Antagonism between $G_o\alpha$ and $G_q\alpha$ in *Caenorhabditis elegans*: the RGS protein EAT-16 is necessary for $G_o\alpha$ signaling and regulates $G_q\alpha$ activity

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To elucidate the cellular role of the heterotrimeric G protein G_o, we have taken a molecular genetic approach in *Caenorhabditis elegans*. We screened for suppressors of activated GOA-1 ($G_o\alpha$) that do not simply decrease its expression and found mutations in only two genes, *sag-1* and *eat-16*. Animals defective in either gene display a hyperactive phenotype similar to that of *goa-1* loss-of-function mutants. Double-mutant analysis indicates that both *sag-1* and *eat-16* act downstream of, or parallel to, $G_o\alpha$ and negatively regulate EGL-30 ($G_q\alpha$) signaling. *eat-16* encodes a regulator of G protein signaling (RGS) most similar to the mammalian RGS7 and RGS9 proteins and can inhibit endogenous mammalian G_q/G_{11} in COS-7 cells. Animals defective in both *sag-1* and *eat-16* are inviable, but reducing function in *egl-30* restores viability, indicating that the lethality of the *eat-16; sag-1* double mutant is due to excessive $G_q\alpha$ activity. Analysis of these mutations indicates that the G_o and G_q pathways function antagonistically in *C. elegans*, and that $G_o\alpha$ negatively regulates the G_q pathway, possibly via EAT-16 or SAG-1. We propose that a major cellular role of G_o is to antagonize signaling by G_q.

[*Key Words*: *C. elegans*; G_o protein; G_q protein;RGS protein; signaling; regulation]

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$G_o\alpha$, a member of the G_i subfamily, is the major heterotrimeric G protein α-subunit of the brain and exists only in species with a nervous system. Although $G_o\alpha$ homologs have been isolated biochemically from several species, including cow (Sternweis and Robishaw 1984; Van Meurs et al. 1987), *Drosophila* (Yoon et al. 1989), *Xenopus* (Olate et al. 1989), hamster (Hsu et al. 1990), and man (Lavu et al. 1988), little is known about the mechanisms through which $G_o\alpha$ functions. To elucidate these mechanisms, we are studying *Caenorhabditis elegans* $G_o\alpha$ (GOA-1), which is 81%–82% identical to mammalian homologs (Lochrie et al. 1991) and is expressed throughout the entire *C. elegans* nervous system (M.R. Koelle, unpubl.; Mendel et al. 1995; Ségalat et al. 1995) and apparently also in some muscles (Mendel et al. 1995; Ségalat et al. 1995). GOA-1 modulates many behaviors, including locomotion and egg laying: mutants defective in *goa-1* function display hyperactive egg-laying and locomotion behaviors, whereas transgenic animals overexpressing wild-type or constitutively activated GOA-1 are lethargic and egg-laying defective (Mendel et al. 1995; Ségalat et al. 1995). Heat shock-induced expression of activated GOA-1 results in lethargy at any developmental stage, indicating that GOA-1 can function throughout the life span of the worm (Mendel et al. 1995).

G protein subunits can function as switches in signal transduction (Simon et al. 1991; Hepler and Gilman 1992). When inactive, the Gα-subunit is bound to GDP and associated with the Gβγ-subunits. Upon activation of an associated transmembrane receptor by a ligand, the α-subunit exchanges GDP for GTP and dissociates from βγ. In this state, the α-subunit is free to interact with effector molecules. GTP hydrolysis inactivates the α-subunit, returning it to Gβγ. Free βγ can also interact with effectors (Birnbaumer 1992). Substitution of leucine for a glutamine in a residue required for GTPase activity (Q205L for GOA-1 and EGL-30) renders the Gα-subunit constitutively activated (Graziano and Gilman 1989).

GOA-1 activity is thought to be regulated by EGL-10 (Trent et al. 1983), which along with the yeast *SST2* gene and GAIP first defined the RGS family of proteins (de Vries et al. 1995; Dohlman et al. 1996; Koelle and Hor-

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vitz 1996). RGS proteins negatively regulate G protein activity (Arshavsky and Pugh 1998; Berman and Gilman 1998) by acting as GTPase activating proteins (GAPs) for Gα-subunits, stabilizing the transition state during hydrolysis, and facilitating a rapid return to the inactive state (Berman et al. 1996a; Hunt et al. 1996; Watson et al. 1996; Faurobert and Hurley 1997). *egl-10* loss-of-function mutant animals have the opposite phenotype as *goa-1* loss-of-function mutants. Eliminating EGL-10 function in a mutant lacking GOA-1 has no additional phenotypic effect, suggesting that EGL-10 may act as a GAP specific for $G_o\alpha$ in *C. elegans* (Koelle and Horvitz 1996). So far, 19 mammalian RGSs have been found (Berman and Gilman 1998), and the *C. elegans* genome project has identified 12 genes containing the RGS core domain (Sulston et al. 1992; the *C. elegans* Sequencing Consortium 1998). Whereas EGL-10 may be an RGS for *C. elegans* $G_o\alpha$, an RGS that regulates *C. elegans* $G_q\alpha$ has not yet been identified.

To identify components in $G_o\alpha$-mediated signaling, we performed random mutagenesis looking for suppressors of constitutively activated $G_o\alpha$ in *C. elegans*, and we isolated mutations in two loci that appear to act downstream of, or parallel to, $G_o\alpha$ based on epistasis analysis: *sag-1*, a new locus, and *eat-16* (Avery 1993). Here, we present an analysis of the function of *eat-16*. We positionally cloned *eat-16* and found that it encodes an RGS homolog with an expression pattern similar to that of GOA-1. Based on double- and triple-mutant analysis involving $G_o\alpha$, $G_q\alpha$, *sag-1*, and *eat-16*, we believe that EAT-16 functions as an RGS for $G_q\alpha$ and that $G_o\alpha$ may negatively regulate $G_q\alpha$-mediated signaling in egg laying and locomotion. Consistent with our in vivo genetic data, EAT-16 can down-regulate the endogenous mammalian G_q/G_{11} when transfected into COS-7 cells. SAG-1 strongly inhibits $G_q\alpha$-mediated signaling and may function downstream of, or parallel to, $G_q\alpha$.

Results

Isolation of sag-1 *and* eat-16 *mutations as suppressors of activated GOA-1*

We performed a genetic screen for extragenic suppressors of *syIs17*, an integrated transgene expressing the constitutively activated *goa-1[Q205L]* mutant gene under the control of a heat shock promoter (hs-G_oQL). Upon heat shock, *syIs17* animals progressively cease locomotion, foraging, feeding, and production and laying of eggs (Mendel et al. 1995). Animals were mutagenized with either ethylmethanesulfonate (EMS; 21,000 haploid genomes) or trimethylpsoralen and UV irradiation (11,000 haploid genomes). The grandprogeny of mutagenized animals were heat-shocked as adults, and moving or foraging mutants were selected. In this manner, 15 independent suppressor strains were isolated that displayed a hyperactive phenotype (see below) in addition to suppressing *syIs17[hs-G_oQL]* (Fig. 1). Fourteen of these mutations mapped to the same region and failed to complement one another, defining a new locus, *sag-1* (suppressor of activated G protein). The other mutation, *sy438*, was allelic to *eat-16(ad702)*, which was isolated previously in a screen for defects in pharyngeal pumping (Avery 1993). *ad702*, the original mutation defining *eat-16*, could also suppress the heat shock-induced lethargy of *syIs17[hs-G_oQL]*, as could *sy438/ad702 trans*-heterozygotes (data not shown).

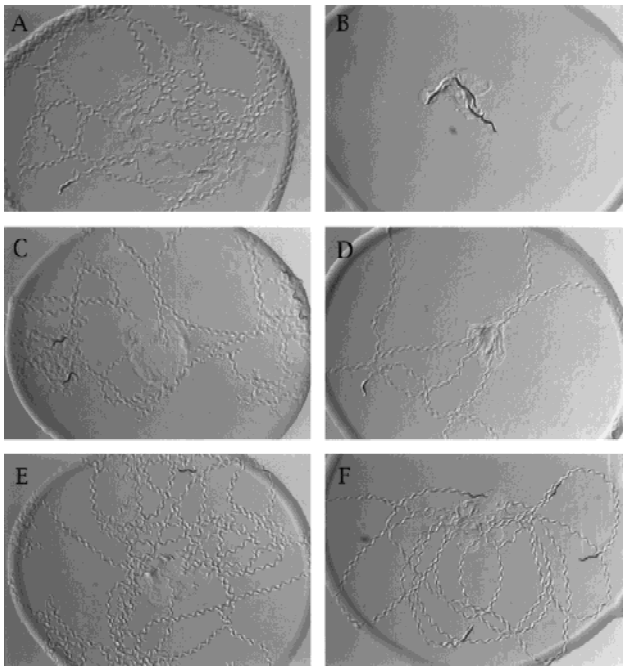

Figure 1. *sag-1* and *eat-16* mutations suppress the lethargy caused by heat shock of *syIs17[hsp::goa-1(QL)]*. Five adult worms were placed in the center of a bacterial lawn, allowed to crawl for 5 min, and photographed. Animals were then heat shocked and photographed 3 hr later. (*A*) *dpy-20 syIs17* animals without heat shock. (*B*) *dpy-20 syIs17* animals after heat shock treatment. (*C*) *dpy-20 syIs17; sag-1(sy428)*. (*D*) The same *dpy-20 syIs17; sag-1(sy428)* animals after heat-shock treatment. (*E*) *eat-16(sy438); dpy-20 syIs17*. (*F*) The same *eat-16(sy438); dpy-20 syIs17* animals after heat shock treatment.

