Designed mini-Gα proteins capable of molecular switch functionalities

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ABSTRACT (UP TO 150 WORDS)

Heterotrimeric (αβγ) G proteins regulate intracellular signaling as molecular switches governed by bound guanine nucleotides. This functionally requires enzymatic GTPase activity and interactions with multiple regulator or downstream "effectors". Truncated "mini-Gα" proteins have been previously engineered and used to bind GPCRs, mostly in structural studies. Here, we design novel mini-Gα proteins with molecular switch functionalities in terms of regulation and partner interactions. These constructs are able to bind and hydrolyze guanine nucleotides *in vitro*, and, notably, be inactivated by RGS proteins with high efficiency. In cells, these mini-Gα proteins can also bind Gβγ and interact with downstream effectors. Such functional mini-G's can be used to investigate G protein function and interactions and can be utilized in synthetic biology efforts.

INTRODUCTION

Heterotrimeric G proteins (αβγ) are molecular switches that regulate numerous intracellular signaling cascades in response to G protein-coupled receptors (GPCRs) {Sprang, 1997 #1;Oldham, 2008 #12}, interacting with downstream effectors that regulate diverse physiological systems {Sprang, 2007 #2;Hurst, 2009 #13;Kimple, 2011 #14}. Within the heterotrimer, the Gα subunit is the actual molecular switch whose functionality depends on binding and hydrolysis of guanine nucleotides. In the inactive conformation, the Gα subunit is bound to GDP and associates with the Gβγ dimer, which prevents Gα interactions with downstream effectors. The Gα switch is activated by the exchange of the bound GDP with GTP, leading to dissociation of the GTP-bound Gα from Gβγ, with either moiety activating downstream partners {Gilman, 1987 #74;Sprang, 1997 #1;Oldham, 2008 #12}. Gα subunits are "turned off" by GTP hydrolysis (GTPase) catalyzed by the Gα subunit itself, yielding the inactive form of Gα-GDP {Sprang, 1997 #1}. The intrinsically slow Gα GTPase activity can be accelerated by Regulator of G protein Signaling (RGS) proteins that determine the actual duration of G protein mediated signaling {Berman, 1996 #17;Hunt, 1996 #18;Watson, 1996 #19;Ross, 2000 #73}. RGS proteins bind activated Gα-GTP, accelerating GTP hydrolysis allosterically, thereby acting as GTPase activating proteins (GAPs).

Engineered "mini-Gα" proteins have been developed, originally for structural studies, by truncating > 40% of the entire Gα subunit and mutating several amino acids to increase stability {Carpenter, 2016 #20;Carpenter, 2016 #21;Nehme, 2017 #22}. These mini-Gα proteins contain only the central Gα "GTPase domain", following deletions of the Gα N-terminus, the entire Gα "helical domain", and additional segments within the GTPase domain. Additionally, 6-8 point mutations were introduced into the GTPase domain to stabilize flexible regions, improve thermostability, and increase affinity to GPCRs. These mini-Gα proteins were optimized for GPCR-binding, so we term them here "GPCR-binding mini-Gα" (GB-mini-Gα) proteins. Over the last decade GB-mini-Gα proteins have been used to solve experimentally dozens of GPCR complexes with GB-mini-Gα proteins, mostly using GB-mini-$G\alpha_s$ and GB-mini-$G\alpha_o$ {Nehme, 2017 #22;Garcia-Nafria, 2018 #58;Byron, 2018 #40;Tsai, 2018 #42;Horing, 2021;#60;Lin, 2023 #43}. Notably, most of the GB-mini-Gα–GPCR complexes that have been solved include βγ subunits, and are nucleotide-free. GB-mini-Gα proteins have also been used as biosensors that selectively bind active GPCRs in living cells {Byron, 2018 #40;Smrcka, 2019 #81}. Previous

studies have shown that fusion of these proteins to fluorescent or luminescent tags, combined with bioluminescence resonance energy transfer (BRET) assays or NanoLuc Binary Technology (NanoBiT), enables measurement of receptor activation and monitoring of subcellularly localized signaling events {Byron, 2018 #40;Wan, 2018 #27;Benkel, 2022 #79;Manchanda, 2024 #59;Harwood, 2025 #80}. All of these previous studies focus on the ability of mini Gα proteins to strongly bind activated GPCRs, however, they do not demonstrate functional downstream interactions. Moreover, previous studies suggested that the GB-mini-Gα proteins are unable to facilitate nucleotide exchange {Carpenter, 2016 #21;Smrcka, 2019 #81}. To our knowledge, GB–mini-Gα proteins have not been tested for their ability to bind and hydrolyze nucleotides or their ability to be regulated by RGS proteins.

Previous structural studies reveal that the functionality of GTP binding and hydrolysis by Gα subunits is mostly encoded within the GTPase domain of Gα subunits, which is common across all members of the G protein superfamily {Sprang, 1997 #1;Sprang, 2007 #2}. On the other hand, the helical domain plays a role in enabling nucleotide exchange and contributes only to interactions with a limited number of effectors and RGS proteins {Sprang, 1997 #44;Rasmussen, 2011 #7;Navot, 2019 #82;Asli, 2021 #57}. The Gα GTPase domain includes three flexible regions designated switch I, II, and III, which "switch" conformation depending on whether GTP or GDP is bound in the active site {Sprang, 1997 #1}. Nucleotide binding was shown to be mediated by conserved motifs in the Gα GTPase domain that include the phosphate-binding loop ("P-loop"), switch I and II, the β5-αG region, and the α5-β6 loop {Sprang, 1997 #1;Vetter, 2001 #28;Sprang, 2016 #8}. Nucleotide hydrolysis requires two conserved Gα catalytic residues, an arginine in switch I and a glutamine in switch II, that are crucial for stabilizing the transition state of GTP hydrolysis {Coleman, 1994 #3;Sprang, 1997 #1;Kosloff, 2003 #9;Thomas, 2004 #10;Mann, 2016 #11}. Note that the Gα switch I connects the Gα helical domain to the GTPase domain {Oldham, 2008 #12;Sprang, 2016 #8}, therefore reasonably to assume that the positioning of the catalytic arginine requires both domains. The Gα active site also contains a Mg^{2+} ion that is needed for nucleotide binding and hydrolysis; this Mg^{2+} ion is coordinated by residues from the Gα P-loop, switch I and II {Sprang, 1997 #1;Sprang, 2016 #8}. An additional region that plays a role in GTP binding and hydrolysis is switch III, which functions indirectly via interactions with switch II to position the catalytic glutamine for GTP hydrolysis {Sprang, 1997 #1;Sunahara, 1997 #34;Sprang, 2016 #8}. Importantly, the GTPase

domain in general, and the three switch regions in particular, are central to interactions with RGS proteins {Tesmer, 1997 #101;Soundararajan, 2008 #91;Kimple, 2009 #36;Kosloff, 2011 #41;Nance, 2013 #63;Mann, 2016 #125;Taylor, 2016 #99;Asli, 2021 #137}. However, The Gα helical domain was shown to determine specific interactions with RGS proteins, which have been suggested to play a role in conferring RGS specificity {Asli, 2021 #57;Asli, 2018 #59;Kasom, 2018 #83}. The GTPase domain also provides all of the Gα binding surface for Gβγ subunits {Sprang, 2007 #95;Oldham, 2008 #142;Rasmussen, 2011 #242} and is critical in interactions with effectors {Sprang, 2007 #95}. In particular, Gα interactions with effectors, are mediated not only by the Gα switch regions, but also by an "effector-binding region" that includes the C-terminal half of switch II, the α3 helix, and the loop that connects the latter to the β5 strand {Sprang, 2007 #95}. Taken together, it remains unclear which structural motifs in the Gα GTPase domain are sufficient to enable nucleotide binding and hydrolysis, as well as interaction with RGS proteins and downstream effectors, and whether GB-mini-Gα proteins contain sufficient functional regions to enable some or all of these functions.

