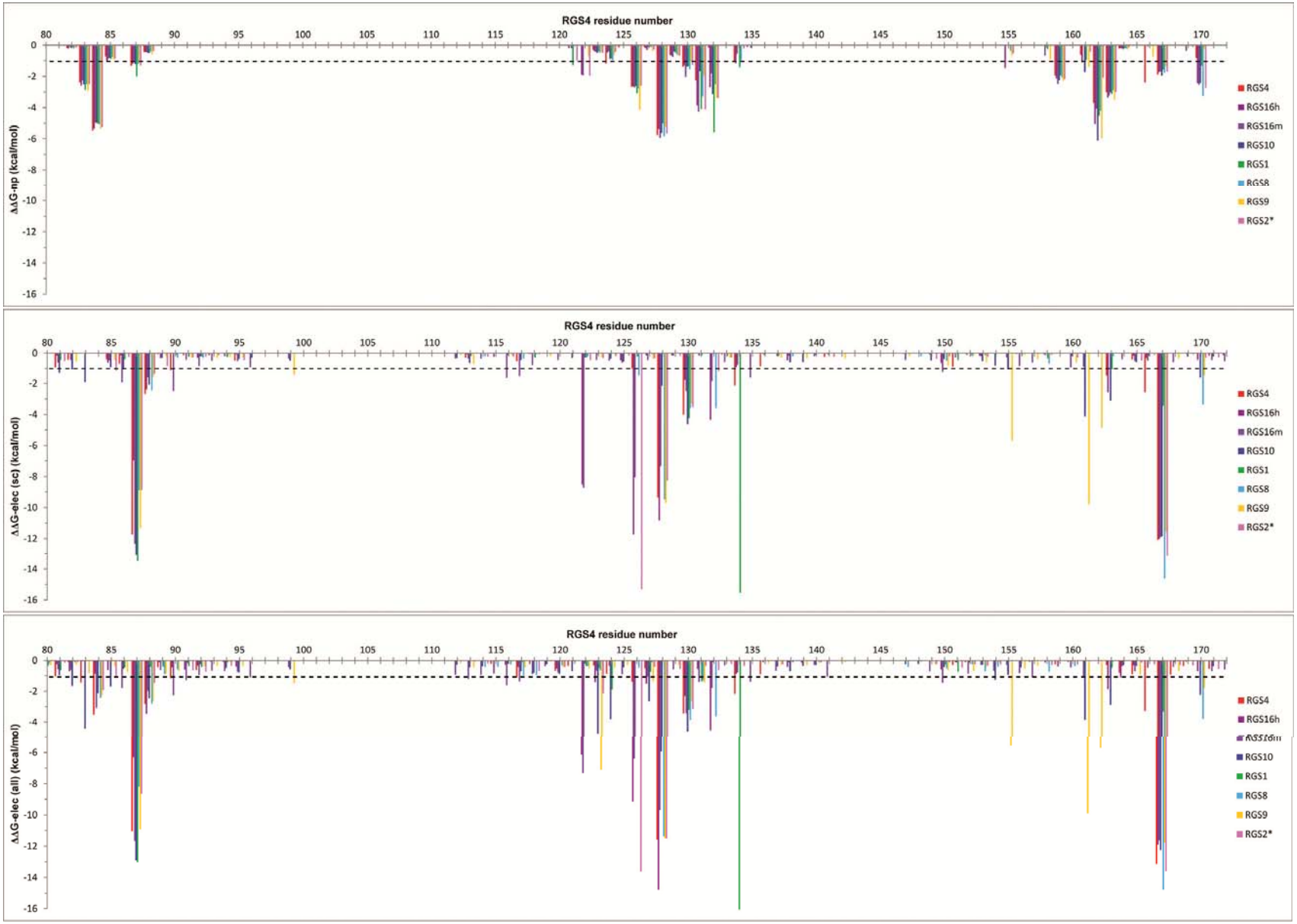
Supplementary Information

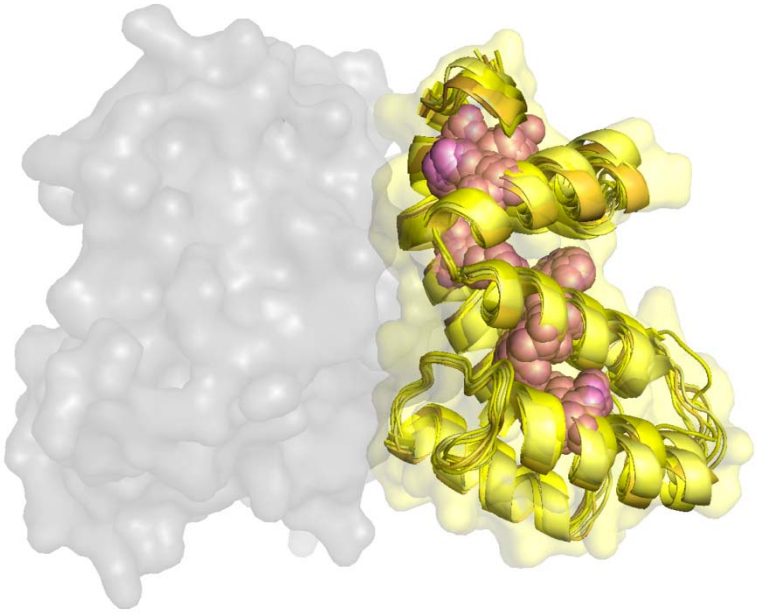
Integrating energy calculations with functional assays to decipher the specificity of G-protein inactivation by RGS proteins

Mickey Kosloff[1], Amanda M. Travis[1], Dustin