Selective Uncoupling of RGS Action by a Single Point Mutation in the G Protein \(\alpha\)-Subunit*

(Received for publication, December 22, 1997, and in revised form, January 14, 1998)

Paul R. DiBello‡‡, Tiffany Runyan Garrison‡‡, Donald M. Apanovitch‡, Ginger Hoffman**, David J. Shuey‡‡, Kimberly Mason‡‡, Mark I. Cockett‡‡, and Henrik G. Dohlman¶¶

Heterotrimeric G proteins function as molecular relays, shuttling between cell surface receptors and intracellular effectors that propagate a signal. G protein signaling is governed by the rates of GTP binding (catalyzed by the receptor) and GTP hydrolysis. RGS proteins (regulators of G protein signaling) were identified as potent negative regulators of G protein signaling pathways in simple eukaryotes and are now known to act as GTPase-activating proteins (GAPs) for G protein \(\alpha\)-subunits in vitro. It is not known, however, if Ga GAP activity is responsible for the regulatory action of RGS proteins in vivo. We describe here a Ga mutant in yeast (gpa1sst) that phenotypically mimics the loss of its cognate RGS protein (SST2). The gpa1sst mutant is resistant to an activated allele of SST2 in vitro and is unresponsive to RGS GAP activity in vitro. The analogous mutation in a mammalian G, \(\alpha\) is also resistant to RGS activity in transfected cells. These mutants demonstrate that RGS proteins act through Ga and that RGS-GAP activity is responsible for their desensitizing activity in cells. The Ga\(^{sst}\) mutant will be useful for uncoupling RGS-mediated regulation from other modes of signal regulation in whole cells and animals.

A wide variety of cellular signals (hormones, neurotransmitters, light, odors) act through a three component system composed of cell surface receptors, heterotrimeric G proteins, and effector proteins (1). The mating pheromones in yeast *Saccharomyces cerevisiae* act through receptors (STE2, STE3 gene products), a G protein \(\alpha\)-heterotrimer (GPA1, STE4, STE18), and a mitogen-activated protein kinase signaling cascade that promotes cell division arrest and fusion (2). If mating is unsuccessful, however, the cells become refractory to pheromone stimulation and will eventually resume normal growth.

RGS\(^1\) proteins have recently been identified as a fourth component of the G protein signaling pathway (2, 3). The founding member of the RGS family, called SST2, was identified in a genetic screen for negative regulators of the pheromone response pathway in yeast (4). Loss of function sst2 mutants render cells supersensitive to a pheromone stimulus and unable to recover from pheromone-induced growth arrest. Dominant gain-of-function alleles of SST2 have the opposite effect, rendering cells insensitive to pheromone stimulation (5). Further genetic and biochemical experiments revealed that Sat2 interacts directly with the G protein \(\alpha\)-subunit, Gpa1 (6).

Behavioral genetic analyses in *C. elegans* uncovered a homologue of Sat2, called EGL-10 (7). egl-10 was shown to negatively regulate goa-1, which encodes the Ga that mediates serotonin-dependent egg laying behavior. Two mammalian homologues, GAIP and RGS10, were identified by their interaction with Ga-subunits in a two-hybrid screen (8, 9). An additional 15 mammalian members of the family were found by expression cloning, degenerate polymerase chain reaction, low stringency hybridization, and as expressed sequence tags (7–11). All of the RGS proteins share a conserved “RGS core domain” of ~120 amino acids, with >20% sequence identity across all species. Several RGS proteins have also been shown to attenuate G protein signaling in cultured cells (12–15) and to partially substitute for the loss of SST2 expression in yeast (10, 12, 15).

RGS proteins were later shown to function as GTPase-activating proteins (GAPs) for Ga-subunits in vitro (9, 11, 16–22). These findings suggest that RGS proteins negatively regulate signaling via their physical association with Ga-subunits. By enhancing the rate of Ga GTP hydrolysis, RGS proteins would shorten the lifetime of the active G protein species and arrest signaling.