Here, we designed novel mini-$G\alpha_o$ proteins that can act as molecular switches that can be regulated by RGS proteins and interact with downstream effectors. We used modeling to select mini-$G\alpha_o$ proteins predicted to be compatible with nucleotide binding and hydrolysis. We tested *in-vitro* which mini-$G\alpha_o$ constructs can bind and hydrolyze nucleotides, and can be inactivated by RGS proteins. We then used BRET-based assays in cells to assess the ability of mini-$G\alpha_o$ proteins to bind Gβγ and downstream effectors, and extended this approach to mini-$G\alpha_{i1}$. In this study, we therefore developed, for the first time, mini-Gα proteins that function as molecular switches in terms of nucleotide binding and hydrolysis, regulation by RGS proteins, and interaction with downstream effectors. These new proteins can be used to better understand the mechanisms of G protein activation and inactivation and serve as synthetic tools to study cellular communication.

RESULTS

GB-mini-Gα$_o$ does not bind nor hydrolyze GTP

We first tested the ability of a previously-engineered GB-mini-Gα protein to bind and hydrolyze nucleotides *in vitro*. We used Gα$_o$ as representative because it is easily expressed and purified and because its GTPase activity can be accurately measured *in vitro* {Lan, 2000 #54;Asli, 2021 #57}. We measured nucleotide binding using intrinsic tryptophan fluorescence within the Gα subunit upon binding the nonhydrolyzable GTPγS. GB-mini-Gα$_o$ did not show any nucleotide binding, while full-length Gα$_o$ showed rapid binding to nucleotides (Fig. 1A). Similar results were observed when measuring nucleotide binding with BODIPY-FL-GTPγS, a fluorescently labeled nonhydrolyzable analog of GTP (Fig. 1B). Using single-turnover GTPase assays with [γ-P^{33}]-labeled GTP, we showed that GB-mini-Gα$_o$ did not showed any GTP hydrolysis beyond the spontaneous hydrolysis of GTP without any protein added. In contrast, full-length Gα$_o$ rapidly hydrolyzes GTP. (Fig. 1C). Note, we tested GTP hydrolysis by GB-mini-Gα$_o$ at a higher temperature, 25˚C, instead of 4˚C as done for full-length Gα$_o$, and still did not observe any increase in hydrolysis rate.

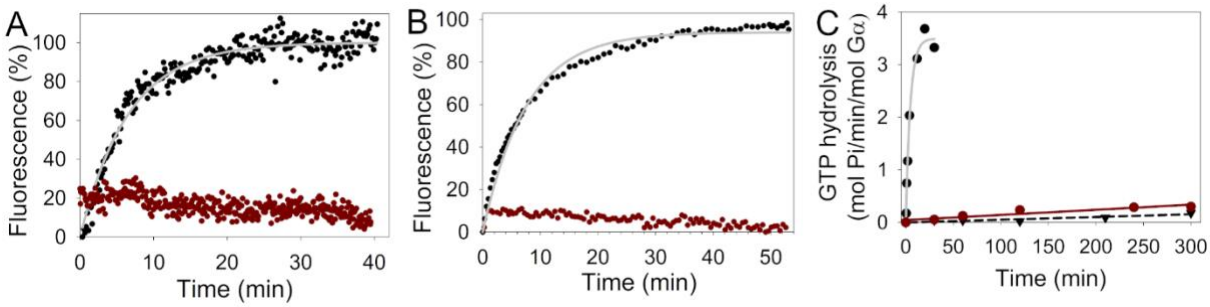

Figure 1: GB-mini-Gα$_o$ does not bind nor hydrolyze GTP. (A) Representative time courses of GTPγS binding to GB-mini-Gα$_o$ (red circles) and to full-length Gα$_o$ (black circles) for reference. The average association rate of GB-mini-Gα$_o$ it was negligible, while for full-length Gα$_o$ with GTPγS was 0.15 ± 0.012 min^{-1}. The GTPγS binding was monitored using intrinsic tryptophan fluorescence at 25°C, initiated by addition of GTPγS (20 μM) to different G proteins (500 nM). **(B)** Representative time courses of BODIPY-FL GTPγS binding to GB-mini-Gα$_o$ (red circles), and to full-length Gα$_o$ (black circles) for reference. The average association rate of GB-mini-Gα$_o$ it was negligible, while for full-length Gα$_o$ with BODIPY-FL GTPγS was 0.15 ± 0.02 min^{-1}. The fluorescence of BODIPY-FL GTPγS (500 nM) was measured at 25°, following the addition of 100 nM Gα subunit. **(C)** Representative time courses of [γ-^{33}P]-GTP hydrolysis with 400 nM GB-mini-Gα$_o$ (red circles) and without any protein (black triangles, dashed line), and with 400 nM Gα$_o$ (black circles) for reference. The basal activity rate of GB-mini-Gα$_o$ protein was 0.001 ± 0.0001 min^{-1}, and the [γ-^{33}P]-GTP hydrolysis without any protein was 0.0005 ± 0.0001 min^{-1}, while for Gα$_o$ was 0.2 ± 0.01 min^{-1}. Note that the GTPase basal activity of Gα$_o$ was measured at 4°C while the experiments with GB-mini-Gα$_o$ and for [γ-^{33}P]-GTP hydrolysis without any protein were held at a higher temperature of 25°C due to the lower activity. Experiments are representative of n≥3, the reaction rate constants (K_{cat}) of full-length Gα$_o$ were calculated using a single-exponential fit to the data, and for GB-mini-Gα$_o$ and spontaneous hydrolysis were calculated using a linear fit using SigmaPlot 10.0.