Linkage tests eliminated the possibility that the suppression of hs-G_oQL could have been due to a deletion of the *syIs17* locus. All 14 *sag-1* mutations were X-linked and resided close to *unc-1*. Three-factor mapping placed *sag-1(sy428)* between *unc-1* and *egl-17*, whereas *eat-16(sy438)* mapped to linkage group (LG) I between *unc-29* and *lin-11* (see Fig. 2; Materials and Methods). In contrast, *syIs17* maps to LG IV (J. Mendel, pers. comm.).

sag-1 *and* eat-16 *mutants resemble* goa-1(lf) *mutants*

sag-1 and *eat-16* mutations not only suppressed the lethargy of hs-G_oQL (Fig. 1) but in a wild-type background conferred a phenotype similar to that of *goa-1* loss-of-function mutants (Mendel et al. 1995; Ségalat et al. 1995). Mutants laid eggs hyperactively, that is, soon after fertilization, resulting in eggs laid as early as uncleaved (Table 1). In addition, eggs were produced more slowly than the wild type (data not shown), resulting in uteri devoid of eggs (Table 1). Forward locomotion of *sag-1* and

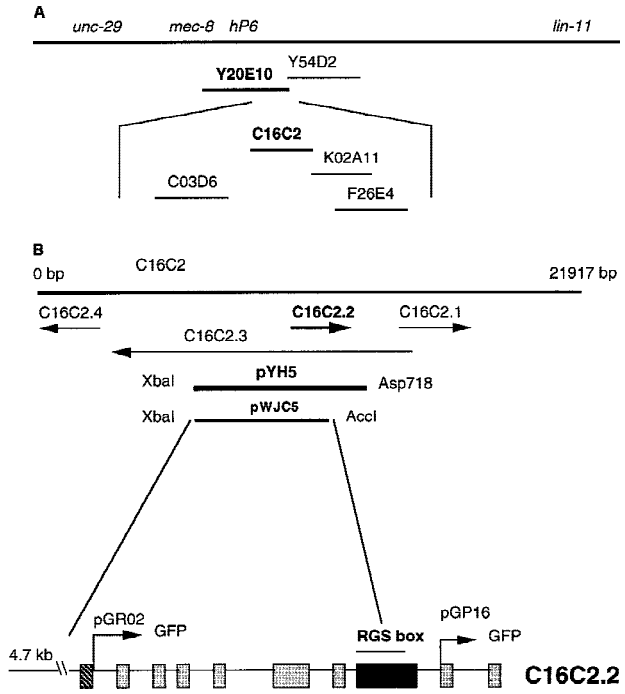

Figure 2. Positional cloning of *eat-16*. (*A*)Physical map of *eat-16*. *Eat-16* was mapped to the *left* half of the interval between *unc-29* and *lin-11* and to the *right* of *hP6* (see Materials and Methods). Two YACs covering this region were injected into *syIs17 dpy-20; eat-16(sy438); lin-15(n765)*. The *eat-16* phenotype was rescued by Y20E10 but not Y54D2. Four cosmids between *hP6* and the *right* end of Y20E10 were tested, and C16C2 rescued *eat-16(sy438)*. (*B*)Subclones of cosmid C16C2; cDNA map of *eat-16* and GFP constructs. Cosmid C16C2 has four open reading frames (*C. elegans* Sequencing Consortium 1998). The rescuing plasmid pYH5 contains the full sequence of C16C2.2 cloned into pBluescript; the first two exons of C16C2.3 are not included. The plasmid pWJC5 is an *Xba*I–*Acc*I fragment from pYH5 cloned into pBluescript; it includes the same promoter region as pYH5, but the coding sequence of EAT-16 is terminated in the middle of RGS domain at amino acid 352. Full-length cDNA sequence of *eat-16* was obtained from clone yk356b3. *eat-16* has 10 exons; the RGS domain is contained within Exon 8, the largest exon. Reporter construct pGP16 includes 4.7 kb of the upstream promoter region and contains most of the *eat-16* coding region, including the RGS domain. Reporter construct pGR02 contains the upstream promoter region but only the first coding exon of *eat-16*.

eat-16 mutants was more rapid than wild type (Table 1). Conversely, pharyngeal pumping rates were impaired (Table 1); therefore, *sag-1* and *eat-16* mutants were somewhat starved and had a pale, scrawny appearance. Thus, the phenotypes of *sag-1* and *eat-16* mutants indicated that these genes might function in a G_o-mediated signaling pathway.

goa-1(n363) null mutants crawl backwards with deeply exaggerated flexions compared with their forward locomotion (J. Mendel, unpubl.); this behavior was not observed in other *goa-1* mutants (J. Mendel, unpubl.) or in *sag-1(sy428)* or *eat-16* mutants. Either the *n363* deletion, which removes more than the entire *goa-1* coding region (Ségalat et al. 1995), also removes a neighboring gene responsible for this phenotype, or the behavior is mediated via a different mechanism than that involving SAG-1 or EAT-16.

Of the 14 *sag-1* mutations, *sy428* was used as the reference allele for all experiments: it displays a strong hyperactive phenotype and appears to be a null or strong reduction-of-function mutation. *sy428* is recessive: One hundred percent of heterozygotes were wild type in appearance and had at least seven eggs in their uteri ($n = 90$), and *sy428/Df* heterozygous animals display a similar phenotype to that of *sy428* homozygotes (see Materials and Methods). *eat-16(ad702)* and *eat-16(sy438)* have similar phenotypes (Table 1) and are reduction-of-function mutations (see below).

SAG-1 and EAT-16 do not affect GOA-1 expression

syIs17[hs-G_oQL] was selected as the parent strain for mutagenesis because of its stability and ease of culture; however, mutations might suppress hs-G_oQL by affecting heat shock-induced protein expression in general. Two experiments addressed this possibility. First, Western blot analysis indicated that mutations in *sag-1* or *eat-16* do not lower heat shock-induced GOA-1 expression (data not shown). Second, we examined the ability of *sag-1(sy428)* and *eat-16(sy438)* mutations to suppress activated GOA-1 under control of its normal regulatory sequences rather than a heat shock promoter. Both *sag-1(sy428)* and *eat-16(sy438)* suppressed the lethargy of *syIs9*, an integrated transgene of $G_o\alpha$ [Q205L] under control of the *goa-1* promoter (G_oQL; Mendel et al. 1995; Table 1). These experiments suggested that SAG-1 and EAT-16 function in GOA-1 signaling rather than in GOA-1 expression.

SAG-1 and EAT-16 function downstream of, or parallel to, GOA-1

eat-16(sy438) suppressed the lethargy and egg-laying defect of *syIs9[G_oQL]* (see above; Table 1), indicating that EAT-16 functions downstream of, or parallel to, GOA-1 and is required for GOA-1 signaling in both behaviors. Suppression of the G_oQL locomotory defect by *sag-1(sy428)* was similarly robust (Table 1), indicating that SAG-1 likely functions downstream of GOA-1 at least with respect to locomotion. *syIs9[G_oQL]; sag-1(sy428)* also laid fewer late-stage eggs than did *syIs9[G_oQL]*, but the egg-laying defect was only partially suppressed (Table 1).

In addition to suppressing activated $G_o\alpha$, *sag-1(sy428)* also significantly suppressed the egg-laying and locomotory defects of reduction-of-function mutations in *egl-30*, a *C. elegans* $G_q\alpha$ homolog that acts antagonistically to $G_o\alpha$, either in parallel or downstream (see below). In all cases, suppression of *egl-30* by *sag-1(sy428)* was stronger than that by *eat-16(sy438)* (Table 2; see below). We infer that SAG-1 and EGL-30 act on a common process and that SAG-1 functions downstream of, or parallel to, EGL-30.

