Caenorhabditis elegans $G\alpha_q$ Regulates Egg-Laying Behavior via a PLCβ-Independent and Serotonin-Dependent Signaling Pathway and Likely Functions Both in the Nervous System and in Muscle

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ABSTRACT

egl-30 encodes the single *C. elegans* ortholog of vertebrate $G\alpha_q$ family members. We analyzed the expression pattern of EGL-30 and found that it is broadly expressed, with highest expression in the nervous system and in pharyngeal muscle. We isolated dominant, gain-of-function alleles of *egl-30* as intragenic revertants of an *egl-30* reduction-of-function mutation. Using these gain-of-function mutants and existing reduction-of-function mutants, we examined the site and mode of action of EGL-30. On the basis of pharmacological analysis, it has been determined that *egl-30* functions both in the nervous system and in the vulval muscles for egg-laying behavior. Genetic epistasis over mutations that eliminate detectable levels of serotonin reveals that *egl-30* requires serotonin to regulate egg laying. Furthermore, pharmacological response assays strongly suggest that EGL-30 may directly couple to a serotonin receptor to mediate egg laying. We also examined genetic interactions with mutations in the gene that encodes the single *C. elegans* homolog of PLCβ and mutations in genes that encode signaling molecules downstream of PLCβ. We conclude that PLCβ functions in parallel with *egl-30* with respect to egg laying or is not the major effector of EGL-30. In contrast, PLCβ-mediated signaling is likely downstream of EGL-30 with respect to pharyngeal-pumping behavior. Our data indicate that there are multiple signaling pathways downstream of EGL-30 and that different pathways could predominate with respect to the regulation of different behaviors.

CAENORHABDITIS elegans $G\alpha_q$ is encoded by *egl-30* (Brundage *et al.* 1996). In mammals, on the basis of sequence similarity the $G\alpha_q$ family is represented by four members: $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and murine $G\alpha_{15}$/human $G\alpha_{16}$ (Wilkie *et al.* 1991). Mutations in *egl-30* were originally identified because of their egg-laying-defective phenotype (Trent *et al.* 1983). Subsequent characterization and identification of additional *egl-30* mutant alleles revealed that *egl-30* is involved with the regulation of many behaviors, including egg laying (Trent *et al.* 1983; Brundage *et al.* 1996), pharyngeal pumping (Brundage *et al.* 1996), movement (Brundage *et al.* 1996), viability (Brundage *et al.* 1996), and spicule protraction (Garcia *et al.* 2001). The vertebrate $G\alpha_q$ family is involved in pertussis-toxin-insensitive regulation of phospholipase Cβ (PLCβ) isoforms (Exton 1996). Like its vertebrate homolog, EGL-30 can stimulate endogenous phospholipase activity in COS-7 cells (Brundage *et al.* 1996).

Given the fact that EGL-30 and $G\alpha_q$ and $G\alpha_{11}$ share >80% identity at the amino acid level and that EGL-30 can functionally complement $G\alpha_q$ (Brundage *et al.* 1996), it is likely that the biochemistry of signaling is well conserved between *C. elegans* and mammals. We employed a genetic approach in *C. elegans* to further characterize EGL-30-mediated signaling pathways. To this end, we screened for mutations that were able to phenotypically suppress a reduction-of-function allele, *egl-30(md186). egl-30(md186)* mutants are lethargic, exhibit slow pharyngeal pumping, and lay eggs at a later stage of development than do wild type.

From our suppressor screen, two intragenic revertants of *egl-30(md186)* that increase *egl-30* function were isolated. We used these mutants and another strong *egl-30* gain-of-function mutant (Doi and Iwasaki 2002) as a tool to explore interactions with genes that encode candidate downstream molecules. Double-mutant strains were constructed to analyze interactions with the PLCβ-signaling pathway and to analyze interactions with respect to the pathway defined as downstream of EGL-30 with respect to synaptic transmission (Lackner *et al.* 1999). We focused primarily on egg-laying behavior since this behavior is regulated by neural input to the vulval muscles by only two classes of motor neurons, the hermaphrodite-specific neurons (HSNs) and the ventral type C neurons (VCs) (White *et al.* 1986; Waggoner *et al.* 1998). We also examined the double mutants with respect to pharyngeal-pumping behavior since *egl-30* mutants display strong pumping defects.

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Our results suggest that PLCβ, the only well-characterized effector for vertebrate $G\alpha_q$ family members, is not the only effector for EGL-30. With respect to egg laying, PLCβ either acts in parallel to *egl-30* or is a minor downstream component of EGL-30-mediated signaling. Consistent with this result, mutations in *itr-1*, which encodes the single inositol 1,4,5 triphosphate (IP_3) receptor, and in a gene that encodes protein kinase C (PKC) also do not significantly modify *egl-30* gain-of-function phenotypes. However, with respect to pharyngeal pumping, each of the mutations in genes that encode components of PLCβ signaling do suppress *egl-30* gain-of-function phenotypes to a similar extent with respect to pharyngeal pumping.

We conclude that in *C. elegans* there are other effector(s) for EGL-30 in addition to PLCβ and that different downstream pathways predominate to control egg laying and pharyngeal pumping. Expression analyses also indicate that there is not complete overlap with respect to EGL-30 localization and EGL-8 localization (Miller *et al.* 1999). It is conceivable that different effector pathways are cell-type specific, and we show that EGL-30 does function in, or via, at least one class of motor neurons that activate the vulval muscles and likely in the vulval muscles themselves as well.