Designing functional mini-Gα$_o$ proteins that can bind and hydrolyze nucleotides

To design a new mini-Gα$_o$ that can function as a molecular switch, we first analyzed available structures of Gα$_{o/i1}$ subunits to define which Gα structural elements are predicted to be necessary and sufficient for nucleotide binding and hydrolysis (Fig. 2). We compared seven structures of Gα$_{i1}$ and Gα$_o$ in different activation states to define a consensus nucleotide binding site for Gα$_o$ (see Materials and Methods). This analysis identified six Gα regions that contribute to nucleotide interactions: the P-loop, the αD-αE loop in the helical domain, as well as switch I, switch II, and the β5-αG and β6-α5 regions (Fig. 2A). We compared full-length Gα$_o$ to GB-mini-Gα$_o$ and saw that four of the Gα$_o$ region that contribute to the consensus nucleotide binding site are present in GB-mini-Gα$_o$: the P-loop, switch II, and the β5-αG and β6-α5 regions (Fig. 2B). These regions have a similar conformation as in the crystal structure of full-length Gα$_o$ in its activated state with the transition-state mimic GDP/AlF_4^- (Fig. 2A cf. B). The other two regions, the αD-αE loop and most of switch I, are either missing or distorted in GB-mini-Gα$_o$. The αD-αE loop is missing due to the truncation of the entire helical domain. However, because this region only interacts with the guanine ring and the ribose sugar through only two atoms, whereas 12 atoms from other motifs in the GTPase domain interact with the same regions, we assume that the αD-αE motif is not required for nucleotide binding or hydrolysis. The N-terminal segment of switch I, which includes Arg_{CAT}, was truncated. The remaining six C-terminal residues of switch I, including the Mg^{2+}-binding T182 residue, are distorted and adopt a completely different conformation compared to this region in full-length Gα$_o$ (Fig. 2B cf. C). Additionally, switch III, which is not part of the consensus Gα nucleotide binding site, was also truncated in GB-mini-Gα$_o$. However, switch III interacts with two switch II residues (R206 and R209, Fig. 2C) and these interactions were shown to play a role in stabilizing switch II in the active Gα conformation. Accordingly, we hypothesize that in addition to the four regions of GB-mini-Gα$_o$ that interact with the nucleotide, a functional mini-Gα$_o$ should also include the entire switch I and III. we further assume that in order to enable switch I, and in particular the Arg_{CAT}, to adopt the same conformation as in full-length Gα$_o$, switch I need to be anchored in place by the αF helix which is within the helical domain and directly connected to switch I. Importantly, the N-terminus of the αF helix includes two residues we term ("EQ" motif) which interact with residues in switch I that are part of the consensus nucleotide binding site and may thereby stabilize the conformation of switch I.

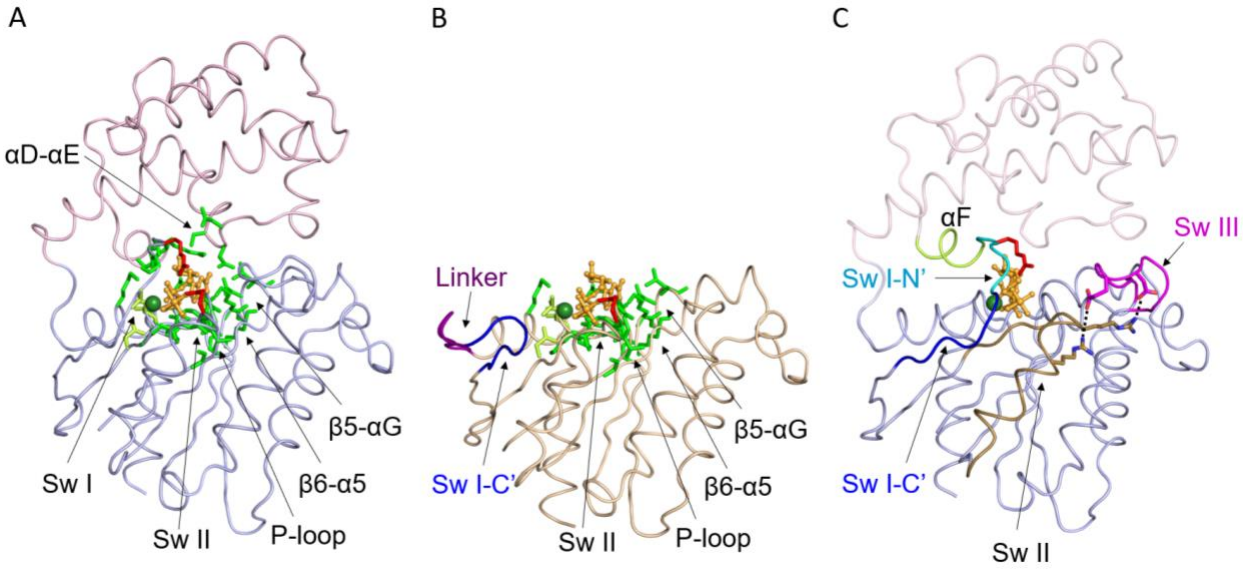

Figure 2: Regions in Gα$_o$ that are either missing or distorted in GB-mini-Gα$_o$. (A) The Gα consensus nucleotide-binding site (see Methods), visualized on the structure of Gα$_o$ (PDB ID: 3C7K). Gα$_o$ is shown as a light purple (GTPase domain) and pink (helical domain) tube, with the N-termini omitted for clarity. The side chain and/or main chain atoms of residues in the Gα consensus nucleotide binding site that are ≤ 5Å from the nucleotide are shown as sticks; residues that interact with the nucleotide are colored green, residues that participate in Mg^{2+} coordination are colored light green, and the two catalytic residues are colored red. These residues are in the following Gα$_o$ regions: the P-loop, the αD-αE loop (αD-αE), switch I (Sw I) region, switch II region (Sw II), β5-αG region (β5-αG), and β6-α5 region (β6-α5). The nucleotide is shown as orange ball and sticks, and the Mg^{2+} ion is shown as a dark green sphere. **(B)** Four regions of the Gα consensus nucleotide-binding site that are present in GB-mini-Gα$_o$: P-loop, Sw II, β5-αG, and β6-α5 (see methods). The nine residues flexible linker connecting residue H57 to T182 ("Linker") are colored purple. The six C-terminus residues of switch I (T182-E187) that are present in the GB-mini-Gα$_o$ are colored blue (Sw I-C'). GB-mini-Gα$_o$ is colored wheat and binding site residues that correspond to the Gα consensus binding site are shown in sticks and colored as in A. **(C)** Structural elements in full-length Gα$_o$ that are predicted to be required for nucleotide binding and hydrolysis and are missing or distorted in GB-mini-Gα$_o$. Gα$_o$ and the GTP analog are shown as in A, but rotated 20° about the y-axis relative to A. Helix αF and the six N-terminal residues of switch I (Sw I-N'), which are absent in GB-mini-Gα$_o$, are colored light green and cyan, respectively. The Arg_{CAT} residue, which is in the middle of switch I-N' is shown as red sticks. Switch I C-terminus residues (Sw I-C`), which is displaced in GB-mini-Gα$_o$, is colored blue. Sw II is colored brown, and switch III (Sw III), which was truncated in GB-mini-Gα$_o$ is colored magenta. The predicted hydrogen bonds between switch II residues and switch III are shown as black dashed lines.

