serving as a positive control, exhibited robust GAP activity on both Gαᵢᵢ and Gαₒ (Fig. 5). These results indicate that G₁₈ does not serve as a GAP toward free Gαᵢᵢ/o subunits.
Novel Regulatory Function of G18

The N-terminal Domain of G18 Acts as a GEF on Goi1—We next assessed whether the N terminus of G18 might have any effects on nucleotide exchange distinct from the established GDI activity of its GoLoco motifs on Goi proteins. Changes in the rate of GDP dissociation from Goi proteins were inferred from changes in the rate of GTPyS binding using a solution-based presteady state assay. As expected, the GoLoco region of G18 (ΔNG18) acted to inhibit GDP dissociation from Goi1, as revealed by an 85% decrease in GTPyS binding to the latter (Fig. 6A). In contrast, the isolated N-terminal segment of G18 (G18ΔC) increased GTPyS binding to Goi1 by ~60%. Interestingly, full-length G18 had essentially no effect on the observed rate of GTPyS binding to Goi1, suggesting that the opposing functions of the two domains balance out under these experimental conditions. These results suggest that G18 can serve as a bifunctional regulator of Goi1, whereby its GoLoco region functions as a GDI and its N-terminal domain acts as a GEF.

We further used a solution-based, steady state GTPase assay to corroborate the putative GEF activity of the G18 N-terminal region on Goi1. Interestingly, full-length G18 significantly promoted GTP turnover (Fig. 7A). In contrast, the N-terminal deletion mutant ΔNG18 decreased GTPase activity (Fig. 7A), which is consistent with its GDI activity. In agreement with the results obtained from presteady state GTPyS binding assays (Fig. 6A), there was a trend toward an increase with G18ΔC (Fig. 7A), and this reached statistical significance when the concentration was raised to 10 μM (data not shown). These results suggest that under steady state conditions with free Goi1, the GEF activity of the N-terminal domain of G18 predominates over the GDI function of its GoLoco region.

The N-terminal Domain of G18 Acts as a GDI on Goi—The effects of G18 and its mutants on Goi activity were also examined. Surprisingly, G18wt inhibited nucleotide exchange on free Goi by ~25% (Fig. 6B). The GoLoco region of G18 (ΔNG18) had no effect on GDP dissociation from Goi, which is consistent with its poor binding to Goi-GDP. In contrast, G18ΔC inhibited GTPyS binding to Goi to the same level as G18wt. These results indicate that G18 has a modest albeit statistically significant GDI effect on Goi, which appears to be primarily attributable to its N-terminal domain.

Furthermore, in the solution-based steady state GTPase assay, both G18wt and G18ΔC decreased the GTP hydrolysis of free Goi, consistent with the observed GDI effects of the full-length protein and the isolated N-terminal domain (Fig. 7B). ΔNG18 also inhibited the GTPase activity of Goi, under these conditions. The reason for the apparent discrepancy regarding the effects of ΔNG18 in Fig. 6B versus Fig. 7B is not clear. Overall our results suggest that the function of the N-terminal domain of G18 may depend on which G protein is involved, i.e. promoting nucleotide exchange at Goi1 but inhibiting at Goi0.

Effects of G18 on Receptor- and Agonist-stimulated G Protein GTPase Activity—The foregoing observations indicate that the activity of G18 is not limited to its GoLoco motifs, as its N-terminal domain also modulates G protein-nucleotide interactions. In addition, these results clearly identify Goi with G18 as a novel interacting partner of G18. However, little is known regarding the activity of G18 (and GoLoco motif-containing proteins in general) within the context of receptor-stimulated G protein function. Therefore, we used a receptor- and agonist-dependent steady state GTPase assay to study the effects of G18 on GTP turnover by overexpressed Goi1 or Goi0 in membranes from Sf9 cells also co-expressing exogenous M2 muscarinic receptor and Gβγ subunits.

