SUPPLEMENTAL DATA

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Purified proteins used in this study. Purified recombinant proteins (2 μg ea) were resolved by SDS-PAGE and visualized by Coomassie Blue staining. Proteins were produced in insect cells unless indicated. Ric-8 proteins are the versions cleaved from amino-terminal GST fusion tags using TEV protease and purified by anion exchange and gel filtration chromatographies. Lane 1. Ric-8A (Figs. 2-4, 6, 7, 4S, 5S, 7S), 2. Ric-8BFL (Figs. 2-7, 4S, 6SA, 7S), 3. Ric-8BΔ9 (Figs. 2-7, 4S, 6SB, 7S), 4. Myristoylated $G\alpha_{i1}$ from *E. coli* (Figs. 1-3), 5. $G\alpha_{s\ short}$ from *E. coli* (Figs. 1-7, 6SA, 6SB, 7S), 6. $G\alpha_{s\ short}$ (data not shown), 7. $G\alpha_q$ (Figs. 1, 2, 4, 5, 7, 4S), 8. $G\alpha_{13}$ (Figs. 1, 2), 9. $G\beta_1\gamma_2$ (Figs. 1, 4).

Fig. S2. Quantitative densitometry analysis of G protein immunoblots. Immunoblots (Fig. 1A) of increasing doses of purified G protein subunit standards were quantified by densitometry analyses using Photoshop CS4 (Adobe Systems, Inc). Purified $G\alpha_{i1}$ was detected with BO84 antiserum[1],specific to $G\alpha_{i1/2}$; $G\alpha_q$ was detected with C-19 antibody (Santa Cruz); $G\alpha_{13}$ was detected with A-20 antibody (Santa Cruz); $G\alpha_{s\ short}$ was detected with 584 antiserum[2]; $G\beta_1$ was detected with B600 antiserum[1], a pan-Gβ antiserum that detects $G\beta_{1\text{-}4}$ subunits. Background-subtracted pixel densities were plotted versus purified G protein subunit (ng). Standard curves were generated by fitting the data to one-phase exponential association functions (GraphPad Prism v5.0). The amounts of individual G protein subunits isolated in GST-Ric-8 pull-down experiments were then determined by solution of the standard curve function using the experimental densitometry value obtained from immunoblots of each G protein subunit. A sample calculation of the amount of $G\alpha_{13}$ isolated from the brain membrane extract with GST-Ric-8A is presented. The measured concentrations of each G protein subunit as they were extracted from rat brain membranes with detergent are reported as ng G protein per mg of total extracted protein, as a percentage of total extracted protein, and the estimated concentration present in the Ric-8 pulldown input samples (nM). Total extracted protein was quantified by Amido-black protein assay. Note: The actual proportions of cross-reacting G protein subtypes present in the extract (*e.g.* $G\alpha_{i1}$ and $G\alpha_{i2}$ with B084 antiserum) could not be calculated because the subtypes could neither be resolved by SDS-PAGE, nor were the precise sensitivities of the antibodies towards each specific G protein subtype known.

Fig. S3. A low amount of $G\alpha_{s\ long}$ was recovered with GST-Ric-8BΔ9 from membrane detergent extracts. A higher exposure of the $G\alpha_s$ immunoblot shown in Fig. 1A showed that Ric-8BΔ9 bound $G\alpha_{s\ long}$ from the detergent membrane extracts.

Fig. S4. Ric-8 proteins interact selectively with $G\alpha_{s\ short}$ or $G\alpha_q$ to affect endpoint GTPγS binding stoichiometry. A, $G\alpha_{s\ short}$ and **B,** $G\alpha_q$ (100 nM ea) were incubated in 30 min (end-point) GTPγS binding reactions at 25 ºC containing 10 μM [^{35}S]GTPγS (SA 10,000 cpm/pmol) and Ric-8 proteins (0, 0.1, 0.5, 1, and 5 μM) as indicated. The amount of Gα bound GTPγS after 30 min was quantified using the GTPγS nitrocellulose filter binding assay.

