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EGL-10 Regulates G Protein Signaling in the C. elegans Nervous System and Shares a Conserved Domain with Many Mammalian Proteins

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Summary

The frequencies of certain periodic behaviors of the nematode C. elegans are regulated in a dose-dependent manner by the activity of the gene *egl-10*. These behaviors are modulated oppositely by the activity of the G protein α subunit gene *goa-1*, suggesting that *egl-10* may regulate a G protein signaling pathway in a dose-dependent fashion. *egl-10* encodes a protein similar to Sst2p, a negative regulator of G protein signaling in yeast. EGL-10 protein is localized in neural processes, where it may function in neurotransmitter signaling. Two previously known and 13 newly identified mammalian genes have similarity to *egl-10* and *SST2*, and we propose that members of this family regulate many G protein signaling pathways.

Introduction

Animals modify their behaviors in response to their experiences by altering the activities of their neurons and muscles. An important challenge in neurobiology is to understand the mechanisms responsible for these changes in activity. One such mechanism involves the action of neurotransmitters through G protein–coupled receptors to alter the properties of target cells. For example, the frequency of the vertebrate heartbeat is regulated by neurotransmitters that act through G proteins to modify the properties of ion channels in the heart muscle and, therefore, the contractile activity of the heart (reviewed by Hille, 1992). In the sea slug Aplysia, a simple form of memory results when the neurotransmitter serotonin acts through a G protein to induce both short- and long-term alterations in the properties of certain sensory neurons (Bailey et al., 1994).

To gain an understanding of the mechanisms that regulate muscle and neuron activity, we have begun a molecular genetic analysis of regulated behaviors of the nematode Caenorhabditis elegans. Several nematode behaviors are regulated in response to the presence of Escherichia coli, which worms eat when cultured in the laboratory. Egg laying, for example, is a periodic behavior greatly increased in frequency by a bacterial lawn (Trent, 1982; E. Sawin and H. R. H., unpublished data). The presence of E. coli also affects the frequency of the body bends that worms display during locomotion, as well as the frequencies of other behaviors (Croll, 1975; Avery and Horvitz, 1990; Thomas, 1990; E. Sawin and H. R. H., unpublished data).

The regulation of egg-laying behavior is particularly well suited for genetic analysis. This behavior is easily studied, since it need not be watched in progress: its occurrence and frequency can be scored after the fact by observing the eggs that have been laid or the unlaid eggs that remain in the adult. As a result, genetic screens for mutants in which egg-laying behavior has been altered can be performed conveniently and on a large scale (Trent et al., 1983; Desai and Horvitz, 1989). In addition, the C. elegans egg-laying system has a simple and well-characterized anatomy (White et al., 1986). Egg laying involves 16 vulval and uterine muscle cells, which are innervated by a pair of serotonergic motor neurons, the HSNs (Desai et al., 1988). Worms can be artificially induced to lay eggs by application of exogenous serotonin or by treatment with serotonin reuptake blockers, which appear to potentiate the action of HSN-released serotonin (Horvitz et al., 1982; Trent et al., 1983).

Two classes of mutations that appear to disrupt the regulation of egg laying have been described. The first class is represented by loss-of-function alleles of the *goa-1* gene; these mutations strongly increase the frequency of egg-laying behavior and also increase the frequencies of locomotory body bends and of other behaviors (Lochrie et al., 1991; Mendel et al., 1995; Ségalat et al., 1995). *goa-1* encodes a protein 80% identical to the mammalian G protein α subunit G_o, suggesting that the frequencies of egg laying and other regulated behaviors are controlled by G protein signaling. *goa-1* is expressed in the neurons and muscles of the egg-laying system and may mediate the actions of serotonin or other neurotransmitters (Mendel et al., 1995; Ségalat et al., 1995).

The second class of mutations consists of alleles of six genes that when mutant severely reduce or abolish egg-laying behavior (Trent et al., 1983; Desai et al., 1988; Desai and Horvitz, 1989). Animals carrying these mutations can lay eggs in response to exogenous serotonin, suggesting that the muscles and other components of the egg-laying apparatus are functional in these mutants and that the neural control of egg laying may be defective. The morphology and serotonin content of the HSNs appear normal in these mutants, but like wild-type worms in which the HSNs have been ablated, the mutants fail to lay eggs in response to serotonin reuptake blockers. This observation suggests that the HSNs in these mutants may be decreased in activity. In this work, we have characterized one of the six genes identified by this second class of mutations, *egl-10*.

Results

egl-10 Controls the Frequencies of Two Behaviors in a Dose-Dependent Manner

We isolated a genomic clone containing the *egl-10* gene using standard methods of genetic mapping and transformation rescue (data not shown; experiments presented below establish that the correct gene was isolated). We analyzed the behavioral abnormalities induced by the *egl-10* loss-of-function mutation *n692* and by overexpression of *egl-10* from a chromosomally

integrated transgene carrying a multicopy array of the *egl-10* gene, *nIs51*. *egl-10(n692)* caused abnormalities similar to those caused by 14 other *egl-10* mutations; one other *egl-10* allele, *n480*, caused similar but less severe abnormalities (Desai and Horvitz, 1989; data not shown). *nIs51* caused abnormalities similar to those caused by other independently isolated, chromosomally integrated *egl-10* transgenes (data not shown). The behavioral abnormalities caused by multicopy *egl-10* transgenes must be the result of overexpression of EGL-10 protein and not of titration of regulatory factors that might bind to the transgene DNA, since similarly generated transgenes carrying a disruption in the *egl-10* reading frame did not induce any noticeable abnormal phenotype (data not shown).

The number of unlaid fertilized eggs that accumulated inside *egl-10(n692)* animals (53 ± 9) was higher than that found in the wild type (18 ± 6); by contrast, *nIs51* animals contained only 1 ± 1 unlaid eggs (Figures 1A–1D). Because animals from these strains all produced similar numbers of eggs (brood sizes were as follows: *egl-10(n692)*, 226 ± 37; wild-type, 318 ± 26; *nIs51*, 263 ± 23), the differences in the steady-state accumulation of unlaid eggs appear to have been caused by differences in rates of egg laying. We conclude that *egl-10* loss-of-function mutants rarely laid eggs, while *egl-10* overexpressers laid eggs more frequently than the wild type.

We used another assay of egg-laying behavior to confirm this conclusion. On average, the more frequently an adult engages in egg-laying behavior, the less time its fertilized eggs will spend in the uterus prior to being laid. Because the fertilized eggs are developing while they spend time in the uterus, adults that engage in egg-laying behavior more frequently lay their eggs at earlier stages of development. We found that *nIs51* animals laid all of their eggs at or before the eight-cell stage of development, that wild-type animals laid 95% of their eggs after the eight-cell stage but before morphogenesis begins at the comma stage, and that *egl-10(n692)* animals laid all their eggs at the comma stage or later (n = 100 for each determination; the eight-cell and comma stages are reached at about 65 and 435 min after fertilization, respectively [Wood et al., 1988]). These differences in the developmental stages of freshly laid eggs must have have been caused by differences in rates of egg laying, not in rates of embryonic development, since by direct observation we found that *nIs51*, *egl-10(n692)*, and wild-type embryos develop at similar rates (data not shown).