Table 1. *Genetic characterization of* sag-1 *and* eat-16 *mutants*

Strain	Egg-laying phenotype: cells per egg	eggs (no.)	eggs in uterus	animals (no.)	Forward locomotion (sine waves/min)	Animals (no.)	Feeding (pumps/min)	Animals (no.)
Wild type (N2)	>10	50	12 ± 1.8	10	25.3 ± 4.9	10	218 ± 21	10
sag-1(sy428)	3.0 ± 1.4	50	1.5 ± 0.9	10	37.4 ± 8.7	10	161 ± 43	10
eat-16(sy438)	2.2 ± 1.1	50	1.3 ± 0.7	10	41.8 ± 6.8	12	156 ± 37	10
sy438/ +	>10	32	13 ± 3	6	N.D.		N.D.	
eat-16(ad702)	2.2 ± 1.0	50	2.8 ± 2.0	10	51.3 ± 8.9	11	110 ± 30	10
ad702/ +	>10	30	12 ± 3	8	N.D.		N.D.	
syIs9 [GoQ205L]	100% late[a]	25	16 ± 4.4	30	12.1 ± 5.1	9	N.D.	
eat-16(sy438); syIs9	0% late[a]	10	7.4 ± 2.9	5	35.9 ± 11.5	10	N.D.	
syIs9; sag-1(sy428)	85% late[a]	27	6.6 ± 2.2	30	40.0 ± 8.1	9		

N.D.Animals were assayed as described in Materials and Methods.
(N.D.) Not determined.
[a]Late-stage eggs are defined as having at least 50 cells (see Materials and Methods).

eat-16 encodes an RGS homolog

To understand the function of *eat-16* in $G_o\alpha$-mediated signaling, we positionally cloned it by transformation rescue. We mapped *eat-16* to the left half of the *unc-29 lin-11* interval and to the right of *mec-8* and *hP6* (Fig. 2A; see Materials and Methods). Rescue was obtained with Y20E10, one of two YACs tested that reside in the left part of the interval between *hP6* (Starr et al. 1989; Lundquist and Herman 1994) and *lin-11* (Freyd et al. 1990), and with C16C2, one of four cosmids tested. C16C2 contains four predicted open reading frames, including C16C2.2, an RGS homolog. C16C2.2 resides entirely within one large intron of its oppositely oriented neighbor C16C2.3 (Fig. 2B). Injection of pYH5, a 7-kb *Asp*718–*Xba*I subclone containing the promoter region and all of C16C2.2 (Fig. 2B) rescued the *eat-16(sy438)* mutant phenotype.

All members of the RGS family have a designated 120-amino-acid RGS core domain (Tesmer et al. 1997). Some of them (RGS7, RGS9, EGL-10, and RGS11) are also highly conserved throughout the amino terminal region, which includes the DEP domain and the GGL domain. The function of the DEP domain is unknown, but the GGL domain is ~34% identical to Gγ and can bind with Gβ in vitro (Snow et al. 1998). Full-length cDNA sequence of C16C2.2 was obtained from the clone yk356b3, a gift from Yuji Kohara (National Institute of Genetics, Mishima Japan. Sequence analysis indicates that C16C2.2 contains all three domains, making it most similar to RGS7, RGS9, and EGL-10 (Fig. 3).

To determine whether the RGS domain of C16C2.2 is necessary to rescue the *eat-16* mutant phenotype, we constructed pWJC5, a truncated genomic clone of *eat-16* that lacks amino acids 353–474. The carboxy-terminal part of the RGS domain (where *sa609* and *sy438* are located; see below) is deleted in this construct (Fig. 2B). Injection of this plasmid failed to rescue the *eat-16* phenotype, indicating that the RGS domain of C16C2.2 is required to suppress the phenotype of activated $G_o\alpha$, and the loss of it causes hyperactive egg laying and locomotion.

sy438 *and* ad702 *are reduction-of-function alleles of eat-16*

To verify that *eat-16* encodes the C16C2.2 RGS protein, we amplified and sequenced C16C2.2 genomic DNA from each *eat-16* mutant (see Materials and Methods). Each strain contained a single point mutation that was then confirmed by sequencing the opposite strand of DNA in the region of the mutation (Fig. 3). *sy438* is a missense mutation that changes a conserved serine at position 400 in the RGS domain to a phenylalanine. *ad702* is an AG → AA mutation in the splice acceptor site before the fourth exon, which is predicted to result in early termination before the RGS domain, although some properly spliced message is likely produced (see Aroian et al. 1993). We also sequenced two other alleles of *eat-16*, *sa609* and *sa735*, kindly provided by M. Robatzek and J. Thomas (University of Washington, Seattle). *sa735* is an AG → AA mutation in the splice acceptor site before the eighth exon (which contains the RGS domain), and *sa609* is another missense mutation within the RGS domain that changes a conserved arginine at position 396 to a cysteine. Both *sa609* and *sa735* confer a phenotype similar to *sy438* and *ad702* (data not shown).

Because the missense mutations confer a phenotype similar to the splice acceptor site mutations, they likely reduce EAT-16 function. Although *sy438* is essentially recessive (Table 1), we noticed that 6.5% of *sy438/+* heterozygotes looked like *sy438* homozygotes ($n = 92$). To test whether this effect was due to semidominance or haploinsufficiency, we examined by Nomarski optics animals heterozygous for the deficiency chromosome *ces-1(n703d) qDf9* (Ellis and Kimble 1995), which deletes *eat-16* and found that 58% of these animals had fewer than six eggs in their uteri and these eggs contained eight or fewer cells ($n = 60$ animals), indicating that the animals were laying eggs hyperactively. When placed in *trans* to *qDf9*, both *sy438* and *ad702* were viable and similar in phenotype to *sy438* and *ad702* homozygotes (data not shown). In contrast, animals bearing

Table 2. *Suppression of* egl-30 *mutant phenotypes by* sag-1 *and* eat-16

Strain	Egg-laying defects						Forward locomotion (sine waves/min)	Animals (no.)
	late-stage eggs (%)[a]	eggs (no.)	eggs about to hatch (%)	eggs (no.)	eggs retained in uterus	animals (no.)		
egl-30(ad805)	100	6[b]	83	6[b]	26.1 ± 5.8	18	N.A.[c]	
ad805 eat-16	75	8[b]	0	8[b]	27.1 ± 6.6	14	N.A.[c]	
ad805; sag-1	4	25	0	25	17.6 ± 4.0	19	17.8 ± 5.1	5
egl-30(ad809)	63	35	0	35	24.2 ± 2.9	21	N.A.[c]	
ad809 eat-16	37	38	0	38	21.6 ± 3.7	22	N.A.[c]	
ad809; sag-1	0	40	0	40	7.2 ± 1.6	21	26.2 ± 5.8	12
egl-30(md186)	77	13	31	13	22.9 ± 4.8	21	10.1 ± 3.7	9
md186 eat-16	20	35	0	35	22.0 ± 3.6	20	14.0 ± 4.7	11
md186; sag-1	3	35	0	35	13.9 ± 3.4	20	19.8 ± 4.1	6
md186 eat-16; sag-1	0	50	0	50	9.4 ± 2.4	20	32.3 ± 8.7	10
egl-30(n686)	90	30	67	30	20.2 ± 4.9	20	16.7 ± 4.4	18
n686 eat-16	53	49	12	49	15.2 ± 3.3	20	29.4 ± 9.7	9
n686; sag-1	4	24	0	24	6.8 ± 2.0	18	35.4 ± 6.7	10

Strains were assayed as described in Materials and Methods. Double mutants were constructed with *sag-1(sy428)* and *eat-16(sy438).*
[a]Late stage was defined as after the comma stage (see Materials and Methods).
[b]Eggs were laid infrequently; therefore they were difficult to harvest.
[c](N.A.) Not available; mutants made few or no sinusoidal waves.

multiple copies of wild-type EAT-16 in the transgene *syEx256* are somewhat egg-laying defective (data not shown). That presumed overexpression has a phenotype opposite that of *sy438* and *ad702* supports the conclusion that *sy438* and *ad702* reduce EAT-16 function.

The expression pattern of EAT-16 is similar to that of GOA-1

To examine expression, we made GFP translational fusions linking GFP either to the amino terminus or carboxyl terminus of *eat-16*. Reporter construct pGP16 (Fig. 2) contains a 7.4-kb *Apa*I–*Bam*HI genomic fragment (including the *eat-16* promoter region) and fuses to GFP-coding sequences in the ninth coding exon predicted by the cDNA sequence; this construct contains the entire amino-terminal region and the RGS domain. Examination of transgenic animals carrying pGP16 using confocal microscopy showed that EAT-16 is expressed in most or all neurons, including the hermaphrodite specific neuron (HSN) required for egg laying, as well as in the vulval and pharyngeal muscles (Fig. 4). Expression was also occasionally seen in the spermatheca and body wall muscles (data not shown). Similar expression patterns were seen when the GFP-coding sequences were fused to the first coding exon of *eat-16*. The expression pattern is consistent with the mutant phenotypes observed and is similar to the GOA-1 expression pattern (Mendel et al. 1995; Ségalat et al. 1995); therefore, *eat-16* might act in many of the same cells as $G_o\alpha$.