Fig S5. Active $G\alpha_{s\ short}$ was recovered with high efficiency after incubation with Ric-8BFL or Ric-8BΔ9 and GTPγS at 25 ºC. $G\alpha_{s\ short}$ (10 μM) was loaded with 100 μM [^{35}S]GTPγS (SA 3000 cpm/pmol) for 30 min at 25 ºC. **A,** Ric-8BFL or **B,** Ric-8BΔ9 (5 μM ea.) were added to the $G\alpha_{s\ short}$ load reactions and incubated for an additional 30 min at 25 ºC. The protein/nucleotide mixtures were centrifuged at 20,000 x g for 5 min to remove particulate, and gel filtered over Superdex 75 and Superdex 200 columns arranged in tandem. The column eluates were fractionated, and protein-containing fractions

were analyzed by Coomassie-stained SDS-PAGE, and scintillation counting to quantify the amount of GTPγS contained in each fraction. The amount of total protein in each fraction was quantified by Bradford assay and the molar percentages of Ric-8B and $G\alpha_{s\ short}$ were determined by ratioing the signals of the respective Coomassie-stained protein bands using IMAGE J lane profile plots (NIH)[3] (vertical rectangular selection for each gel lane) and back calculating from the amount of total protein present in each fraction while factoring in the molecular mass of each.

Fig. S6. Ric-8 proteins do not activate heterotrimeric G protein steady state GTPase activities. A, $G\alpha_{s\ short}$ or **B,** $G\alpha_q$ (50 nM ea, total 1 pmol per assay) were pre-incubated with or without $G\beta_1\gamma_2$ (500 nM) for 15 min at 22 ºC. Ric-8A, Ric-8BFL or Ric-8BΔ9 proteins (0 or 500 nM ea) were added and steady-state GTPase reactions were initiated by the addition of 500 nM or 50 μM [γ-^{32}P]GTP (SA 10,000 – 80,000 cpm/pmol). Triplicate aliquots were quenched at 7.5 min in acidic charcoal suspension and processed. The pmols of phosphate (Pi) released per min for each condition were plotted using GraphPad Prism v5.0. Results are presented as the mean ± S.E.M. of three experiments. Note: most error bars are smaller than the actual plotted symbols.

Fig S7. Ric-8A binds $G\alpha_q$-GTPγS. $G\alpha_q$ (10 uM) and Ric-8A (5 uM) were incubated with 100 μM [^{35}S]GTPγS (SA 5,000 cpm/pmol) for 15 min at 25 ºC. The protein/nucleotide mixture was centrifuged to remove particulate, and gel filtered over Superdex 75 and Superdex 200 gel filtration columns arranged in tandem. The column eluate was fractionated, and protein-containing fractions were analyzed by Coomassie-stained SDS-PAGE and scintillation counting to quantify the amount (pmol) of GTPγS contained in each fraction. The lines beneath the Coomassie-stained SDS-PAGE show the relative positions that the Ric-8A:$G\alpha_q$ complex, monomeric Ric-8A, and monomeric $G\alpha_q$ eluted from the gel filtration columns (l to r is decreasing molecular weight).

Fig. S8. Ric-8BFL-stimulated $G\alpha_{s\ short}$ GTPγS release did not go to completion when challenged with GDP. $G\alpha_{s\ short}$ (100 nM) was loaded to completion with 10 μM [^{35}S]GTPγS for 30 min at 30 ºC. $G\alpha s_s$ GTPγS release was initiated by addition of unlabeled GDP (100 μM) (○) and/or Ric-8A (●), Ric-8BFL (■), or Ric-8BΔ9 (▲) (500 nM ea). Reactions were quenched at the indicated time points and the amount of GTPγS that remained bound to $G\alpha_{s\ short}$ was measured using the nitrocellulose filter binding method [4,5]. Ric-8BFL-stimulated $G\alpha_s$ GTPγS release went to completion in the presence of excess GTPγS (Fig. 7), but did not in the presence of excess GDP. Therefore, Ric-8BFL-stimulated GTPγS release is a combination of the processes of nucleotide-free Ric-8BFL:$G\alpha_{s\ short}$ production and $G\alpha_{s\ short}$ GTPγS for GTPγS futile nucleotide exchange. Results shown are in duplicate and are representative of three independent experiments.