We found that altering the dosage of the *egl-10* gene by just one copy was sufficient to alter the frequency of egg laying. Animals heterozygous for the *egl-10* loss-of-function mutation or animals heterozygous for a chromosomal deficiency for the *egl-10* locus laid eggs less frequently than the wild type, but more frequently than *egl-10* mutant homozygotes (Figure 1D). Our experiments also showed that the *egl-10(n692)* mutation behaves genetically like a deficiency for the *egl-10* locus (we compared animals of genotypes +/*Df* with +/*egl-10* and *egl-10*/*Df* with *egl-10*/*egl-10*; Figure 1D). Thus, the strong *egl-10* mutations, such as *n692*, appeared to result in a strong loss-of-function of the *egl-10* gene, a conclusion confirmed below by molecular analysis.

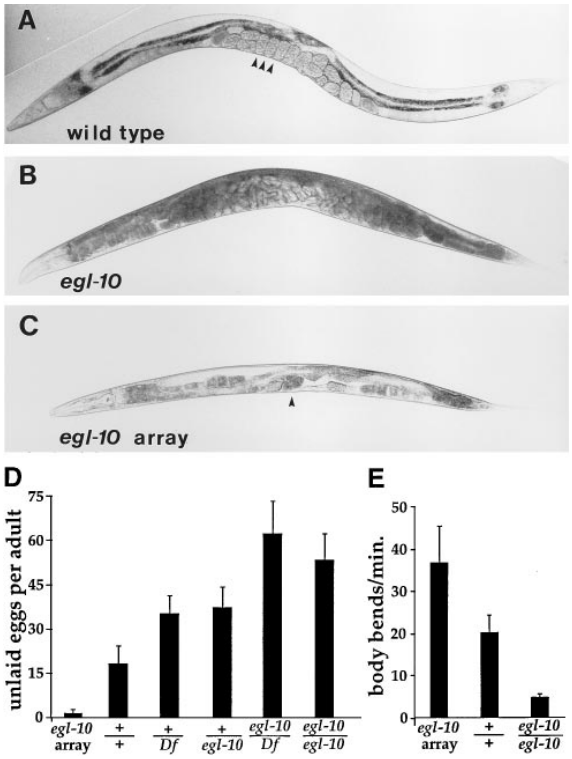

Figure 1. Effects of *egl-10* Gene Dosage on Egg-Laying and Locomotion Behaviors

(A) A wild-type adult hermaphrodite. Arrowheads point to 3 of the 16 unlaid fertilized eggs inside the adult.

(B) An *egl-10(n692)* adult. The body of this animal was packed with about 50 unlaid eggs, most of which had progressed to a later stage of development than the unlaid eggs found in wild-type adults.

(C) An adult carrying the *nIs51* transgenic array of extra wild-type copies of the *egl-10* gene. This animal contained only one unlaid fertilized egg (arrowhead).

(D) Steady-state levels of unlaid eggs in animals carrying various doses of the *egl-10* gene. Values represent the means and standard deviations measured from at least 20 adults of each genotype. *egl-10* array, animals homozygous for the *nIs51* transgene, which contains multiple copies of the wild-type *egl-10* gene. *Df*, the chromosomal deficiency *arDf1*, which deletes the *egl-10* gene. *egl-10*, *egl-10(n692)*, the canonical loss-of-function allele of *egl-10*. All animals in this experiment were also heterozygous for the recessive marker mutation *unc-42(e270)*, which was necessary to verify some genotypes and was included in the others for consistency.

(E) Rates of locomotion behavior of animals carrying various doses of the *egl-10* gene. Values represent the means and standard deviations measured from 10 adults of each genotype. *egl-10* array, animals homozygous for the *nIs51* transgene. *egl-10*, the *egl-10(n692)* mutation.

The rate of locomotion behavior was also affected by *egl-10* gene dosage (Figure 1E). As worms move forward, sinusoidal bends initiated at the head of the worm propagate backward through the body. Transgenic worms overexpressing *egl-10* initiated such body bends more frequently (37 ± 8/min) than the wild type (20 ± 4/min), while *egl-10* loss-of-function mutants initiated

Table 1. Genetic Interaction between *goa-1* and *egl-10*

Genotype	Developmental Stage of Freshly Laid Eggs[a]			Locomotion Rate of Young Adults (body bends/min)
	One- to Eight-Cell	Nine-Cell to Comma	Postcomma	
Wild type	5	95	0	20 ± 4
egl-10(md176)	0	0	100	3 + 1
goa-1(n363)	95	5	0	39 ± 5
goa-1(n363; egl-10(md176)	95	5	0	42 ± 7

[a] For each strain, 100 freshly laid eggs were observed, and each was placed into one of three categories based on its developmental stage. Wild-type embryos reach the eight-cell stage of development at 65 min post-fertilization and the comma stage of development at 435 min (Wood et al., 1988). Embryonic development occurred at similar rates for each of the strains used in this experiment (data not shown), and differences shown here reflect different delay times between fertilization and laying of eggs caused by different frequencies of egg-laying behavior.

body bends less frequently (5 ± 1/min). *egl-10* also affected the frequency of foraging movements of the nose of the worm, which were more frequent in *egl-10* overexpressers than in the wild type, while they were less frequent in *egl-10* null mutants (data not shown).

EGL-10 Appears to Function in Signal Transduction Upstream of the G Protein GOA-1

The C. elegans G protein GOA-1 has effects on behavior opposite those of EGL-10. Loss-of-function *goa-1* mutants, like *egl-10* overexpressers, are hyperactive for egg-laying, foraging, and locomotion behaviors, while *goa-1* overexpressers, like *egl-10* loss-of-function mutants, show sluggish egg laying, foraging, and locomotion (Mendel et al., 1995; Ségalat et al., 1995). These results suggest that *egl-10* and *goa-1* might function in a common signal transduction pathway, perhaps mediating the actions of neurotransmitters in the C. elegans nervous system that regulate the frequencies of egg laying, locomotion, and foraging. Given the opposite effects of *goa-1* and *egl-10* mutations, these genes might have opposite roles, one promoting signaling and the other negatively regulating signaling.

To investigate the relationship between the *goa-1* and *egl-10* genes, we examined the phenotypes of double mutant animals carrying null mutations in both. Described below are the results we obtained using the *goa-1(n363)* and *egl-10(md176)* mutations, both of which almost completely delete the coding sequences of their respective genes and therefore constitute null mutations (Ségalat et al., 1995; this work). Four other combinations of *goa-1* and *egl-10* alleles gave similar results (data not shown).

