Multiple RGS proteins alter neural G protein signaling to allow *C. elegans* to rapidly change behavior when fed

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Regulators of G protein signaling (RGS proteins) inhibit heterotrimeric G protein signaling by activating G protein GTPase activity. Many mammalian RGS proteins are expressed in the brain and can act in vitro on the neural G protein G_o, but the biological purpose of this multiplicity of regulators is not clear. We have analyzed all 13 RGS genes in *Caenorhabditis elegans* and found that three of them influence the aspect of egg-laying behavior controlled by G_o signaling. A previously studied RGS protein, EGL-10, affects egg laying under all conditions tested. The other two RGS proteins, RGS-1 and RGS-2, act as G_o GTPase activators in vitro but, unlike EGL-10, they do not strongly affect egg laying when worms are allowed to feed constantly. However, *rgs-1; rgs-2* double mutants fail to rapidly induce egg-laying behavior when refed after starvation. Thus EGL-10 sets baseline levels of signaling, while RGS-1 and RGS-2 appear to redundantly alter signaling to cause appropriate behavioral responses to food.

[*Key Words*: RGS protein; heterotrimeric G protein; neurotransmission; *C. elegans*]

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Heterotrimeric G proteins mediate the effects of a vast array of hormones and neurotransmitters by acting as molecular switches that alternate between active and inactive forms (Hamm 1998). Signaling is initiated when an activated cell-surface receptor stimulates a G protein to bind GTP and is terminated when the G protein hydrolyzes GTP to return to the inactive GDP-bound form. Genetic experiments have shown that RGS proteins act as inhibitors of G protein signaling that can bind to G protein α subunits (De Vries et al. 1995; Dohlman et al. 1995; Druey et al. 1996; Koelle and Horvitz 1996). In vitro, RGS proteins accelerate the GTPase activity of G protein α-subunits, thus driving them to their inactive GDP-bound form (for review, see Berman and Gilman 1998). Mammals have ≥20 proteins containing the ~120 amino acid RGS (regulators of G protein signaling) domain that defines RGS proteins. The RGS domain folds into a nine-helix structure that binds the Gα subunit to stimulate its GTPase activity (Tesmer et al. 1997). Although many RGS proteins consist of little more than an RGS domain, a subset of them also contain a large amino-terminal conserved region of unknown function, as well as a G gamma-like (GGL) domain that is able to bind a specific G protein beta subunit (Snow et al. 1998).

Despite many in vitro studies of RGS–G protein interactions, it remains largely unclear how cells benefit by using RGS proteins, rather than simply using G proteins with higher intrinsic GTPase activities to allow appropriate termination of signaling. The use of RGS proteins might be explained if they were themselves regulated to allow signaling to be altered. A current challenge is to understand whether RGS proteins are indeed regulated and, if so, for what purpose. A further puzzle concerns the G protein specificities of RGS proteins. Although a few RGS proteins have distinct G protein specificities in vitro, the majority of those studied behave similarly to each other, acting preferentially on members of the $G_{i/o}$ subfamily of G proteins and, to a lesser extent, on G_q (for review, see Hepler 1999). If the in vivo specificities of RGS proteins are the same as those measured in vitro, it is unclear why cells need multiple regulators of the same G proteins. Analysis of RGS expression also raises this issue. For example, the G protein G_o is expressed throughout the brain, and at least nine RGS genes are also expressed in the brain, some broadly distributed and others restricted to certain areas (Gold et al. 1997).

Genetic studies have the potential to clarify the roles of RGS proteins by revealing their genuine in vivo functions and G protein targets. The most detailed analysis to date is that of the yeast Sst2p RGS protein. This acts on the G protein Gpa1p, which mediates mating pheromone signaling (Apanovitch et al. 1998). *sst2* mutations have two effects. First, they cause yeast to be supersensitive to the pheromone, such that they respond to a concentration that is ~200-fold lower than that required for wild-type yeast (Chan and Otte 1982). Second,

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whereas wild-type yeast desensitize to mating pheromone after prolonged exposure, *sst2* mutants fail to desensitize and thus cannot terminate the mating responses if mating fails. Desensitization is explained, at least in part, by the fact that pheromone signaling induces higher expression of Sst2p, which then feeds back to inhibit Gpa1p signaling (Dietzel and Kurjan 1987). Thus Sst2p is required both to set the baseline level of signaling sensitivity in pheromone-naive yeast and to adjust the level of sensitivity after pheromone exposure.

Two *Caenorhabditis elegans* RGS genes have been analyzed and shown to act on the homologs of the G proteins G_o and G_q (known as GOA-1 and EGL-30, respectively). The RGS protein EGL-10 inhibits signaling by G_o, which in turn inhibits egg-laying and locomotor behaviors (Mendel et al. 1995; Ségalat et al. 1995; Koelle and Horvitz 1996), whereas the RGS protein EAT-16 inhibits signaling by G_q, which has effects that are the opposite of those caused by G_o (Fig. 1; Brundage et al. 1996; Hajdu-Cronin et al. 1999; Lackner et al. 1999; Miller et al. 1999). Studies of EGL-10 and EAT-16 have shown that these RGS proteins have roles in setting baseline levels of signaling, but have not provided evidence that they are regulated to adjust signaling levels.

The biological purpose of RGS control of G_o and G_q in *C. elegans* remains obscure. Egg laying in *C. elegans* is strongly regulated, stopping when animals are starved and resuming when they are fed (Trent 1982). This allows worms to deposit their fertilized eggs where and when food is available for their progeny. Because the rate of egg laying is set by the balance between G_o and G_q signaling, and because this balance is determined by RGS control, RGS proteins are ideally positioned to adjust signaling to alter egg laying. However, there is as yet no evidence that either EGL-10 or EAT-16 is regulated by starvation or feeding in a manner that could account for changes in egg-laying behavior. Another puzzle derives from the fact that many RGS genes other than *egl-10* and *eat-16* have been identified in the *C. elegans* genome sequence. Because in vitro studies of mammalian RGS proteins show that most can act on G_o and G_q, the question arises as to whether the additional RGS proteins in *C. elegans* also regulate G_o and G_q. If so, for what purpose?

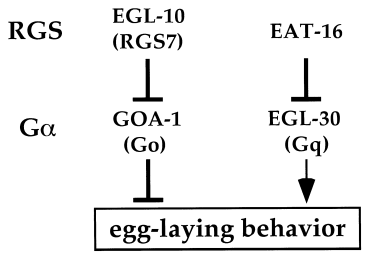

Figure 1. Model for RGS and G protein control of egg laying in *C. elegans*. Mammalian orthologs are indicated in parentheses below the names of the *C. elegans* proteins. No mammalian ortholog of EAT-16 has yet been identified. Genetic experiments show that G_o and G_q signaling antagonize each other, and that G_q acts either downstream of or, as drawn here, parallel to G_o (Hadju-Cronin et al. 1999).

This study was designed to identify the RGS proteins that regulate G_o and G_q signaling in *C. elegans* and to understand the biological roles of these proteins. We take a functional-genomics approach, surveying all the RGS genes of *C. elegans* for effects on egg-laying behavior. We identify RGS-1 and RGS-2 as potential regulators of G_o and use a recently developed gene knockout technology to delete the *rgs-1* and *rgs-2* genes. We find that these RGS genes redundantly adjust signaling when animals are fed to allow rapid induction of egg-laying behavior. Our results suggest that multiple RGS proteins control G_o and G_q to set baseline and regulated levels of signaling.