Does RGS GAP activity account for the negative regulatory properties of these proteins in vivo? Proving this model would require that RGS knockout mutants, and Ga mutants that disrupt RGS interaction, exhibit the same phenotype. RGS mutations have been obtained in yeast and nematodes, but not in mammals. Indeed, constructing knockout mutants in mammals will be complicated by the fact that there are so many closely related (and possibly redundant) RGS isoforms. An RGS-insensitive Ga mutant has not been reported in any system.

Here, we report the identification and characterization of a yeast Ga mutation that specifically disrupts Sat2 regulation in

---

1. The abbreviations used are: RGS, regulator of G protein signaling; GAP, GTPase-activating protein; GAIP, G protein \(\alpha\)-interacting protein; GST, glutathione S-transferase; 5-HT, serotonin; IP\(_3\), inositol triphosphate; GTP\(^\gamma\)S, guanosine 5’-3-O-(thio)triphosphate.
2. Apanovitch, D. M., Slep, K. C., Sigler, P. B., and Dohlman, H. G. (1998) Biochemistry, in press.
vivo and in vitro. An analogous mutation in Grqα is similarly insensitive to RGS action in cultured cells. An RGS-uncoupled Ga mutation proves that Ga is the primary target of RGS in cells. Ga mutants of this type will be extremely useful for determining the overall contribution of RGS proteins to signal regulation in vivo.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Antibodies—**Established methods were used for the growth and genetic manipulation of yeast and bacteria (23). *Escherichia coli* strain DH10B was used for the maintenance of plasmids; strain BL21 (DE3) was used for expression of recombinant Gpa1 and RGS. *S. cerevisiae* strains used in this study were: YHD436 (MATa ura3–52 lys2–801am ade2–101oc trp1–D63 his3–D200 leu2–D1 bar1::hisG mf1::hisG mf2::hisG FUS1-LacZ-URA3) for mutagenesis and screening, YGS5 (24) or YRGS5 (YGS5, sst2–D2) (24) for halo assays, and BJ2168 (25) for purification of Sst2-GST. Yeast expression plasmids were pRS315, pRS316, pAD4M, pRS315-GPA1, pAD4M-GPA1, pRS315-gpa1sst, pAD4M-gpa1sst (24), pRS315-SST2, pRS315-SST2-1 (5), pAD4M-GST (24), pCDNAm-pGq (38). pcDNAamp-Grqsst was constructed by oligonucleotide-directed mutagenesis in pcDNAamp-GrqEE2 (26) (Altered Sites, Promega). pAD4M-SST2-GST was provided by K. Blumer, Washington University. Antibodies to Gpa1 (27), EE (Babco), and GST (J. Steitz, Yale University) are described elsewhere. Molecular modeling of the Gi1α-GDP-AlF42/RGS4 complex (23) (PDB accession number 1AGR) was performed using SYBYL (Tripos, St. Louis, MO).

**Mutagenesis Screening and Pheromone Assays—**YHD436 cells were mutagenized with ethyl methanesulfonate (Sigma) as described (28) and assayed for α-factor supersensitivity using a reporter gene (β-galactosidase) assay on nitrocellulose colony lifts (29). A genomic DNA library in YCp50 was used for complementation cloning (ATCC 77162). The halo assay was performed as described (29).

**Sst2-GST/Gpa1 Binding, GTPase Assays, and Mammalian Cell Culture Assays—**Sst2-GST binding experiments were performed as described (6) with the following modifications: 50 mM Hepes (pH 7.5) and 0.1% Tween were used instead of 40 mM triethanolamine and 1% Triton X-100. Washes were performed with binding buffer at various salt concentrations instead of in phosphate-buffered saline. Binding and wash buffers contained 5 mM MgCl2 and either 10 mM GDP or 10 μM GDP, 30 μM AlCl3, 10 mM NaF. Purification of recombinant Go and RGS proteins (>95% homogeneity), as well as guanine nucleotide binding

---

**Fig. 1. Pheromone response of GPA1 and gpa1sst.**

A. YRG55 (gpa1Δ, sst2Δ) cells expressing either GPA1 or gpa1sst, and either wild type SST2 or vector alone, were plated and exposed to filter discs containing 10 μg α-factor. Cells were allowed to grow for 48 h on selective medium before being photographed. B. YRG55 (gpa1Δ) cells expressing either GPA1 or gpa1sst, and either the dominant gain-of-function SST2-1 mutant or vector alone was plated as described in A.