We found that *goa-1*; *egl-10* double mutants are phenotypically indistinguishable from *goa-1* single mutants, consistent with the functioning of *egl-10* upstream of or parallel to *goa-1* to regulate signaling through the G protein GOA-1. *goa-1*; *egl-10* double mutants showed hyperactive egg-laying behavior similar to that of *goa-1* single mutants (Table 1). We measured the frequency of egg-laying behavior by examining the developmental stages of freshly laid eggs; the accumulation of unlaid eggs was not measured in this experiment, since the severely reduced production of eggs by *goa-1* mutants (Mendel et al., 1995; Ségalat et al., 1995) would bias the results of this assay. *goa-1*; *egl-10* double mutants also showed hyperactive locomotion behavior similar to that shown by *goa-1* single mutants (Table 1). Finally, the hyperactive foraging behavior and reduced brood size observed in *goa-1* single mutants also appeared similar in *goa-1*; *egl-10* double mutants (data not shown).

egl-10 Sequence, Gene Structure, and Mutant Alleles

Using *egl-10* genomic DNA as a probe, we isolated a 3.2 kb cDNA and then determined its sequence, which contains a 555 amino acid open reading frame (Figure 2A). We also determined the sequence of the *egl-10* genomic DNA and deduced the structure of the *egl-10* gene by comparing the two sequences (Figure 2B). The 3.2 kb cDNA appears to be full length, since it contains a sequence identical to that of the C. elegans *trans*-spliced leader SL1 (Krause and Hirsh, 1987) at its 5′ end, as well as a poly(A) tract at its 3′ end, and matches the length of the 3.2 kb RNA detected by Northern blot hybridization (Figure 2C).

We identified alterations in this open reading frame associated with 14 of the 15 existing *egl-10* alleles (Figure 2), confirming that we had correctly identified the *egl-10* gene. Two spontaneous alleles, *md176* and *md1179*, appear to be null mutations of *egl-10* by molecular criteria. Specifically, multiple polymerase chain reaction (PCR) primer pairs failed to amplify any portion of *egl-10* exons 2–7 from *md176* or *md1179* genomic DNA, suggesting that this region is deleted or severely rearranged in these alleles. While exon 1 remains intact in these alleles, it encodes only the first seven amino acids of the EGL-10 protein. These mutants also failed to express detectable EGL-10 protein as assayed by Western blotting or antibody staining of mutant animals (Figure 3; data not shown). One *egl-10* allele, *n953*, appeared to contain no alterations from the wild type in the *egl-10* coding sequences; this allele may contain alterations in other parts of the gene, since it did not express detectable EGL-10 protein as assayed by a Western blot (data not shown).

EGL-10 Protein Is Localized in Neural Processes and Is Subcellularly Localized in Body Wall Muscle Cells

We raised polyclonal antibodies against recombinant EGL-10 protein. When affinity purified, these antibodies

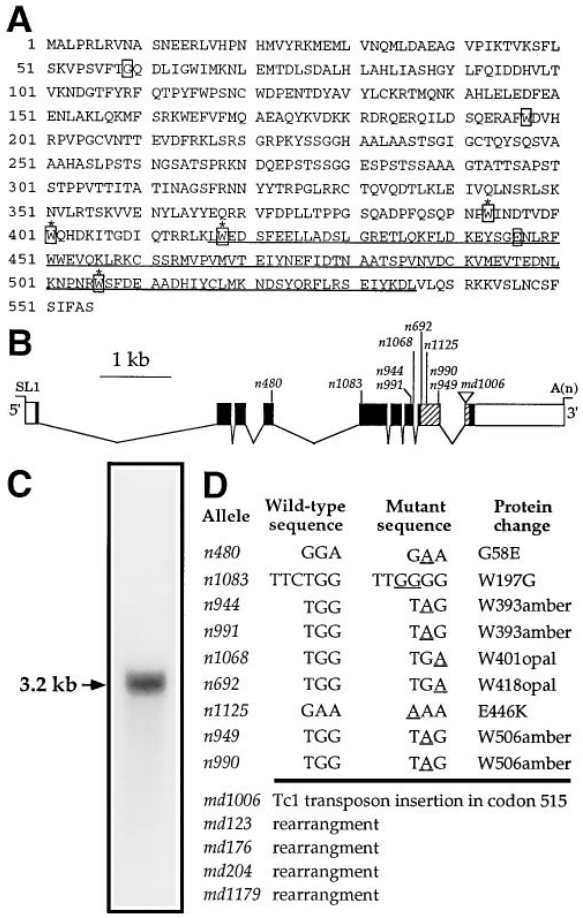

Figure 2. EGL-10 Sequence, Exon Structure, Northern Blot Analysis, and Mutant Allele Sequences

(A) Predicted amino acid sequence of the EGL-10 protein derived from the *egl-10* cDNA sequence. Amino acid residues altered in missense mutants are indicated by plain boxes; boxes with asterisks indicate the positions of stop codons in nonsense mutants. The conserved RGS domain is underlined.

(B) *egl-10* exon structure and positions of *egl-10* mutations. Boxes show the positions of exons as determined by comparison of cDNA and genomic sequences. The 5′ and 3′ untranslated regions are indicated by open boxes. The coding sequences are shown as solid boxes, except for the RGS domain coding sequences, which are hatched. A 5′ SL1 *trans*-spliced leader and a 3′ poly(A) tail are indicated at the ends of the transcript. The positions of nine point mutations are indicated by lines above the boxes labeled with their allele designations. The insertion site of a Tc1 transposon in the *md1006* mutant is marked by a triangle. Five other alleles are not indicated; four are complex rearrangements, and the remaining mutation, *egl-10(n953)*, causes no alterations in the *egl-10* coding sequences.

(C) Northern blot analysis of *egl-10*. Poly(A)$^+$-selected RNA (10 μg) prepared from a mixed-stage culture of wild-type C. elegans was subjected to electrophoresis, blotted, and probed with ^{32}P-labeled *egl-10* cDNA. The position of the single 3.2 kb *egl-10* transcript is indicated by the arrow.

(D) *egl-10* mutations. The codons altered and positions of induced amino acid substitutions or stop codons are indicated for nine ethyl methanesulfonate-induced mutants (top). *n1083* carries a silent-third position change in codon 196 as well as a tryptophan to glycine change in codon 197. Five spontaneous *egl-10* alleles are listed at the bottom. *md1006* carries an insertion of the 1.6 kb Tc1 transposon in codon 515. The remaining spontaneous alleles have rearrangements of the *egl-10* locus detectable by Southern blots.

recognized two major proteins on Western blots of total C. elegans proteins (Figure 3A). The larger of these proteins is the product of the *egl-10* gene, since this protein was absent from extracts of the *egl-10* null mutant *md176* (Figure 3A), as well as from extracts of 12 other *egl-10* mutants (data not shown). This larger protein was detected at a reduced abundance in the weak *egl-10* mutant *n480* and was present at normal abundance in *egl-10(n1125)* animals, which carry a missense mutation that alters amino acid 446. The 47 kDa protein recognized by the anti-EGL-10 antibodies is not affected by *egl-10* mutations and thus is not encoded by the *egl-10* gene (Figure 3A).