Results

Overexpression of four of the 13 RGS genes of C. elegans *affects G_o/G_q-controlled egg-laying behavior*

To identify RGS genes controlling G_o and G_q in *C. elegans*, we generated transgenic strains that overexpressed each of the 13 RGS genes identified in the *C. elegans* genome sequence and analyzed these animals for defects in egg laying. This overexpression strategy is based on the observation, made in every previous genetic analysis of an RGS gene, that transgenic overexpression of the RGS gene induced phenotypic defects opposite to those caused by null mutations in the same RGS gene. This observation has been made in five cases: two from *C. elegans* (Koelle and Horvitz 1996; Hajdu-Cronin et al. 1999), two from yeast (Dohlman et al. 1995; Versele et al. 1999), and one from *Aspergillus nidulans* (Yu et al. 1996). These results suggest that RGS proteins are generally present at levels that partially inhibit their G protein targets, and that RGS overexpression can increase this inhibition.

RGS genes were overexpressed by injecting genomic clones for each into *C. elegans* to produce multicopy extrachromosomal transgenic arrays of the injected DNA. The RGS genes were thus expressed from their own promoters, presumably in their normal temporal and spatial expression patterns, but overexpressed because of the high copy number of the transgene DNA (see below for analysis of overexpression levels). The transgenic strains were examined in assays for behavioral defects associated with changes in G_o and G_q signaling. Rates of egg-laying behavior were measured using two assays that gave similar results for each strain tested. We quantitated the accumulation of unlaid eggs in adults and the developmental stages of the freshly laid eggs. The unlaid-egg assay is presented in Figure 2. Increased accumulation indicates reduced egg laying, presumably because of G_q inhibition, whereas reduced accumulation indicates a high rate of egg laying, presumably because of G_o inhibition.

Although most transgenes had no significant effect on egg laying, we found that four RGS transgenes did cause changes relative to the control (Fig. 2). *egl-10* overexpression resulted in hyperactive egg laying, whereas *eat-16*

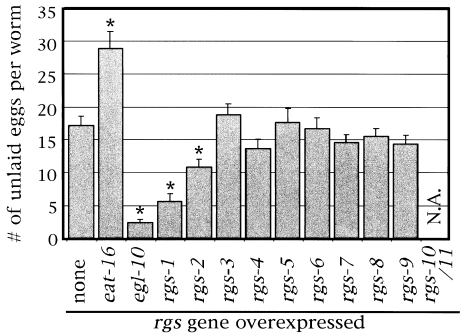

Figure 2. Effects of transgenic overexpression of each of the 13 *C. elegans* RGS genes on egg-laying behavior. Each *C. elegans* RGS gene was overexpressed under its own promoter from multicopy extrachromosomal transgenes. Empty vector transgenes serve as the control (none). The number of unlaid eggs per adult worm is indicated as a mean ±95% confidence interval, determined for each gene from ≥50 animals (10 each from five independent transgenic strains). The adjacent, dicistronic genes *rgs-10* and *rgs-11* were overexpressed together. *rgs-10/11* cooverexpression resulted in sick animals in which egg laying could not accurately be assessed. Asterisks indicate values that are statistically different from the control ($P < 0.05$). N.A., not applicable.

overexpression resulted in reduced egg laying, which is consistent with the previously determined functions of these RGS proteins as inhibitors of G_o and G_q, respectively. In addition, we identified the *rgs-1* and *rgs-2* genes as potential inhibitors of G_o. Overexpression of either reduced the accumulation of unlaid eggs and thus appeared to cause hyperactive egg laying, similar to that seen in *goa-1* null mutants. The effects of *rgs-2* were weaker than those of either *egl-10* or *rgs-1*. In addition to affecting egg-laying behavior, G_o signaling also controls the rate of locomotion (Mendel et al. 1995; Ségalat et al. 1995). We noted strongly hyperactive locomotion in *egl-10* and *rgs-1* overexpressors, similar to that seen in *goa-1* null mutants (data not shown). We did not observe any other behavioral defects in the *egl-10*, *rgs-1*, or *rgs-2* overexpressors beyond those also seen in *goa-1* mutants. The only defect that we noted in animals that overexpressed the other 10 RGS genes was decreased viability in strains that overexpressed the dicistronic gene *rgs-10/rgs-11*.

Overexpression of rgs-1 *mimics the phenotype of mutants lacking the G protein G_o, whereas* rgs-2 *overexpression shows similar but weaker effect*

We investigated the effects of *rgs-1* and *rgs-2* overexpression in more detail by analyzing strains of *C. elegans* in which the unstable extrachromosomal transgenic arrays overexpressing these RGS genes were stably integrated into the chromosomes. To show that RGS proteins were indeed overexpressed in the stable transgenic strains, we generated polyclonal antibodies against recombinant RGS-1 and RGS-2 proteins and used them to probe Western blots of extracts from control and transgenic strains (Fig. 3A). The RGS-1 antibody detected a 25-kD protein that was overexpressed more than 10-fold in the stable *rgs-1* transgenic strain and that was absent from the *rgs-1* mutant. Although the RGS-2 protein was below our limit of detection in the wild-type strain, a 19-kD RGS-2 protein was detected in extracts of the *rgs-2* transgenic strains.

We also showed that the effects of the *rgs-1* transgene were actually attributable to overexpression of the RGS-1 protein, as injection of an *rgs-1* transgene modified by inserting a frame-shift mutation in the *rgs-1* coding sequences failed to induce any of the phenotypic defects caused by the wild-type *rgs-1* transgene (data not shown).

To further analyze changes in egg-laying behavior induced by RGS-1 and RGS-2 overexpression, we examined freshly laid eggs from the stable transgenic strains, an assay that measures hyperactive egg laying (Koelle and Horvitz 1996). The eggs of *C. elegans* are fertilized internally and normally accumulate in the uterus, where they sit for ~2 hr and reach the ~100-cell stage before being laid. In mutants that show hyperactive egg laying, however, eggs are laid shortly after fertilization resulting in freshly laid eggs that are often at early stages of development (≤4 cells). The frequency of egg laying can thus be indirectly assessed by examining freshly laid eggs and determining their developmental stages. Whereas wild-type animals or sluggish egg layers such as *egl-10* mutants lay virtually no early-stage eggs, hyperactive egg layers such as *eat-16* mutants or *goa-1* mutants lay mostly early-stage eggs (Fig. 3B). Hyperactive egg laying induced by overexpression of *rgs-1* is as strong as that seen in *goa-1* null mutants, whereas overexpression of *rgs-2* also induced hyperactive egg laying, but at a much lower level.

All the behavioral defects that have been described in *goa-1* null mutants and *egl-10* overexpressors (Mendel et al. 1995; Ségalat et al. 1995; Koelle and Horvitz 1996) also occur in RGS-1 overexpressors. In addition to hyperactive egg laying, these animals also display rapid foraging behavior, deep body bends, and abnormally fast locomotion (Fig. 3D).

These results indicate that *rgs-1* and *rgs-2* might act by inhibiting *goa-1* signaling. To test this idea, we constructed animals carrying the *rgs-1* overexpression transgene as well as a transgene expressing a GTPase-defective GOA-1(Q205L), which would be expected to be insensitive to RGS proteins (Berman et al. 1996). The *rgs-1* transgene by itself causes hyperactivity in egg laying and locomotion (Fig. 3), whereas the GOA-1(Q205L) transgene alone causes sluggish locomotion and blocks egg laying (Mendel et al. 1995). In animals carrying both transgenes, the effects of *rgs-1* overexpression were blocked. The animals moved sluggishly and did not lay early-stage eggs. These results suggest that the effects of *rgs-1* overexpression occur mostly or entirely through inhibition of GOA-1 signaling.

