Molecular architecture of Ga\textsubscript{o} and the structural basis for RGS16-mediated deactivation

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Heterotrimeric G proteins relay extracellular cues from heptahelical transmembrane receptors to downstream effector molecules. Composed of an α subunit with intrinsic GTPase activity and a βγ heterodimer, the trimeric complex dissociates upon receptor-mediated nucleotide exchange on the α subunit, enabling each component to engage downstream effector targets for either activation or inhibition as dictated in a particular pathway. To mitigate excessive effector engagement and comitant signal transmission, the Go subunit’s intrinsic activation timer (the rate of GTP hydrolysis) is regulated spatially and temporally by a class of GTPase accelerating proteins (GAPs) known as the regulator of G protein signaling (RGS) family. The array of G protein-coupled receptors, Ga subunits, RGS proteins and downstream effectors in mammalian systems is vast. Understanding the molecular determinants of specificity is critical for a comprehensive mapping of the G protein system. Here, we present the 2.9 Å crystal structure of the enigmatic, neuronal G protein Go\textsubscript{a}, in the GTP hydrolytic transition state, complexed with RGS16. Comparison with the 1.89 Å structure of apo-RGS16, also presented here, reveals plasticity upon Go\textsubscript{a} binding, the determinants for GAP activity, and the structurally unique features of Go\textsubscript{a} that likely distinguish it physiologically from other members of the larger Ga family, affording insight to receptor, GAP and effector specificity.

Many extracellular cues ranging from photons to neurotransmitters are detected with high specificity by G protein-coupled receptors that in turn elicit an intracellular response by promoting GTP exchange on the α subunit of a heterotrimeric G protein. The heterotrimeric G protein, composed of an α subunit exhibiting endogenous GTPase activity and a heterodimeric βγ subunit, dissociates, enabling each component to activate downstream effectors until GTP is hydrolyzed on the α subunit and the heterotrimeric complex reforms. The α subunit’s endogenous GTP hydrolysis rate is relatively slow, therefore the cell uses GTPase accelerating proteins (GAPs) to increase the rate to suit the time scale and magnitude needed for a specific physiological response.

The regulators of G protein signaling (RGS) proteins are a class of heterotrimeric G protein GAP first identified in Saccharomyces cerevisiae (Sst2) and Caenorhabditis elegans (Egl10) (1, 2). Studies, both biochemical and structural, have shown an overall preference for RGS domains to bind Go subunits in their transition state (mimicked by the analog GDP·AlF\textsubscript{4}\textsuperscript{−}) and to accelerate GTPase activity by stabilizing the transition state of hydrolysis, thereby optimizing the endogenous GTPase activity of the Go subunit without directly contributing to the hydrolytic mechanism (3–5). RGS proteins serve to quench the G protein signal temporally and spatially, either independently, or coupled (in cis or in trans) to an effector (6, 7). Thirty-seven RGS proteins have been identified in the human genome, cataloged into eight subfamilies based on the protein family the RGS domain resides delineated as RGS subfamilies R4, R7, R12, RZ, and RhoGEF RGS (rgRGS); G protein-coupled receptor kinases (GRKs); sorting nexins; and Axin (8–10). Each member displays a unique expression and localization pattern (11). With the number of RGS proteins greatly exceeding the number of Ga subunits, RGS proteins are likely to be finely tuned, titrated, and localized to regulate specific signaling pathways within a Ga subunit’s repertoire of effector targets. To mediate the specificity and fidelity requisite for accurate signal transmission, the Go subunit and its binding partners (GPCR, Gβγ, RGS, and effector) must have specific reciprocating molecular determinants to minimize the convergence of independent signaling pathways. Thus, a comprehensive understanding of the molecular basis for Ga engagement with its activators, regulators, and effectors is critical for elucidating specificity determinants. RGS subfamilies pair with distinct cognate Ga substrates via unique stereochemical binding determinants. The RGS subfamilies R4, R7, and R12 engage G\textsubscript{a16} (3, 12, 13); the R4 subfamily also engages G\textsubscript{a15} (14); the rgRGS subfamily engages G\textsubscript{a12,13} (15); and the RZ subfamily engages G\textsubscript{a} and G\textsubscript{α12} (3, 16, 17). It is noted that Go–RGS interaction modes observed in R4, R7 and R12 (5, 7) [see accompanying article by Soundararajan et al. (18)] contrast with the unique interlocking geometry observed between p115RhoGEF rgRGS and the G\textsubscript{a334} chimera (19) and the effector-like binding mode observed between GRK2 and G\textsubscript{α16} (20).