EAT-16 does not regulate GOA-1

RGS proteins have been shown to facilitate the inactivation of Gα-subunits by GTP hydrolysis (Berman et al. 1996a; Hunt et al. 1996; Watson et al. 1996; Faurobert and Hurley 1997); therefore, EAT-16 likely regulates one or more Gα-subunits in *C. elegans*. We thought it unlikely that $G_o\alpha$ would be the target for EAT-16 based on the following arguments. First, the *syIs17* parent strain expresses multiple copies of constitutively activated GOA-1(Q205L) upon heat shock, which would likely be insensitive to a wild-type RGS for G_o (Berman et al. 1996b). Reducing the function of this RGS would render the excess activated subunits even more immune to down-regulation and therefore would not suppress the hs-G_oQL phenotype. Second, if EAT-16 negatively regulates GOA-1, we would expect the *eat-16* reduction-of-function phenotype to resemble that of activated $G_o\alpha$; instead, *eat-16* mutants resemble *goa-1* hypomorphs. Finally, *eat-16* appears to act downstream of, or parallel to, *goa-1* based on double-mutant analysis with *syIs9[G_oQL]*.

However, the similar expression patterns of EAT-16 and GOA-1 encouraged us to further test the possibility that EAT-16 regulates GOA-1. We overexpressed wild-type EAT-16 in a *goa-1* null mutant background and found that the transgene *syEx256[eat-16(+)]* suppressed the hyperactive egg-laying behavior of *goa-1(n363)*: Ten percent of eggs laid by *goa-1; syEx256* animals were premature (n = 48), compared with 97% of eggs laid by their nontransgenic siblings (n = 34) (see Materials and Methods). If EAT-16 preferentially regulates GOA-1, we would have seen no suppression of *goa-1(n363)*, because the *n363* lesion deletes the entire *goa-1* coding region (Ségalat et al. 1995). Therefore, we conclude that the major function of EAT-16 is not to regulate GOA-1 activity.

Genetic evidence that EAT-16 regulates EGL-30 $G_q\alpha$

Because GOA-1 was an unlikely target for EAT-16, we considered other *C. elegans* Gα-subunits. Two of many

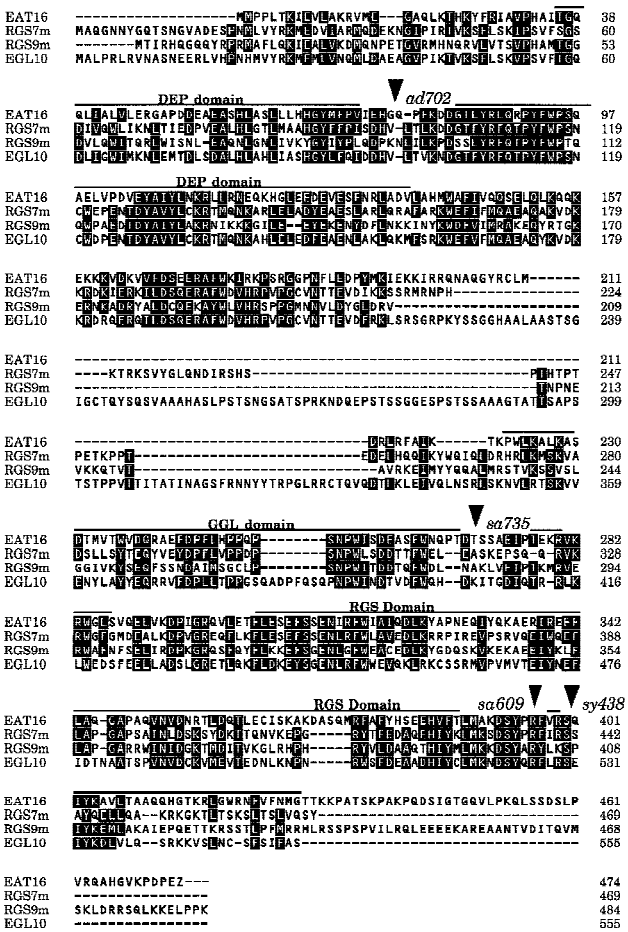

Figure 3. Sequence alignment of EAT-16 with RGS7, RGS9, and EGL-10. Regions of sequence similarity are highlighted. EAT-16 is 42% identical to mouse RGS7 in the RGS domain, 39% identical in the DEP domain, and 45% identical in the GGL domain; 41% identical to mouse RGS9 in the RGS domain, 37% identical in the DEP domain, and 34% identical in the GGL domain; and 30% identical to *C. elegans* EGL-10 in the RGS domain, 40% identical in the DEP domain, and 39% identical in the GGL domain. Arrowheads indicate the sites of *eat-16* mutations. *ad702* is AG → AA in the splice acceptor site before exon 4, *sa735* is AG → AA in the splice acceptor site before exon 8, *sa609* is Arg-396–Cys, and *sy438* is Ser-400–Phe.

Gα-subunits identified in *C. elegans* have been shown to affect the same sets of behaviors as $G_o\alpha$: EGL-30, the $G_q\alpha$ homolog (Brundage et al. 1996), and GSA-1, the $G_s\alpha$ homolog (Korswagen et al. 1997). Because the crystal structure of $G_s\alpha$ as well as biochemical evidence suggests that $G_s\alpha$ is not regulated by an RGS (Berman et al. 1996b; Tesmer et al. 1997; Natochin and Artemyev 1998), we focused our attention on $G_q\alpha$. Whereas the putative null mutation, *ad810*, is lethal (Brundage et al. 1996), reduction-of-function mutations in *egl-30* result in a lethargic and egg laying-defective phenotype roughly opposite to that of *goa-1* reduction-of-function or null mutations. *ad809* is a splice-donor site mutation, and *ad805* and *n686* are splice-acceptor site mutations; all result in reduced copies of full-length EGL-30 (Brundage et al. 1996). *md186* is a missense mutation that reduces EGL-30 activity (Miller et al. 1996; L. Brundage, pers. comm.). The phenotypes of these reduction-of-function mutants vary in severity with *ad805* having the strongest phenotype (Brundage et al. 1996). Because *egl-30* and *eat-16* reduction-of-function mutations have essentially opposite phenotypes, we asked whether EAT-16 might regulate EGL-30 activity.

We reasoned that if EAT-16 accelerates $G_q\alpha$ GTPase activity, reducing EAT-16 function should allow more $G_q\alpha$-subunits to remain active, thereby alleviating the phenotype of a $G_q\alpha$ hypomorph, whereas reducing EAT-16 function in a null $G_q\alpha$ background should have no phenotypic effect. To test this hypothesis, we built double mutants between *eat-16(sy438)* and several *egl-30* mutations. Although *sy438* did not suppress the lethality of the putative null allele *ad810* (see Materials and Methods), we found that *sy438* partially suppressed the egg-laying defect of all hypomorphs tested and partially suppressed the locomotory defect of *n686* (Table 2). These results support the hypothesis that EAT-16 inhibits EGL-30 activity.

To examine whether multiple copies of EAT-16 could compensate for EGL-30 overexpression, we overexpressed EAT-16 (using the transgene *syEx256*) in two different *egl-30* transgenic strains (see Materials and Methods). Animals bearing *syIs36[egl-30(+)]*, an integrated transgene overexpressing wild-type EGL-30 (L. Brundage, pers. comm.), move and lay eggs hyperactively (see

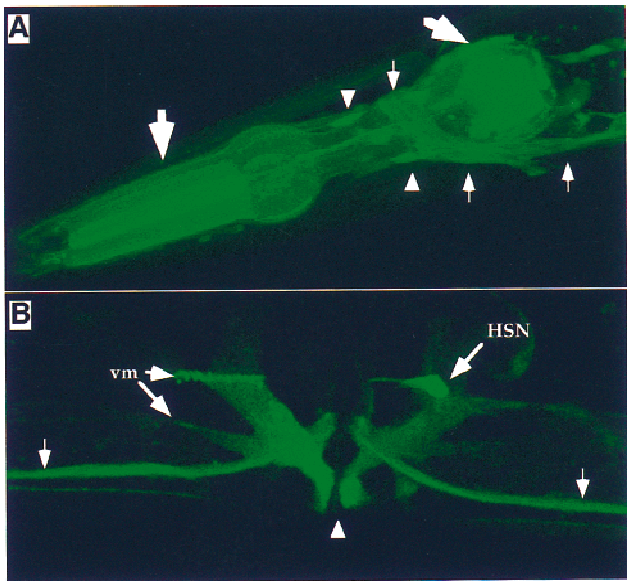

Figure 4. Expression of *eat-16* in *C. elegans*. Transgenic animals carrying the *eat-16*::GFP reporter construct pGP16 were examined by confocal fluorescence microscopy. Similar expression patterns were seen using the reporter construct pGR02. (*A*) Adult head region, showing fluorescence in the cell bodies (arrowheads) and processes (small arrows) of many neurons, as well as in pharyngeal muscles (large arrows). (*B*) Adult vulva region. Vulval opening is indicated by the arrowhead. Fluorescence is detected in the HSN neuron, vulva muscles (vm), and ventral cord neurons (small arrows).