Another RGS protein, EGL-10, was previously shown to be an inhibitor of the G protein GOA-1 (Koelle and Horvitz 1996). We therefore tested whether overexpression of RGS-1 or RGS-2 could correct the sluggish egg-laying in *egl-10* null mutants. The accumulation of unlaid eggs (the Egl phenotype) that occurs in *egl-10* mu-

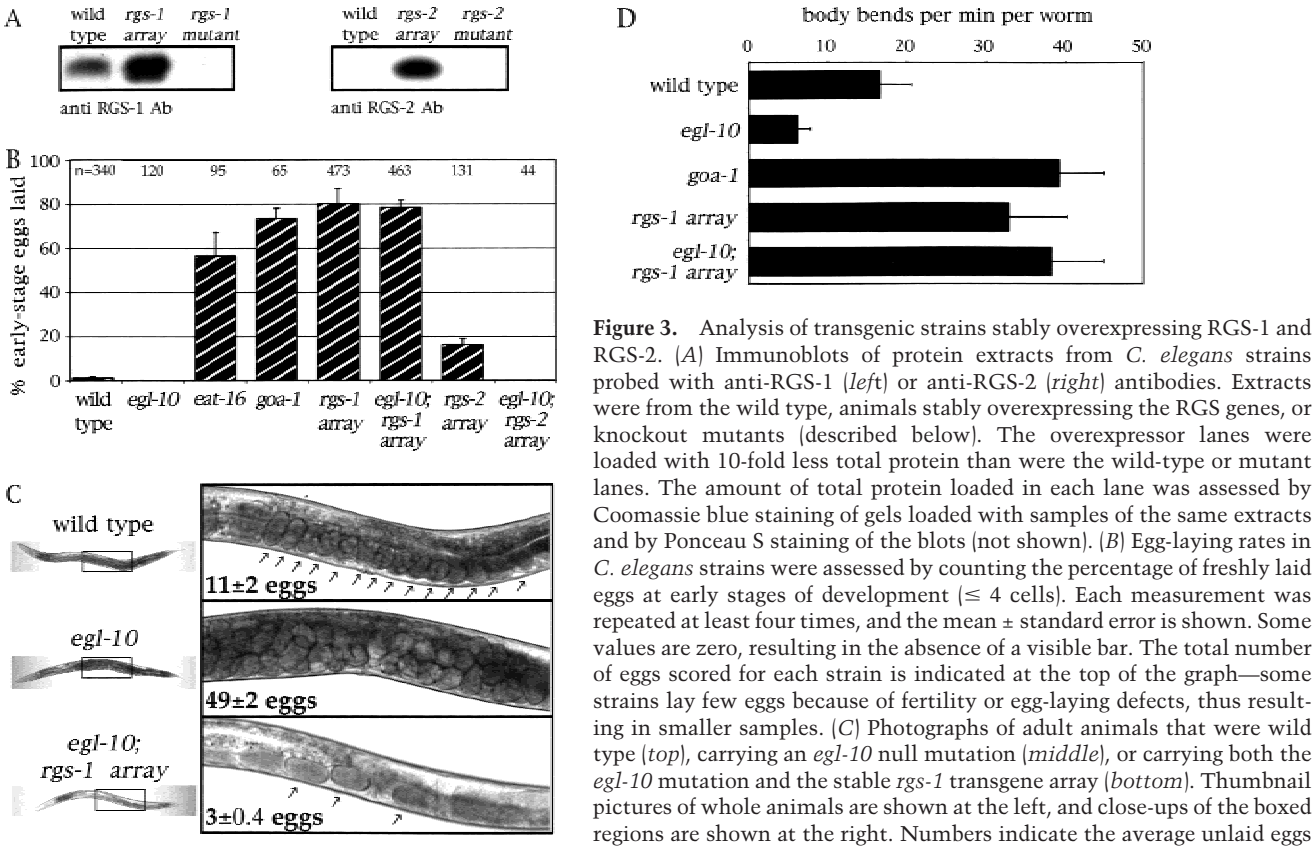

Figure 3. Analysis of transgenic strains stably overexpressing RGS-1 and RGS-2. (*A*) Immunoblots of protein extracts from *C. elegans* strains probed with anti-RGS-1 (*left*) or anti-RGS-2 (*right*) antibodies. Extracts were from the wild type, animals stably overexpressing the RGS genes, or knockout mutants (described below). The overexpressor lanes were loaded with 10-fold less total protein than were the wild-type or mutant lanes. The amount of total protein loaded in each lane was assessed by Coomassie blue staining of gels loaded with samples of the same extracts and by Ponceau S staining of the blots (not shown). (*B*) Egg-laying rates in *C. elegans* strains were assessed by counting the percentage of freshly laid eggs at early stages of development (≤ 4 cells). Each measurement was repeated at least four times, and the mean ± standard error is shown. Some values are zero, resulting in the absence of a visible bar. The total number of eggs scored for each strain is indicated at the top of the graph—some strains lay few eggs because of fertility or egg-laying defects, thus resulting in smaller samples. (*C*) Photographs of adult animals that were wild type (*top*), carrying an *egl-10* null mutation (*middle*), or carrying both the *egl-10* mutation and the stable *rgs-1* transgene array (*bottom*). Thumbnail pictures of whole animals are shown at the left, and close-ups of the boxed regions are shown at the right. Numbers indicate the average unlaid eggs per strain ±95% confidence interval. Arrows point to individual unlaid eggs, except in the middle panel in which the body is fully packed and the eggs are too numerous to indicate individually. (*D*) Locomotion rates were assessed by counting body bends per min. For each strain, 30 worms were observed for 3 min each and the mean ±95% confidence interval is shown. The transgenes and mutations used for this Figure were: *vsIs1* ("*rgs-1* array"), *vsIs4* ("*rgs-2* array"), *rgs-1(nr2017)*, *rgs-2(vs17)*, *egl-10(md176)*, *eat-16(ad702)*, and *goa-1(n363)*.

tants was completely corrected by overexpression of RGS-1 (Fig. 3C). In fact, overexpression of RGS-1 in an *egl-10* mutant background still leads to hyperactive egg-laying behavior, as shown by the reduced accumulation of unlaid eggs (Fig. 3C) and by the high percentage of early-stage eggs laid (Fig. 3B). We also found that the sluggish locomotion seen in *egl-10* null mutants could be rescued by RGS-1 overexpression (Fig. 3D and data not shown). The effects of RGS-2 overexpression were consistently weaker than those of RGS-1 overexpression. RGS-2 overexpression neither corrected the Egl phenotype of *egl-10* mutants (data not shown) nor did it lead to hyperactive egg laying in the absence of EGL-10 (Fig. 3B).

In summary, we found that overexpression of RGS-1 mimics all the behavioral defects seen in *goa-1* null mutants. Overexpression of EGL-10 also causes the same behavioral defects (Koelle and Horvitz 1996), and overexpression of RGS-1 rescues all the behavioral defects seen in *egl-10* null mutants. These results suggest that RGS-1, like EGL-10, is able to inhibit signaling by GOA-1, and that overexpressed RGS-1 may substitute for EGL-10. RGS-2 overexpression causes weaker effects than overexpression of either EGL-10 or RGS-1 and does not substitute for EGL-10. RGS-2 may function differently than EGL-10, or the effects of RGS-2 overexpression may simply be too weak to overcome the absence of EGL-10. Although RGS-2 overexpression generates only weak effects on egg laying, its sequence similarity to RGS-1 and the overlapping expression patterns of RGS-1 and RGS-2 (see below) led us to continue to characterize both genes as potentially redundant regulators of GOA-1.

rgs-1 *and* rgs-2 *expression overlaps with that of* G_o *in the* C. elegans *nervous system*

To examine the expression patterns of *rgs-1* and *rgs-2*, we fused the promoters of these genes to the coding sequences for the green fluorescent protein (GFP) and examined transgenic animals carrying these constructs by confocal fluorescence microscopy. We saw *rgs-1*::GFP expression in most or all neurons (Fig. 4A) and *rgs-2*::GFP expression in a subset that included ventral cord and head- and tail-ganglia neurons (Fig. 4B). We did not observe *rgs-2*::GFP fluorescence in the hermaphrodite-specific neurons (HSNs), which also synapse onto the egg-laying muscles. We observed *rgs-2*::GFP fluorescence in some non-neuronal cells, including the pharyngeal muscles and uterine muscles, the latter of which are used for egg laying. The *rgs-1* and *rgs-2* expression patterns thus appear to overlap each other, and also to over-