G\textsubscript{α} is a member of the Ga family, which includes G\textsubscript{a12,3} and G\textsubscript{α16}. Although G\textsubscript{α16} is the most abundant Ga subunit in the human brain, little is known about the pathways it is involved in, in stark contrast to our understanding of G\textsubscript{a12,3} and G\textsubscript{α16}. Much of the work on G\textsubscript{α16} to date has implicated a role for its cognate Gβγ subunit in the activation of Ca\textsuperscript{2+} channels (21). Additional evidence points to G\textsubscript{α16} involvement in signaling from A1-adenosine receptors (22), dopamine D2 receptors (D2R) (23) and µ-opioid signaling (24). A G\textsubscript{α16} effector molecule has been reported, GRIN1, which promotes growth cone neurite extension in the mammalian brain (25). The implicated role of G\textsubscript{α16} as a mediator and regulator of core neurological and cognitive GPCR-coupled pathways positions G\textsubscript{α16} as a prime target for pharmaceutical intervention. A molecular understanding of downstream signaling components is a key step toward identifying potential therapeutic points of drug intervention used
alone or in combination with GPCR-targeted drugs to minimize side effects (26).

Here, we report the first crystal structure determination of \(G_{\text{G}}\), presented in the transition state of hydrolysis, complexed with the R4 subfamily RGS protein RGS16 (27). \(G_{\text{o}}\) displays a number of unique determinants that likely affords it specificity in receptor, RGS, and effector engagement. We discuss the conserved nature of the RGS GAP mechanism, structural aspects that confer specificity, and the plasticity of the RGS domain required to engage and maintain binding during the transformation of \(G_{\text{t}}\) from the GTP-bound state to the transition state by comparing the \(G_{\text{t}}\)-GDP-AlF\(_4\) complex to our independently determined structure of apo-RGS16, also presented here. The \(G_{\text{o}}\)-RGS interactions noted in our determination of \(G_{\text{o}}\), complexed to RGS16 dovetails with specificity findings presented in an accompanying article by Soundararajan et al. (18), facilitating the first examination of an RGS protein complexed to different \(G_{\text{o}}\) subunits.

Results and Discussion

Architecture of the \(G_{\text{o}}\)-RGS16 Complex. Mouse \(G_{\text{o}}\) was complexed with mouse RGS16 and purified over gel filtration in the presence of GDP and AlF\(_4^-\) to simulate the transition state of GTP hydrolysis. The complex was crystallized in the space group P3\(_2\)1 with two protomers in the asymmetric unit. The structure was determined by molecular replacement, using coordinates from the \(G_{\text{t}}\)-GDP-AlF\(_4^-\)-RGS4 structure (5) and refined to a resolution of 2.9 Å. Crystallographic, phasing, and refinement statistics are presented in supporting information (SI) Table S1. The architecture of the \(G_{\text{o}}\)-GDP-AlF\(_4^-\)-RGS16 complex resembles those observed in \(G_{\text{t}}\)-GDP-AlF\(_4^-\)-RGS4 and \(G_{\text{f1}}\)-GDP-AlF\(_4^-\)-RGS9 structures (Fig. 1) (5, 7). The RGS domain is positioned almost exclusively on the \(G_{\text{o}}\) switches regions I and II. This is the first structural determination of the \(G_{\text{o}}\) subunit; thus, it cannot be compared with previously solved states of \(G_{\text{o}}\), but, when compared with the solved structures of \(G_{\text{t}}\) and \(G_{\text{f1}}\), the \(G_{\text{o}}\) switch conformations most closely resembles that observed in the transition state structures: \(G_{\text{t}}\)-GDP-AlF\(_4^-\) and \(G_{\text{f1}}\)-GDP-AlF\(_4^-\) (28, 29). \(G_{\text{o}}\) retains the overall domain architecture observed in previously solved \(G_{\text{o}}\) structures delineated by a Ras-like GTPase domain encompassing three nucleotide-dependent switch regions and a helical domain, inserted within the Ras-like domain and tethered by two linker regions (28, 30, 31). RGS16, like RGS4 and RGS9, is delineated by nine helices, \(\alpha1\)-\(\alpha9\), segregated into two subdomains: one formed by helices \(\alpha1\), \(\alpha2\), \(\alpha3\), \(\alpha8\), and \(\alpha9\) and the other by helices \(\alpha4\), \(\alpha5\), \(\alpha6\), and \(\alpha7\) arranged in an antiparallel four-helix bundle (5, 7). The two subdomains are united by a conserved hydrophobic interface that constitutes the structure’s core. RGS16 is a conserved member of the R4 RGS subfamily and retains central conserved elements that define RGS domains except for one component, Thr-158, which replaces the conserved isoleucine or valine found in the equivalent position across all other RGS members (Fig. S1). Thr-158 is buried in the hydrophobic core between the two subdomains. It enhances stabilization through a hydrogen bond to the residue 154 backbone carbonyl and engages the side chain of Phe-93 in a unique geometry independently confirmed by Soundararajan et al. (18).