REFERENCES

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2. Mumby, S. M., and Gilman, A. G. (1991) Synthetic peptide antisera with determined specificity for G protein α or β subunits. in *Methods Enzymol.* (Roger, A. J., and Jackie, D. C. eds.), Academic Press. pp 215-233
3. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) *Biophotonics International* **11**, 36-42
4. Sternweis, P. C., and Robishaw, J. D. (1984) *J. Biol. Chem.* **259**, 13806-13813
5. Tall, G. G., Krumins, A. M., and Gilman, A. G. (2003) *J. Biol. Chem.* **278**, 8356-8362

Figure S1

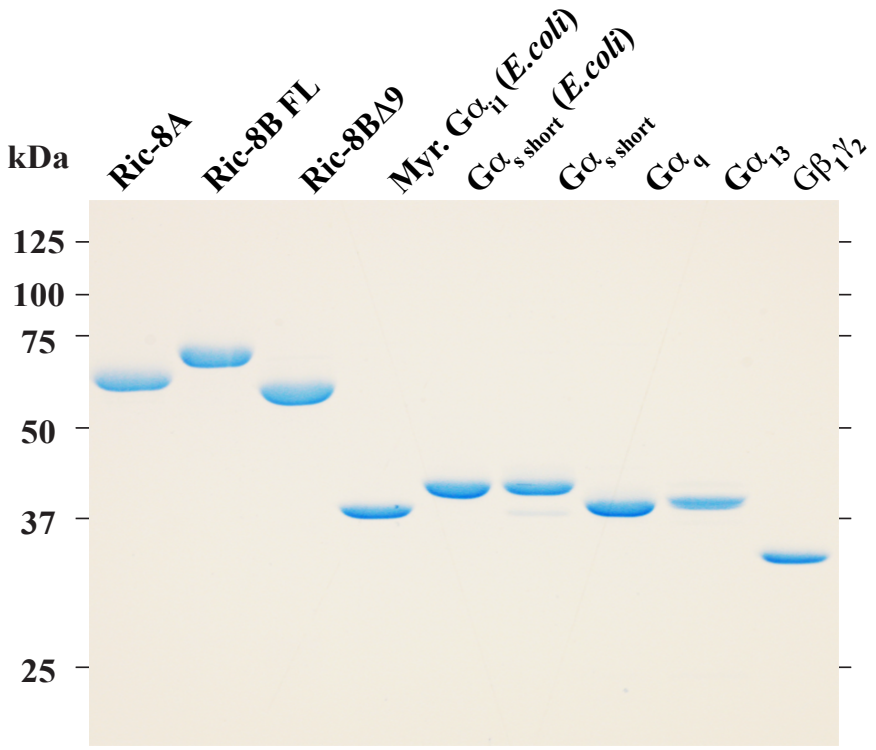
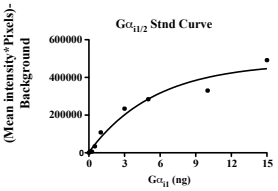
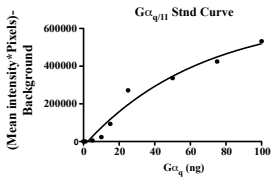
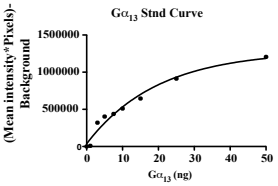

kDa
Ric-8A
Ric-8B FL
Ric-8BΔ9
Myr. Gαi1 (E.coli)
Gαs short (E.coli)
Gαs short
Gαq
Gα13
Gβ1γ2
125
100
75
50
37
25

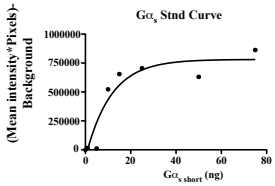
Figure S2

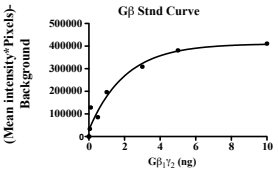
Fig. 2S Quantitative Densitometry Analysis of G protein Immunoblots











Standard Curves (One-phase association):