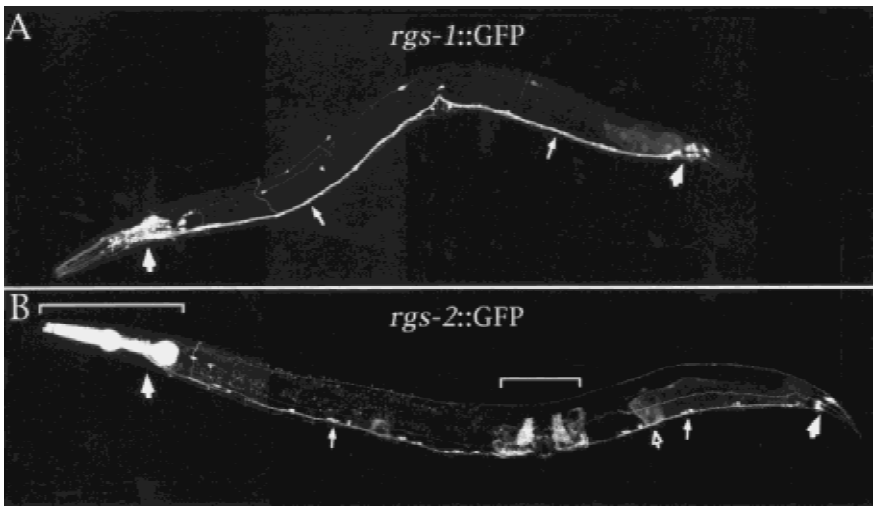

Figure 4. Expression patterns of *rgs-1* and *rgs-2*. Transgenic animals carrying fusions of the *rgs-1* or *rgs-2* promoter regions to the coding sequences for the GFP were examined by fluorescence confocal microscopy. (*A*) *rgs-1*::GFP fluorescence is seen in most or all neurons. Large arrows indicate the head and tail ganglia at the left and right, respectively. Two small arrows point to the ventral nerve cord that runs the length of the animal. The only nonneuronal cells labeled are several posterior intestinal cells that are faintly fluorescent in some animals. (*B*) *rgs2*::GFP fluorescence is seen in a subset of neurons and in certain muscle cells. Large and small filled arrows indicate the same structures as in panel *A*. The bracket at the left lies over the pharynx; the bracket at the middle of the animal lies over the uterine muscles. The open arrow indicates faint fluorescence sometimes seen in the spermatheca.

lap the expression pattern of their potential target, the G_o protein GOA-1, which is expressed in all neurons of the animal (Mendel et al. 1995; Ségalat et al. 1995).

RGS-1 and RGS-2 encode highly similar, small RGS proteins

To express recombinant RGS-1 and RGS-2 for biochemical studies, we obtained cDNAs containing the complete coding sequences for these proteins. Figure 5 shows the RGS-1 and RGS-2 protein sequences encoded by the cDNAs, as well as the gene structures deduced by comparison with the *C. elegans* genome sequence.

The RGS-1 and RGS-2 proteins have 201 and 169 amino acid residues, respectively, and each sequence contains a 119–amino acid RGS domain, but no other features conserved with other proteins in the sequence database. There are no clear orthologs of RGS-1 and RGS-2 among the mammalian RGS proteins identified to date. The RGS domain sequences of the two proteins are 77% identical, making them much more similar to each other than either is to any other RGS domain sequence found in *C. elegans* (Fig. 5C).

RGS-1 and RGS-2 are GTPase activators of the C. elegans *G_o protein in vitro*

Because the genetic experiments and expression patterns described above suggested that RGS-1 and RGS-2 might act on the G_o protein GOA-1, we tested whether RGS-1 and RGS-2 can act as GTPase activators of GOA-1. The proteins were expressed in *Escherichia coli* fused to glutathione S-transferase (GST), partially purified on glutathione agarose, cleaved by a protease to remove the GST tag, and finally obtained in a high state of purity after ion exchange chromatography. A Coomassie-stained gel of the purified proteins is shown in Figure 6A.

We preloaded purified GOA-1 with radiolabeled GTP and monitored the GTP hydrolysis rate of GOA-1 by measuring the release of the radioactive γ phosphate as a function of time (Fig. 6B). GOA-1 by itself had a relatively slow rate of GTP hydrolysis, as has been described for other Gα proteins (Berman and Gilman 1998). In the presence of RGS-1 or RGS-2, however, GTP hydrolysis by GOA-1 was markedly enhanced, and the reactions went to completion before the first time point of the experiment. RGS-1 and RGS-2 by themselves had no detectable GTPase activity. These results suggest that the genetic results described above, in which overexpression of RGS-1 or RGS-2 mimicked the effects of GOA-1 loss-of-function mutations, could be explained by the direct action of RGS-1 and RGS-2 on the GOA-1 protein to inhibit its ability to signal.

Knockout mutants of rgs-1 *and* rgs-2

The experiments described above are not sufficient to determine the true physiological functions of the RGS-1 and RGS-2 proteins. Overexpression in living animals may cause these proteins to carry out abnormal activities. The in vitro studies show that RGS-1 and RGS-2 can act on GOA-1, but do not show that GOA-1 must be their true Gα-target in vivo, and do not reveal the biological purpose for which they might regulate GOA-1. Most previous studies of RGS proteins have used the same experimental approaches and suffer from the same limitations. To rigorously determine the in vivo functions of *rgs-1* and *rgs-2*, we produced knockout mutants of *C. elegans* deleted for these genes. The high sequence similarity of RGS-1 and RGS-2, their overlapping expression patterns, similar overexpression phenotypes, and similar biochemical activities all suggested that these two proteins might function redundantly. Therefore, we decided to study both knockouts, individually and in combination. We used an adaptation of a recently devel-

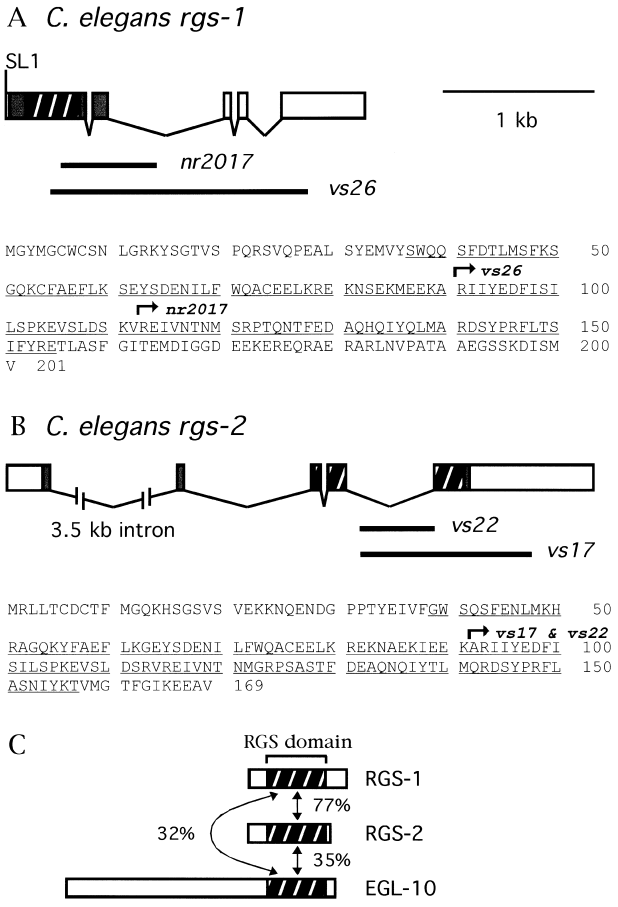

Figure 5. Gene structures, protein sequences, and knockout mutations of *rgs-1* and *rgs-2* . (*A*) Gene structure, protein sequence, and knockout mutations for *rgs-1*. Exons are boxed, protein coding sequences are filled and the RGS domain coding region is hatched. An SL1 trans-spliced leader found at the 5′ end of the cDNA sequence is indicated. Thick lines below the exon structure indicate the extents of the genomic regions deleted in two mutant alleles. The protein sequence deduced from the cDNA sequence is at the bottom, with the RGS domain underlined. Arrows indicate the regions of protein for which the coding sequences are removed by the indicated deletion mutations. (*B*) Gene structure, protein sequence, and knockout mutations for *rgs-2*. Features are shown in the same manner as in panel *A*. The *vs22* mutation removes the beginning of exon five and thus shifts the reading frame for the remainder of the RGS domain coding sequences. (*C*) Schematic diagrams of the predicted RGS-1, RGS-2, and EGL-10 proteins. The RGS domains are hatched, and percent sequence identities between them are indicated.