Brundage et al. 1996). *syIs36/+; syEx256* transgenic animals displayed various phenotypes (probably due to mosaicism of the *syEx256* transgene), ranging from hyperactive (similar to *syIs36*) to slightly egg-laying defective (similar to *syEx256*); however, suppression of the pale, scrawny phenotype of *syIs36[egl-30(+)]* was observed in 50% of animals (n = 189). In contrast, overexpression of EAT-16 did not suppress the phenotype of overexpression of activated EGL-30(Q205L) under control of a heat shock promoter (L. Brundage, C. Bastiani, P.W. Sternberg, and M.I. Simon, unpubl.; data not shown). The Q205L mutation renders α-subunits insensitive to regulation by an RGS protein (Berman et al. 1996b). That we see suppression of wild-type, but not constitutively activated, EGL-30 by EAT-16 is consistent with a model in which EAT-16 inactivates EGL-30.

EAT-16 reduces endogenous G_q/G_{11} activity in COS-7 cells

The M1 receptor is coupled specifically to G_q/G_{11} in mammalian cells, and the activity of G_q/G_{11} can be measured by a PLCβ-IP_3 assay (Berstein et al. 1992; Wu et al. 1992; Offermanns et al. 1994). We cotransfected COS-7 cells with expression constructs of M1 receptor and *eat-16* (Fig. 5A) and observed that the addition of EAT-16 to the system significantly reduces the PLCβ activity caused by endogenous G_q/G_{11}. A similar result was obtained when we cotransfected EGL-30 and EAT-16 (Fig. 5B; no M1 receptor was added), but because the stimulation of PLCβ activity by EGL-30 is not much greater than the background of endogenous G_q/G_{11}, we cannot infer from this experiment that EAT-16 down-regulates EGL-30.

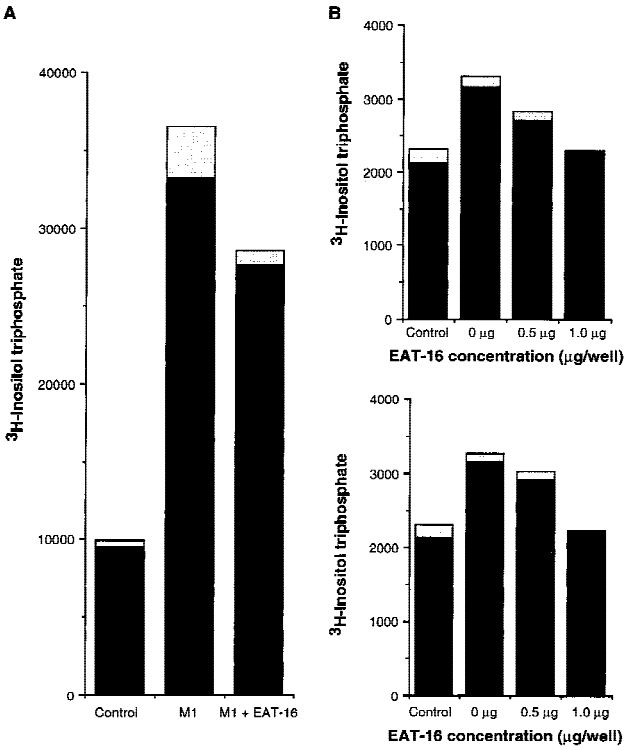

Figure 5. EAT-16 down-regulates the PLCβ activity of endogenous G_q/G_{11} in COS-7 cells. (*A*) M1 receptor cotransfection. COS-7 cells were transfected with either control vector pCIS (*left* lane), 0.25 μg of M1 receptor (middle lane), or 0.25 μg of M1 receptor + 0.25 μg of EAT-16 (*right* lane). The total concentration of DNA was normalized to 1.0 μg per well using pCIS vector. (The M1 receptor activates endogenous G_q/G_{11}.) Shown is the measured [^{3}H]Inositol phosphate level 48 hr after transfection. Higher concentrations of EAT-16 (up to 1.5 μg per well) gave similar results (data not shown). (*B*) EGL-30 cotransfection. EAT-16 was cotransfected at various concentrations with EGL-30 into COS-7 cells. Total DNA concentration was normalized to 2.0 μg per well with pCIS. (No M1 receptor was added in this experiment.) PLCβ activity was caused by both endogenous G_q/G_{11} and EGL-30. (*Top*) COS-7 cells were transfected with control vector pCIS (*left*), 0.5 μg of EGL-30 (second from *left*), or 0.5 μg of EGL-30 with various concentrations of EAT-16 (*right* lanes). (*Bottom*) Same conditions as at *top* except that 1.0 μg of EGL-30 was transfected. (Shaded bars) S.D.; (solid bars) average.

Reducing EGL-30 function restores viability to eat-16; sag-1 *mutants*

We constructed a strain that segregates *eat-16; sag-1* double mutants and found that >99% of the double mutants die (see Materials and Methods), arresting during larval development (Fig. 6A). Because each suppressor mutation results in a starved phenotype, one might argue that the lethality is caused by an additive starvation effect. However, *goa-1(n363)* has a more severe phenotype than either suppressor, and *goa-1(n363); sag-1(sy428)* double mutants are viable. Therefore SAG-1 and EAT-16 appear to act synergistically and are functionally redundant for survival.

Because *sag-1(sy428)* significantly suppresses the phenotype of *egl-30* hypomorphs (see above; Table 2), it seemed likely that SAG-1 and EAT-16 function synergistically to reduce EGL-30 signaling. If so, lowering EGL-30 signaling might suppress the lethality of *eat-16; sag-1* double mutants. To test this hypothesis we constructed the *egl-30(md186) eat-16(sy438); sag-1(sy428)* triple mutant (see Materials and Methods) and found that it is viable to adulthood (Fig. 6C); this result indicates that excess EGL-30 activity is responsible for the lethality of *eat-16; sag-1* double mutants. The lethargic and egg laying-defective phenotype of *egl-30(md186)* (Fig. 6B) was almost completely suppressed in the triple mutant (Table 2; Fig. 6C).

$G_o\alpha$ antagonizes $G_q\alpha$ in C. elegans

Reduction-of-function mutations in *goa-1* and *egl-30* have essentially opposite phenotypes (Mendel et al. 1995; Ségalat et al. 1995; Brundage et al. 1996), suggesting that $G_o\alpha$ and $G_q\alpha$ function antagonistically in *C. elegans*. *egl-30(ad805) goa-1(n363)* double mutants are lethargic and egg laying-defective like *egl-30(ad805)* animals (L. Brundage, P.W. Sternberg, and M.I. Simon, unpubl.), indicating that EGL-30 functions downstream of,

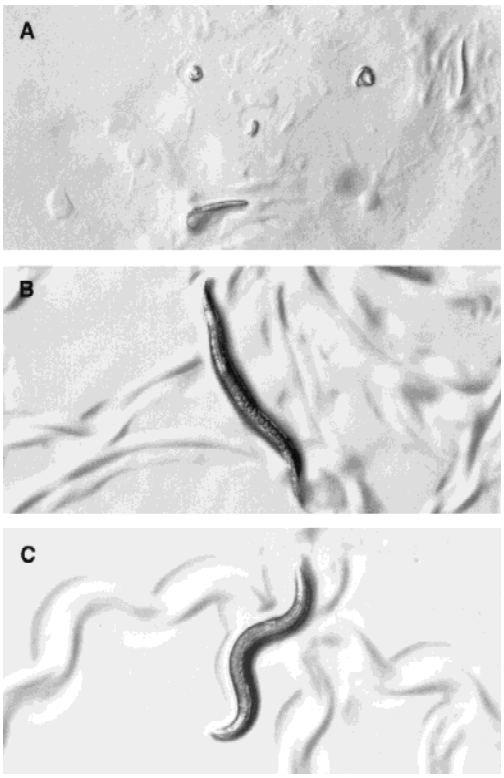

Figure 6. Reducing EGL-30 function restores viability to *eat-16; sag-1* double mutants. All animals were photographed at the same magnification. (*A*) *eat-16(sy438); sag-1(sy428)* double mutants arrest as young larvae. (*B*) *egl-30(md186)* mutants are le thargic and egg-laying defective and leave flattened tracks (Miller et al. 1996). (*C*) *egl-30(md186) eat-16(sy438); sag-1(sy428)* triple mutants are viable and active, leaving sinusoidal tracks and laying eggs.

or parallel to, GOA-1. Although on a gross level the double mutant resembled the *ad805* single mutant, *ad805 n363* had more active egg laying than *ad805* alone: Fifteen percent of *ad805 n363* eggs (n = 34) versus 100% of *ad805* eggs (n = 12) were laid >5 hr after fertilization, respectively. The partial suppression of *ad805* by *n363* might be due to the presumably low level of wild-type EGL-30 activity expressed by the splice acceptor site mutant *ad805* (especially because the *egl-30* null phenotype is lethal; Brundage et al. 1996), which would be enhanced when GOA-1 activity is reduced. Nonetheless, the partial suppression of the egg-laying defect of *egl-30(ad805)* by *goa-1(n363)* is similar to that by *eat-16(sy438)* (see Table 2), consistent with GOA-1 and EAT-16 both negatively regulating EGL-30.