Based on the structural analysis described above, we derived the principles required to develop functional mini-Gα$_o$ proteins capable of binding and hydrolyzing nucleotides, and used them to design and model a set of mini-Gα$_o$ constructs with AlphaFold2 (Fig. 3). Accordingly, we understand that in designing our mini-Gα$_o$ constructs we need to connect the α1 helix, which is part of the GTPase domain, to the αF helix. Importantly, the C-terminal end of the α1 helix we termed ("ED" motif) interacts with the β2 strand preceding switch I, and should also stabilize the conformation of switch I. We tested the distance between the C-terminal end of the α1 helix and

the αF helix and found that at least three residues are required as a linker to connect this region. Accordingly, we designed mini-Gα$_o$ constructs in which the α1 helix was connected to the αF helix via flexible linkers of varying lengths composed of glycine and serine residues. This variable region, which starts at the C-terminus of the α1 helix and connects to switch I, was termed the α1–switch I ("α1–sw I") linker. We modeled these constructs using AlphaFold2, and compared them to the structure of full-length Gα$_o$ in its active conformation. As expected, models with linker less than three residues were unable switch I to adopt the same conformations as full-length Gα$_o$ (Fig. 3C). In these constructs, the Cα atoms of the N' of switch I, were at least 10 Å away from their corresponding positions in full-length Gα$_o$. construct with α1-sw I linker of six or less residues were predicted to unable switch I to adopt the same conformations as full-length Gα$_o$ (Fig. 3B). In these constructs, the Cα atoms of the N' of switch I, including Arg$_{CAT}$ and the Mg^{2+} binding T182, were at least 2 Å away from their corresponding positions in full-length Gα$_o$. In contrast, mini-Gα$_o$ constructs with α1-sw I linkers of seven or more amino acids were predicted to enable switch I to adopt the same conformations as full-length Gα$_o$ (Fig. 3C). In all of these constructs, the Cα atoms of the N' of switch I, including Arg$_{CAT}$ and the Mg^{2+} binding T182, were < 1.5 Å to their corresponding positions in full-length Gα$_o$. We therefore proceeded to test the ability of the latter constructs to bind and hydrolyze nucleotides experimentally.

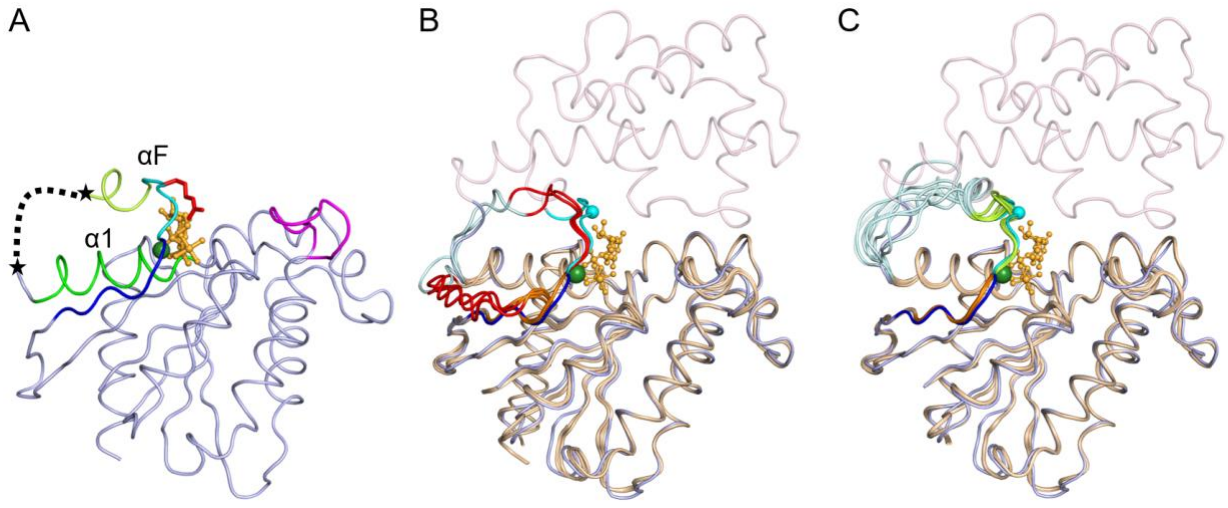

Figure 3: Mini-Gα$_o$ constructs with α1-sw I linkers ≥ seven residues adopt similar conformations to those seen in full-length Gα$_o$. (A) Principles for designing a functional mini-Gα$_o$. The α1 helix is colored green, switch I and III, and αF helix, are colored as in Fig. 2C. The asterisks mark the C-terminal end of the α1 helix and the N-terminus of the αF helix. The linker that should enable the αF helix to constrain switch I to adopt the same conformation as full-

length $G\alpha_o$ is marked with a black dashed line **(B)** Representative AlphaFold2 models of mini-$G\alpha_o$ constructs with α1-sw I linkers of up to six amino acids. In these models the positions of Cα atoms of nucleotide-binding residues in N` of switch I do not match the corresponding positions in full-length $G\alpha_o$. The $G\alpha_o$ crystal structure of $G\alpha_o$–RGS16 complex (PDB ID 3C7K) was superimposed with the AlphaFold2 models of mini-$G\alpha_o$ constructs. The $G\alpha_o$ subunit and the nucleotide are shown as in Fig. 2A. Residues of full-length $G\alpha_o$ in the N` of the switch I region in the consensus nucleotide binding site are colored cyan. Residues in the C` of switch I are colored blue. The mini-$G\alpha_o$ proteins are shown as a wheat tube. Residues of mini-$G\alpha_o$ in the N` of switch I are colored red, and in the C` of switch I are colored orange. Residues of the α1-sw I linker are colored light blue. The Cα of the $G\alpha_o$ catalytic arginine (Arg_{CAT}) is shown as a cyan sphere. **(C)** Representative AlphaFold2 models of mini-$G\alpha_o$ constructs with α1-sw I linkers of ≥ seven amino acids whose switch I backbone adopts conformations similar to that seen in full-length $G\alpha_o$. The full-length $G\alpha_o$ and the GTPase domains of mini-$G\alpha_o$ models are shown as in A. Residues of mini-$G\alpha_o$ in the N` of switch I are colored green, in the C` of switch I are colored orange, and in the α1-sw I linker and αF helix are colored light blue. The Cα of the $G\alpha_o$ catalytic arginine (Arg_{CAT}) is shown in sphere.

Identification of designed mini-$G\alpha_o$ constructs with molecular switch functionality

We expressed and purified mini-$G\alpha_o$ proteins that include an α1-sw I linker of six or more amino acids and tested their ability to bind and hydrolyze nucleotides. We used the intrinsic tryptophan fluorescence assay to measure the ability of these mini-$G\alpha_o$ proteins to bind GTPγS (Fig. 4). The time courses of binding showed that all of these mini-$G\alpha_o$ proteins exhibited a time-dependent increase in fluorescence, with binding rate constants varying between 0.03 min^{-1} and 0.35 min^{-1}. Surprisingly, mini-$G\alpha_o$, which includes a short α1-sw I linker of five amino-acids, binds GTPγS with a binding rate of 0.05 min^{-1}. Mini-$G\alpha_o$ proteins with α1-sw I linkers of 7-12 amino acids were able to bind GTPγS with binding rates ranging from 0.03 to 0.06 min^{-1} (Fig. 4). On the other hand, mini-$G\alpha_o$ proteins with α1-sw I linkers of similar length exhibited binding rates ~2-3-fold higher, of ~0.1 min^{-1}. The highest binding rate was measured for mini-$G\alpha_o$, which included the longest α1-sw I linker of 13 amino acids – 0.35 min^{-1}. We did not observe a correlation between the α1-sw I linkers and nucleotide binding, and therefore proceeded to test GTP hydrolysis for all constructs.