oped gene knockout technology in which the progeny of several hundred thousand mutagenized animals are screened by PCR to identify rare individuals in which a gene of interest has suffered a deletion of ~0.5–3 kb (Liu et al. 1999). We produced two independent deletion mutations for each RGS gene. The regions deleted are indicated in Figure 5. We present results obtained with the alleles *rgs-1(nr2017)* and *rgs-2(vs17)*. Each experiment shown was repeated with the alleles *rgs-1(vs26)* and *rgs-2(vs22)*. These repetitions yielded similar results (data not shown), verifying that the defects seen were indeed the result of deletion of the *rgs-1* and *rgs-2* genes and not attributable to any genetic background mutations that might exist. Because each deletion mutation removes a large portion of the coding sequences for the RGS domain (*nr2017*, *vs26*, and *vs17*) or shifts the reading frame in the middle of the RGS domain (*vs22*), each mutation is likely to be a null allele.

rgs-1*and* rgs-2 *knockout mutations cause only weak egg-laying defects in well-fed worms*

In the egg-laying assays presented in Figures 2 and 3, the animals were grown and examined in the presence of abundant food. Although *egl-10* and *eat-16* mutations have strong effects on egg laying under these conditions, *rgs-1* and *rgs-2* mutations cause only mild defects. Figure 7 shows that the numbers of unlaid eggs in well-fed *rgs-1* and *rgs-2* mutants are similar to those of the wild type, in comparison with the strong defect seen in *egl-10* mutants. *rgs-1; rgs-2* double mutants are also similar to the wild type under these conditions and *rgs-1; egl-10; rgs-2* triple mutants appear similar to *egl-10* single mutants. Because no strong synthetic behavioral defects are revealed in these double and triple mutants, *rgs-1, rgs-2*, and *egl-10* do not have obvious redundant functions in controlling egg laying under well-fed conditions.

The slight increase in accumulated eggs in *rgs-1* single mutants relative to the wild type (Fig. 7) is statistically significant ($P < 0.0005$). This effect is additive with the strong defects induced by *egl-10* null mutations and it is suppressed by a *goa-1* null mutation (data not shown). These results are consistent with RGS-1 causing a mild inhibition of GOA-1 signaling under well-fed conditions, whereas most of the inhibition of GOA-1 is attributable to EGL-10. The slight decrease in accumulated eggs in *rgs-2* mutants is also reproducible and may be caused in part by a mild defect in egg production. Other mutants shown in Figure 7 had brood sizes close to that of the wild type (see Materials and Methods).

rgs-1 *and* rgs-2 *redundantly regulate signaling to allow starved animals to rapidly induce egg laying when fed*

Because analysis of egg laying in well-fed animals failed to identify strong functions for RSG-1 and RSG-2, we examined the mutants for defects in the regulation of egg laying caused by the removal and return of food. Worms lay eggs regularly on petri dish in the presence of a lawn of bacteria, the food used for laboratory culture of *C. elegans*. However, as shown below, egg-laying behavior virtually ceases if worms are starved by removing them from bacteria and resumes only if the animals are returned to bacteria. In this way, worms can lay their eggs when and where food is available for their progeny. Because RGS-1 and RGS-2 are potential regulators of GOA-1 based on their overexpression phenotypes, we reasoned that RGS-1 and/or RGS-2 might exist to alter GOA-1 signaling in response to starving or feeding, thereby altering egg laying under these conditions.

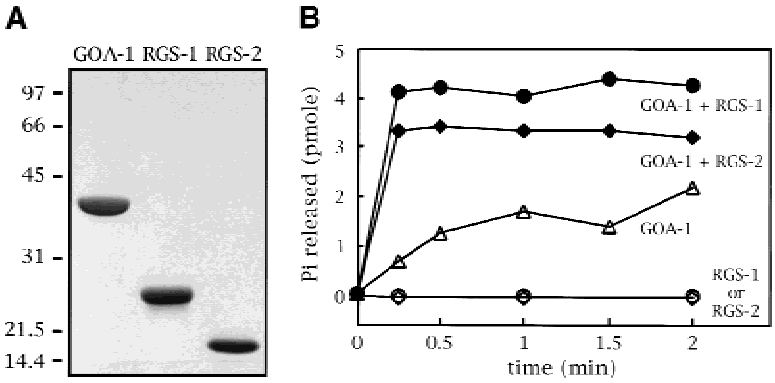

Figure 6. Activation of the GTPase activity of GOA-1 by RGS-1 and RGS-2. (*A*) Coomassie-stained SDS-PAGE gel showing 4 µg each of the purified preparations of GOA-1, RGS-1, and RGS-2. The positions of molecular weight markers are indicated, with their sizes in kD. (*B*) Rates of GTP hydrolysis by the purified proteins were measured in a single-turnover assay. 0.6 µM GOA-1 was assayed either alone or in the presence of 0.2 µM RGS-1 or RGS-2. RGS-1 and RGS-2 were tested by themselves for GTPase activity (*bottom curves*) at 2 µM. The data shown represent the average of two experiments. The top two curves plateau at different levels because of differences in the amounts of GTP-loaded GOA-1 present at the outset of each assay.

We found that RGS-1 and RGS-2 are redundantly required to induce egg laying when starved animals are fed. Figure 8A shows the rates of egg laying by wild-type or mutant animals that have been (1) maintained in constant food (nonstarved); (2) removed from food for 2 hr (starved); or (3) removed from food for 2 hr and then returned to food (starved/refed). Wild-type animals strongly regulate egg laying under these conditions, such that virtually no eggs are laid by starved animals, and refeeding starved animals returns egg laying to the same rate as that seen in nonstarved animals. *rgs-1* single mutants also show strong regulation of egg laying. *rgs-2* mutants show weak defects. Their baseline (nonstarved) level of egg laying is slightly reduced, probably because of the mild defect in egg production noted above, and they do not fully return to their baseline level of egg laying when starved and refed.

A dramatic defect, however, is seen in *rgs-1; rgs-2* double mutants. These animals show a reduced baseline level of egg laying, just as seen in the *rgs-2* single mutant, and they are able to stop laying eggs when starved. However, the double mutants fail to induce egg laying when refed after starvation (Fig. 8A). This result suggests a model in which feeding induces the activities of both RGS-1 and RGS-2, thus inhibiting signaling by the G protein GOA-1 and causing an increase in egg-laying behavior. Egg laying can be induced in wild-type animals by treatment with the neurotransmitter serotonin, which is normally released from the HSNs onto the egg-laying muscles to stimulate their contraction (Desai et al. 1988). Serotonin also stimulates *rgs-1; rgs-2* double mutants to lay eggs, even after starvation (data not shown), suggesting that the *rgs-1* and *rgs-2* mutations may interfere with activity of the egg-laying neurons rather than with the responsiveness of the egg-laying muscles to serotonin. In addition to the synthetic egg-laying defect seen in *rgs-1; rgs-2* double mutants, these animals also have smaller bodies than do the wild type or the single mutants (data not shown). RGS-1 and RGS-2 therefore have at least two redundant functions.