$Y = Y_0 + (Ymax - Y_0) * (1-e^{-kx})$

$X = \frac{\ln ((1-((Y-Y_0)/(Ymax-Y_0))))}{-k}$

X = ng Gα or ng Gβ

Y = (Mean intensity*Pixels)-Background

Sample Calculation GST-Ric-8A membrane extract pulldown

Total $G\alpha_{13}$ present in the 150 µl TEV protease elution:

ng $G\alpha_{13}$ detected by immunoblotting
= ln((1-((245460.6-37623)/(1285000-37623))))/-0.05051
= 3.61 ng $G\alpha_{13}$

3.61 ng $G\alpha_{13}$ / 5 µl of sample loaded on gel = 0.72 ng $G\alpha_{13}$ / µl TEV eluate

0.72 ng/µl * 150 µl = 108.25 ng $G\alpha_{13}$

Measured Concentrations of G proteins Obtained from Rat Brain Membrane Detergent Extraction

Subunit	Amount in extract (ng/mg)	% of Total Extract	nM
$G\alpha_{i1/2}$	166.7	0.017	31.7
$G\alpha_{q/11}$	484.1	0.048	87.6
$G\alpha_{13}$	135.9	0.014	24.3
$G\alpha_{s\,long}$	61.3	0.006	10.4
$G\beta_{1-4}$	1434.1	0.14	294.6

Figure S3

Higher Exposure of Figure 1A $G\alpha_s$ Immunoblot

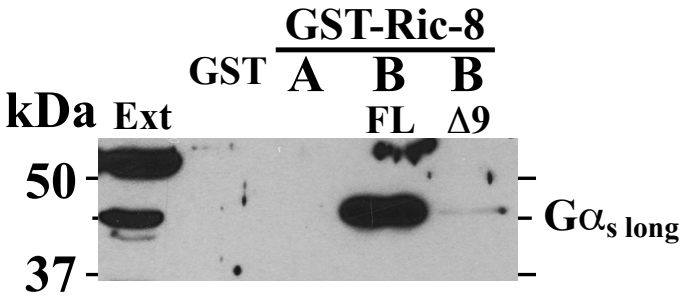


Figure S4

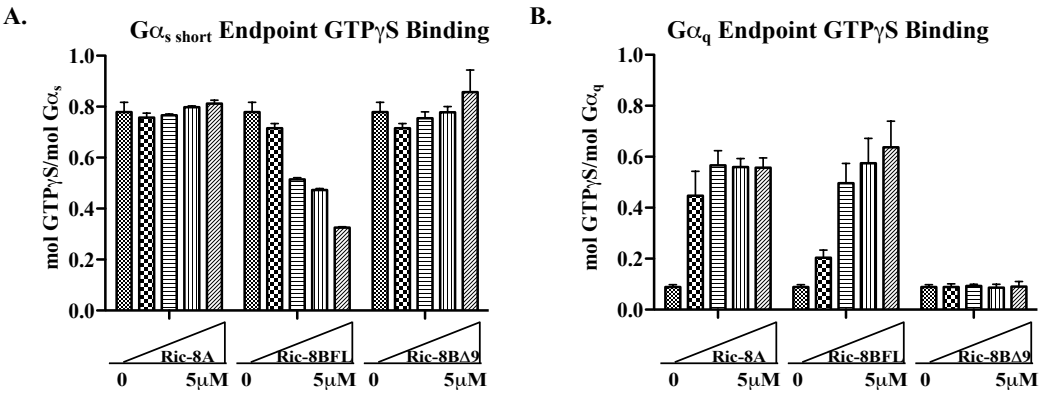
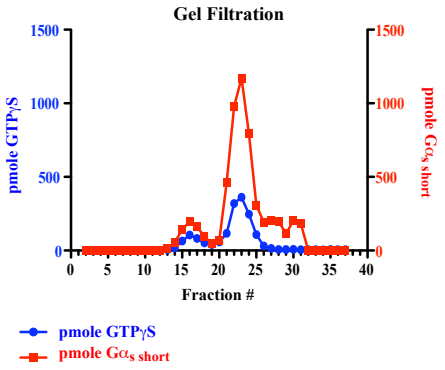

