Supporting Information

Slep *et al.* 10.1073/pnas.0801569105

SI Materials and Methods

Mouse RGS16, residues 53–180, was subcloned into a modified pGEX-2T vector (GE Healthcare) to yield a thrombin-cleavable GST-fusion of $H_6RGS16_{53–180}$. Plasmid was transformed into BL21 (DE3) pLysS *E. coli* and grown to an $O.D._{600}$ of 3.0 in terrific broth (TB) supplemented with 50 μg/ml ampicillin at 27°C. Protein expression was induced with 0.5 μM IPTG for 6 h at 27°C. Cells were harvested and resuspended in buffer A (25 mM Tris pH 8.0, 300 mM NaCl, 0.1% β-ME, 1 mM PMSF, 5% glycerol, 5 mM $MgCl_2$). Lysozyme, DNaseI and $MgSO_4$ were added to final concentrations of 0.1 mg/ml, 10 μg/ml and 2.5 mM respectively and incubated at 4°C for 1 h to initiate lysis. Lysis was completed by sonication, Triton X-100 added to 0.5% and the lysate clarified by centrifugation at 36,000 $\times$ *g* for 30 min. Supernatant was applied to 10 ml of glutathione S-Sepharose (GE Healthcare) equilibrated in buffer B (buffer B = buffer A without PMSF), washed with 250 ml of buffer B and bound protein step-eluted with 75 ml of buffer C (buffer C = buffer B plus 50 mM glutathione pH 8.0). $CaCl_2$ and human α-thrombin were added to 2.5 mM and 2.7 μg/ml respectively and incubated for 18 h at 4°C. Imidazole was added to 20 mM and the digested fusion protein was applied to 20 ml of Ni^{2+}-NTA resin (Qiagen) equilibrated in buffer D (buffer D = buffer B + 20 mM imidazole). Resin was washed with 400 ml of buffer D and bound protein was step eluted with 100 ml of buffer E (buffer E = buffer D plus 0.3 M imidazole). Protein was exchanged into buffer F (50 mM Tris pH 8.5, 0.1% β-ME, 25 mM NaCl and 5 mM $MgCl_2$) and loaded onto a Mono Q 10/10 column (GE Healthcare) preequilibrated in buffer F. Protein was eluted with a linear gradient from 25 to 45 mM NaCl >75 ml. Fractions containing pure $H_6RGS16_{53–180}$ were pooled, exchanged into 5 mM Tris pH 8.0, 0.1% β-ME, 5% glycerol, 200 mM NaCl, concentrated to 17.0 mg/ml, flash frozen in liquid nitrogen and stored at −80°C.