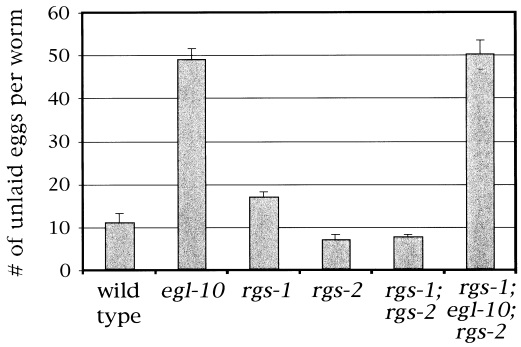

Figure 7. Accumulation of unlaid eggs under well-fed conditions in RGS mutants. The number of unlaid eggs per worm was determined from 30 worms for each strain. Mean ±95% confidence interval is shown. The mutations used were *egl-10(md176)*, *rgs-1(nr2017)*, and *rgs-2(vs17)*.

The blocked induction of egg laying that is seen in *rgs-1; rgs-2* double mutants is temporary. Figure 8B shows the results of a time course in which the rate of egg laying was measured in 30-min periods after the return of starved animals to food. The block of egg laying is virtually complete in the first 30 min, but is largely gone after 2 hr. This suggests that a mechanism that is not dependent on RGS-1 or RGS-2 eventually allows the induction of egg laying when animals are fed, and that the RGS proteins are responsible mainly for the rapid phase of the normal response.

The RGS proteins EGL-10 and EAT-16 were identified based on their strong effects on egg laying under well-fed conditions (Koelle et al. 1996; Hadju-Cronin et al. 1999). *egl-10* mutants lay eggs infrequently, whereas *eat-16* mutants are hyperactive egg layers (Fig. 3; Hadju-Cronin et al. 1999). We tested these mutants in the starving and feeding assay (Fig. 8C). Unlike *rgs-1; rgs-2* double mutants, the *egl-10* and *eat-16* mutants induce egg laying when refed after starvation. Both mutants also lay some eggs even when starved. In the case of *egl-10*, this is probably attributable to the fact that the animals are bloated with excess unlaid eggs and may be unable to retain any more. In the case of *eat-16*, hyperactive egg-laying behavior appears to continue even in the absence of food. Therefore, unlike *rgs-1* and *rgs-2*, the RGS genes *egl-10* and *eat-16* are not required for the induction of egg laying after starved animals are refed.

If RGS-1 and RGS-2 cause changes in egg-laying be-

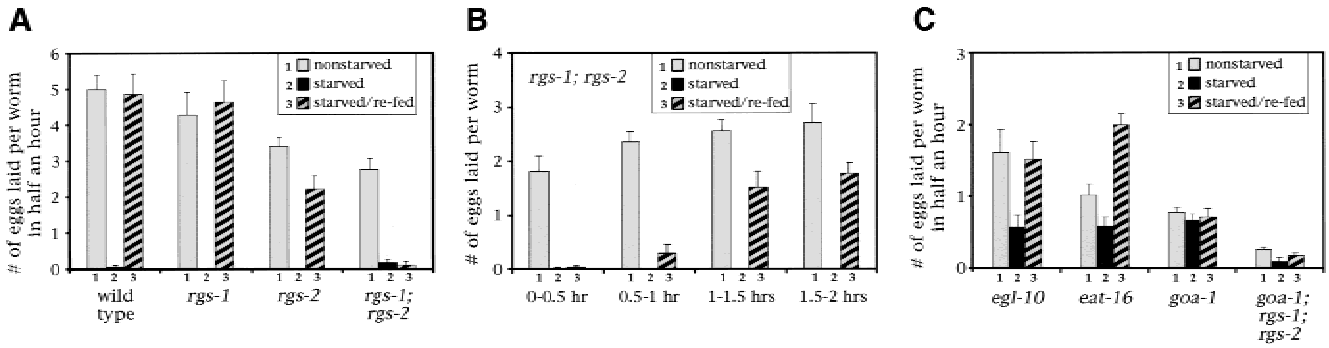

Figure 8. Egg-laying behavior responses to starving and feeding in wild-type and mutant strains. (*A*) The strains indicated were preconditioned for 2 hr on plates either containing bacteria (to feed the animals) or not containing bacteria (to starve them). Animals were then moved to assay plates either with or without bacteria and the number of eggs laid in 30 min was counted. (Nonstarved) Both the preconditioning and assay plates had bacteria; (starved) both plates lacked bacteria; (starved/refed) the animals were preconditioned without bacteria and moved to assay plates with bacteria. Each value was determined by measuring 80 animals (8 experiments with 10 animals each) and is shown as a mean ± standard error. The mutations used were *rgs-1(nr2017)* and *rgs-2(vs17)*. The mutations *rgs-1(vs26)* and *rgs-2(vs22)* were also tested and gave similar results (data not shown). The values for starved animals were often zero and thus did not result in visible bars in the graph. (*B*) *rgs-1;rgs-2* double mutants were assayed as in panel *A*, except that animals were left on the assay plates for 2 hr, and the number of eggs laid during each 30-min period within the 2 hr was determined. (*C*) The mutants indicated were assayed as in panel *A*. The mutations used were *egl-10(md176), eat-16(ad702)*, and *goa-1(n363)*. The absolute levels of egg laying in these mutants are reduced relative to the wild type, either because of sluggish egg-laying behavior (*egl-10*) or because the animals have relatively few available eggs in their uteri (*eat-16* and *goa-1*).

havior by regulating GOA-1, then a *goa-1* null mutation should abolish these changes. In fact, *goa-1* null mutants show no changes in rates of egg laying when starved or refed. Furthermore, *goa-1; rgs-1; rgs-2* mutants behave like *goa-1* mutants, with similar egg-laying rate under all conditions (Fig. 8C). However, interpretation of these results is complicated by the fact that *goa-1* mutants are partially sterile (average brood size, 36; Ségalat et al. 1995) and have few eggs to lay. Thus egg production, rather than egg-laying behavior, may limit the number of eggs laid. The fertility of *goa-1; rgs-1; rgs-2* mutants is even lower. Analysis of these mutants thus does not serve as a strong test of the idea that RGS-1 and RGS-2 act via regulation of GOA-1. However, the other data presented in this work do indicate that RGS-1 and RGS-2 act to inhibit GOA-1 and show that RGS-1 and RGS-2 are required to induce egg laying when starved animals are fed. These results suggest a model in which feeding after starving induces RGS-1 and RGS-2 activity, thus inhibiting GOA-1 signaling to induce egg laying.

Discussion

The discovery of a large family of RGS proteins, many of which can act as GTPase activators of the same G proteins in vitro, has raised the issue of whether one G protein might be regulated by several RGS proteins in vivo. If so, for what biological purpose? In this work, we have taken advantage of the completed genome sequence of *C. elegans* to survey the in vivo functions of all of its RGS proteins. Our results suggest that three RGS proteins, EGL-10, RGS-1, and RGS-2, all act on GOA-1, the *C. elegans* homolog of G_o. The combined action of these regulators allows *C. elegans* to set the frequencies of egg-laying behavior in a manner appropriate for both constant and changing supplies of food.