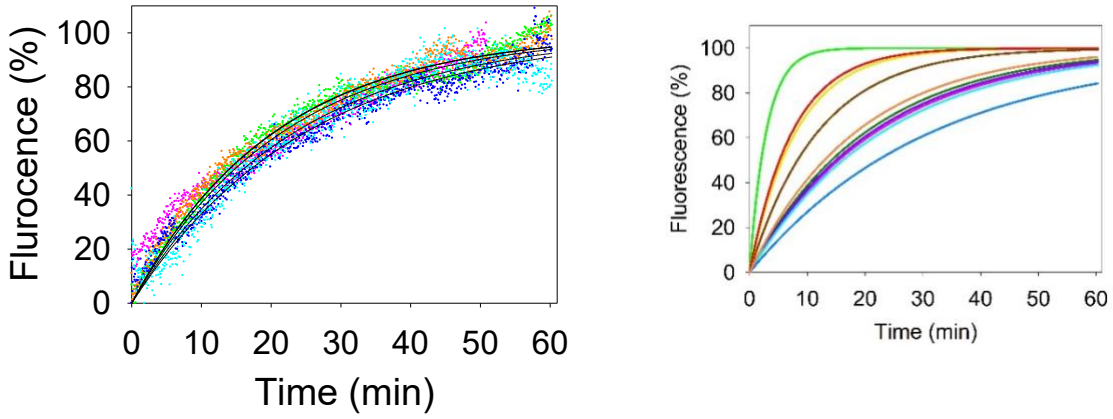

Figure 4: Mini-Gα$_o$ proteins bind nucleotides in a time-dependent manner. Time courses of GTPγS binding to mini-Gα$_o$-S5 (magenta), mini-Gα$_o$-S6 (blue), mini-Gα$_o$-S7 (brown), mini-Gα$_o$-S8 (red), mini-Gα$_o$-S9 (purple), mini-Gα$_o$-S10 (dark green), mini-Gα$_o$-S11 (yellow), mini-Gα$_o$-S12 (orange), mini-Gα$_o$-S13 (cyan), and mini-Gα$_o$-S14 (green) were monitored using intrinsic tryptophan fluorescence at 25°C. Excitation was set at 280 nm, and emission was measured at 340 nm. The binding reaction was initiated by the addition of GTPγS (20 μM) to different G protein variants (500 nM). The rate constants for GTPγS binding are as follows: mini-Gα$_o$-S5 = 0.05 min^{-1}, mini-Gα$_o$-S6 = 0.03 min^{-1}, mini-Gα$_o$-S7 = 0.085 min^{-1}, mini-Gα$_o$-S8 = 0.12 min^{-1}, mini-Gα$_o$-S9 = 0.045 min^{-1}, mini-Gα$_o$-S10 = 0.05 min^{-1}, mini-Gα$_o$-S11 = 0.1 min^{-1}, mini-Gα$_o$-S12 = 0.06 min^{-1}, mini-Gα$_o$-S13 = 0.05 min^{-1}, and mini-Gα$_o$-S14 = 0.35 min^{-1}. Reaction rate constants were calculated using a single-exponential fit in SigmaPlot 10.0.

To test the ability of mini-Gα$_o$ proteins to hydrolyze GTP, we measured their GTP hydrolysis rates using the single-turnover GTPase assay. As expected, mini-Gα$_o$, which includes the short α1-sw I linker of five amino-acids, was not able to hydrolyze nucleotides (Fig. 4. The GTP hydrolysis rates catalyzed by all mini-Gα$_o$ proteins that contained the ED-motif, ranged from 0.0005 to 0.002 min^{-1} – negligible GTPase activity, which is essentially the same as that measured for GB-mini-Gα$_o$ (Fig. 5A). In contrast, a mini-Gα$_o$ protein with an α1-sw I linker of eight amino acids, or mini-Gα$_o$ with a seven or ten amino acids linker and containing the EQ-motif, exhibited GTP hydrolysis rates ~ 6-fold faster, ranging from 0.006 to 0.008 min^{-1} (Fig. 5B). Mini-Gα$_o$ proteins with α1-sw I linkers of ten or 13 amino acids, showed ~ 30-fold higher GTPase rates (Fig. 5C).

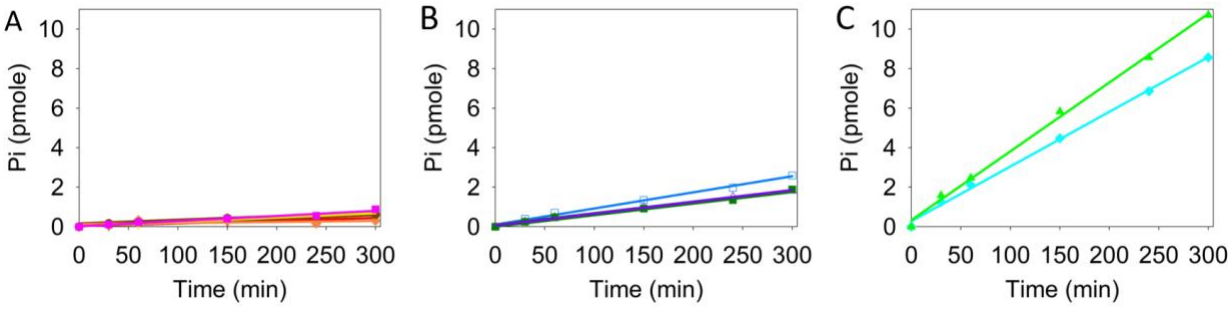

Figure 5: Time courses of [γ-^{33}P]-GTP hydrolysis with mini-Gα$_o$ proteins. (A) Representative single-turnover GTPase assays of using 400 nM mini-Gα$_o$ with α1-sw I linker of five amino-acids (magenta squares), mini-Gα$_o$ with α1-sw I linker of seven amino-acids and include the ED motif (brown circles), mini-Gα$_o$ with α1-sw I linker of nine amino-acids and include the ED and EQ motifs (down red triangles) mini-Gα$_o$ with α1-sw I linker of ten amino-acids and include the ED motif (down yellow triangles) , mini-Gα$_o$ with α1-sw I linker of twelve amino-acids and include the ED and EQ motifs (orange diamonds) **(B)** Representative single-turnover GTPase assays of using 400 of mini-Gα$_o$ mini-Gα$_o$ with α1-sw I linker of seven amino-acids and include the EQ motif (open blue squares), mini-Gα$_o$ with α1-sw I linker of eight amino-acids (open purple triangles), mini-Gα$_o$-S10 with α1-sw I linker of ten amino-acids and include the EQ motif (dark green squares). **(C)** Representative single-turnover GTPase assays of using 400 nM of mini-Gα$_o$ with α1-sw I linker of ten amino-acids (Cyan diamonds), and mini-Gα$_o$ with α1-sw I linker of thirteen amino-acids (green triangles). The reaction rates constant are as follows: mini-Gα$_o$-S5=0.002±0.0005 min^{-1}, mini-Gα$_o$-S6=0.008±0.0001 min^{-1}, mini-Gα$_o$-S7=0.002±0.0005 min^{-1}, mini-Gα$_o$-S8=0.0015±0.0005 min^{-1}, mini-Gα$_o$-S9=0.006±0.0002 min^{-1}, mini-Gα$_o$-S10=0.006±0.0005 min^{-1}, mini-Gα$_o$-S11=0.002±0.0003 min^{-1}, mini-Gα$_o$-

S12=0.0005±0.0001 min^{-1}, mini-Gα$_o$-S13=0.03±0.002 min^{-1}, and mini-Gα$_o$-S14=0.04±0.003 min^{-1}. Experiments were performed at 25˚C due to the low GTPase activity of these proteins. Experiments are representative of n≥2, the GTP hydrolysis rates were calculated using a linear fit in SigmaPlot 10.0.