E. Bosch[2], David P. Siderovski[2] and Vadim Y. Arshavsky[1]

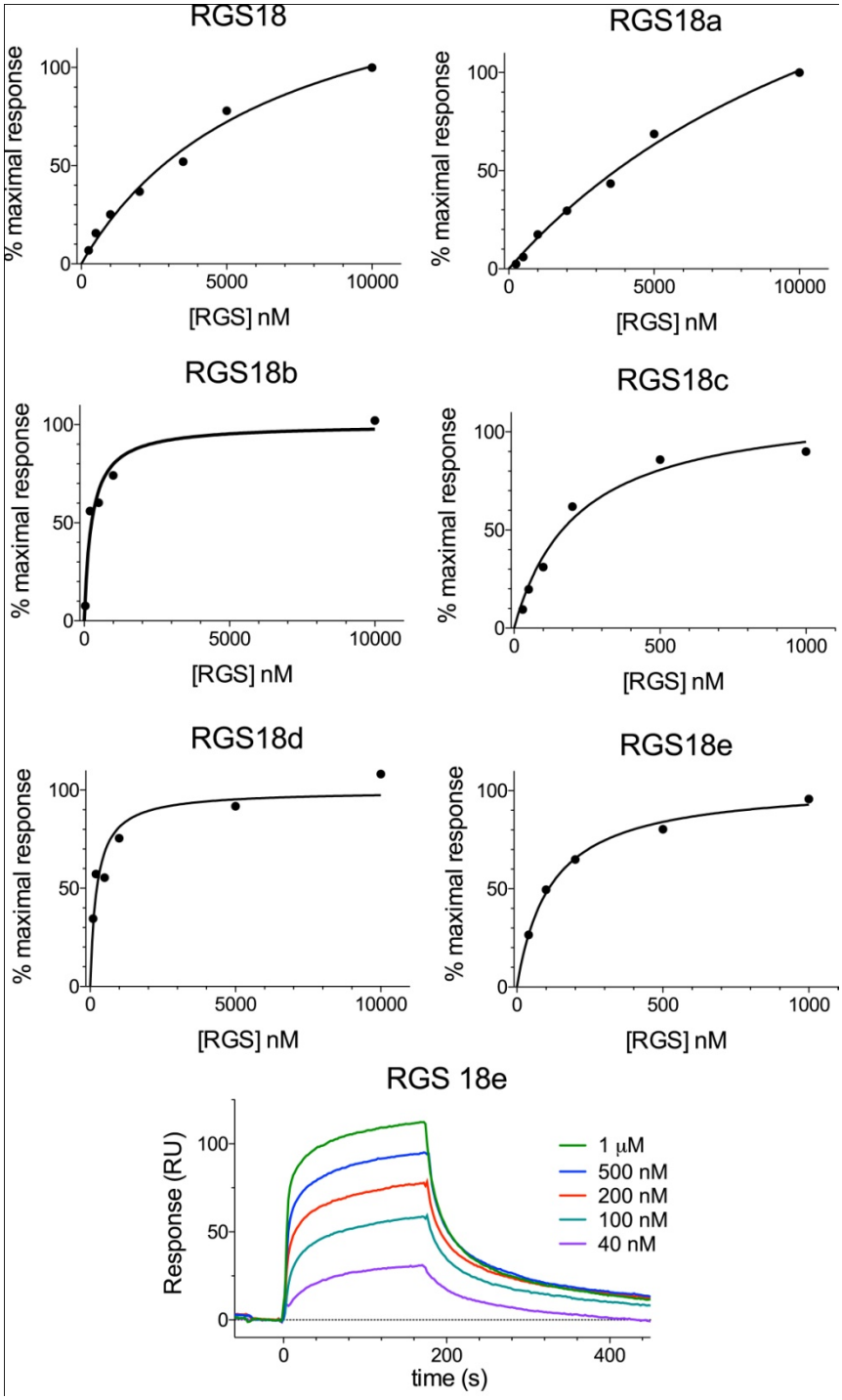
[1]*Duke Eye Center, Duke University Medical Center, Durham, NC 27710, USA.* [2]*Department of Pharmacology, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.*