A.
Gαs short Endpoint GTPγS Binding
mol GTPγS/mol Gαs
1.0
0.8
0.6
0.4
0.2
0.0
Ric-8A
Ric-8BFL
Ric-8BΔ9
0
5μM
0
5μM
0
5μM
B.
Gαq Endpoint GTPγS Binding
mol GTPγS/mol Gαq
1.0
0.8
0.6
0.4
0.2
0.0
Ric-8A
Ric-8BFL
Ric-8BΔ9
0
5μM
0
5μM
0
5μM

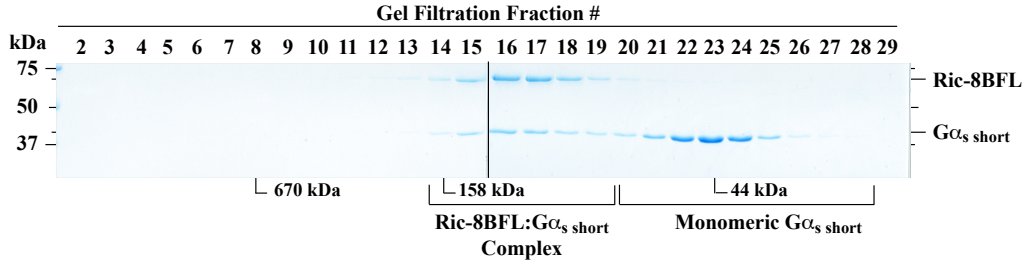
Figure S5

Recovery of $G\alpha_{s\ short}$ protein after incubation with Ric-8BFL + GTPγS at 25ºC for 30 min.

A. Ric-8BFL + $G\alpha_{s\ short}$

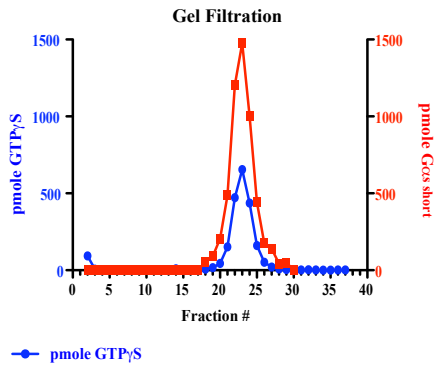


Fraction #	Bradford Assay Total Protein µg/µl	IMAGE J Estimation of Ric-8B:$G\alpha_{ss}$	Bradford Assay Estimation of $G\alpha_{ss}$ (pmol)	GTPγS (pmol)	Fractional GTPγS binding (mol/mol)
14	0.038	68.1 : 31.9	55.49	18.19	0.33
15	0.080	69.1 : 30.9	113.25	64.81	0.57
16	0.123	69.8 : 30.2	169.47	105.26	0.62
17	0.099	69.3 : 30.3	136.15	81.44	0.60
18	0.064	67.9 : 32.1	93.72	51.26	0.55
19	0.031	54.3: 45.7	64.35	40.99	0.64
20	0.034	28 : 72	110.33	58.68	0.53
21	0.042	6.8 : 93.2	433.05	116.97	0.27
22	0.088	3.9 : 96.1	941.59	319.09	0.34
23	0.105	3.5 : 96.1	1130.71	362.15	0.32
24	0.071	0 : 100	792.93	246.47	0.31
25	0.028	0 : 100	308.08	107.76	0.35
26	0.017	0 : 100	191.92	30.69	0.16
27	0.019	0 : 100	207.07	14.95	0.07
28	0.018	0 : 100	196.97	6.87	0.03
Total	**428.6 µg**		**4945.07**	**1625.58**	
Input	**450 µg**		**5000**	**4945.07**	
			99% (recovery)	**~33% (GTPγS bound)**	



Recovery of $G\alpha_{s\ short}$ protein after incubation with Ric-8BΔ9 + GTPγS at 25ºC for 30 min.