For complex formation, the RGS16 construct was reengineered to remove the noncleavable H_6-tag. RGS16 RGS domain, residues 53–180, was subcloned into pGEX-2T creating a thrombin-cleavable GST tag that, postcleavage, would leave an N-terminal glycine artifact. Protein was expressed and purified according to the H_6-$RGS16_{53–180}$ construct except that the Ni^{2+}-NTA column was omitted from the procedure and the final, purified protein was exchanged into 5 mM Tris pH 8.0, 25 mM NaCl, 5 mM $MgCl_2$, 0.1% β-ME, 5% glycerol, concentrated to 49 mg/ml, frozen in liquid nitrogen and stored at −80°C. Mouse $G\alpha_o$ in pET15b (Novagen)(gift from John Sondek and Rachelle Gaudet) was expressed in BL21 (DE3) pLysS cells, grown in 2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM KH_2PO_4 pH 7.2 at 30°C to an OD_{600} of 0.4 and induced for 12 h with 30 μM IPTG and 1 μg/ml chloramphenicol. Cells were harvested, resuspended in buffer G (50 mM Tris pH 8.0, 100 mM NaCl, 0.1% β-ME, 10% glycerol, 1 mM PMSF), lysed via sonication and clarified by centrifugation at 36,000 $\times$ *g* for 30 min. Supernatant was loaded onto 20 ml of Ni^{2+}-NTA resin preequilibrated in buffer H (50 mM Tris pH 8.0, 500 mM NaCl, 0.1% β-ME, 10 μM GDP), washed with 15 column volumes of buffer H, and eluted with a 0–150 mM imidazole gradient >400 ml in buffer H. Fractions containing H_6-$G\alpha_o$ were pooled, diluted 3-fold with buffer I (buffer I = buffer H without NaCl), loaded onto a Q Sepharose Fast Flow column (GE Healthcare), washed with buffer I supplemented with 30 mM NaCl and eluted with a 30–500 mM NaCl gradient in buffer I >400 ml. Fractions containing $G\alpha_o$ were pooled and the ion exchange protocol repeated by using a MonoQ 10/10 column. Fractions containing $G\alpha_o$ were pooled, exchanged into buffer J (5 mM Tris pH 8.0, 10% glycerol, 0.1% β-ME, 100 mM NaCl, 30 mM NaF, 1 mM $AlCl_3$, 20 mM GDP, 5 μM $MgCl_2$) and 5 units of Endo-Lys C (Roche Molecular Biochemicals) were added and allowed to proteolyze $G\alpha_o$ for 48 h at 4°C. An equal volume of buffer I was added, the sample was filtered over Ni^{2+}-NTA resin and the flow through applied to a MonoQ 10/10 column. The anion exchange purification protocol was repeated identically as before except buffers were supplemented with 10 mM NaF, 20 μM $AlCl_3$, 100 mM $MgCl_2$ and 20 μM GDP. Fractions containing $G\alpha_o$ were pooled, concentrated and purified over a Superdex 75 16/60 column (GE Healthcare) in buffer J. Fractions containing $G\alpha_o$ were pooled, concentrated to 26 mg/ml, frozen in liquid nitrogen and stored at −80°C. N-terminal protein sequencing was performed at the Yale Keck microsequencing facility yielding the sequence NLKEDGISA, indicative of Endo Lys C cleavage between K21 and N22. To obtain a 1:1 stoichiometric ratio of $G\alpha_o$:RGS16, a 1.5 molar excess of concentrated RGS16 was added to 150 μl of 2.7 mM $G\alpha_o$ in buffer J, mixed, and allowed to incubate on ice for 1 h. Sample was injected onto a Superdex 75 16/60 column equilibrated in buffer J and a 1:1 $G\alpha_o$:RGS16 complex was separated from excess RGS16. Fractions containing the complex were pooled, concentrated to 26 mg/ml, frozen in liquid nitrogen and stored at −80°C.

Crystallization, Data Collection, Structure Determination, and Refinement. $H_6RGS16_{53–180}$ was crystallized by the hanging drop method, using 1.5 μl of 13 mg/ml $H_6RGS16_{53–180}$ and 1.5 μl of mother liquor (34% PEG 400, 150 mM ammonium citrate, 100 mM cacodylate pH 6.5, 500 μM $KCl_6Ir(IV)$) equilibrated against 1 ml of mother liquor at 4°C. Crystals were transferred to mother liquor supplemented with 5% glycerol and frozen in liquid nitrogen. Data were collected at the NSLS X25 beamline above the iridium edge at a wavelength of 1.10 Å, using a Brandeis B4 CCD detector and a 100° K cryo stream. Data were processed and scaled by using HKL2000 (1). Crystals belong to the space group $P2_12_12_1$. Because no iridium sites were found in the crystal, a molecular replacement solution was obtained by using the CNS package (2) and the RGS4 RGS domain (PDB 1AGR (3)) as a search model. Two molecules were found in the asymmetric unit correlating with a solvent content of 36%. Model building used the program O (4). Refinement was performed with the CNS package and monitored with a random 10% of the data that was removed from the working set and used as a test set for cross-validation (5). Model refinement used the MLF target function, rounds of torsion angle molecular dynamics simulated annealing (6), B factor refinement and rebuilding in O with σA-weighted difference Fourier maps. The final model includes RGS16 residues 57–180 (chain A), 53–180 (chain B) and 179 water molecules.