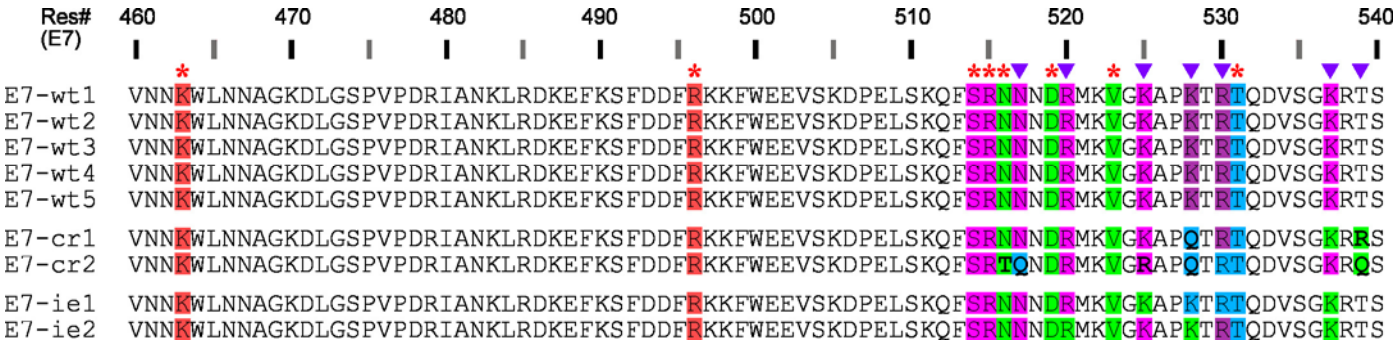
Supplementary Figure 1: Per-residue energetic contributions to the interaction of RGS domains with Gα subunits, calculated as described in Methods. np=non-polar, sc=side-chain, all=full-residue. The dashed lines mark the 1 kcal/mol threshold used to determine substantial contributions to the interaction.



Supplementary Figure 2: RGS4 residues mutated in previous studies that belong to the conserved RGS domain hydrophobic core. Superimposed RGS domain structures are depicted as ribbon diagrams with residues corresponding to Phe79, Phe91, Trp92, Ile114, Phe149, Met160, and Phe168 in RGS4, shown as pink spheres.



Supplementary Figure 3: Determination of RGS18 mutants' binding affinity for $G\alpha_o$. SPR was performed as described in Supplementary Methods. Sensorgrams were used in equilibrium saturation binding analyses to determine binding affinities for all RGS18 mutants. Precise K_D values for RGS18 (wild-type) and RGS18a could not be determined because saturation was not reached at the concentrations tested. Individual sensorgrams from a representative series of RGS18e injections are shown at the bottom.



Supplementary Figure 4: Colicin E7 residues contributing substantially to the interactions with immunity proteins. The figure shows the residue-level sequence map (as in Fig. 6a) of the E7 proteins. Energy calculations were performed on the following structures (PDB IDs): Wild-type E7–Im7 complexes (wt1–5: 7CEI, 2JAZ, 2JB0, 2JBG, 1ZNV); Computationally-redesigned E7–Im7 (cr1–2: 1UJZ, 2ERH); E7 bound to Im9 proteins evolved *in vitro* to bind E7 with high affinity (ie1–2: 3GJN, 3GKL). The sequences in the multiple sequence alignment are taken from the crystal structures. Residues that contribute substantially to the interaction are color-coded according to the type of energy contribution (see legend in Fig. 6). Consensus analysis was applied to the five wild-type proteins and Significant & Conserved and Modulatory positions were determined for all nine structures as in Figures 2 and 6.