Mini-Gα$_o$ proteins can be efficiently inactivated by RGS16

We used dose response analysis in single turnover conditions of the radioactively-labeled GTPase assay to test the ability of mini-Gα$_o$ proteins to be inactivated by RGS16 (Fig. 6). RGS16 was used as a representative for high-activity RGS proteins, as in previous studies {Asli, 2018 #56;Asli, 2021 #57}. Mini-Gα$_o$ proteins with α1-sw I linkers of seven or more amino acids, but without the ED-motif, were readily inactivated by RGS16 with EC_{50} values of 15 to 90 nM (Fig. 6A). These values reflect GAP activities that are comparable to the GAP activity of RGS17 towards full-length Gα$_o$ (EC_{50} of 30 nM) and almost reach the activity of RGS16 GAP towards full-length Gα$_o$ (EC_{50} of 4 nM). On the other hand, RGS16 had no effect on GTP hydrolysis by GB-mini-Gα$_o$, even in the presence of 15 μM of RGS16 (Fig. 6B). RGS16 GAP activity towards mini-Gα$_o$, which includes the short α1-sw I linker of five amino-acids, as well as towards any mini-Gα$_o$ proteins that included the ED-motif was very low, and was measurable only at higher RGS concentrations of 500 nM or higher (Fig. 6B). Together with the absence of basal GTPase of any mini-Gα$_o$ that contains the ED-motif, these results suggest the ED-motif inhibits GTPase activity with or without RGS presence.

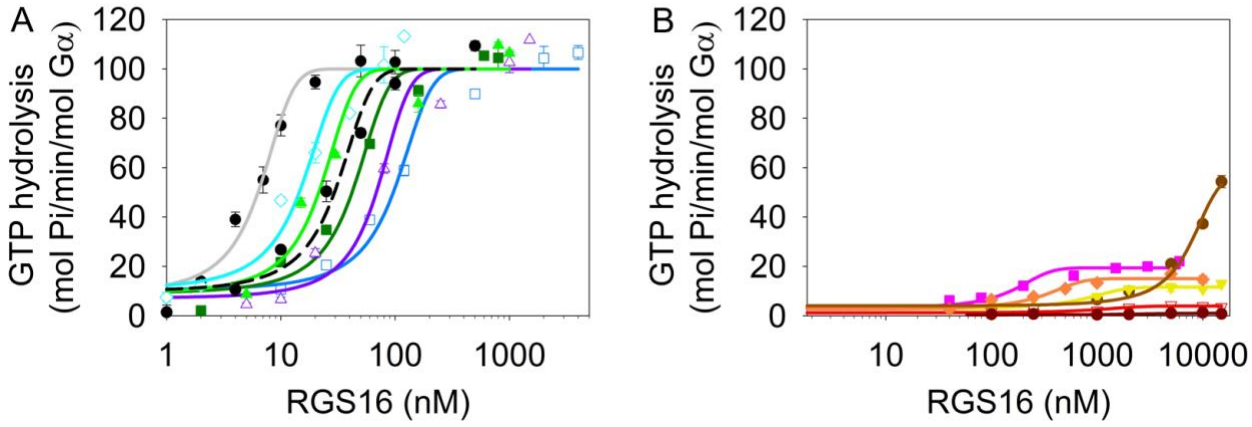

Figure 6: Mini-Gα$_o$ proteins with α1-sw I linkers ≥ seven residues and without the ED motif can be efficiently inactivated by RGS16. (A) Dose-response analysis of RGS16 activity toward mini-Gα$_o$ with α1-sw I linker of seven amino-acids and include the EQ motif (open blue squares), mini-Gα$_o$ with α1-sw I linker of eight amino-acids (open purple triangles), mini-Gα$_o$ with α1-sw I linker of ten amino-acids and include the EQ (dark green squares), mini-Gα$_o$ with α1-sw I linker of ten amino-acids (Cyan diamonds), and mini-Gα$_o$ with α1-sw I linker of thirteen amino-acids

(green triangles). RGS16 activity towards wild-type $G\alpha_o$ is shown for reference (black circles and gray line), EC_{50} value of wild-type $G\alpha_o$ with RGS16 was 4±1 nM. RGS17 activity toward wild-type $G\alpha_o$ is shown also for reference (Black circles, dashed line). **(B)** Dose-response analysis of RGS16 activity toward GB-mini-$G\alpha_o$ (dark red circles) mini-$G\alpha_o$ with α1-sw I linker of five amino-acids (magenta squares), mini-$G\alpha_o$ with α1-sw I linker of seven amino-acids and include the ED motif (brown circles), mini-$G\alpha_o$ with α1-sw I linker of nine amino-acids and include the ED and EQ motifs (down red triangles), mini-$G\alpha_o$ with α1-sw I linker of ten amino-acids and include the ED motif (down yellow triangles), mini-$G\alpha_o$ with α1-sw I linker of twelve amino-acids and include the ED and EQ motifs (orange diamonds). EC_{50} values are as follows: wild-type $G\alpha_o$ = 30±5 nM (with RGS17), mini-$G\alpha_o$-S6 = 90±10 nM, mini-$G\alpha_o$-S9 = 70±10 nM, mini-$G\alpha_o$-S10 = 40±10 nM, mini-$G\alpha_o$-S13 = 15±5 nM, and mini-$G\alpha_o$-S14 = 20±5 nM. Note that all the experiments were done in 4 ˚C because of the faster reaction rates in the presence of RGS proteins. EC_{50} values for dose response analysis of the net RGS-induced GTPase activity were calculated using three parameter sigmoidal curves using SigmaPlot 10.0. Data presented are mean ± s.e.m. of experiments performed in triplicate, representative of at least two independent biological replicates each.

Mini-Gα$_o$ can bind Gβγ subunits and interact with the Rap1Gap effector

We tested the ability of Mini-$G\alpha_o$ protein to bind Gβγ by bioluminescence resonance energy transfer (BRET) in cells using a GRK-Gβγ-based sensor.

DISCUSSION

In this study, we designed functional mini-Gα$_o$ proteins that are capable of binding and hydrolyzing nucleotides and, surprisingly, can be inactivated efficiently by RGS proteins. These mini-Gα$_o$ proteins can be expressed well, purified to homogeneity and are stable at room temperature. We also showed that the mini-Gα$_o$ that was previously engineered to bind GPCRs {Nehme, 2017 #22} is not able to bind and hydrolyze nucleotides. Our analysis suggests that this inability stems from the truncations in the switch I and switch III regions in this previously engineered mini-Gα$_o$, but also from the inability of switch I residues to reach a conformation compatible with nucleotide binding and hydrolysis.