$G\alpha_o$·GDP·AlF_4^-·RGS16 crystals were grown by the hanging drop method at 4°C. 1.5 μl of $G\alpha_o$·GDP·AlF_4^-·RGS16 at 20 mg/ml was mixed with 1.5 μl of mother liquor from a 1-ml well containing 15% PEG 8000 and 200 mM Tris pH 8.5. Crystals were transferred to a stabilizing solution containing mother liquor plus 5% ethylene glycol. Ethylene glycol was increased in 5% increments to a final concentration of 30%. Crystals were then transferred to a solution containing mother liquor supplemented with 30% ethylene glycol and 1 mM $(Me)_3PbOAc$ for 30 seconds and then frozen in liquid nitrogen. Data were collected at the NSLS X25 beamline above the lead edge at a wavelength

of 1.15 Å, using a Brandeis B4 CCD detector and a 100 K cryo stream. Data were processed and scaled by using HKL2000 (1). Crystals belong to the space group $P3_221$. No lead sites were found in the crystal so a molecular replacement solution was obtained by using the CNS package (2) and the $G\alpha_{i1}$·GDP·AlF_4^-·RGS4 structure (PDB 1AGR (3)) as a search model. Two molecules were found in the asymmetric unit. Model building and refinement followed a protocol similar to that outlined for the RGS16 apo structure. The final model includes complex 1: $G\alpha_o$ residues 35–56, 61–194, 198–346 (chain A), RGS16 residues 58–177 (chain B); complex 2: $G\alpha_o$ residues 35–345 (chain C), RGS16 residues 64–176 (chain D); 2 GDP, 2 Mg^{2+}, 2 AlF_4^- and 6 water molecules. Data collection, phasing information and refinement statistics are presented in Table S1. Figures were generated by using PyMol (DeLano Scientific).

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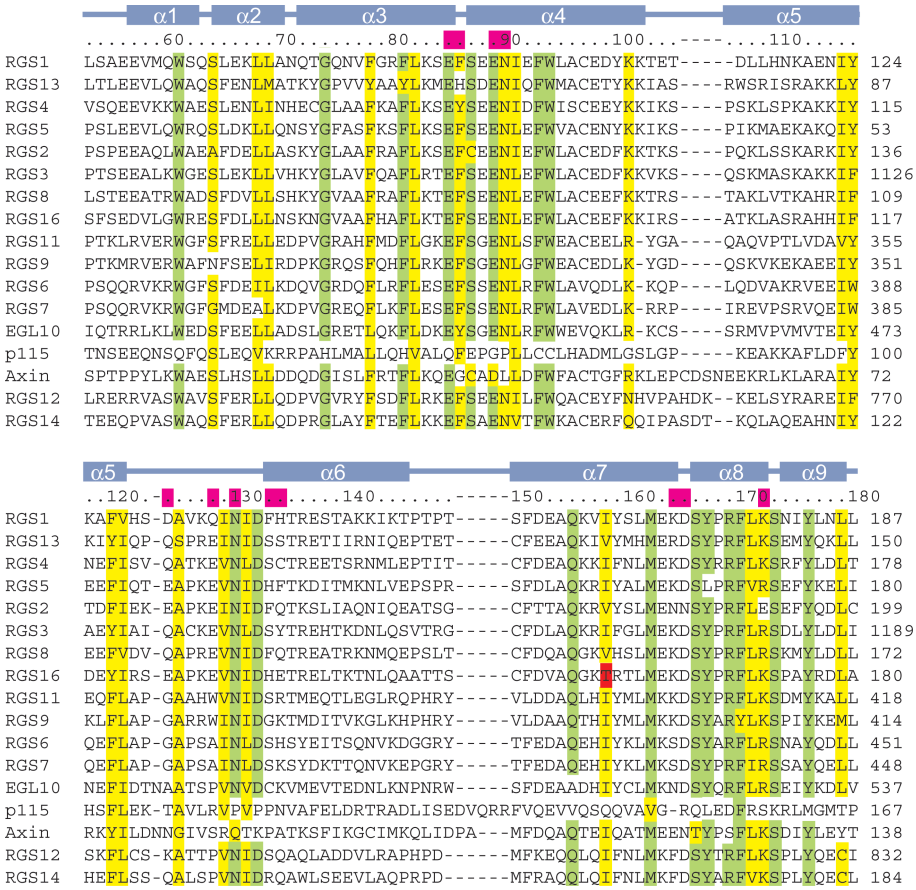