As with reducing gene function, overexpression of wild-type or activated GOA-1 and EGL-30 have opposite phenotypic effects (Mendel et al. 1995; Ségalat et al. 1995; Brundage et al. 1996). We reasoned that if the lethargy of *syIs17[hs-G_oQL]* is due to excessive negative regulation of $G_q\alpha$ activity by activated $G_o\alpha$, then simultaneously overexpressing $G_q\alpha$ might suppress this lethargy. We tested this hypothesis by overexpressing both Gα-subunits in wild-type animals (see Materials and Methods) and found that *syIs36[egl-30(+)]*, which overexpresses multiple copies of wild-type EGL-30, suppressed the heat shock-induced lethargy of *syIs17-[hsG_oQL]* (89 of 90 animals tested). Supporting these observations, our identification of genes involved in EGL-30 signaling as suppressors of activated GOA-1 suggests that $G_o\alpha$ negatively regulates $G_q\alpha$ activity in *C. elegans*.

Discussion

To elucidate the largely unknown role of $G_o\alpha$ in signal transduction, we screened for suppressors of activated GOA-1, the *C. elegans* $G_o\alpha$ homolog. Because in *C. elegans* loss-of-function mutations occur at a frequency of 1 in 5000 to 1 in 2000 EMS-mutagenized gametes (Brenner 1974), our screen of 21,000 EMS-mutagenized gametes should be fairly representative of the *C. elegans* genome. In this EMS screen we isolated 13 mutations in *sag-1* and 1 mutation in *eat-16*. The frequency with which we identified *sag-1* mutations is consistent with their being reduction-of-function mutations. Mutations of *eat-16* appear to be reduction-of-function alleles based on both genetic and molecular criteria. The low frequency with which *eat-16* mutations were isolated suggests that weak mutations in EAT-16 might not suppress *syIs17[hs-G_oQL]* well enough to be detected in our screen. In addition, Avery (1993) did not isolate any mutations in *eat-16* in an F_2 EMS mutagenesis of similar size; *ad702* was isolated in an F_1 screen designed to recover mutations at 100% efficiency (Avery 1993). These results and ours indicate that mutations in *eat-16* are not easily recoverable; perhaps only mutations affecting the RGS domain (one exon) confer starvation and suppression of hs-G_oQL. *eat-16* and *sag-1* mutants display a hyperactive phenotype similar to that of *goa-1* loss-of-function mutants and are required for GOA-1 signaling. The simplest interpretation of our results is that EAT-16 and/or SAG-1 function as effectors for GOA-1; however, it is also possible that the effectors of GOA-1 either do not mutate to a viable phenotype or are numerous and functionally redundant.

EAT-16 and EGL-10 distinguish between G_q and G_i/G_o subfamilies

Several lines of evidence indicate that EAT-16 does not inhibit $G_o\alpha$. *eat-16* reduction-of-function mutations suppress the phenotype of an overexpressed, constitutively activated form of GOA-1 and phenotypically resemble *goa-1* loss-of-function mutants; moreover, overexpression of EAT-16 compensates for a complete deletion of *goa-1*. We have presented genetic and biochemical data consistent with a model in which EAT-16 regulates the $G_q\alpha$ homolog EGL-30. A missense mutation in the RGS domain of *eat-16* alleviates the phenotypes of several *egl-30* reduction-of-function mutations but not that of the putative null allele *ad810*. Overexpression of EAT-16 can suppress the phenotype caused by overexpression of wild-type, but not constitutively activated, EGL-30. Co-

transfection of EAT-16 and M1 receptor in COS-7 cells significantly reduces PLCβ activity resulting from endogenous mammalian G_q/G_{11}, and a similar result was observed in cells cotransfected with EAT-16 and EGL-30. These results taken together argue that EAT-16 functions as a GAP for EGL-30.

EGL-10 and EAT-16, which are homologous to each other in both the amino-terminal region and the RGS domain, have similar GFP expression patterns but opposite phenotypic effects, indicating that they are selectively regulating different G proteins within the same cell. Eliminating EGL-10 function in a *goa-1* null background has no additional phenotypic effect, suggesting that EGL-10 regulates $G_o\alpha$ (Koelle and Horvitz 1996). Our results indicate that EAT-16 does not regulate $G_o\alpha$ activity but instead regulates $G_q\alpha$ activity. Previous in vitro experiments on RGS proteins have provided some evidence that RGS proteins can act on G_q (Heximer et al. 1997; Zhang et al. 1998), but most RGS proteins examined could also act on $G_{i/o}$ family members (Heximer et al. 1997; Zhang et al. 1998). Our results provide in vivo evidence that RGS7 homologs can distinguish among major families of Gα-subunits.

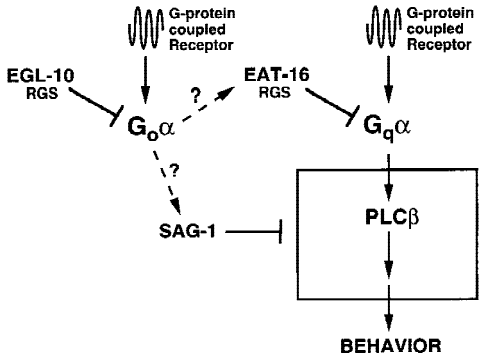

Figure 7. A model for regulation of behavior by GOA-1, EAT-16, and SAG-1. $G_o\alpha$ regulates behavior by antagonizing EGL-30-mediated signaling, either via EAT-16 and/or SAG-1 or an unknown effector. SAG-1 and EAT-16 function downstream, or parallel to, $G_o\alpha$ and operate synergistically to negatively regulate $G_q\alpha$ signaling. EAT-16 regulates EGL-30 by accelerating EGL-30 GTPase activity. SAG-1 also negatively regulates EGL-30 signaling and likely functions downstream of EGL-30, based on the stronger suppression of *egl-30* hypomorphs by *sag-1(sy428)*.

Negative regulation of G_q by G_o

Analysis of double mutants involving *goa-1* and *egl-30* indicates that $G_o\alpha$ and $G_q\alpha$ function antagonistically in *C. elegans*. Although it is possible that G_q and G_o antagonize each other by positively and negatively regulating a common target, such as intracellular calcium, the identification of genes required for $G_o\alpha$ signaling that negatively regulate $G_q\alpha$ signaling argues that $G_o\alpha$ regulates behavior by modulating $G_q\alpha$ activity. Because we identified only one gene (*eat-16*) apparently upstream of *egl-30* in a fairly extensive screen for downstream targets of GOA-1, the number of steps between G_o and the G_q pathway might be small. In that view, G_o might antagonize G_q directly or might antagonize a downstream target of G_q, perhaps via SAG-1. A third possibility is that G_o antagonizes G_q signaling via EAT-16, in which case EAT-16 might be a direct effector for $G_o\alpha$ as well as being an RGS for $G_q\alpha$. G_o could modulate the activity of other Gα-subunits by activating one or more RGS proteins such as EAT-16 that in turn could down-regulate other Gα-subunits.

The data presented here suggest a model for the functions of GOA-1, EGL-30, EAT-16, and SAG-1 (Fig. 7). GOA-1 negatively regulates EGL-30 activity, possibly via EAT-16 or SAG-1. EGL-10 selectively regulates GOA-1 activity, whereas EAT-16 selectively regulates EGL-30 activity. EGL-30 has been shown to activate PLCβ in COS-7 cells (Brundage et al. 1996); therefore, second messengers generated from stimulation of PLCβ by $G_q\alpha$ are probably produced downstream of EGL-30. Reducing EAT-16 function would result in elevated levels of active EGL-30 subunits, which would in turn result in higher levels of second messengers and increased mobilization of internal calcium stores (Berridge 1993). SAG-1 negatively regulates the EGL-30 pathway. Whereas moderate overexpression of wild-type EGL-30 has been shown to cause hyperactive egg-laying and locomotion behaviors (Brundage et al. 1996), more intense overexpression of EGL-30 results in lethality (L. Brundage, P.W. Sternberg, and M.I. Simon, unpubl.). We have shown that the *eat-16(sy438); sag-1(sy428)* double mutant is also inviable. The synthetic lethality of *sag-1* and *eat-16* mutations, as well as the lethality caused by expressing multiple copies of EGL-30, could be due to an excessive production of second messengers and/or excessive calcium release downstream of activated EGL-30. Reducing EGL-30 activity (and hence the level of downstream signaling) restores viability to *eat-16; sag-1* mutants.