The RGS16 GAP Mechanism. The RGS16 RGS domain engages the \(G_{\text{o}}\) switch regions via a conserved interface that buttresses their transition state conformation. The interaction is structurally similar to that observed in \(G_{\text{t}}\)-RGS4 and \(G_{\text{f1}}\)-RGS9 structures (5, 7). Although the total group of interactions between the RGS domain and the \(G_{\text{o}}\) switch regions is not identical between these pairs, key conserved RGS residues are used in identical interactions (Fig. 2A). Three critical RGS16 residues are involved near the \(G_{\text{o}}\) active site. RGS16 Asn-90 forms a hydrogen bond to the hydroxyl group of the \(G_{\text{o}}\)-Thr-183 side chain. In the \(G_{\text{o}}\)-GDP-AlF\(_4^-\) structure, this switch I threonine is rotated ~60° from switch II (29). The orientation observed in the \(G_{\text{o}}\)-RGS16 structure allows the Thr-183 side chain to contact switch II residues Lys-211 and Glu-208. This clamp switches I and II together, further stabilizing the transition state conformation from that observed in the \(G_{\text{o}}\)-GDP-AlF\(_4^-\) structure. A second RGS16 residue, Asp-165, positioned next to switch I, forms a hydrogen bond to the Thr-183 peptide amide. This interaction orients the \(G_{\text{o}}\)Thr-182 backbone carbonyl into the ideal geometry for hydrogen bonding to the nucleophilic water. A third RGS16 residue, Asn-130, is inserted between \(G_{\text{o}}\) residues Lys-181 and Glu-208. Interactions with these two \(G_{\text{o}}\) side chains dictate the orientation of the Asn-130 side chain. In this conformation, the Asn-130 side chain amide forms a hydrogen bond with the \(G_{\text{o}}\) Gln-205 side chain carbonyl. This resolves the torsional ambiguity of the glutamine side chain, orienting it for both stabilization of the nucleophilic water and the planar intermediate. Through these means, RGS16, upon binding \(G_{\text{o}}\) in the activated GTP-bound state, reorients \(G_{\text{o}}\) switches and the residues involved in GTP hydrolysis from their GTP-bound state into their transition state conformation.

Posttranslation modifications have been implicated in increased RGS16 GAP activity. One reported modification is palmitoylation of Cys-98 (Cys-97 in mouse RGS16) (32). Cys-97...
**Go–RGS Specificity Determinants.** Interactions between the RGS domains and Go subunits determined to date are centered primarily on Go switch regions I and II. Residues in switches I and II that contribute to RGS binding are nearly invariant across Go subunits and do not vary at all across Go members Goα12, Goα13, Goα3, and Goα (Fig. S2). Outside the Goα family, RGS specificity is likely to be modulated by the substitution of a lysine at position 181 (relative to Goα) with either a proline or alanine as found in Goαq and Goα12/13 family members or a leucine as found in the RGS-GAP incompetent family Goαr. RGS–Go interactions, however, are not limited exclusively to the nucleotide-dependent switch regions, but may also involve electrostatic interactions with components of the helical domain (Fig. 2B and Fig. S2) (7). A higher degree of variation, both within the composition of the Go helical domain and the RGS residues that contact it, provides a platform for specificity proximal to, but independent of, the nucleotide-dependent switch regions.