The addition of G18wt to carbachol-activated M2+Goi1 or M2+Goi0 membranes yielded little or no change in agonist-dependent GTPase activity (Fig. 8, A and B), notwithstanding its demonstrated effects in solution-based assays. Mutant forms of G18 similarly lacked activity under these conditions (data not shown). This apparent lack of effect could reflect a masking of changes in nucleotide exchange rates by the relatively slow intrinsic hydrolytic activities of Goi and Goi0 in the presence of the receptor. To ensure that GTP hydrolysis per se was not rate-limiting, cyclical GTP turnover was also measured in the presence of purified RGS4, which accelerates the hydrolytic step (Fig. 5). Indeed, the inclusion of RGS4 in these assays revealed effects of G18wt on both Goi1 and Goi0, which were inhibited, respectively, by ~60 and 80% at the maximally obtainable concentration of G18wt (Fig. 8, A and B). Another conceivable explanation is that this observation may reflect an effect of G18 on RGS4 activity. We used a presteady state GTPase assay to test this possibility and found that G18 had little or no effect on the GAP activity of RGS4 on either Goi1 or Goi0 (Fig. 8, C and D).
To determine which regions of G18 might contribute to its effects on receptor-stimulated GTP turnover by G_{i1} and G_{o}, mutants bearing truncations and/or inactivating GoLoco point substitutions were also evaluated. Compared with full-length G18wt, N-terminal-truncated G18 (ΔN-G18) produced a similar inhibitory effect on receptor-activated G_{i1} (Fig. 9A) but a greatly reduced effect on G_{o} (Fig. 9B). In contrast, mutation of the GoLoco motifs (G18-mGL) substantially reduced activity on G_{i1} (Fig. 9C) but caused only a minor change in the inhibitory effect of G18 on G_{o} GTPase activity (Fig. 9D). Despite the evident GEF effect of G18ΔC on isolated G_{o} in solution (Fig. 6A), such activity was not observed in membranes in the presence of agonist-activated receptor plus G_{o} (Fig. 9E), suggesting that the GEF activity of the receptor may exceed that of the N-terminal domain of G18. G18AC instead produced a marginal inhibitory effect on receptor-activated G_{i1} (Fig. 9E) and a more pronounced inhibitory effect in corresponding experiments with G_{o} (Fig. 9F). The latter observation reinforces the notion that G18ΔC has the potential to act as a GDI toward G_{o}. Overall, the inhibitory effect of full-length G18 on M2+G_{i1} GTPase activity is attributable primarily to its GoLoco motifs, whereas the effect on M2+G_{o} seems to derive mostly from its N-terminal domain.

**DISCUSSION**

G18 was first identified within the major histocompatibility complex class III region on chromosome 6 and, thus, may be unique. A comparable enigma exists with the R12 subfamily of RGS proteins, most of which contain a GoLoco motif that can produce GDI effects on G_{o} and also an RGS domain that accelerates GTPase activity (7).

The most widely recognized GEF effects on heterotrimeric G proteins are those produced by agonist-activated GPCRs, but beyond this classical paradigm a variety of non-receptor GEFs has also been identified including Ric-8A (32, 33), Ric-8B (34), CSPα (35), GIV (36), AGS1/Dexras1 (37), GAP-43/neuromodulin/B-50 (38), and the yeast protein Arr4 (39). The primary amino acid composition of the N-terminal domain of G18 does not resemble any of the previously identified GEFs; however, there are structural attributes of G18 that could conceivably contribute to interactions with G proteins. For example, the N-terminal segment of G18 is fairly widely distributed. Little is known about the biological function of G18, and a clear understanding of this is difficult without accurate knowledge of its biochemical behavior. The most significant finding described herein is the identification of the N-terminal region of G18 as a novel binding partner of G_{o} proteins. Surprisingly, this domain acts as a GEF on G_{o}, but as a weak GDI on G_{i1}. To our knowledge, this is the first example of a single domain that has distinct regulatory effects toward different Gα proteins. Another unusual property of G18 is that it contains multiple G protein binding domains that produce dissimilar effects on the activity of a common target, and the ability of G18 both to promote and impede GDP dissociation from G_{o} respectively, via its N-terminal and GoLoco regions appears to be involved in the control of host immune defense and inflammatory responses (30, 31). Such a role is further suggested by its relatively high expression levels in the spleen (Fig. 2) and other immune tissues (3), although overall it appears to be fairly widely distributed. Little is known about the biological function of G18, and a clear understanding of this is difficult without accurate knowledge of its biochemical behavior.
partner for heterotrimeric G proteins. The mechanism by which G18 confers GEF activity on free G$_{i1}$ requires further study, but the presence of multiple arginine residues, particularly those at positions 31, 34, and 46 (which would line up in a PPII helix), could conceivably provide the cationic interface needed to promote nucleotide exchange (46).