We stained wild-type and *egl-10* mutant worms with the affinity-purified anti-EGL-10 antibodies. We observed staining in the nerve ring (Figure 3B), ventral nerve cord (Figure 3D), and dorsal nerve cord (data not shown) of wild-type animals, but saw no neural staining in *egl-10* mutants (Figure 3C). The stained structures consisted of bundles of neural processes and were at the locations of the majority of the chemical synapses in the animal (White et al., 1986). In neurons, EGL-10 protein appeared to be localized exclusively to processes; no staining was seen in the neural cell bodies of wild-type animals. Animals at all stages of development from first stage larvae to adults showed similar staining of neural processes. The localization of EGL-10 protein to structures in which chemical synapses are made is consistent with a role for EGL-10 in intercellular signaling.

We also used the EGL-10 antibodies to stain worms that overexpress EGL-10 from a multicopy array of *egl-10* transgenes (Figures 3F and 3G). EGL-10 was detected in neural cell bodies as well as neural processes of these animals, either because overexpression raised the level of EGL-10 protein in cell bodies above the threshold of detection or because overexpression of EGL-10 exceeded the capacity of neurons to localize the protein to processes. Figure 3F shows that a large number of neurons in the major ganglia of the head region expressed EGL-10. In addition, our examination of the ventral cord neurons, lateral neurons, and tail ganglia suggested that most if not all neurons in C. elegans expressed EGL-10. In particular, the HSN motor neurons, which control egg-laying behavior and appear to be functionally defective in *egl-10* mutants, expressed EGL-10 (Figure 3F).

A second staining pattern present in wild-type animals consisted of spots arranged in linear arrays within the body–wall muscle cells (Figure 3D). Although this staining was not absent from *egl-10* null mutants, we nevertheless believe that the EGL-10 protein is localized to these muscle structures, since the muscle stain was more intense in EGL-10 overexpressing animals (data not shown) and was reproduced by *egl-10*::GFP transgenes (see below). The residual antibody stain seen in the muscles of *egl-10* mutants may have been caused by the presence of a cross-reactive protein (perhaps the 45 kDa protein detected in our Western blots) that is colocalized with EGL-10. The body–wall muscles are used in locomotion behavior (Wood et al., 1988), the frequency of which is controlled by *egl-10*. Every body–

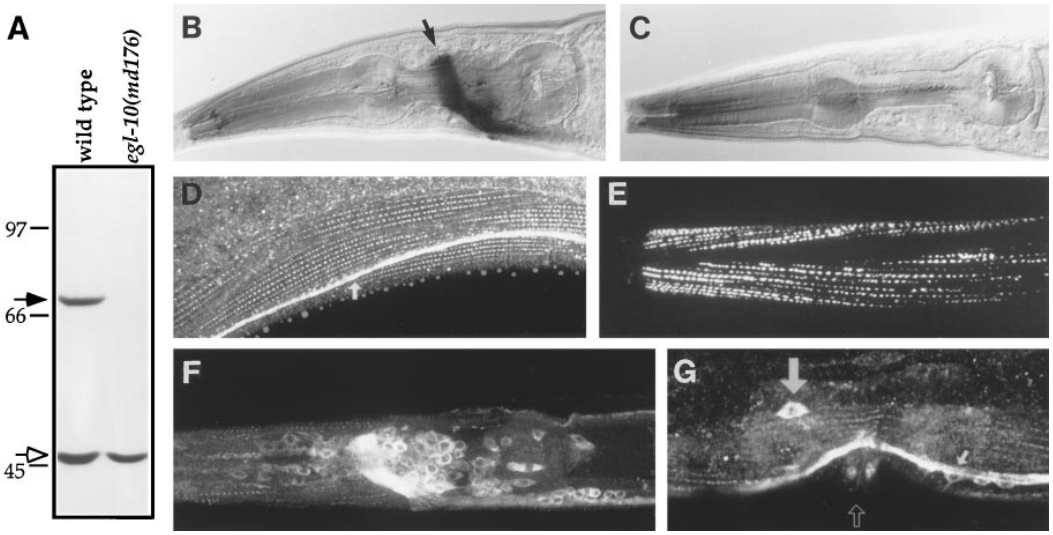

Figure 3. EGL-10 Protein Expression

(A) Western blot analysis of protein extracts from wild-type and *egl-10(md176*) worms probed with the affinity-purified anti-EGL-10 polyclonal antibodies. The closed arrow indicates the position of the EGL-10 protein detected in wild-type but not in *egl-10* mutant extracts. The open arrow indicates the 47 kDa protein that cross-reacted with the EGL-10 antibodies but was not a product of the EGL-10 gene. The positions of molecular weight markers are indicated, with their sizes in kilodaltons.

(B) Anti-EGL-10 antibody staining of the head of a wild-type adult hermaphrodite. The dark immunoperoxidase stain labeled the neural processes of the nerve ring (arrow).

(C) Anti-EGL-10 antibody staining of the head of an *egl-10(md176)* adult hermaphrodite, prepared in parallel to (B) and lacking any specific staining.

(D) Anti-EGL-10 immunofluorescence staining in the midbody region of a wild-type adult. The fluorescence here and in (E)–(G) appears white on a black background, the reverse of the staining in (B) and (C). The arrow points to the brightly stained ventral cord neural processes. Body–wall muscle cells on either side of the ventral cord contained brightly stained spots arranged in linear arrays. Body–wall muscles throughout the animal showed similar staining.

(E) Fluorescence in the head of a transgenic adult carrying a fusion of the *egl-10* promoter and N-terminal coding sequences to the GFP gene. The fusion protein is localized in spots within the body–wall muscles similar to those seen in (D). GFP fluorescence was also present in neural processes and cell bodies out of the plane of focus.

(F) Anti-EGL-10 antibody staining in the head of a transgenic worm carrying the *nIs51* multicopy array of wild-type *egl-10* genes. The processes of the nerve ring were visible, as in (B), but overexpression of EGL-10 caused the protein also to be detected in cell bodies.

(G) Anti-EGL-10 antibody staining in the vulva region of *nIs51* worms. The open arrow points to the vulva. The large closed arrow indicates the HSN neuron. The small closed arrow points to the ventral cord and associated neural cell bodies.

wall muscle cell stained, but no staining was detected in other types of muscle cells, even in animals overexpressing EGL-10 (data not shown). The body–wall muscle stain superimposed on structures visible in Nomarski optics called dense bodies, which function as attachment sites between the body–wall muscles and the cuticle that surrounds them (Wood et al., 1988). Each dense body is flanked by membranes of the sarcoplasmic reticulum, and our observations at the light microscope level cannot distinguish between localization of the stain to the dense bodies or to the sarcoplasmic reticulum. The significance of the localization of EGL-10 to these structures is unclear.