Goα–RGS complex structures determined to date have all used different Go subunits and different RGS members. In an accompanying article by Soundararajan et al. (18), the human cognate pair Goαt/RGS16 is presented, enabling us to directly compare the interactions between Goα family members (Goαi1 and Goαi3) bound to the same RGS member. Analysis of contacts between RGS16 and Goi members shows that a core set of interactions involving 11 pairs of residues is constant for both Goαi1 and Goαi3 (Fig. 2B). A number of Goαi1–RGS16 pairs are unique, including interactions formed between Arg-236–Asp-137 and Arg-90–Glu-135. In the latter pair, an arginine, specific to Goαi1 and Goαi3, spans the interaction distance that could not be attained by the equivalent Goαo residue, Thr-90. Unique Goαi1–RGS16 interactions include an interaction with the GTPase domain Thr-182–Asp-165; interactions with the helical domain including αA residues Asp-65–Lys-172, Ser-75–Lys-164, and Arg-86–Glu-134; and an interaction between RGS16 Lys-164 and Thr-117 on the Goαo αB–αC loop. In addition, residues are used in van der Waals contacts between Goα and RGS16, including Goαo His-236 (an alanine or valine residue in Goαi family counterparts) that contacts the side chain of RGS16 Asp-132 forming a 4 Å electrostatic interaction between its εN group and the Asp-132 δ carbonyl. As discussed below, prime differences between Goαo and Goαi3 reside in the helical domain, specifically in terms of side chain diversity and the organization of the αB–αC region. The differential set of interactions between RGS16 and Goαo vs. Goαi3 is reflected in the total area of solvent accessible surface buried upon complex formation. Analysis of the two Goαi1/RGS16 structure protomers shows each complex burying 738 Å² and 802 Å², respectively. In contrast, Goαo–RGS16 structure protomers bury 897 Å² and 873 Å², respectively, an average of 115 Å² more than those observed in the Goαi1/RGS16 structures, the majority of the differential due to RGS domain–Goαo helical domain contacts. The Goαo helical domain’s role in conferring specificity is likely to be one of a number of factors involved. Although binding determinants between the helical domain and the RGS domain α7 and α8 helices have been observed in Goαi1/RGS4 (5), Goαi1–RGS9 (7), and the Goαi2/RGS8 and Goαi3–RGS10 structures (see ref. 18), no contacts were observed between the Goα helical domain and the RGS domain in the Goαi1–RGS1 and Goαi3–RGS16 structures.

**Conformational Changes in RGS16.** The independently determined structure of RGS16 alone and in complex with Goαo allows analysis of any conformational changes that occur upon binding the Goα subunit. A least-squares fitting of the two RGS16 structures over the RGS domain shows little variation in the backbone of the segments that contact Goα (Fig. 3). Minor movement is noted along the backbone at the α7–α8 linker and N-terminal to α6. Examination of the RGS16 backbone outside
the binding region shows a minor reorientation of the N- and C-terminal subdomain formed by α1, α2, α3, α8, and α9. In the apo state, this region is pivoted away from the Gαo binding face. This is primarily due to movements in α1, α2, and α9. The N- and C-terminal subdomain is more highly ordered in the apo state with electron density evident for more residues at the N and C termini, potentially indicative of subdomain plasticity when complexed with Gαo. RGS16 side chains involved in Gαo switch stabilization undergo a variety of conformational changes. Residues along α3 and α4, including Glu-85, Phe-86, Glu-89, and Asn-90 undergo modest changes in orientation. In contrast, significant conformational changes are noted for Glu-125 and Asp-165 along the α6 helix, including His-133 and Glu-134 and along the α1 helix, where the Leu-161 side chain rotates toward the binding interface interior, and Asp-165 and Arg-169, linked in a stabilized triad with Glu-85 via the Arg-169 δ-guanido group. This triad undergoes a planar shift of their hydrogen-bonding network facilitated by a rearrangement of Arg-169 into an alternative rotamer via rotations along the Cα–Cβ and Cδ–Ne bonds. The orientation of this triad mediates the critical interaction between RGS16 Asp-165 and the switch I Thr-183 backbone amide, which in turn positions the Thr-182 backbone carbonyl into optimal geometry for transition state stabilization of the attacking water. The plasticity observed in the RGS16 RGS domain between apo and Gαo-complexed states has also been observed in RGS9 and
the independently determined structures of RGS16 in the companion article by Soundararajan et al. (18). Domain and side chain rearrangements are a key mechanistic feature of the RGS domain. Although the RGS domain has the highest affinity for Go in the transition state, it must be able to recognize and bind the activated GTP-bound subunit and engage it as it reconfigures the complex into the transition state. Subsequently, after releasing Go-GDP, it must reconfigure, priming itself for subsequent engagement of another activated Go subunit.