**Fig. 2. GTP binding and GTPase assays of wild type Gpa1 and Gpa1sst.**

A. A purified Gpa1 or Gpa1sst was incubated with [35S]GTP-γS (5 μM) at 30 °C, and the percent of bound nucleotide was measured at the indicated times by membrane filtration. Symbols: ■ Gpa1; ● Gpa1sst.

B. Gpa1, Gpa1sst, or RGS (GAIP, as a negative control) was incubated with [γ-32P]GTP (5 μM) at 30 °C. Liberated 32Pi was measured at the indicated times by charcoal adsorption. Symbols: ■ Gpa1; ● Gpa1sst; ● RGS.

C. Gpa1 and Gpa1sst (250 nM) were incubated with [γ-32P]GTP (1 μM) for 30 min at 30 °C. The samples were placed in ice for 5 min, and Mg2+ (an essential cofactor) was added to initiate the reaction, either in the presence or absence of RGS (GAIP, 20 μM) (t = 0). 32Pi was measured as described in B. Symbols: ■ Gpa1; ● Gpa1 + RGS; ● Gpa1sst; ● Gpa1sst + RGS. Data shown are the average of duplicate measurements and are representative of at least two independent experiments.
and hydrolysis assays, were performed as described previously (19). Mammalian cell culture, transfections, and second messenger assays are described elsewhere (38). Data were analyzed using analysis of variance for a randomized block design, with log transformation, followed by pairwise comparisons employing the least significant difference method.

RESULTS AND DISCUSSION

In the course of a large scale genetic screen designed to identify new desensitization components in yeast, we isolated a novel allele of GPA1, designated gpa1<sup>1st</sup> (for supersensitive allele of gpa1). Sequencing revealed a single missense mutation resulting in a Gly-to-Ser substitution at position 302. This glycine is conserved among Ga-subunit family members and is located in the first of three switch regions known to undergo a conformational change upon GTP hydrolysis (30–36).

Since the gpa1<sup>1st</sup> mutant mimics the loss of SST2, we investigated its ability to be regulated by SST2. First we compared the pheromone response in cells expressing GPA1 or gpa1<sup>1st</sup>, using the growth inhibition (“halo”) assay (29). In SST2<sup>−</sup> cells, the pheromone response through gpa1<sup>1st</sup> was potentiated compared with GPA1. In the absence of SST2, however, the wild type and mutant forms of Gpa1 responded equally (Fig. 1A). In cells expressing SST2<sup>−</sup>–1 (a dominant mutant that promotes pheromone desensitization), there was a striking difference between wild type and mutant GPA1 (Fig. 1B). Cells expressing GPA1 and SST2<sup>−</sup>–1 exhibited a greatly attenuated response to pheromone, resulting in a “filled in” halo. However, cells containing SST2<sup>−</sup> and gpa1<sup>1st</sup> responded no differently than cells containing gpa1<sup>1st</sup> alone. Clearly, the Gly-to-Ser mutation blocks the negative regulatory effect of SST2 in vivo. Since there is no functional difference between Gpa1 and Gpa1<sup>1st</sup> in the absence of Sst2 expression, we conclude that Gpa1<sup>1st</sup> is fully competent to transmit a pheromone signal and interacts normally with Gβγ and the receptor.