Transgenic animals carrying fusions of the *egl-10* promoter and N-terminal coding sequences to the reporter green fluorescent protein (GFP) (Chalfie et al., 1994) showed fluorescence in body–wall muscle cells in the same pattern seen in animals stained with the EGL-10 antibody (Figure 3E). These experiments demonstrated that the N-terminal 122 amino acids of EGL-10, when fused to GFP, were sufficient to localize the fusion protein to the dense body/sarcoplasmic reticulum–like structures. The EGL-10::GFP fusion proteins were also expressed in neurons (data not shown) but, like overexpressed full-length EGL-10 protein, were not tightly localized to processes, preventing us from identifying the regions of EGL-10 responsible for localization of EGL-10 to neural processes.

EGL-10 Is Similar to Sst2p, a Negative Regulator of G Protein Signaling in Yeast

The 555 amino acid EGL-10 protein contains a 120 amino acid region near its C-terminus with similarity to several proteins in the sequence databases (Figure 4A). The similarities with the C. elegans C05B5.7 protein and the human RGS1 (previously known as BL34 and 1R20) and RGS2 (previously known as G0S8) proteins extend across the entire 120 amino acid region; this region is 34%–55% identical in pairwise comparisons among EGL-10 and these other proteins. An additional C. elegans protein, C29H12.3, consists almost entirely of two highly diverged repeats of this domain. The first 43 and last 29 amino acids of the conserved 120 amino acid region are similar to sequences found in the yeast protein Sst2p and the Aspergillus nidulans protein FlbA. Sst2p and FlbA are 30% identical to each other over

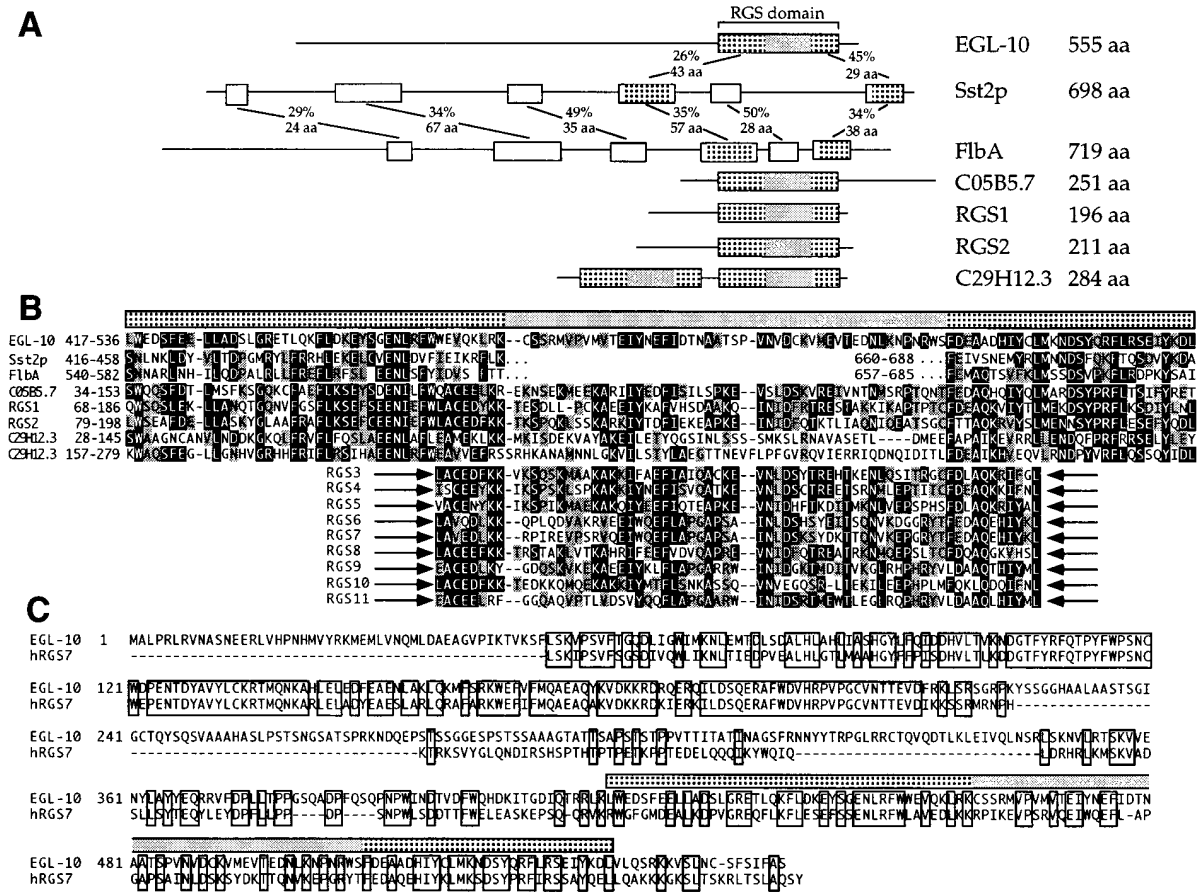

Figure 4. EGL-10 Contains a Conserved Domain Found in Proteins from Yeast, C. elegans, and Mammals

(A) Schematic comparison of EGL-10 and related proteins. Boxes represent regions of conserved sequence. Stippled regions are conserved among all the proteins shown, grey shaded regions are conserved in the metazoan sequences but not in the fungal Sst2p and FlbA sequences, and open boxes represent regions conserved only between Sst2p and FlbA. Lines between the EGL-10, Sst2p, and FlbA schematics are labeled with the percent identities and lengths in amino acids (aa) of the various conserved regions.

(B) Alignment of RGS domain sequences in EGL-10 and related proteins. Stippled and grey shaded bar (top) overlies sequences corresponding to the stippled and grey shaded regions of the schematics in (A). Numbers indicate the positions of the amino acids shown from each protein. The bottom nine sequences are conceptual translations of PCR products amplified from rat brain cDNA. The most highly conserved amino acids are outlined in black; less conserved residues are outlined in grey. Arrows indicate the positions corresponding to the PCR primers used; translations of the primer sites are not shown, since the primer annealing sequences may have been corrupted during amplification with degenerate primers. The partial sequences of the human RGS12, RGS13, RGS14, and RGS15 proteins, identified by expressed sequence tags, are not shown.

(C) Alignment of the EGL-10 and human RGS7 protein sequences. Amino acids identical in both sequences are boxed. The RGS domain is indicated by a stippled and grey shaded bar, as in (B). The RGS7 sequence is derived from an incomplete cDNA and therefore lacks N-terminal sequences.

their entire lengths and show higher conservation in several short regions (Figure 4A); it is two of these more highly conserved regions that show similarity to the conserved domain found in EGL-10, C05B5.7, RGS1, RGS2, and C29H12.3. Alignments of all of these conserved sequences are shown in Figure 4B. This figure also shows alignments with the sequences of nine additional mammalian EGL-10 homologs whose isolation is described below.