Our newly designed mini-Gα$_o$ proteins contain the GTPase domain, the entire switch I and switch III regions, but also include motifs from full-length Gα$_o$ that we predicted play a role in GTP binding and hydrolysis functionality. AlphaFold2 predictions suggested that all mini-Gα$_o$ proteins with α1-sw I linkers of seven or more amino acids can adopt switch I conformations that are essentially the same as those of full-length Gα$_o$. In the experimental testing of these proteins we showed that all such mini-Gα$_o$ proteins were able to bind nucleotides to some degree. However, more substantial differences among these proteins were seen in their basal GTPase activities, and more so, in the ability of RGS proteins to accelerate GTPase activity. We showed that for substantial basal GTPase activities, α1-sw I linkers of ten or more amino acids are needed, in the absence of the ED and EQ motifs. In contrast, to enable GTPase activity that is efficiently accelerated by RGS proteins, mini-Gα$_o$ proteins needed α1-sw I linkers of seven or more amino acids, but without the ED-motif. The presence of the ED-motif prevents the GTPase activity of mini-Gα$_o$ proteins, while the presence or absence of the EQ-motif did not seem to affect this activity directly. Thus, mini-Gα$_o$-S6, mini-Gα$_o$-S9, mini-Gα$_o$-S10, mini-Gα$_o$-S13, and mini-Gα$_o$-S14 reached full functionality, with RGS16 able to reach GAP activities that are similar to the activities measured towards full-length Gα$_o$.

In a broader context, our newly developed mini-Gα$_o$ proteins are the first engineered mini-Gα proteins with broader molecular switch functionalities. Although they contain only the GTPase domain, they are able to bind and hydrolyze nucleotides and are capable of being inactivated efficiently by RGS proteins. These novel functional G proteins can be used to better understand how Gα subunits function as molecular switches and enable us to pinpoint contributions of specific G protein regions to interactions with RGS proteins. Moreover, these

mini-Gα proteins can be used as tools to control and manipulate engineered synthetic signaling pathways in cells and as building blocks for entirely new synthetic signaling networks.

ACKNOWLEDGEMENTS

This work was supported by the Israel Science Foundation (grant 1454/13), the International Development Research Centre (IDRC), the Israel Science Foundation (ISF), and the Azrieli Foundation (grant 3512/19), and by a grant from the Council for Higher Education through the Data Science Research Center at the University of Haifa.

AUTHOR CONTRIBUTIONS

SHM designed and performed experiments, analyzed data and wrote the manuscript, MAS designed experiments, supervised research and revised the manuscript, MK designed experiments, analyzed data, supervised research, and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

MATERIALS AND METHODS

Protein structures and models

We used the following 3D structures in our analysis and visualization of Gα proteins (with PDB codes for each structure): $G\alpha_o$ bound to the transition state analog GDP-AlF_4^- in complex with RGS16 (3C7K) {Slep, 2008 #60}, $G\alpha_{i1}$ bound to GDP-AlF_4^- in complex with RGS4 (1AGR) {Tesmer, 1997 #48}, $G\alpha_{i1}$ bound to GTPγS (1GIA) {Coleman, 1994 #3}, $G\alpha_{i1}$ monomer bound to GppNHp (1CIP) {Coleman, 1999 #63}, two $G\alpha_{i1}$ monomers bound to GDP with slightly different conformations (1GDD, 1BOF) {Coleman, 1998 #62;Wall, 1995 #5}, $G\alpha_{i1}$ bound to GDP in complex with Gβγ (1GP2) {Mixon, 1995 #4}. GB-mini-$G\alpha_o$ in complex with serotonin 5-HT1B receptor (6G79) {Garcia-Nafria, 2018 #58}.

We generated 3D models of GB-mini-$G\alpha_o$, including residues missing in the experimental structure, and of the designed mini-$G\alpha_o$ proteins using the AlphaFold2 Colabfold server {Jumper, 2021 #64;Mirdita, 2022 #65} (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb

Structure-based definition of the consensus Gα nucleotide binding site

We compared structures of $G\alpha_{i1}$ and $G\alpha_o$ in different activation states to define a consensus nucleotide binding site for $G\alpha_o$. We used seven structures of $G\alpha_o$ and $G\alpha_{i1}$ described above as they representing different stages of activation. These structures included the only available crystal structure of activated $G\alpha_o$, in complex with RGS16, and six representative crystal structures of $G\alpha_{i1}$ Three of the latter structures are of activated $G\alpha_{i1}$ bound to different analogs of GTP, while the other three structures are of inactive $G\alpha_{i1}$ bound to GDP, in monomeric form or in complex with Gβγ. In each of these structures, we defined a consensus nucleotide binding site for $G\alpha_o$ as all Gα residues that are ≤ 5Å from the nucleotide in at least one of these seven structures. This $G\alpha_o$ consensus binding site contained 34 amino acids across six regions of the Gα subunit: the P-loop ($G\alpha_o$ 40-48, and $G\alpha_o$ 51) and the αD-αE loop ($G\alpha_o$ 150-152), as well as the switch I ($G\alpha_o$ 76-82), switch II ($G\alpha_o$ 201-205), β5-αG ($G\alpha_o$ 270-274), and β6-α5 ($G\alpha_o$ 324-327) regions. About a third of these residues are located in the P-loop, enveloping the phosphates of the nucleotide, and include one residue that also interacts with the Mg^{2+} ion, and one residue that interacts with the ribose sugar. The αD-αE loop is the only part of

the Gα binding site that originates from the Gα helical domain. This loop contains three residues that interact with the ribose of the nucleotide, with one of these residues also interacting with the guanine base. The switch I region is a particularly central element in the Gα binding site. It includes seven residues that interact with all three parts of the nucleotide: three residues are at hydrogen bond distances to the ribose ring, one of which also interacts with the guanine base via a salt bridge, and four residues are in the vicinity of the phosphates. The switch I region also includes the catalytic arginine, (Arg_{CAT}, R179 in $G\alpha_o$) that is critical for GTP hydrolysis and a threonine residue that coordinates the Mg^{2+} ion ($G\alpha_o$ T182). The switch II region contains five residues that surround the β and γ phosphates of the nucleotide – one of these residues also coordinates the Mg^{2+} ion and another is the catalytic glutamine (Gln_{CAT}) residue. Lastly, the β5-αG and β6-α5 regions envelop the guanine base, with one of the residues in the β5-αG region also interacting with the ribose.