Sst2 is thought to act by binding to Gpa1 and stimulating its GTPase activity. Therefore, genetic uncoupling of SST2 and gpa1<sup>1st</sup> should accompany a physical and/or functional uncoupling of the two proteins. To test this, we purified each of the proteins and compared the ability of Gpa1 and Gpa1<sup>1st</sup> to bind and hydrolyze GTP. We first measured the rate of [γ<sup>35</sup>S]GTPγS binding and steady state [γ<sup>32</sup>P]GTP hydrolysis and found no difference between the wild type and mutant forms of Gpa1 (Fig. 2, A and B). We then compared the ability of each protein to catalyze the rate-limiting hydrolytic step of the reaction, using a single turnover assay in the absence or presence of a purified RGS protein (GAIP, Fig. 2C). GAIP is functionally equivalent to Sst2, but is more stable and can be purified in much larger quantities. In the absence of RGS, the initial k<sub>cat</sub> of hydrolysis was ~0.006 min<sup>−1</sup> for both wild type and mutant. With the addition of RGS, however, the rate of hydrolysis was greatly accelerated (at least 20-fold) for Gpa1 but not at all for Gpa1<sup>1st</sup>. A more accurate determination of the RGS-stimulated GTPase rate could not be made, as the reaction was essentially complete at the first time point. Thus Gpa1<sup>1st</sup> can bind and hydrolyze GTP normally, but is completely unresponsive to RGS GAP activity. These results are consistent with the in vivo experiments where, in the absence of Sst2 expression, Gpa1<sup>1st</sup> can signal as well as the wild type.

Despite the complete loss of GAP activity in vitro, Gpa1<sup>1st</sup> does not equal the loss of SST2 in vivo. There are at least two possible explanations for this difference. First, Sst2 could regulate proteins other than Gpa1. Such interactions could involve the N-terminal 300 amino acid region of Sst2, a domain that is not found in any other RGS protein. A less likely alternative is that Gpa1<sup>1st</sup> is weakly activated by RGS in vivo but not in vitro.

The X-ray crystal structure has recently been solved for RGS4 complexed with G<sub>i</sub>α and GDP-AlF<sub>4</sub><sup>−</sup>, a transition state mimic (37). The structure suggests a mechanism in which RGS promotes hydrolysis by stabilizing the transition state conformation of Ga. A key prediction of this model is that loss of GAP activity should accompany a loss of RGS binding. Accordingly, we compared the ability of mutant and wild type Gpa1 to bind Sst2 in vitro. Yeast lysates containing an Sst2-GST fusion protein were adsorbed onto glutathione-Sepharose and mixed with similarly prepared lysates containing Gpa1 or Gpa1<sup>1st</sup>. The resin was washed at various NaCl concentrations, in the presence of either GDP or GDP-AlF<sub>4</sub><sup>−</sup> (Fig. 3). At 50 mM NaCl, both the wild type and mutant were retained by Sst2-GST, but binding was not AlF<sub>4</sub><sup>−</sup>-dependent and is therefore likely to be nonspecific or nonfunctional. At higher salt concentrations (150–250 mM) Gpa1 was selectively retained when GDP-AlF<sub>4</sub><sup>−</sup> was present, but Gpa1<sup>1st</sup> did not bind at all. At very high concentrations (350 mM), neither protein was retained. Thus the inability of the RGS protein to act as a GAP for Gpa1<sup>1st</sup> appears to be due to a weakened protein-protein interaction.

Since we have shown that Gpa1<sup>1st</sup> can specifically block RGS action both in vivo and in vitro, we examined if a similar mutation in a mammalian Gα would also block RGS action in cultured cells. We created a Gly<sup>188</sup>→Ser mutation in G<sub>i</sub>α (G<sub>i</sub>α<sup>1st</sup>) and examined its sensitivity to RGS7 in cells co-transfected with the SHT<sub>2</sub> receptor. This particular combination of signaling proteins was chosen because they have overlapping expression patterns in the brain and are known to interact in cells (38). In cells expressing wild type G<sub>i</sub>α, 5-HT stimulation resulted in a typical calcium response (using Fura-2), which was attenuated ~40% by RGS7 expression (Fig. 4A). In cells expressing G<sub>i</sub>α<sup>1st</sup>, however, the response was completely refractory to RGS7 (Fig. 4B). To confirm the results obtained by calcium release, measurements of agonist-induced [3H]inositol...
trisphosphate (IP₃) production were performed on the same cells. Co-expression of RGS7 with wild type Gq,α reduced maximal IP₃ generation by ~30%, while co-expression with Gq,αsst had no effect (Fig. 4C). Thus, like Gpa1sst, Gq,αsst effectively couples to receptor and Gbgα, yet is resistant to the effects of RGS regulation. Since signaling in this case is mediated by Gq,α, rather than Gβγ (as it is in yeast), we can also conclude that effector coupling is unaltered by the Gly₁₈₈ → Ser mutation.