The similarity of EGL-10 to Sst2p is of particular interest, since Sst2p functions as a regulator of the G protein–mediated pheromone response pathway in yeast (reviewed by Sprague and Thorner, 1992; Kurjan, 1993). EGL-10 and Sst2p therefore appear to be members of an evolutionarily conserved family of regulators of G protein signaling. We have named the conserved region found in EGL-10, Sst2p, and other proteins the RGS domain (regulator of G protein signaling similarity domain) and refer to mammalian proteins containing this domain as RGS proteins.

Little is known about the functions of the other genes that have sequence similarity to *egl-10*. *flbA* mutants of A. nidulans are defective in the development of conidiophores, specialized spore-bearing structures (Lee and Adams, 1994). The *C05B5.7* and *C29H12.3* genes were identified by the C. elegans genome sequencing project (Wilson et al., 1994). *RGS1* is a human gene expressed specifically in activated B lymphocytes (Murphy and

Norton, 1990; Hong et al., 1993; Newton et al., 1993). *RGS2* is a human gene identified by a clone from a blood monocyte cDNA library (Siderovski et al., 1994).

A Large Family of Mammalian Genes Is Similar to *egl-10* and *SST2*

Do proteins similar to EGL-10 and Sst2p exist in mammals to regulate many or all G protein signaling pathways? As noted above, the two human proteins RGS1 and RGS2 contain conserved RGS domain sequences similar to those present in EGL-10 and Sst2p. However, since RGS1 appears to be expressed specifically in B lymphocytes, it cannot play a general role in all mammalian G protein signaling pathways. The expression of RGS2 has not been analyzed.

To identify additional mammalian genes encoding RGS domains, we designed degenerate oligonucleotide primers to contain all possible codons for the amino acids found in the EGL-10, RGS1, and RGS2 proteins at the positions indicated in Figure 4B and used these primers to perform PCR amplifications from rat brain cDNA. We identified nine novel rat genes that encode RGS domains. Conceptual translations of the amplified fragments from these nine genes are shown in Figure 4B, aligned with the previously known members of the EGL-10/Sst2p protein family. We named the rat genes *Rgs3* through *Rgs11*. The rat RGS domains have structures similar to those of the C. elegans and human members of the EGL-10/Sst2p family; unlike the fungal proteins Sst2p and FlbA, the rat RGS domains contain a conserved region of 48 amino acids that lies immediately between conserved regions found in both the fungal and metazoan sequences (Figure 4).

We identified additional human genes encoding RGS domains by searching a database of expressed sequence tags. This search identified matches to five previously defined genes (*RGS1*, *RGS2*, and apparent human orthologs of the rat *Rgs4*, *Rgs5*, and *Rgs7* genes) as well as partial sequences (data not shown) of four novel genes, which we have named *RGS12* through *RGS15*.

RGS7 shares sequence similarity with *egl-10* outside of the RGS domain, unlike other RGS domain proteins for which extended sequences are available. We therefore obtained and determined the sequence of a human *RGS7* cDNA. While incomplete at its 5′ end, this 1.9 kb cDNA contains a 420 codon open reading frame that encodes a protein with similarity to EGL-10 throughout its length (Figure 4C). The predicted RGS7 protein is 53% identical to EGL-10, with the highest conservation (75% identity) occurring in the N-terminal 174 amino acids of the RGS7 sequence. The 119 amino acid RGS domain of RGS7, by contrast, is 46% identical to the corresponding C-terminal region of EGL-10. EGL-10 contains a 79 amino acid serine/alanine-rich insertion relative to RGS7 between these conserved N- and C-terminal regions. The conserved N-terminal region of EGL-10 functions to localize the protein within muscle cells, and the corresponding region of RGS7 could play a similar role in intracellular localization. It is possible that RGS7 is the human protein most similar to EGL-10,

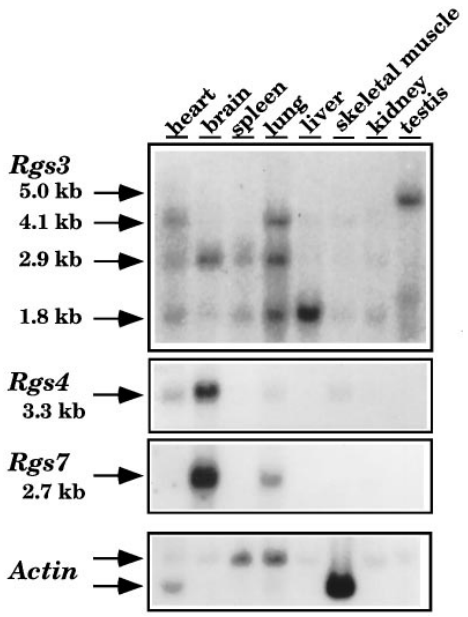

Figure 5. Northern Blot Analysis of Rat *Rgs* Gene Expression in Various Tissues

A Northern blot carrying about 2 μg of poly(A)$^+$-selected RNA prepared from various rat tissues was serially hybridized with three *Rgs* gene probes and, as a control for RNA loading, with a β-actin probe. The *Rgs3* probe detected four different transcripts, while the *Rgs4* and *Rgs7* probes each detected a single transcript. The β-actin probe detected two transcripts; the upper band represents nonmuscle actin, while the lower band is muscle actin.

and it is therefore a candidate to play a functional role analogous to EGL-10 in regulating signaling by G_o. However, we have not obtained sequence outside of the RGS domain for 12 other *Rgs* genes, and they may also have extended similarity with *egl-10*.

To examine patterns of *Rgs* gene expression, we hybridized rat *Rgs* gene probes to Northern blots carrying RNA prepared from various rat tissues (Figure 5). *Rgs4* and *Rgs7* were both expressed at high levels in brain and at lower or undetectable levels in other tissues. *Rgs3* produced a number of different transcripts and was expressed in a wide variety of tissues.

Discussion

EGL-10 May Function in a Signal Transduction Pathway Upstream of the G Protein GOA-1

What aspect of G protein signaling might be affected by EGL-10? Null mutations in the *egl-10* gene and in the G protein gene *goa-1* result in opposite effects on behavior, and the double mutants carrying null mutations in both genes are indistinguishable from the *goa-1* single mutants. This result strongly excludes the possibility that the activity or expression of the EGL-10 protein is regulated as part of an effector mechanism downstream of the G protein GOA-1. Two types of molecular models remain consistent with the genetic data. In the first, EGL-10 functions to regulate GOA-1 by directly or indirectly modifying the expression or activity of GOA-1

or of a molecule upstream of it. Within this type of model, our experiments do not distinguish between EGL-10 acting in the production of or in the response to signaling molecules. However, EGL-10 most likely plays a role in signal response, since Sst2p, the yeast protein with sequence similarity to EGL-10, affects the response to rather than the production of yeast mating pheromone (Chan and Otte, 1982b). In the second type of model, EGL-10 and GOA-1 function in parallel to each other and do not influence the expression or activity of one another. As an example of the second type of model, GOA-1 could regulate an effector mechanism that synthesizes a second messenger molecule, while EGL-10 might function in a process that degrades the second messenger.