Multiple RGS proteins control G_o signaling in C. elegans

EGL-10 was previously shown to function in vivo by inhibiting GOA-1, the *C. elegans* homolog of G_o (Koelle and Horvitz 1996). Several lines of evidence suggest that RGS-1 and RGS-2 also induce behavioral change by negatively regulating G_o. First, overexpression of RGS-1 or RGS-2 in well-fed animals, like overexpression of EGL-10, mimics the behavioral defects of a G_o null mutant, and these are the only behavioral defects we have noted (Fig. 3). RGS-1 or EGL-10 overexpression induces defects as strong as those seen in the G_o null mutant, whereas RGS-2 overexpression causes weaker defects. Second, the effects of RGS-1 overexpression can be abolished by expression of GOA-1(Q205L), a GTPase-deficient mutant of GOA-1. Third, some neurons that express GOA-1 also express RGS-1 and RGS-2 (Fig. 4). Fourth, purified RGS-1 and RGS-2 proteins both stimulate the GTPase activity of purified GOA-1 (Fig. 5). Fifth, well-fed *rgs-1* mutants show a modest but significant decrease in egg laying (Fig. 7), which is consistent with RGS-1 causing a modest inhibition of G_o under these circumstances (compared with the much greater inhibition caused by EGL-10). Overexpression of RGS-1 through the increase of the copy number of its gene thus magnifies this modest effect into a large one, and can even compensate for the lack of EGL-10 in *egl-10* mutants (Fig. 3). Defects in well-fed *rgs-2* single mutants are more subtle than those of *rgs-1* mutants. Sixth, the major role of RGS-1 and RGS-2 is to induce egg laying when starved animals are fed, a function that these two RGS proteins carry out redundantly (Fig. 8). The G_o null mutation appears to abolish the effects of feeding and starving on egg laying, suggesting that the behavior may be normally regulated through this G protein. Finally, a genetic study of all the

Gα-proteins encoded in the *C. elegans* genome has identified only one *C. elegans* G protein, namely G_o, that inhibits egg laying, making G_o the best candidate to be itself inhibited by RGS-1 and RGS-2 to cause the observed effects (Jansen et al. 1999).

C. elegans *adjusts behavior in response to food by adjusting G protein signaling*

In this article we show that worms demonstrate robust behavioral changes when they are removed from and returned to food. Egg-laying behavior is almost completely suppressed after animals are starved for 2 hr, but returns to its starting levels within 30 min after they are returned to food.

Previous work has shown that signaling through the G_o protein GOA-1 stimulates egg laying, whereas signaling through the G_q protein EGL-30 inhibits this behavior (Fig. 1), and that the rate of egg laying is set by the balance between signaling by these two G proteins (Mendel et al. 1995; Ségalat et al. 1995; Brundage et al. 1996; Hadju-Cronin et al. 1999). Receptors for neurotransmitters that control egg laying, such as serotonin, acetylcholine, and certain neuropeptides, may directly stimulate signaling by GOA-1 and/or EGL-30 (Desai et al. 1988; Weinshenker et al. 1995; Waggoner et al. 1998, 2000). In response to feeding or starving, animals might alter release of these or other neurotransmitters to alter egg laying. Indeed, the release of the FLP-1 neuropeptide appears to be required for animals to properly halt egg laying when starved (Waggoner et al. 2000).

We have identified an additional mechanism by which animals alter signaling to control egg laying. As an alternative to altering the release of the neurotransmitters that couple to GOA-1 and/or EGL-30, animals might change the ability of these G proteins to signal in response to neurotransmitter release. Our work shows that this latter mechanism stimulates egg laying when starved animals are refed. A feeding-induced signal apparently stimulates RGS-1 and RGS-2 activity to reduce the efficiency of GOA-1 signaling, thus increasing egg-laying behavior.

Different RGS proteins control baseline and regulated levels of G_o signaling

Our results begin to address why many RGS proteins with similar G protein specificities might exist in higher organisms such as *C. elegans* and mammals. We first consider yeast, which has only two RGS proteins, one specific for each of the two Gα-proteins in this organism (Apanovitch et al. 1998; Versele et al. 1999). The yeast RGS protein Sst2p, in particular, has been well studied (for review, see Dohlman et al. 1998). Sst2p inhibits Gpa1p, the G protein that mediates signaling by mating pheromone. In yeast that have never been exposed to pheromone, Sst2p partially inhibits Gpa1p signaling and thus sets the baseline level of sensitivity to pheromone. Once yeast are exposed to pheromone, Gpa1p signaling induces Sst2p expression, which feeds back to further inhibit Gpa1p and thus desensitizes yeast to pheromone. In this way Sst2p sets both baseline and regulated levels of signaling by Gpa1p.

In *C. elegans*, RGS proteins appear to set baseline and regulated levels of signaling by the G protein G_o, but the roles played by just one RGS protein in yeast appear to be distributed among three different RGS proteins in *C. elegans.* The RGS protein EGL-10 has a very strong effect on egg laying under all circumstances, but feeding and starving still alter the residual egg laying seen in *egl-10* mutants. These results suggest that the baseline levels of signaling by G_o are set by EGL-10 activity, and that other factors besides EGL-10 are responsible for changing signaling to alter egg laying when worms are starved or fed. In contrast, the RGS proteins RGS-1 and RGS-2 have little effect on baseline levels of signaling, as indicated by their mild effects on egg laying in animals that are given constant food. However, RGS-1 and RGS-2 are redundantly required for the rapid induction of egg laying when animals are fed, suggesting that they are induced to alter signaling under these circumstances. We have not yet determined the molecular mechanism by which RGS-1 and RGS-2 activity might be induced upon feeding. Their expression could be controlled transcriptionally, as in the case of *SST2* (Dietzel and Kurjan 1987) or their activity could be altered at other levels. Analysis with RGS-1 and RGS-2 antibodies and observation of *rgs-1::GFP* and *rgs-2::GFP* reporters has not yet revealed changes in animals that have been starved and fed (M.Q. Dong, unpubl.).

Our results suggest the logic that underlies the use of the many RGS proteins in mammals and in *C. elegans*. First, these proteins show some level of functional redundancy, as exemplified both by the redundant effects of RGS-1 and RGS-2 in inducing egg laying when starved animals are refed and by the partial redundancy between EGL-10 and RGS-1 in well-fed animals. RGS proteins thus appear to be typical of many other protein families, which often have more members in higher eukaryotes than in yeast, and which often show overlapping expression patterns and some redundancy in higher eukaryotes. In addition, it appears that many RGS proteins may exist in higher eukaryotes so that individual RGS proteins can be specialized to adjust signaling under particular circumstances. In the case of G_o in *C. elegans*, EGL-10 appears to play the major role in setting the baseline level of signaling, whereas RGS-1 and RGS-2 appear to be specialized to modify signaling when animals are fed. It is possible that additional RGS proteins also regulate G_o by altering signaling levels in particular cells and under circumstances that we have not examined. In general, the use of different RGS proteins to alter signaling in particular circumstances may explain the existence of the large number of these proteins.

Two classes of RGS proteins emerge from the genetic studies that have so far been performed on higher eukaryotes. EGL-10 and EAT-16 are members of the class of large RGS proteins that have an amino-terminal conserved region, as well as G gamma-like (GGL) and RGS

domains. These proteins appear to set the baseline level of signaling by their respective Gα targets. The second class includes the small RGS proteins RGS-1 and RGS-2, which consist of little more than an RGS domain. These proteins mainly adjust signaling under particular circumstances and do not affect baseline signaling. The mammalian RGS proteins that are known to be transcriptionally regulated, and thus to have the potential to adjust signaling, are also members of the small RGS protein class (Druey et al. 1996; Pepperl et al. 1998; Miles et al. 2000). It remains to be seen if, in general, the large RGS proteins always function to set baseline signaling levels, whereas the small RGS proteins always function to adjust signaling under various circumstances. It is interesting to note that in this study we found that overexpressing the small RGS-1 protein can compensate for the loss of EGL-10 despite the fact that RGS-1 lacks the amino terminal and GGL domains found in EGL-10.

Materials and methods

Detailed protocols for many of the methods used in this work are available electronically at http://info.med.yale.edu/mbb/koelle/.