The available crystal structure of GB-mini-$G\alpha_o$ (PDB ID: 6G79) is missing 12 residues in the region that replaces the helical domain, including a nine-residue flexible linker (GGSGGSGG) connecting H57 to T182. It is also missing three residues at positions E290–P292 (numbered according to the Gαo sequence). We used AlphaFold2 to model the entire GB-mini-$G\alpha_o$ protein. All five models produced by AlphFold2 were similar to one another and to the conformation of the crystal structure of GB-mini-$G\alpha_o$ (PDB ID: 6G79). This was evidenced by the low global RMSD (~1.5 Å) of the models when superimposed on the crystal structure of GB-mini-Gαo, except that the models include coordinates for the nine-residue flexible linker (GGSGGSGG), which are missing in the experimental structure. By comparing the regions of full-length $G\alpha_o$ that contribute to the consensus nucleotide binding site to GB-mini-$G\alpha_o$ we identified four $G\alpha_o$ regions that are part of the consensus binding site and are present in GB-mini-$G\alpha_o$ and adopt the same conformation as the crystal structure of the full-length $G\alpha_o$ – the P-loop, switch II, β5-αG, and β6-α5 regions. 3D structural visualization and superimposition were carried out with the molecular graphics program PyMOL (http://pymol.org). To quantify differences between structures we calculated root mean square deviation (RMSD) values, the average distance between the Cα atoms of the superimposed residues, using the TM-align server (https://zhanggroup.org/TM-align/).

Protein expression, purification, and mutagenesis

All proteins were expressed as N-terminally His_6-tagged proteins. The N-terminally His_6-tagged rat $G\alpha_o$ clone was a gift from Vadim Arshavsky (Duke University). The RGS domain of human RGS16 (residue 86–205) was expressed in the pNIC-SGC1 vector (Addgene). Expression and purification of $G\alpha_o$ and RGS16 proteins were conducted as described in {Asli, 2021 #57}. GB-mini-$G\alpha_o$ construct was expressed in the PET-15b vector, and the mini-$G\alpha_o$ proteins were produced using the QuikChange site-directed mutagenesis kit (Invitrogen) with primers designed using the Primer Design Program (www.genomics.agilent.com). Expression and purification of mini-Gα proteins were conducted as described in {Carpenter, 2017 #66}.

In all our mini-$G\alpha_o$ constructs we mutating to an aspartate the alanine residue that precedes switch III (the equivalent position to Ala277 in full-length $G\alpha_o$), as the equivalent mutation was shown to stabilize the nucleotide binding pocket in GB-mini-Gα proteins and improved both purification and yield {Carpenter, 2016 #21;Nehme, 2017 #22}. Indeed, this Ala-to-Asp mutation enabled us to purify this protein to over 95% purity. Based on the 3D location of A227 in full-length $G\alpha_o$, we assumed this mutation will not impact the interactions with the nucleotide. The yield and purity of all proteins was validated by SDS-PAGE electrophoresis and Coomassie staining. We also tested the thermal stability of all these mini-$G\alpha_o$ proteins – the Tm values for all constructs were 38-41ºC. Protein concentrations were determined by measuring absorption at A_{280} nm, using extinction coefficients predicted with ProtParam (Swiss Institute for Bioinformatics) with the sequence of each expressed protein.

Protein thermostability analysis using Differential Scanning Fluorimetry

Differential scanning fluorimetry measurements to determine the stability of expressed proteins were performed using a label-free fluorimetric analysis with the Prometheus NT.48 nanoDSF instrument (NanoTemper Technologies GmbH, Germany). Protein unfolding was measured by detection of changes in tryptophan and tyrosine fluorescence at emission wavelengths of 330 and 350 nm when applying a temperature gradient. Samples were prepared on ice and mixed with DSF buffer (50 mM HEPES pH 7.5, 0.05% v/v polyoxyethylene, 5 mM EDTA, 12 mM NaCl, 36 mM $MgCl_2$, 1 mM GDP, 0.2 µM NaF, 0.3 mM $AlCl_3$, 5 µg/ml BSA, 1 mM dithiothreitol). 10 µl of each sample were loaded into “standard capillaries” (NanoTemper Technologies). DSF unfolding was performed by increasing the temperature from 15° C to 95° C

at a rate of 1° C/min. The melting temperature (Tm) was defined as the inflection point of the melting curve as analyzed by the NanoTemper Technologies instrument software.

Guanine nucleotide binding measurements

Intrinsic tryptophan fluorescence was used to measure guanine nucleotide binding to $G\alpha_o$ and mini-$G\alpha_o$ proteins. The experiments were conducted with a Microplate Reader (SPARK 10M, Tecan) using black-bottomed 96-well plates (NUNC). Time-based assays were conducted with excitation and emission wavelengths set at 280 and 340 nm, respectively. Assays were initiated by addition of 10 μM GTPγS to pre-incubated 500 nM Gα protein in assay buffer containing 50 mM Tris PH 7, 1 mM $MgCl_2$, and 1 mM DTT. Fluorescence values were recorded every 10 seconds, and time-dependent curves were calculated using linear fit to the data in SigmaPlot 10.0.

Single-turnover GTPase assays and RGS GAP activity measurements

We used single-turnover GTPase assays to measure the basal GTPase activity of $G\alpha_o$ and mini-$G\alpha_o$ {Ross, 2000 #67;Kosloff, 2011 #55}. $G\alpha_o$ subunit and mini-$G\alpha_o$ proteins were loaded with radioactively labeled GTP by incubating for 15 min at 25˚C with 1 μM [γ-^{33}P]-GTP (Hartmann Analytic GmbH) in Reaction Buffer (50 mM HEPES, pH 7.5, 0.05% polyoxyethylene (v/v), 5 mM EDTA, 5 mg/ml BSA, 1 mM dithiothreitol). For $G\alpha_o$ subunit, the exchange reaction was followed by cooling on ice for 5 min, and the GTP hydrolysis reaction was measured at 4˚C, for $G\alpha_o$ as preformed previously {Ross, 2000 #67;Kosloff, 2011 #55;Asli, 2021 #57}. On the other hand, because the basal GTPase activity of all the mini-$G\alpha_o$ proteins were extremely slow we increased the experiments temperature to 25˚C in order to be able to quantify basal activity. We initiated the experiments for both $G\alpha_o$ and mini-$G\alpha_o$ by raising the free magnesium concentration to 5 mM (using $MgCl_2$), together with 100 μM cold GTP (final concentration). Aliquots were taken at different time points and quenched with 5% charcoal in 50 mM $Na_2H_2PO_4$ (pH 3), followed by centrifugation at 12,000 g for 5 min at room temperature. The supernatants were transferred to 3 ml liquid scintillation liquid and analyzed using a Tri-Carb 2810 TR scintillation counter (Perkin Elmer).

We used dose-response analysis with a range of RGS concentrations to measure GAP activity towards $G\alpha_o$ subunit and mini-$G\alpha_o$ proteins. Gα proteins were incubated with 1 μM [γ-

^{33}P or γ-^{32}P]-GTP for 15 min at 25˚C and then cooled on ice for 5 min. Hydrolysis was initiated by adding 10 µl RGS protein in different concentrations in assay buffer containing 5 mM $MgCl_2$, together with 100 µM cold GTP to a tube containing 20 µl Gα subunit (500 nM) on ice. Each reaction was terminated after 45 sec by adding 100 µl 5% perchloric acid and quenched with 700 µl 10% (w/v) charcoal slurry in 50 mM phosphate buffer (pH 7.5), followed by centrifugation at 12,000 g for 5 min at room temperature. 200 µl of the supernatants were transferred to 3 ml liquid scintillation and analyzed using a Tri-Carb 2810 TR scintillation counter (Perkin Elmer).

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