Genetic studies of yeast are consistent with the hypothesis that the Sst2p protein plays a role in G protein signaling analogous to that of EGL-10. Yeast respond to mating pheromones using a G protein signaling pathway that has been well characterized genetically (for reviews see Sprague and Thorner, 1992; Kurjan, 1993). Upon initial exposure to mating pheromone, *SST2* loss-of-function mutants are about 200-fold more sensitive to the pheromone than the wild type (Chan and Otte, 1982a). In addition, *SST2* loss-of-function mutations block the desensitization to mating pheromone that normally occurs after prolonged exposure to the pheromone (Chan and Otte, 1982b). Sst2p protein therefore appears to function as a negative regulator of the mating pheromone signaling pathway. Dominant gain-of-function mutations in *SST2* inhibit pheromone response and thus have an effect opposite that of the loss-of-function mutations (Dohlman et al., 1995). The opposite effects of the loss-of-function and gain-of-function *SST2* alleles may be analogous to the opposite effects in C. elegans of *egl-10* null mutations and *egl-10* overexpression. Overexpression of the wild-type *SST2* gene does have an effect opposite to that of an *SST2* null mutation, but this effect is not as great as that of gain-of-function alleles of *SST2* (Dohlman et al., 1995).

A number of genetic experiments have sought to determine the level in the pheromone signaling pathway at which the Sst2p protein acts (Blinder and Jenness, 1989; Hasson et al., 1994; Dohlman et al., 1995). Each of these experiments has been interpreted as consistent with Sst2p protein negatively regulating signaling by acting either upstream of the G protein or as a regulator of the G protein itself and inconsistent with models in which Sst2p is directly or indirectly regulated by the G protein. The yeast experiments also appear to preclude models with a parallel activity of the Sst2p protein and the G protein in which Sst2p acts directly or indirectly on a molecule downstream of the G protein. For example, dominant SST2 mutations that block pheromone-induced signaling in a wild-type background cannot block the constitutive signaling induced by mutations in G protein subunits (Dohlman et al., 1995).

While Sst2p is known to negatively regulate signaling in yeast, our experiments in C. elegans do not yet assign a positive or negative sign to the action of EGL-10 in signaling. In principle, *goa-1* null mutations may either block signaling or, if signaling is mediated by G protein $\beta\gamma$ subunits (Neer, 1995) that are negatively regulated by the GOA-1 α subunit, could lead to constitutive signaling. Thus, *egl-10* loss-of-function mutations, having the opposite effects, could also act either positively or negatively on signaling.

EGL-10 and Sst2p might function in any of several mechanisms that have been proposed to regulate receptor or G protein activity. One such mechanism depends on phosphorylation of the receptors by specific receptor kinases (reviewed by Inglese et al., 1993). However, EGL-10 and Sst2p are unlikely to function in this mechanism, since Sst2p appears to act independently of receptor phosphorylation in yeast (Konopka et al., 1988; Reneke et al., 1988). Other proposals are that G protein α subunits may be regulated by their reversible palmitoylation (reviewed by Ross, 1995), that G proteins are regulated by their interactions with phosducin, a protein that is itself regulated by phosphorylation (Bauer et al., 1992; Lee et al., 1992), and that G proteins may be regulated by GTPase-activating proteins (Angleson and Wensel, 1993; Otto-Bruc et al., 1994).

Members of a Large Family of EGL-10/Sst2p Homologs May Regulate G Protein Signaling in Mammals

We describe in this work a novel conserved domain shared by the EGL-10 and Sst2p proteins, termed the RGS domain. At least 15 mammalian genes encode proteins containing RGS domains: the previously described human *RGS1* and *RGS2* genes, the nine rat genes we isolated in our degenerate PCR screen, and four additional human genes identified by expressed sequence tags. In addition, RGS domains are encoded by at least two C. elegans genes besides *egl-10* and by the A. nidulans *flbA* gene.

The EGL-10 and Sst2p proteins share sequence similarity only in their RGS domains, and it is therefore likely that their apparent common function in regulating G protein signaling depends on this domain. Since no molecular function is known for any of the other proteins that contain RGS domains, it is possible that all of these proteins act to regulate G protein signaling. Alternatively, the RGS domain may perform a function that can be used both in G protein signaling and in other processes.

If RGS domain proteins all function to regulate G protein signaling, why might there be so many? One possibility is suggested by the highly specific expression patterns of several RGS domain proteins: expression of the yeast Sst2p is strongly induced by mating pheromone (Dietzel and Kurjan, 1987); the *RGS1* gene is expressed specifically in activated B lymphocytes (Murphy and Norton, 1990; Hong et al., 1993); the rat *Rgs4* and *Rgs7* genes identified in this work are expressed at high levels specifically in the brain. Organisms may be able to modify their responses to signaling molecules at specific times and in specific tissues by using a number of RGS domain proteins, each expressed in a restricted spatial and temporal pattern. Another possibility is that RGS domain proteins with overlapping expression patterns are able to regulate only certain G protein signaling pathways, perhaps by being restricted to interacting

with only a subset of G proteins or G protein–coupled receptors. By using a number of different RGS domain proteins with such specific effects, organisms could independently regulate different signaling pathways, even those acting simultaneously in the same cells.

Experimental Procedures

Detailed protocols for many of the methods used in this work are available electronically at http://www.dartmouth.edu/artsci/bio/ambros/protocols/worm_protocols.html.

Behavioral Assays

Rates of egg-laying behavior were assayed by two different methods. While measuring the frequency at which eggs are actually laid would seem the most obvious method, for many genotypes this frequency is limited by the rate of egg production, not by the rate of egg-laying behavior, and this method was therefore not used. In our first method, the number of unlaid fertilized eggs that accumulated inside of adult animals was measured. This method was used only to compare strains of which adults produce about the same number of eggs, ensuring that large differences between strains in the steady-state accumulation of unlaid eggs reflected differences in rates of egg laying rather than in rates of egg production. L4 larvae were isolated at the white-crescent stage (an approximately 2 hr period of development when the maturing vulva appears as a large white crescent when viewed with a dissecting microscope) and were allowed to develop 36 hr at 20°C. The resulting adults were incubated for 10 min in microtiter wells containing 50 μl of 1% sodium hypochlorite. This treatment dissolves the bodies of adult worms and releases their fertilized eggs, which are protected by their egg shells (Wood et al., 1988). Eggs were viewed in an inverted microscope (Zeiss Axiovert 10) and counted.

In the second egg-laying assay, we determined the developmental stages of freshly laid eggs. White-crescent stage L4 animals were allowed to develop 29 hr at 20°C, and 20 adults were transferred to a fresh petri plate at 20°C. After 30 min, the developmental stage of each freshly laid egg, determined by viewing in a high magnification dissecting microscope (Wild Makroskop M420), was identified as being in one of three categories: one- to eight-cell stage; nine-cell stage to comma stage (Wood et al., 1988); postcomma stage. By direct observation, we timed embryonic development and determined that it occurred at similar rates for each strain analyzed by this method (data not shown). Differences between strains measured by this assay thus reflect differences in the rates of egg laying rather than differences in the rates of egg development or in the rates of egg production.