Consistent with the present results, previous studies have shown the GoLoco region of G18 to selectively bind to and impede GDP dissociation from inactive G$_{i1}$ (3, 24). However, the GoLoco motifs in proteins such as Pcp2 and Rap1GAP appear not to be selective between G$_{i1}$ and G$_{o}$ (10, 47, 48). Also it is not obvious that the potential effects of activating agents have necessarily been tested in all studies on GoLoco-G interactions. The present results indicate that the binding of the GoLoco region of G18 to G$_{o}$ can be induced, albeit modestly, by AlF$_4$ (Fig. 3, fourth lane). Nothing analogous to this observation could be found in the literature; however, the Drosophila GoLoco protein Pins has been shown to bind to both active and inactive G$_{o}$ (in this case Drosophila G$_{o}$ purified from bacteria) and to regulate G$_{o}$-dependent GPCR signaling (49).

All of the G protein binding functions of G18 appear to be sensitive to the activation states of their G targets (Fig. 3). N-terminal domain binding seems to be selective for the transition state of both G$_{i1}$ and G$_{o}$. These interactions appear to be of primary importance for the binding of full-length G18wt in the presence of AlF$_4$ as G protein binding was greatly reduced or eliminated in the absence of the N-terminal domain. However, both the GEF (on G$_{i1}$) and GDI (on G$_{o}$) functions of the N-terminal domain of G18 must ultimately be viewed within the context of the entire protein, including its three

![Figure 9](https://example.com/fig9.png)
Novel Regulatory Function of G18

GoLoco motifs. Important considerations include 1) which, if any, domain has a predominant effect on a particular G protein either as it signals at the plasma membrane or performs other functions in the cell interior, 2) whether G protein binding is mutually exclusive or can occur simultaneously to both the N-terminal domain and one of the GoLoco motifs, and 3) whether an individual G protein can bind to different G18 domains at different points within its GTPase cycle.

The effect of G18 on a G protein may depend on its cellular localization and/or other binding partners. We observed that the N-terminal GEF effect negates (Fig. 6A) or overrides (Fig. 7A) the GoLoco GDI effects on Goi1, whereas the ability of G18 to inhibit receptor-stimulated G11 activity is unaffected by the removal of the N-terminal domain (Fig. 9A). This suggests that perhaps free intracellular Goi1 would be activated by the N-terminal GEF function of G18, whereas the GoLoco motifs would inhibit receptor-dependent Goi1 activation at the plasma membrane.

Together, the four G protein binding functions within G18 have the potential to produce complex effects on G protein activity. It is unclear whether the N-terminal and GoLoco domains might either impede or facilitate the other’s binding to Ga or whether the different domains can act sequentially as Ga goes through its GTP cycle. If they act independently, then multiple G proteins could be affected at the same time. Kimple et al. (24) have shown that the first and third GoLoco motifs within N-terminal-truncated G18 can simultaneously bind individual G proteins and, thus, function as independent GDIs, although this could potentially differ in the presence of other G protein regulators. The idea that the N-terminal domain might be able to access GoLoco-associated Ga is suggested by evidence that Ric-8A can exert its GEF activity on Ga while the latter is coupled to the GoLoco region of AGS3 (33). Analogously, GPCRs and Gβγ must act in concert for agonist-stimulated nucleotide exchange to occur (50–52). Although it is an intriguing possibility, the present results do not directly speak to whether the N-terminal and GoLoco domains of G18 might bind simultaneously to either Goi1 or Ga (or alternatively inhibit one another), and thus, further studies will be required to address this issue.

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