B. Ric-8BΔ9 + $G\alpha_{s\ short}$



Fraction #	Bradford Assay Total Protein µg/µl	IMAGE J Estimation of Ric-8B:$G\alpha_{ss}$	Bradford Assay Estimation of $G\alpha_{ss}$ (pmol)	GTPγS (pmol)	Fractional GTPγS Binding (mol/mol)
20	0.037	56.7 : 43.3	89.11	44.49	0.50
21	0.044	13.9 : 86.1	419.63	152.15	0.36
22	0.108	4.4 : 95.6	1204.55	472.16	0.39
23	0.133	1.6 : 98.4	1453.64	653.80	0.45
24	0.090	0 : 100	1002.53	436.86	0.44
25	0.040	0 : 100	441.92	160.96	0.36
26	0.016	0 : 100	174.24	51.68	0.30
27	0.013	0 : 100	138.89	20.67	0.15
28	0.004	0 : 100	42.93	7.98	0.19
Total	**242.16 µg**		**4967.43**	**2000.75**	
Input	**450 µg**		**5000**	**4967.43**	
	Frac. 5-19 (185.8 µg)		**99% (recovery)**	**~40% (GTPγS bound)**	

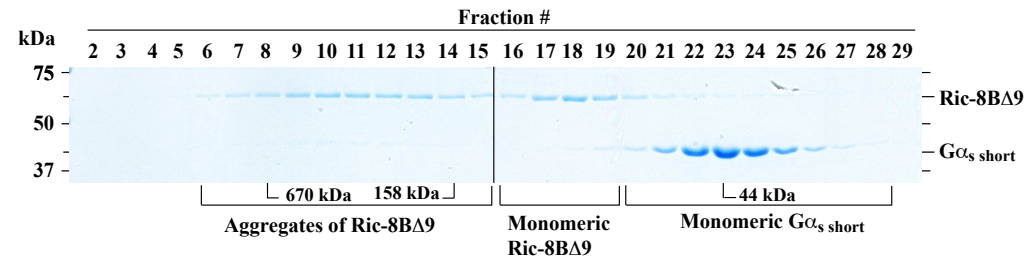


Figure S6

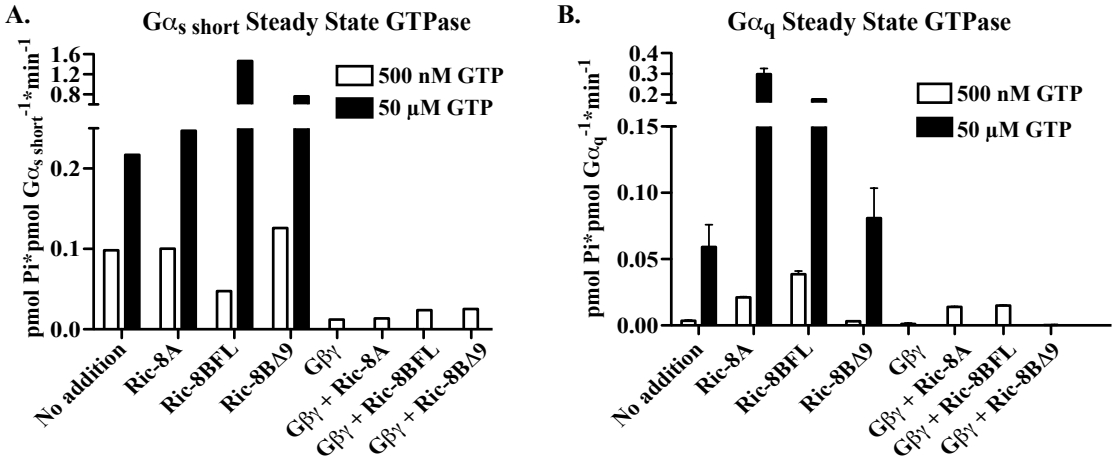

A.
Gαs short Steady State GTPase
pmol Pi*pmol Gαs short-1*min-1
1.6
1.2
0.8
0.2
0.1
0.0
500 nM GTP
50 µM GTP
No addition
Ric-8A
Ric-8BFL
Ric-8BΔ9
Gβγ
Gβγ + Ric-8A
Gβγ + Ric-8BFL
Gβγ + Ric-8BΔ9
B.
Gαq Steady State GTPase
pmol Pi*pmol Gαq-1*min-1
0.4
0.3
0.2
0.15
0.10
0.05
0.00
500 nM GTP
50 µM GTP
No addition
Ric-8A
Ric-8BFL
Ric-8BΔ9
Gβγ
Gβγ + Ric-8A
Gβγ + Ric-8BFL
Gβγ + Ric-8BΔ9