Fig. S1. Sequence alignment of RGS domains highlighting RGS16-Gα_o binding determinants. Alignment was performed in CLUSTALW and modified manually (7). Secondary structure and amino acid numbering for mouse RGS16 is indicated above the alignment. Eighty-eight percent identity and 88% similarity are highlighted in green and yellow respectively. The divergent residue Thr-158 is highlighted in red. Amino acids that form contacts with Gα_o are indicated above the alignment and highlighted magenta. Sequences from top to bottom correspond with GenBank accession nos. NP_056626.1, O14921, P49799, NP_033089.2, AAF34625.1, P49796, P49804, P97428, AAC69175.1, O46469, AAD34717.1, O54829, P49809, NP_004697.2, NP_003493, O14924, and P97492, respectively.

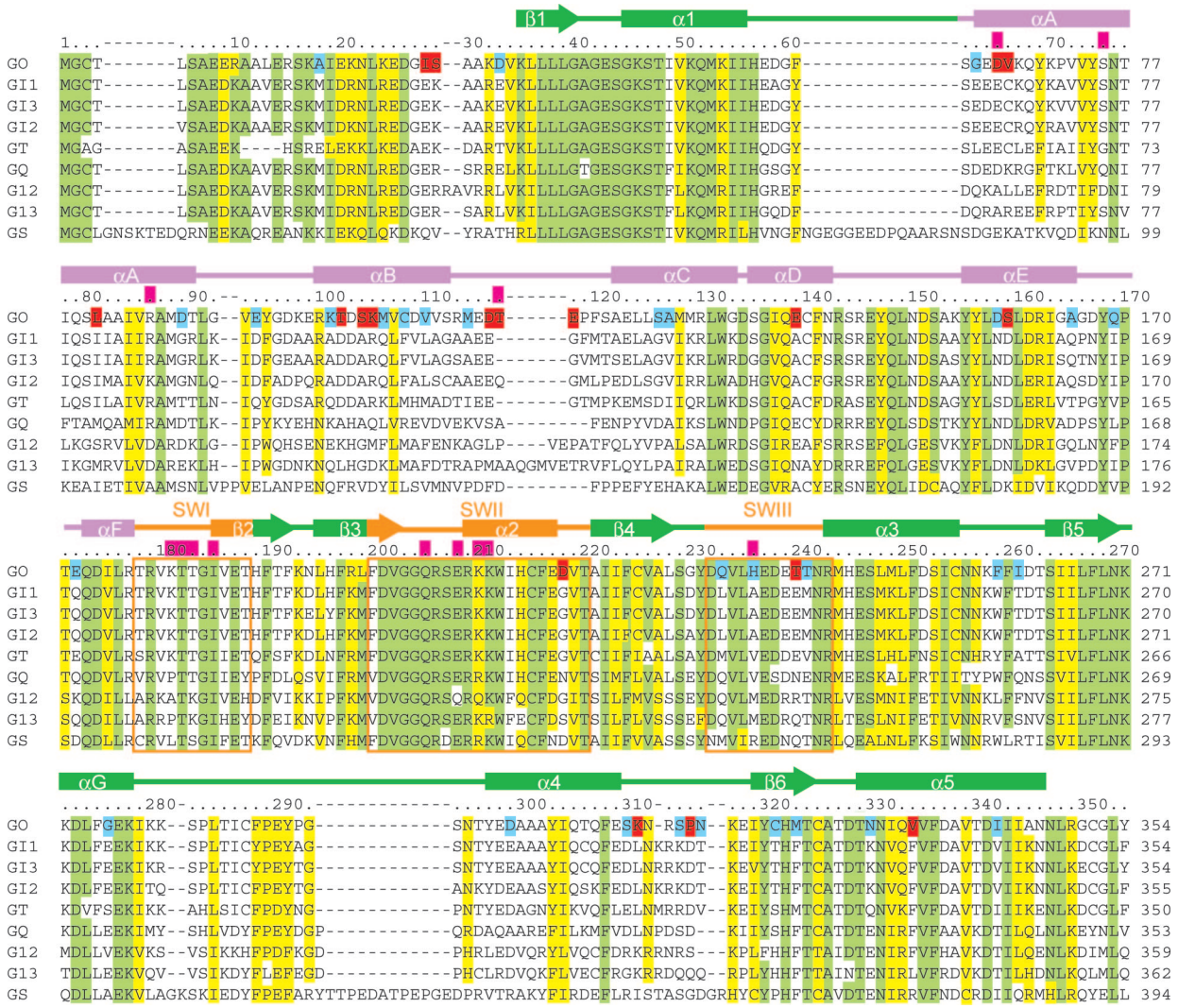

Fig. S2. Sequence alignment of heterotrimeric Gα subunits. Alignment performed by using CLUSTALW and modified manually (7). Protein sequences (from top to bottom) are from GenBank or the PDB with accession nos. NP_034438, P10824, NP_037238, NP_112297, CAA26285, 2BCJ_Q, 1ZCA_B, 1ZCB_A, and P04896, respectively. Secondary structure and amino acid numbering for $G\alpha_o$ is indicated above the alignment and color coded as in *A*. Eighty-nine percent identity and 89% similarity are highlighted in green and yellow, respectively. Residues identical across $G\alpha_{i1-3}$ and $G\alpha_t$ but not identical in $G\alpha_o$ are highlighted in red. Residues identical across $G\alpha_{i1-3}$ but not identical in $G\alpha_o$ are highlighted in blue. Amino acids that form contacts with RGS16 are indicated above the alignment and highlighted magenta.

Table S1. Crystallographic data collection and refinement statistics