To determine how the Gly to Ser substitution can disrupt Go-RGS interactions in such a selective manner, we employed molecular modeling using the coordinates of the RGS4-Gi₁₈ crystal structure (37). The conserved Gly in Gq,α (Gly₁₈₅) is located directly opposite Glu₈₃ in RGS4 at the binding interface (Fig. 5). Buried surface area in this region accounts for 120 Å², or 22%, of the Go binding site. Substituting Gly with Ser would introduce a hydroxyl group less than 1 Å from the backbone.
carbonyl of Glu\textsuperscript{83}, an energetically unfavorable position both electrostatically and sterically (compare Fig. 5, B versus C). When mapped onto the crystal structure of G\textsubscript{i1,α}-RGS4, however, the same substitution shows no crowding at the βγ binding interface, no interference with guanine nucleotide binding, and no effect on the conformational changes that occur during GTP hydrolysis. In a direct test of this model, the corresponding mutation in G\textsubscript{i1,α} was shown to cause a >1000-fold reduction in RGS4 binding (by flow cytometry measurements) and >1000-fold reduction in GAP activity.\textsuperscript{3}

RGS proteins were first identified through genetic studies carried out in yeast (4,5). G proteins were identified as potent targets of RGS regulation, through enzymological studies carried out in mammals (9,19,21). With this first description of a mutation that selectively blocks Go interaction with RGS, their cellular target and mechanism of action are now firmly established. The next major challenge will be to disrupt Go-RGS interactions in animals. Knockout mutations of the RGS isoforms may be impractical, so Go\textsuperscript{α} mutants could be used instead to determine which signaling pathways are subject to RGS regulation and how desensitization of G proteins compares with desensitization of receptors. Thus we believe that Go\textsuperscript{α} mutants will prove useful for determining how RGS proteins regulate signaling, not just in cultured cells but also in whole animals.

Acknowledgments—We thank Takatoshi Karasawa for plasmid construction, Deborah Nassernk for technical assistance, Charlie Boone for discussion, and Steven Sprang for RGS4-G\textsubscript{i1,α} coordinates.