**Structural Features of Goα.** Goα is a member of the Go family and is most closely related to Goi1 (34, 35). A comparison of identical vs. nonidentical residues between Goα and the Go family shows that the majority of differences occur in the helical domain, primarily in the αb helix and the αb–c loop (Fig. 4C and Fig. S2). Although no evidence exists that the helical domain serves any function in regard to effector coupling, the differences between Goα and Goi1 raise that possibility. A least-squares fitting of the two molecules reveals a large difference in the αb–αc region. The difference relative to Goi1 and Goα is primarily the result of a proline insertion N-terminal to αC, displacing the αb–αc region ~6 Å from its comparable position in Goi1 and Goα (Fig. 4A and B). Temperature factors for this region of Goα are slightly elevated above the average main chain temperature factor, likely indicative of mobility within this region. The αb–αc region is a central point of divergence among all Goα subunits, particularly for the Goi12/13 family caused by insertions in the αb–αc loop (Fig. 1B). The structures of chimeric Goi12 and Goi13 with intact Goi12 and Goi13 helical domains reveal a loop for Goi12 and an additional helix for Goi13 that extends from αb and αc toward the switch regions of the GTPase domain (Fig. 4B) (36). It is likely that diversity within the αb–αc region is a critical element for GEF, effector, and GAP specificity given its proximity to the nucleotide-dependent switch regions. In strong support for this observation is the finding that GoLoco guanine nucleotide dissociation inhibitor (GDI) motifs target Goα subunits with specificity determinants mediated by the Go helical domain and the GoLoco C-terminal region. GoLoco motifs from RGS12, RGS14, and AGS3 exert GDI activity on Goi1–3, but not Goα (37–39). The helical domain’s role as a determinant in GDI GoLoco specificity was confirmed through analysis of a Goi1–Goα chimera in which the helical domain of Goi1 was replaced with the Goα helical domain, thus preventing GoLoco-mediated GDI activity on the Goi1 GTPase domain (39).

To delineate residues that functionally distinguish Goi1 from other Goi family members, we mapped residues that are identical across Goi1 and Goα but are not identical in Goα and a second tier of residues that are identical across Goi1,3, but are not identical in Goα (Figs. 1B and 4C). The majority of residues unique to Goi1 under these criteria map to the helical domain along the αb–αc region. Additional unique residues reside on the opposite face of the Go subunit, although most also diverge from Goi1,3 in Goα. Although the nucleotide switch regions are highly conserved, a number of unique residues occur in Goα, including Asp-218 in switch II and Glu-233, His-236, Thr-250, and Thr-251 in switch III. The threonine residues on switch III reside proximal to RGS16, implicating them as key nucleotide-dependent determinants for effector–GAP coupling. His-236 of switch III (alanine or valine in Goα family counterparts) engages RGS16 as mentioned above, contributing van der Waals contacts and electrostatic interactions with RGS16 Asp-132. Gln-233 is buried between the GTPase domain and the helical domain. Asp-218 of switch II (a glycine in Goi family counterparts) is solvent exposed, and, although distal to the RGS domain (14 Å separation), it may also contribute to nucleotide-dependent effector–GAP coupling determinants.

**Conclusion**

The heterotrimeric G protein α subunit is a molecular switch efficiently designed to transmit signaling information from upstream receptors to downstream effectors with high fidelity. G protein α subunits must orchestrate a unique signal transduction pathway amidst hundreds of G protein-coupled receptors and scores of downstream effectors. Functionally, this requires the encoding of higher level specificity. The requirements placed on the Go subunit include time-dependent activation (endogenous GTPase activity), conformational change to regulate binding partners (nucleotide-dependent switch regions), and binding specificity (unique structural determinants). To regulate a subunit activation temporally and spatially, GAP proteins, including members of the RGS family, are used to bind and enhance the α subunit’s endogenous GTPase activity. Although RGS proteins quench G protein activity *in vitro*, it is critical at the cellular level to ensure signal transmission to downstream effectors before inactivation. This paradigm is best illustrated in the visual system where effector–GAP coupling between phosphodiesterase γ and RGS9-GB3 ensures efficient, temporally regulated Goα-mediated signal transmission. To extend this paradigm to other Go subunits, specific effector binding determinants provide great insight into the neuronal mechanisms the pair govern.

**Materials and Methods: Protein Expression and Purification**

Details regarding the expression and purification of H6RGS1653-180,Goα, complex formation, crystallization, data collection, structure determination, and refinement are in SI Materials and Methods.

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