Identification and transgenic overexpression of C. elegans *RGS genes*

Analysis of the *C. elegans* genome sequence identified 13 predicted genes with RGS domain similarity, including the previously studied *egl-10* and *eat-16* genes. The remaining genes were named as follows (designations from the genome sequence are given in parentheses): *rgs-1* (*C05B5.7*), *rgs-2* (*F16H9.1a*), *rgs-3* (*C29H12.3*), *rgs-4* (*Y38E10A.v*), *rgs-5* (*B0336.4*), *rgs-6* (*C41G11.3*), *rgs-7* (*F56B6.2*), *rgs-8* (*F52D2.2*), *rgs-9* (*ZC53.7*), *rgs-10* (*F45B8.2*), *rgs-11* (*F45B8.1*). Mammalian RGS genes are given the names *RGS-1*, *RGS-2*, and so on, but the RGS numbering schemes in mammals and worms do not indicate the complex evolutionary relationships between these gene sets. The *rgs-3* gene encodes a protein with two highly similar RGS domains. For each gene, genomic DNA containing the coding sequences and approximately 5 kb of flanking DNA on either side (presumed to contain the promoters and regulatory sequences) were subcloned into the plasmid pBluescript (Stratagene). The source of genomic DNA was either cosmid clones obtained from the *C. elegans* genome project, or (for *rgs-4*) a PCR product amplified from total genomic DNA. *rgs-10* and *rgs-11* are highly similar, adjacent genes predicted to be transcribed from the same promoter to form a dicistronic transcript. They were subcloned and overexpressed together as a pair. Transgenic strains were generated by injecting RGS subclones (or pBluescript as a control) at 80 ng/μl into *lin-15(n765ts)* animals along with a *lin-15* rescuing plasmid as a coinjection marker. Extrachromosomal transgenes were chromosomally integrated by irradiating transgenic animals with γ-rays and the resulting strains were outcrossed at least four times to provide a clean genetic background. The integrated *rgs-1* transgene array used in this work has the allele designation *vsIs1*. The integrated *rgs-2* transgene array is *vsIs4*. The integrated transgene expressing GOA-1(Q205L) used was *syIs9* (Mendel et al. 1995).

Behavioral assay

Measurement of accumulation of unlaid eggs, staging of freshly laid eggs, and measurement of locomotion rates were performed as described previously (Koelle and Horvitz 1996). We generally used accumulation of unlaid eggs to quantify the failure to lay eggs, and laying of early-stage eggs (≤4 cells) to quantify hyperactive egg laying. The use of both assays, along with measurements of brood size, ensures that changes in egg-laying behavior can be differentiated from changes in egg production (Koelle and Horvitz 1996). Brood sizes for strains shown in Figure 7 were: Wild type, 247 ± 12; *egl-10*, 226 ± 37; *rgs-1*, 253 ± 34; *rgs-2*, 220 ± 35; and *rgs-1; rgs-2*, 167 ± 41. All assays were performed on animals selected as late L4 larvae and aged at 20°C to produce precisely staged adults. The post-L4 aging times used were 36 hr (Fig. 2) and 30 hr (Figs. 3,7,8).

To measure responses to starving and feeding, staged adult animals were allowed to feed or starve for 2 hr at 20° C by transferring them to NGM plates either with or without a lawn of OP50 bacteria as food (Wood 1988). Ten of the starved worms were then transferred to a bacteria-free plate, and another 10 starved worms were transferred to a plate with a lawn of OP50. As a control, 10 nonstarved worms were put onto a plate with a lawn of OP50. The three assay plates were each ringed by a circle of 4M fructose solution as an osmotic barrier to prevent worms from leaving the agar surface. All three groups of worms were allowed to lay eggs for 30 min and the number of eggs laid was counted. For time course experiments the animals were allowed to continue to lay eggs and the accumulation of eggs was counted every 30 min. Each determination was repeated eight times and the results were averaged.

Antibodies

RGS-1 and RGS-2 were expressed in *E. coli* as glutathione S-transferase fusion proteins, purified, and injected into rabbits to raise polyclonal antisera. RGS-1 and RGS-2 were also expressed in *E. coli*, fused to the maltose binding protein, and these fusion proteins were used to affinity purify the antisera as described by Koelle and Horvitz (1996). For Western blots, the RGS proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescence detection reagents (Pierce).

GFP transgenes

GFP reporter constructs were constructed by inserting genomic DNA fragments from *rgs-1* or *rgs-2* into the vectors pPD95.69 and pPD95.67 (provided by Dr. Andrew Fire, Carnegie Institute of Washington). These constructs contained the promoter regions and 5′ coding sequences of the RGS genes, such that a coding exon for each gene was fused in frame to the coding sequence for GFP. The *rgs-1* transgene contained sequences from –2399 to +3 of the *rgs-1* gene (coordinates relative to the translation start site of *rgs-1*). The *rgs-2* transgene contained sequences from –4770 to +3592 (relative to the *rgs-2* translation start), and thus included the large first intron of *rgs-2*.

cDNA sequences of rgs-1 *and* rgs-2

A 1131-bp *rgs-1* cDNA clone was obtained from the library of Barstead and Waterston (1989) and extended at its 5′ end by an additional 179 bp using the 5′-RACE technique (Frohman et al. 1988). The resulting cDNA sequence appears to be full length as it has an SL1 trans-spliced leader at its 5′ end (Krause and Hirsh 1987), a poly(A) tail at its 3′ end, and it matches the length of the single 1.3-kb message seen on Northern blots. An *rgs-2* expressed sequence tag identified an *rgs-2* cDNA clone (yk78b6), which we obtained from Yuji Kohara. The 1562-bp sequence of this clone contains a complete open reading frame and is similar

in length to the two *rgs-2* transcripts of 1.4 and 1.6 kb seen on Northern blots. The structural differences between the two transcripts have not been investigated. The GenBank accession numbers for the *rgs-1* and *rgs-2* sequences are AF220159 and AF220265, respectively.

Protein purification and GTPase assays

RGS-1, RGS-2, and GOA-1 were expressed and purified using the same strategy. The proteins were expressed in *E. coli* as fusion proteins consisting of glutathione S-transferase, followed by a His(6) tag, followed by a cleavage site for the tobacco etch virus (TEV) protease (Parks et al. 1994), followed by the protein of interest. The fusion proteins were partially purified on glutathione agarose, cleaved by a His(6)-tagged recombinant TEV protease to remove the GST–His(6) tag, passed through Ni–NTA resin to absorb the released GST–His(6) tag as well as the His(6)–TEV protease and any uncleaved fusion protein, and further purified by anion exchange chromatography. Single-turnover GTPase assays were performed as described by Berman et al. (1996).

C. elegans *gene knockouts*

A library of frozen mutant worms derived from 460,000 trimethlypsoralen/UV mutagenized animals was screened by PCR for deletions in the *rgs-1* and *rgs-2* genes. Library construction and screening methods were adapted from Liu et al. (1999) and are described in detail at http://info.med.yale.edu/mbb/koelle/. The *rgs-1(nr2017)* mutation came from a similarly prepared library of ethylnitrosourea-mutagenized animals. Deletion mutants were outcrossed at least four times to the wild type to produce clean genetic backgrounds. *rgs-1(nr2017)* is a 638-bp deletion of sequences whose limits are CGAGAAATTGTCAACACTAAC. . .GTTTGGAATGGTTTATCAGTT. The deleted material is replaced by the following 35-bp insertion: TATGTTTAAGTTAAGTTTATAGTTTAAGTTTAAAG. *rgs-1(vs26)* is a 1409-bp deletion of sequences with limits GTATCATCTATGAAGATTTCA. . .GTGTAACTAATATGCAAAAGT. *rgs-2(vs17)* is a 1136-bp deletion of sequences with limits ATATATATATCTCATTACTGG. . .AATCAAGTGTAACACTAATAT. *rgs-2(vs22)* is a 495-bp deletion with limits ATATATATATATCTCATTACT. . .TTTCAGGCTCGCATTATCTAC.

Acknowledgments

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