Crystallographic/model parameter	Crystal structures: $G\alpha_o$·GDP·AlF_4^-·RGS16	Crystal structures: RGS16
X-ray diffraction data		
Space group	$P3_221$	$P2_12_12_1$
Unit cell dimensions, a, b, c; Å	96.4, 96.4, 235.6	47.4, 71.7, 72.3
Resolution, Å	50–2.9	50–1.89
Reflections, total	27,489	20,395
Redundancy	7.5 (3.0)	2.5 (1.9)
Completeness, %	83.0 (48.2)	94.6 (75.7)
R_{sym}, %	7.8 (36.3)	4.3 (11.0)
I/σ	20.2 (2.0)	21.3 (5.2)
Molecular replacement		
Search model (PDB code)	$G\alpha_{i1}$·GDP·AlF_4^- ·RGS4 (1AGR)	RGS4 (1AGR)
Rotation 1, θ1, θ2, θ3; deg.	201.0, 49.4, 190.7	359.2, 24.0, 180.1
Translation 1 *x*, *y*, *z*; Å	−36.0, 71.3, 16.0	3.14, 19.0, 29.85
σ above mean monitor value	2.3	2.5
Rotation 2, θ1, θ2, θ3; deg.	208.5, 43.3, 186.1	355.7, 25.1, 180.5
Translation 2, x, y, z; Å	54.8, 73.0, −99.8	−21.7, −15.1, 31.2
σ above mean monitor value	3.0	3.6
Refinement statistics		
Reflections, total no.	24,012	19,238
Reflections, working set	21,645	17,342
Reflections, test set	2365	1896
R_{work}, %	25.1	22.2
R_{free}, %	30.5	26.8
Non-hydrogen atoms	6,889	2,235
Waters	6	179
Average B-factor, Å^2 (min/max)	93.1 (19.0/180.0)	14.3 (1.6/41.9)
rmsd bond length, Å	0.009	0.005
rmsd bond angles, deg.	1.4	1.0
rmsd dihedral angles, deg.	20.5	18.0
rmsd improper angles, deg.	0.8	0.8

Numbers in parentheses refer to the highest resolution shell in the structural refinement unless otherwise noted. $R_{sym} = \Sigma_{hkl}\Sigma_j|I_j(hkl) - <I(hkl)>|/I_j(hkl)$. $R_{work} = \Sigma_{hkl}||F_o(hkl)| - |F_c(hkl)||/\Sigma_{hkl}|F_o(hkl)|$. $R_{free} = \Sigma_{hkl}||F_o(hkl)| - |F_c(hkl)||/\Sigma_{hkl}|F_o(hkl)|$, calculated from reflections (≈10% of total) delineated as a test set of randomly selected data.