Supplementary Table 1: Sequence identities among Human RGS domains

RGS sub-families:				R4						R7				R12			RZ		
RGS domains	RGS1 72-187	RGS2 83-199	RGS3 1073-1198	RGS4 62-178	RGS5 64-180	RGS8 56-171	RGS13 34-150	RGS16 65-181	RGS18 86-202	RGS6 336-441	RGS7 333-448	RGS9 298-413	RGS11 299-414	RGS10 33-148	RGS12 715-832	RGS14 67-184	RGS17 84-200	RGS19 90-206	RGS20 262-378
RGS1 72-187		53	54	55	53	54	49	52	53	40	40	47	43	46	41	40	40	45	43
RGS2 83-199	53		55	52	56	60	50	58	56	37	39	37	34	37	37	37	38	41	42
RGS3 1073-1198	54	55		61	59	62	47	60	53	41	40	41	42	44	42	39	43	47	47
RGS4 62-178	55	52	61		57	58	50	56	53	34	34	39	38	43	40	41	40	43	46
RGS5 64-180	53	56	59	57		59	42	56	56	38	42	44	41	41	37	42	40	41	45
RGS8 56-171	54	60	62	58	59		48	73	55	37	40	41	44	43	41	41	40	48	47
RGS13 34-150	49	50	47	50	42	48		46	42	31	34	34	34	31	33	31	37	42	44
RGS16 65-181	52	58	60	56	56	73	46		52	39	39	41	43	40	43	43	40	47	45
RGS18 86-202	53	56	53	53	56	55	42	52		38	36	40	41	41	37	39	44	44	49
RGS6 336-441	40	37	41	34	38	37	31	39	38		80	48	47	37	37	38	39	37	36
RGS7 333-448	40	39	40	34	42	40	34	39	36	80		48	48	37	39	39	39	38	39
RGS9 298-413	47	37	41	39	44	41	34	41	40	48	48		62	44	41	42	39	39	35
RGS11 299-414	43	34	42	38	41	44	34	43	41	47	48	62		44	45	44	39	43	41
RGS10 33-148	46	37	44	43	41	43	31	40	41	37	37	44	44		53	54	41	38	39
RGS12 715-832	41	37	42	40	37	41	33	43	37	37	39	41	45	53		63	37	37	37
RGS14 67-184	40	37	39	41	42	41	31	43	39	38	39	42	44	54	63		34	40	37
RGS17 84-200	40	38	43	40	40	40	37	40	44	39	39	39	39	41	37	34		69	76
RGS19 90-206	45	41	47	43	41	48	42	47	44	37	38	39	43	38	37	40	69		76
RGS20 262-378	43	42	47	46	45	47	44	45	49	36	39	35	41	39	37	37	76	76	

[a] Sequence identities were calculated using t-coffee v.4.67

[b] the numerical range marks the first and last residue of the RGS domain in each RGS protein according to Uniprot annotation

Supplementary Table 2: "Hot spots" in RGS proteins identified by Rosetta's computational alanine scanning

	Number of predicted hot spots [b]	Number of predicted hot spots identified in our study as Significant & Conserved	Number of predicted hot spots identified in our study as Modulatory	Number of predicted hot spots that correspond to experimental "neutral residues" [c]
$G\alpha_{i1}$-RGS4	9	6	1	2
$G\alpha_{i1}$-RGS16	5	5	-	-
$G\alpha_{o}$-RGS16	8	6	-	2
$G\alpha_{i3}$-RGS10	8	7	1	-
$G\alpha_{i1}$-RGS1	8	8	-	-
$G\alpha_{i3}$-RGS8	9	7	2	-
$G\alpha_{t/i}$-RGS9	8	7	1	-
$G\alpha_{i3}$-RGS2* [d]	11	8	1	2

[a] Hot spot calculations were performed as in refs. 1,2.