REFERENCES

1. Neer, E. J. (1995) Cell \textbf{80}, 249–257
2. Dohlman, H. G., and Thorner, J. (1997) \textit{J. Biol. Chem.} \textbf{272}, 3871–3874
3. Iyengar, R. (1997) Science \textbf{273}, 42–43
4. Chan, R. K., and Otte, C. A. (1982) \textit{Mol. Cell. Biol.} 2, 11–20
5. Dohlman, H. G., Apaniok, D., Chen, Y., Song, J., and Nussknern, D. (1995) \textit{Mol. Cell. Biol.} 15, 3635–3643
6. Dohlman, H. G., Song, J., Ma, D., Courchene, W. E., and Thorner, J. (1996) \textit{Mol. Cell. Biol.} 16, 5194–5209
7. Koelle, M. R., and Horvitz, H. R. (1996) Cell \textbf{84}, 115–125
8. DeVries, L., Mouli, M., Wurmser, A., and Farquhar, M. G. (1995) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{92}, 11916–11920
9. Hunt, T. W., Fields, T. A., Casey, P. J., and Peralta, E. G. (1996) \textit{Nature} \textbf{383}, 175–177
10. Chen, C., Zheng, B., Han, J., and Lin, S. C. (1997) \textit{J. Biol. Chem.} \textbf{272}, 8677–8685
11. Zeng, L., Paffo, F., Zhang, T., Hsu, W., Vasiek, T. J., Perry, W. S., Lee, J. J., Tlgihman, S. M., Gumbiner, B. M., and Costantini, F. (1997) \textit{Cell} \textbf{90}, 181–192
12. Dreyer, K. M., Blumer, K. J., Vang, V. H., and Kehrl, J. H. (1996) \textit{Nature} \textbf{379}, 742–746
13. Yan, Y., Chi, P. P., and Bourne, H. R. (1997) \textit{J. Biol. Chem.} \textbf{272}, 11924–11927
14. Huang, C., Hepler, J. R., Gilman, A. G., and Mumber, S. M. (1997) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{94}, 6159–6163
15. Siderovski, D. P., Hessel, A., Chung, S., Mak, T. W., and Tyers, M. (1996) \textit{Curr. Biol.} \textbf{6}, 211–212
16. Wieland, T., Chen, C. K., and Simon, M. I. (1997) \textit{J. Biol. Chem.} \textbf{272}, 8853–8856
17. Chen, C. K., Wieland, T., and Simon, M. I. (1996) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{93}, 12885–12889
18. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) \textit{J. Biol. Chem.} \textbf{271}, 27209–27212
19. Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) \textit{Cell} \textbf{86}, 445–452
20. Nekrasova, E. R., Berman, D. M., Bustandi, R. R., Hamm, H. E., Gilman, A. G., and Arshavsky, V. Y. (1997) \textit{Biochemistry} \textbf{36}, 7633–7643
21. Watson, N., Linder, M. E., Dreyer, K. M., Kehrl, J. H., and Blumer, K. J. (1996) \textit{Nature} \textbf{383}, 172–175
22. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{94}, 428–432
23. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) \textit{Current Protocols in Molecular Biology}, Wiley-Interscience, New York
24. Song, J., Hirschman, J., Gunn, K., and Dohlman, H. G. (1996) \textit{J. Biol. Chem.} \textbf{271}, 20273–20283
25. Jones, E. (1991) \textit{Methods Enzymol.} \textbf{194}, 428–453
26. Medina, R., Grihina, G., Meleni, E. G., Muth, T. R., and Berlot, C. H. (1996) \textit{J. Biol. Chem.} \textbf{271}, 24720–24727
27. Dohlman, H. G., Goldsmith, P., Spiegel, A. M., and Thorner, J. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{90}, 9688–9692
28. Sikorski, R. S., and Boeke, J. D. (1991) \textit{Methods Enzymol.} \textbf{194}, 302–318
29. Sprague, G. F., Jr. (1991) \textit{Methods Enzymol.} \textbf{194}, 21–37
30. Noell, J. P., Hamm, H. E., and Sigler, P. B. (1992) \textit{Nature} \textbf{354}, 658–663
31. Lambright, D. G., Noell, J. P., Hamm, H. E., and Sigler, P. B. (1994) \textit{Nature} \textbf{369}, 621–628
32. Sondak, J., Lambright, D. G., Noell, J. P., Hamm, H. E., and Sigler, P. B. (1994) \textit{Nature} \textbf{372}, 276–279
33. Lambright, D. G., Sondak, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) \textit{Nature} \textbf{379}, 311–319
34. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) \textit{Science} \textbf{265}, 1405–1412
35. Wall, M. A., Coleman, D. E., Lee, E., Iniguez, L. J., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) \textit{Cell} \textbf{83}, 1047–1058
36. Mizuno, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G., and Sprang, S. R. (1995) \textit{Science} \textbf{270}, 954–960
37. Tesmer, J. J., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) \textit{Cell} \textbf{89}, 251–261
38. Shaei, D. J., Betty, M., Jones, P. G., Khawaja, X. Z., and Cockett, M. I. (1998) \textit{J. Neurochem.}, in press