Behavior is modulated through a network of G proteins

Our results are consistent with a model in which a network of G protein pathways within cells can affect behavior by both positive and negative cross talk. Although synergistic effects between $G_{i/o}$ and G_q pathways have been observed (for review, see Selbie and Hill 1998), our results indicate negative regulation of $G_q\alpha$ or its downstream targets by $G_o\alpha$. That G_o and G_q function antagonistically in some way was implied from the opposite phenotypes of *goa-1* and *egl-30* mutations (Brundage et al. 1996). The isolation and analysis of GOA-1 suppressors involved in $G_q\alpha$ signaling support the model that $G_o\alpha$ functions to modulate behavior by down-regulating the G_q pathway in *C. elegans* and perhaps in other species as well. These results are analogous to the stimulatory and inhibitory effects of G_s and G_i on adenylyl cyclase (Hepler and Gilman 1992), raising the possibility that antagonistically acting G protein subunits are more universal than previously thought.

Materials and methods

Nematodes were cultured and handled according to standard procedures (Brenner 1974). All experiments were performed at 20°C except where otherwise noted. The following mutations and strains were used in this study for mapping experiments and double-mutant constructions: LGI *egl-30(ad805)*, *egl-30(ad809)*, DA1096 *egl-30(ad810)/szT1 [lon-2(e678)]* (Brundage et al. 1996), *egl-30(md186)*, *egl-30(n686)*, *unc-55(e402)*, MT363 *goa-1(n363)* (Ségalat et al. 1995), *dpy-5(e61)*, *unc-29(e1072)*, SP1726 *unc-29(h1) hP6 dpy-24(s71)* (a gift from J.A. Powell-Coffman, Iowa State University, Ames, IA), *mec-8(e398)*, *lin-11(n566)*, JK1553 *ces-1(n703d) qDf9/unc-29(e1702) lin-11(n566)* (Ellis and Kimble 1995). LGII: *unc-4(e120)*. LGIII: *unc-32(e189)*. LGIV: *dpy-20(e1282ts)*, PS1681 *dpy-20 syIs17[hsp::goa-1(Q205L)]* (Mendel et al. 1995), *unc-31(e169)*. LGV: *unc-42(e270)*, *him-5(e1490)*. LGX: TY2137 *meDf6; yDp13* (Akerib and Meyer 1994), PS1104 *egl-17(e1313) sli-1(sy143) unc-1*(e719), *dpy-3(e27)*, *unc-20(e112)*, *lin-15(n765ts)*. Linkage unknown: *syIs9[goa-1(Q205L)]* (Mendel et al. 1995), *syIs36[egl-30(+)]* (L. Brundage, P.W. Sternberg, and M.I. Simon, unpubl.).

Genetic screen

dpy-20(e1282) syIs17[hsp::goa-1(Q205L)] animals (Mendel et al. 1995) were mutagenized with ethylmethanesulfonate (21,000 haploid genomes) or trimethylpsoralen + UV irradiation (Yandell et al. 1994; 11,000 haploid genomes). F_2 progeny were heat-shocked (33°C, 30 min) as adults; moving animals were selected the next morning. All suppressors were backcrossed three times to the *syIs17* parent strain, with the suppression of heat shock-induced lethargy used as the criterion for scoring; the reference alleles were then outcrossed to N2 for characterization by selecting for the empty uterus and pale, scrawny appearance. *eat-16(sy438)* was originally isolated with another linked mutation that was removed by recombination during mapping experiments.

Characterization of mutants and double-mutant strains

To characterize the egg laying phenotype, animals were examined 24–28 hr after selecting them as L4 larvae, except for *syIs9[goa-1(Q205L)]* and *egl-30(n686)* strains, which were selected from mixed stage plates as gravid young adults before excess egg retention. A large number of staged adults were placed on a plate with *Escherichia coli* OP50. Newly laid eggs were harvested every 10–20 min and examined at 125× or with Nomarski optics (for *syIs9* strains). Cells in premature or wild-type eggs could be easily counted at this magnification. Later stage eggs were categorized qualitatively as follows: 20–50 cells (2–3 hr after fertilization), ~50 cells, precomma (gastrulation is beginning, before comma stage), comma (~5 hr after fertilization), twofold (~7 hr after fertilization), threefold (~9 hr after fertilization), and about to hatch. Eggs were considered premature if they contained eight or fewer cells. To count the number of eggs in the uterus, adults were examined at 125× magnification 24 hr after selecting as L4 larvae. N2, *eat-16*/+, *syIs9*, and Egl strains were bleached (as in Koelle and Horvitz 1996) to facilitate counting eggs. Hyperactive mutants were examined without bleaching.

To calculate pharyngeal pumps per minute, similarly staged adults were placed on individual plates seeded with OP50 and left undisturbed at least 15 min before counting. Pharyngeal pumps were counted for 2 min by pressing a counter once every three pumps; then, the numbers were multiplied by 1.5 to yield pumps per minute.

To calculate forward locomotion rate, staged adult animals were observed under conditions that maximize forward locomotion and minimize other behaviors (J. Mendel, pers. comm.): Two hundred microliters of a 5-ml OP50 culture was spread over the entire surface of a fresh 60-mm NGM plate preincubated at 20°C. Plates were left uncovered for the lawns to dry. After drying (which generally took ~1 hr), the plates were stored with lids on and used within 2 hr after drying. The result was a very thin lawn that covered the entire plate. Animals were left undisturbed on the lawns at least 5 min and then observed for 2 min. Seconds elapsed per sine wave (counting anterior flexing just posterior to the pharynx) were recorded using software written for this purpose by Hou-Pu Chou and Chieh Chang. Only forward flexing was counted, and waves right before or after a reversal were not included. Entries for all animals were then converted to waves/second and averaged. Averages and standard deviations were multiplied by 60 to yield waves per minute.

Because the presence of excess eggs in the uterus might affect locomotion rate, *egl-30* and *syIs9* strains were not staged as above; instead, young gravid adults with a single row of eggs in the uterus were selected from mixed-stage plates. Because of *syIs9* animals' tendency to travel in a circular manner (J. Mendel, unpubl.), one side of the body would often make a more visible flexion and the other side would not flex much, if at all; counting was done using the side that made the deeper flexions. Occasional animals did not move normally and may have been harmed during transfer; these animals' data were not included in the totals.

Characterization of sag-1/meDf6

meDf6; yDp13 males were mated to *unc-4(e120); sag-1(sy428) dpy-3(e27)* hermaphrodites and all nonUnc cross-progeny were selected as L4 larvae and examined 24 hr later. Cross-progeny were either non-Dpy or Dpy. *meDf6* deletes *sag-1* and *dpy-3*, and *yDp13* likely covers *sag-1* as well as *dpy-3*; therefore, Dpy progeny were assumed to be *sag-1 dpy-3/meDf6*, and Dp-bearing non-Dpy animals were examined in parallel as a control. All 16 *meDf6/sag-1(sy428) dpy-3(e27)* heterozygotes examined had empty uteri, and at least 15 of them suppressed the *syIs17[hs-G_oQL]* lethargy (the sixteenth animal crawled off the plate after heat shock treatment and could not be scored).

Mapping experiments

sag-1(sy428) was mapped between *egl-17* and *unc-1* by selecting Egl non-Unc recombinant progeny from *sy428/egl-17(e1313) sli-1(sy143) unc-1(e719)* heterozygous animals; 14 of 16 recombinants carried the *sy428* mutation. The following three-factor crosses determined the map position of *eat-16(sy438)*. First, *eat-16* was mapped between *unc-29* and *lin-11* by selecting recombinants from *unc-29 lin-11/eat-16; dpy-20 syIs17* heterozygotes. Nine of 13 Lin non-Unc recombinants carried *sy438* (scored by heat shock). Then, *sy438* was mapped right of *mec-8* by selecting Eat non-Dpy recombinants from *dpy-5 eat-16/mec-8; dpy-20 syIs17* heterozygotes. One of seven recombinants carried *mec-8*. (Recombinants were scored for *sy438* by the hyperactive phenotype and by heat shock.) Finally, *sy438* was mapped right of *hP6* by building + *mec-8* + *eat-16*+/*unc-29* + *hP6* + *dpy-24* heterozygotes and selecting for non-Mec recombinants with empty uteri. Unc progeny from these recombinants were homozygosed, and the presence of *hP6* was determined by PCR amplification (Williams et al. 1992) using a mixture of three primers, 618 Tc1 primer (Williams et al. 1992), and two primers designed by J.A. Powell-Coffman (pers. comm.): hP6 (5′-TAG-

ATTTTGATCGTCTTCG) and hP62 (5′-TGTCTCGCCTACGATCTGATATTGC). Two of 10 Eat non-Mec recombinants carried *hP6*.