[b] Residues with a predicted $\Delta\Delta G_{binding}$ > 1 kcal/mol (i.e. mutation to alanine predicted to destabilize the complex by >1 kcal/mol)

[c] Residues whose mutation to alanine did not impair RGS function in refs. 3-7.

[d] C106S/N184D/E191K gain-of-function triple mutant.

[e] The particular Modulatory residues identified by Rosetta are characterized by considerable surface area buried upon complex formation (>50Å).

Supplementary Methods

Protein expression, purification and GTPase assays. Clones for the RGS domains of human RGS1, RGS7, RGS8, RGS10, RGS14, RGS16, RGS17, RGS18 were provided by the Structural Genomics Consortium (Oxford, UK). RGS domains were expressed in the pLIC-SGC1 vector as N-terminally His_6-tagged fusion proteins [8]. The N-terminally His_6-tagged rat $G\alpha_o$ clone was a gift from Nikolai Artemyev (U. Iowa). Proteins were expressed and purified following ref. 8 as follows: Proteins were expressed in E. coli BL21(DE3) cells and grown in 0.5 liters of LB broth at 37°C until an $OD_{600\ nm}$ of ~0.6. The temperature was then reduced to 29°C and protein expression was induced by addition of 0.5 mM isopropyl-D-thiogalactopyranoside. Cells were harvested by centrifugation after 4 h followed by freezing the pellet at -80°C. Bacterial pellets were resuspended in Lysis Buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM b-mercaptoethanol, protease inhibitor cocktail (Roche)) and the cells were lysed using a Sonifier 450 (Branson Ultrasonics), followed by centrifugation at 27,000 g for 30 min at 4°C. The supernatant was equilibrated to 500 mM NaCl and 20 mM imidazole and loaded onto Ni-NTA agarose beads (Qiagen) in a glass column at 4°C. The column was washed with >20 volumes of Wash Buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole) and the tagged proteins were eluted with Elution Buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 100 mM imidazole). The eluate was dialyzed against Dialysis Buffer (50 mM Tris, pH 8.0, 40% (v/v) glycerol, 50 mM NaCl, 5 mM b-mercaptoethanol). All purified proteins were estimated to be >95% pure, as assessed by SDS-PAGE electrophoresis. Protein concentration was determined by $A_{280\ nm}$ using predicted extinction coefficients (ProtParam, Swiss Institute for Bioinformatics).

Single turnover GTPase assays. Single turnover GTPase assays using recombinant $G\alpha_o$ and various RGS proteins were conducted as in refs. 9,10. Briefly, $G\alpha_o$ in Reaction Buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 0.05% (v/v) polyoxyethylene, 5 mM EDTA, 5 mg ml^{-1} BSA, 1 mM dithiothreitol) was incubated for 15 min at 25°C with 1 mM [γ-^{32}P]GTP and cooled on ice for 5 min. GTP hydrolysis was initiated by 10 mM $MgCl_2$ with 100 mM cold GTP (final concentrations) with or without RGS proteins at 4°C. Aliquots, taken at 7–8 different time points, were quenched with 5% charcoal in 50 mM $Na_2H_2PO_4$ (pH 3), followed by centrifugation and liquid scintillation analysis of $^{32}P_i$ in supernatants. GTPase rates were determined from single exponential fits to the time courses. k_{gap} rate constants were determined by subtracting the basal GTPase rate (without RGS protein) from the GTPase rate measured in the presence of the RGS protein [9,10].

Surface Plasmon Resonance (SPR) Assays. $G\alpha_o$ was expressed with a C-terminal Biotin tag and immobilized to a streptavidin chip as described [11]. SPR binding experiments were conducted using a Biacore 3000 or 2000 biosensor (GE Healthcare) after equilibrating the sensor surfaces, pump, and fluidic systems with 10 mM HEPES (pH 7.4), 150 mM NaCl, 6 mM $MgCl_2$, 0.05% (v/v) Nonidet P-40, and GDP–AlF_4^- (100 mM GDP, 20 mM NaF, 30 mM $AlCl_3$). Maximal resonance units achieved with each injection, as a percentage of the highest measurement, were plotted against RGS concentration. K_D values were calculated by fitting the dose response curves to a single site binding equation using GraphPad Prism 5.0, and K_D values from independent experiments were combined as weighed averages with respect to their corresponding error in a maximum likelihood framework [12].

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