We assayed rates of locomotion behavior by measuring the frequency at which young adult animals initiated body bends. NGM plates with reproducibly thin lawns of OP50 bacteria (Brenner, 1974) were prepared by growing the bacterial lawns overnight at 37°C and using the plates immediately. White-crescent stage L4 animals were placed individually on such plates, allowed to develop 24 hr at 20°C, and assayed. Because adult animals begin producing eggs only shortly before the stage at which we assayed locomotion, animals with egg-laying defects did not carry burdens of unlaid eggs grossly different from that of wild-type animals. Egg-laying defects are thus unlikely to indirectly affect the worm locomotion assay. For each animal, we counted the number of body bends by direct observation for a total of 9 min in three 3 min blocks with 30 min between each block. A body bend was scored each time a bend reached a maximum just posterior to the pharynx, either on the dorsal or ventral side of the animal.

Transgenic Animals

Germline transformation was performed according to the method of Mello et al. (1991), and the resulting extrachromosomal transgenes were chromosomally integrated by irradiating transgenic animals with γ-rays. The *egl-10* genomic fragment in the *nIs51* transgene is the 15 kb SmaI–FspI fragment of cosmid W08H11 from the C. elegans physical map (Wilson et al., 1994). The animal shown in Figure 3E carried a transgene in which the 11.2 kb NotI–BamHI fragment of W08H11 was inserted into a GFP reporter gene vector (Chalfie et al., 1994).

Molecular Biology of *egl-10*

The *egl-10* cDNA was obtained from the library of Barstead and Waterston (1989). *egl-10* mutant allele sequences were determined directly from PCR products amplified from the *egl-10* coding sequences of the various mutants. Genomic DNA from the five spontaneous *egl-10* alleles was also analyzed on Southern blots by probing with clones spanning the *egl-10* gene. For Northern blot analysis, RNA was purified and analyzed as described by Sambrook et al. (1989) from worms grown in liquid culture and purified by floating in 30% sucrose, as described by Wood et al. (1988).

Antibodies

The *egl-10* cDNA was fused at its BamHI site to a modified pATH10 E. coli expression vector (Rimm and Pollard, 1989) to express a fusion protein containing TrpE, then amino acids 122–555 of EGL-10, followed by six histidine residues. Polyclonal antibodies were prepared by immunizing rabbits with the purified fusion protein. Anti-EGL-10 antibodies from 2 ml of antiserum were purified by binding to a nitrocellulose filter strip carrying 1 mg of EGL-10 fusion protein and eluting the specifically bound antibodies with 100 mM glycine–HCl (pH 2.5). The antibodies were purified further by preabsorption against TrpE protein expressed from the pATH10 vector. Finally, the antibody was preabsorbed against an acetone precipitate of C. elegans proteins from the mutant *egl-10(n1068)*, prepared as described by Catty (1988).

For Western blots, the EGL-10 protein was visualized using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) and chemiluminescent detection reagents (Amersham). Immunofluorescence staining was as described by Finney and Ruvkun (1990) and was viewed in a Bio-Rad MRC-500 confocal microscope. For immunoperoxidase staining, a horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (Bio-Rad) was used at a dilution of 1:60, and the stain was developed using metal-enhanced diaminobenzidine reagents (Pierce).

Identification and Characterization of Mammalian Genes Encoding RGS Domains

Two 5′ primer pools were used with two 3′ primer pools in all four possible combinations. The primers contained the base inosine (I) at certain positions to allow promiscuous base pairing. The 5′ primers were the following: *5E*, G(G/A)IGA(G/A)AA(T/C)(A/T/C)TIGA(G/A)TT(T/C)TGG; *5R*, G(G/A)IGA(G/A)AA(T/C)(A/T/C)TI(A/C)GITT(T/C)TGG. The 3′ primers were the following: *3T*, G(G/A)TAIGA(G/A)T(T/C)ITT(T/C)T(T/C)CAT; *3A*, G(G/A)TA(G/A)CT(G/A)T(T/C)ITT(T/C)T(T/C)CAT.

Amplification reactions were performed in 50 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM each of dATP, dCTP, dGTP, and dTTP, 1 U of Taq polymerase, 2 μM each PCR primer pool, and 1.5 ng of rat brain cDNA as a template (purchased from Clontech). The reaction conditions were the following: initial denaturation at 95°C for 3 min, followed by 40 cycles of 40°C for 1 min, 72°C for 2 min, 94°C for 45 s, and a final incubation of 72°C for 5 min; 2 μl of each initial amplification reaction was used as a template for further 40 cycle amplification reactions under the same conditions. The approximately 240 bp PCR products were subcloned into pBluescript (Stratagene), generating plasmid libraries for the amplification products from each of the four primer pairs. Clones from each library were were divided into classes with different restriction maps, and the sequences of several clones from each restriction map class were determined. A total of 121 clones were mapped, and sequences were determined for 47. Each of the nine genes identified by this approach was thus isolated at least twice. Three of the four primer pairs identified a gene not identified by any other primer pair.

Labeled *Rgs* gene probes were serially hybridized to a Northern blot (purchased from Clontech) carrying approximately 2 μg of poly(A)$^+$ RNA from each of various rat tissues. A human β-actin cDNA probe was used to control for loading of RNA.

The human *RGS7* cDNA 32047 was obtained from the Washington University/Merck and Company EST Project.

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GenBank Accession Numbers

Accession numbers for the sequences reported in this paper are as follows: *egl-10*, U32326; *Rgs3*, U32434; *Rgs4*, U32327; *Rgs5*, U32435; *Rgs6*, U32436; rat *Rgs7*, U32328; human *RGS7*, U42439; *Rgs8*, U32432; *Rgs9*, U32433; *Rgs10*, U32437; *Rgs11*, U32438. Accession numbers for representative expressed sequence tags from human *RGS* genes are as follows: *RGS4*, R12757, F07186; *RGS5*, D31257, R35272; *RGS12*, R35472, T57943; *RGS13*, T94013; *RGS14*, R11933; *RGS15*, T92100.

Note Added in Proof

While this paper was in press, De Vries et al. (1995) reported the identification of a human RGS protein, GAIP, that interacts in a yeast two-hybrid system with the G protein $G\alpha_{i3}$ and is distinct from the 15 mammalian RGS proteins discussed in this work: De Vries, L., Mousli, M., Wurmser, A., and Farquhar, M. G. (1995). GAIP, a protein that specifically interacts with the trimeric G protein $G\alpha_{i3}$, is a member of a protein family with a highly conserved core domain. Proc. Natl. Acad. Sci. USA *92*, 11916–11920.