Figure S7

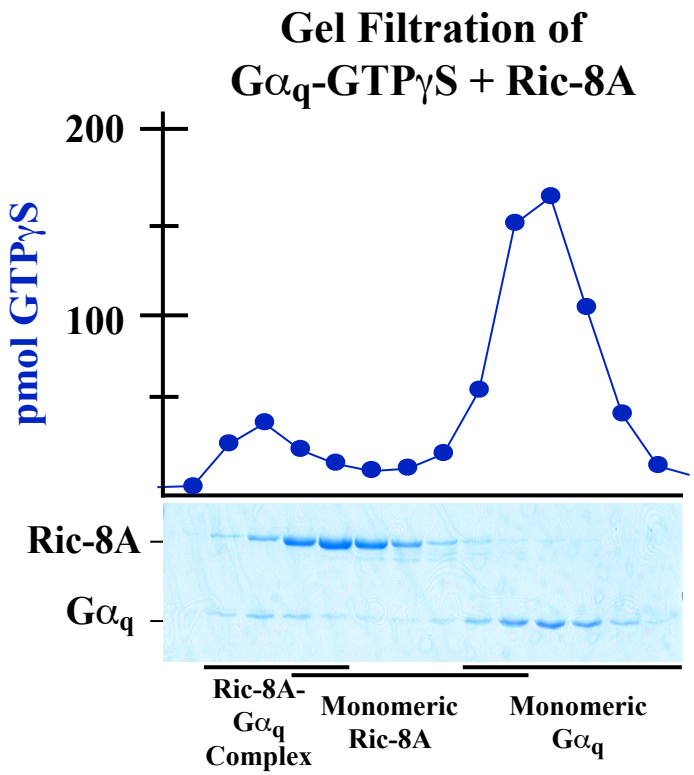
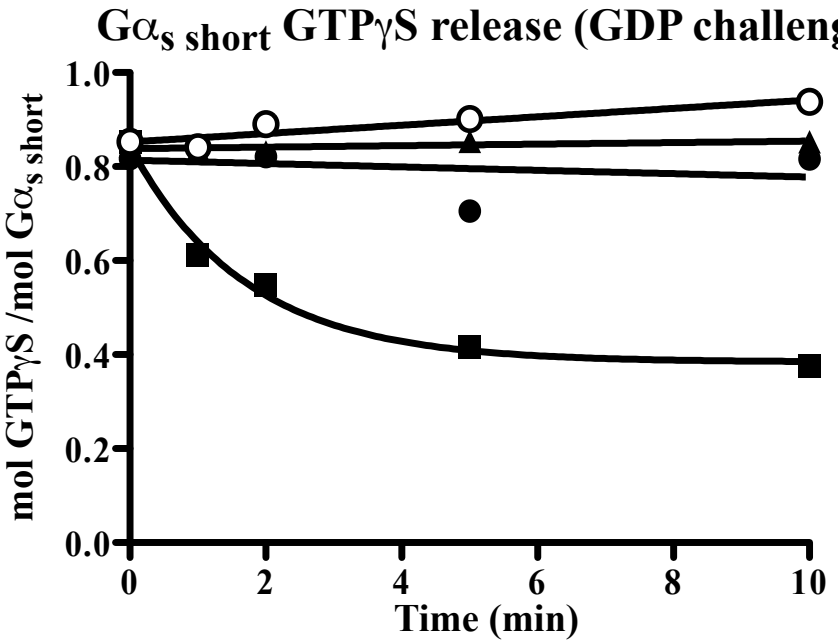

Gel Filtration of
Gαq-GTPγS + Ric-8A
200
100
pmol GTPγS
Ric-8A
Gαq
Ric-8A-
Gαq
Complex
Monomeric
Ric-8A
Monomeric
Gαq

Figure S8


Gαs short GTPγS release (GDP challenge)
mol GTPγS /mol Gαs short
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Time (min)