Transformation rescue

Animals were microinjected according to standard protocols (Mello et al. 1991; Mello and Fire 1995). The *lin-15* rescuing plasmid pbLH98 at 50 ng/µl (Huang et al. 1994) was used as the coinjection marker for all rescue experiments. pBluescript was included as carrier DNA to bring total DNA concentrations to 150–200 ng/µl. Strains bearing the temperature-sensitive *lin-15(n765)* mutation were cultured at 15°C before injection; afterwards, they were cultured at 22°C–23°C for 4–5 days, and non-Muv transformants were selected. Rescue was scored after at least one generation by heat-shocking non-Muv animals and looking for no suppression. Y20E10, C16C2, and three other cosmids contained within Y20E10 were each injected at ~50 ng/µl into *eat-16(sy438); dpy-20 syIs17; lin-15(n765)* animals. Subclone pYH5 was injected at a concentration of 30 ng/µl, and pWJC5 was injected at a concentration of 25 ng/µl.

Sequencing of eat-16 *mutations*

A 3-kb genomic DNA fragment was amplified (Williams et al. 1992) from *ad702* mutant animals in three independent reactions and from *sy438* animals in 10 independent reactions using the *Expand* long-range PCR kit (Boehringer Mannheim) with the following primers (from 5′ to 3′): AGACAGCTTCGTCGTATGTCTCAC ("P1") and GCAGTGTTGGGTGGTTCGAGATTG ("P2"); the products from each strain were gel-purified (Qiagen) and pooled. The *ad702* fragment was amplified a second time with P2 and the nested primer TGTCGAGCTGATTGAGACACGCTG ('S1') in 10 independent reactions; the products were purified as above and pooled. For both strains, PCR fragments were cloned into pGEM vectors (Stratagene), and the entire predicted gene product was sequenced (Kretz et al. 1989) in two plasmids per strain. The point mutations were then confirmed in the second strand and in both strands of three additional plasmids for *sy438* and four additional plasmids for *ad702*. For *sa735* and *sa609* mutants, products amplified as above in three independent PCR reactions were gel-purified, pooled, and sequenced directly.

Sequencing of eat-16 *cDNA*

Full-length cDNA sequence of *eat-16* was obtained from clone yk356b3, kindly provided by Yuji Kohara. Phage clones were excised in vitro and amplified in SOLR cells (Maniatis et al. 1982). Purified phagemids were then sequenced by the primers used for sequencing the *eat-16* mutations and primers for the T3 and T7 promoters. The splicing pattern was obtained by comparing yk356b3 with wild-type *eat-16* genomic sequence obtained from the GenBank database.

GFP-tagged expression of eat-16

Genomic DNA fragments including the *eat-16* promoter region and some coding exons were cloned into GFP expression vectors provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu (pers. comm.). Reporter construct pGP16 contains the 7.4-kb *Apa*I–*Bam*HI fragment and fuses to GFP-coding sequences in the ninth coding exon of *eat-16*. Reporter construct pGR02 contains the same upstream sequence but fuses to GFP-coding sequences in the first coding exon of *eat-16*. Both constructs were injected at 80 ng/µl into *lin-15(n765)* animals along with the *lin-15* rescuing plasmid pL15EK at 50 ng/µl (Clark et al. 1994) as a coinjection marker.

Double-mutant constructions

egl-30 eat-16 linked double mutants were constructed as follows: *dpy-5 eat-16*/++ males were mated to *egl-30* hermaphrodites (or *egl-30/szT1* heterozygotes, in the case of *ad810*). Non-Egl F_1 progeny were picked individually and removed the following day to synchronize the F_2 progeny. Plates with Dpy F_2 progeny were saved, and Eat non-Dpy animals were selected based on the empty uterus phenotype (which was transitory in these recombinants due to the semidominance of the *egl-30* alleles; see Brundage et al. 1996). Egl non-Dpy F3 progeny were saved, and the presence of *eat-16(sy438)* was confirmed by mating with N2 males and reisolating Eat non-Egl F_2 recombinants from all of several F_1 cross-progeny.

In the case of *ad810*, two Eat non-Dpy recombinants segregated Eat, Eat Dpy and arrested larvae; no viable Egl progeny were seen. Recombinants were mated with *szT1* males to balance the lethal chromosome. A parallel comparison of the lethal progeny of *ad810 sy438/szT1* and *ad810/szT1* was done by placing 16 worms of each strain on a plate with a thin bacterial lawn and removing them the next day. Dead larvae on both plates were observed over the course of several days and appeared similar.

Construction of double mutants between unlinked genes was straightforward; the strains were all confirmed either by complementation tests or by crossing with N2 males and reisolating both mutations. *egl-30(ad805) goa-1(n363); him-5(e1490)* was built by L. Brundage.

Transgenic strains

Strains containing *eat-16* transgenes were constructed by following the marker *lin-15(n765)* for *syEx256*, whereas G_o and G_q transgenes were followed by using *dpy-20(e1282)* as a rescuing marker. For example, a cross between *dpy-20* males and *dpy-20; lin-15; syEx256* hermaphrodites was kept for one day at 20°C to allow mating to occur and then cultured at 15°C to reduce the severity of the Dpy phenotype of the F_1 progeny. The resulting *dpy-20; lin-15; syEx256* males were mated to *dpy-20; syIs36; lin-15* hermaphrodites at 20°C, and non-Lin progeny were saved. The *goa-1(n363); syEx256* strain was constructed by mating *dpy-20*/+; *lin-15; syEx256* males to *goa-1(n363); lin-15* hermaphrodites and saving non-Dpy, non-Muv transgenic F_2 animals whose Lin (i.e., nontransgenic) progeny were all homozygous for *goa-1(n363)*. To test animals expressing both G_o and G_q transgenes, *dpy-20(e1282) syIs17* males were mated to *dpy-20(e1282); syIs36* hermaphrodites, and the resulting male progeny were heat-shocked.

Synthetic lethality of eat-16 *and* sag-1

To build the *eat-16; sag-1* double mutant, *mec-8 lin-11*/++; *sag-1* males were mated to *dpy-5 eat-16* hermaphrodites. Non-Dpy F_1 progeny were picked to individual plates, and homozygous Sag F_2s were picked from plates with Mec Lin progeny. To score penetrance of the lethality, 20 *dpy-5* + *eat-16*+/+*mec-8* + *lin-11; sag-1* L4 heterozygotes were placed on individual plates and transferred daily for 4 days. The total number of progeny was counted at the L4-Adult stage, yielding 2193 heterozygous, 936 Mec, and 52 Dpy animals. Dpy animals were saved and followed for a generation to determine their genotype.

Of the 50 viable Dpy animals, 48 were either *dpy++/dpy mec lin* or *dpy++/++lin* recombinants, one was a spontaneous male whose genotype could not be determined, and one escaped to adulthood and produced a few inviable progeny, a 0.1% survival rate. Arrested larvae were seen among the progeny of all 20 heterozygous mothers.

egl-30 eat-16; sag-1 *triple-mutant construction*

+eat-16+/mec-8 + lin-11; him-5; sag-1 males were mated to *egl-30(md186) eat-16* hermaphrodites, and F_1 progeny with empty uteri were picked to individual plates. From two plates that segregated Eat, Egl, and dead larvae but no Mec Lin (i.e., *egl eat/+ eat; sag-1/+*), 26 Egl F_2 progeny were picked to individual plates. None of the Egls produced lethal progeny. Seven of 26 Egls gave only Egl-30-like progeny, but on 18 plates, about one-fourth of the progeny were active, and one plate had only active progeny. Homozygosity of *sag-1* was confirmed by mating *dpy-20 syIs17* males with the triple mutant; male progeny were heat-shocked, and all were active 8 hr following heat shock ($n = 36$). As a control, *egl-30(md186) eat-16(sy438)* animals were also crossed to *syIs17* males; the male progeny resulting from this cross were lethargic 8 hr following heat shock ($n = 20$). Homozygosity of *eat-16* was confirmed by sequencing across the portion of the *eat-16* locus containing the *sy438* mutation in both strands, using a protocol similar to that described above.

COS-7 cell transfection and IP_3 assay

A 2.4-kb *Xho*I–*Xba*I fragment from yk356b3 (EAT-16 cDNA) was cloned into the pCIS vector to make pWJC4 for COS-7 cell transfection. M1 receptor (Bo Yu, pers. comm.) and EGL-30 (L. Brundage, pers. comm.) were also cloned in the same vector. Cells were transfected as described (Wu et al. 1992; Liu and Simon 1996) and seeded (1×10^5 per well) to 12-well plates the night before transfection. The pCIS vector was used as carrier DNA to normalize the total concentration of DNA in each well to 1.0 µg (for M1 receptor experiments) or 2.0 µg (for EGL-30 transfections). Lipofectin (5 µl) was added to each well. M1 receptor was activated by Carbachol (1 µM), and IP_3 assays were done as described (Liu and Simon 1996). Each transfection was performed in duplicate.

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