MATERIALS AND METHODS

Manipulation of *C. elegans*: Maintenance and manipulation of *C. elegans* were as described (Brenner 1974). The following alleles were used for behavioral analyses and for double-mutant constructions: LGI, *egl-30(md186)I* (Miller *et al.* 1996), *egl-30(ad805)I* (Brundage *et al.* 1996), and *unc-13(e51) unc-122(n2916) I* (Brenner 1974); LGII, *tph-1(mg280)II* (Sze *et al.* 2000); LGIV, *dpy-20(e1282)IV* (Hosono *et al.* 1982), *itr-1(sa73) IV* (Iwasaki *et al.* 1995), *dpy-9(e12)IV* (Brenner 1974), and *tpa-1(k530)IV* (Tabuse and Miwa 1983); LGV, *egl-8(md1971)V* (Miller *et al.* 1999), *egl-8(n488)V* (Trent *et al.* 1983), *egl-8 (sa47)V* (Thomas 1990), and *egl-1(n487)V* (Trent *et al.* 1983). The *unc-13* strain used in this study (CB51) also contains a linked *unc-122* mutation. This linked mutation was uncovered in Richmond *et al.* (1999) and was presumably used in all similar studies prior to this discovery (Lackner *et al.* 1999). In general, mutations were followed in crosses on the basis of their own visible phenotypes. For strains harboring *tpa-1, dpy-9(e12)* was used as a balancer, and for strains with *itr-1, dpy-20(e1282)* was used as a balancer *in trans*. For example, *egl-30(gf)/+* males were mated to *dpy-9* hermaphrodites. Since *egl-30(md186sy676)* is semidominant, it was possible to identify heterozygous male progeny. Hyperactive (*egl-30(md186sy676)/+*; *dpy-9/+*) F_1 males were mated with *tpa-1* mutant hermaphrodites. A total of 10 hyperactive (*egl-30(md186sy676)/+*) F_1 progeny were selected and picked onto individual plates. A total of 25 candidate *egl-30(md186sy676)* homozygous animals were selected only from plates that segregated *dpy-9*. Plates that segregated only hyperactive non-Dpy progeny were examined for progeny that segregated hyperactive animals of which approximately one-fourth had a modified phenotype. In the cases of *itr-1* and *tpa-1*, both mutations slightly modified the movement of *egl-30(gf)* homozygotes. *egl-30(gf); itr-1(sa47)* animals were scrawnier than *egl-30(gf)* homozygotes and in *egl-30(gf); tpa-1* double mutants a synthetic phenotype was observed such that a tail spike and vacuolization of some of the nerve ring ganglia was apparent in the double mutant, but not in either single mutant. In all cases, double mutants were backcrossed to N2 and, after picking F_1's to individual plates, each single mutant was reisolated in the F_2 progeny.

Construction and integration of functional GFP-tagged EGL-30, *hsp-16*::*egl-30(QL)*, and overexpression and integration of the wild-type *egl-30* locus: Standard molecular biological techniques were performed as described (Sambrook *et al.* 1989). For construction of an in-frame green fluorescent protein (GFP) fusion just 5′ to the *egl-30* initiator ATG, the following primers were used to amplify a GFP S65T mutant from a pRSET vector (kind gift from Roger Tsien): 5′-AAT AAAGGCCTAAATGGCCATGAGTAAAGGAGAAGAACTT TTC-3′/5′-TTAGCAGGCCATGGCGGCCAAGCATTTGTAT AGTTCATCCATGC-3′. The PCR product was gel purified (QiaexII, QIAGEN, Valencia, CA), digested with SfiI, and cloned into the SfiI site of pLB2 (Brundage *et al.* 1996). The resulting construct, pCB50, was injected into *egl-30(md186); dpy-20(e1282)* at a concentration of 5 ng/μl. Animals were microinjected according to standard procedures (Mello *et al.* 1991; Mello and Fire 1995). The *dpy-20* rescuing plasmid, pMH86, was injected at 20 ng/μl (Han and Sternberg 1990) and was used as the coinjection marker. pBluescript was included as carrier DNA to bring the total DNA concentration to 200 ng/μl.

A fusion of the *egl-30(Q205L)* cDNA was cloned into pPD49.78 as a *Kpn*1-*Sac*I fragment to generate pLB24. pLB24 was at injected at 50 ng/μl as described for pCB50.

Transgenic arrays (pLB24 and pCB50) were chromosomally integrated as described (Way *et al.* 1991), except that animals were treated with 38 Gy from an X-ray source. The pLB24 integrant was named *syIs38*, and the pCB50 integrant was named *syIs105*. Construction of and expression from the extrachromosomal array that was integrated (*syEx125*) to make *syIs36* by the procedures described for other integrated lines (Brundage *et al.* 1996). The integrated lines were outcrossed a minimum of six times with *dpy-20(e1282)*. The integration of the transgenic arrays generally increased the expressivity of the phenotype observed in the nonintegrated lines. In the case of *syIs38*, the severity of the phenotype after heat shock also increased.

Expression analysis: An EGL-30 antibody was generated in rabbits against the C-terminal peptide: KDTILQHNLKEYNLV (Quality Controlled Biochemicals). The antibody was affinity purified with the peptide used for immunization using SulfoLink Coupling Gel (Pierce, Rockford, IL) according to the manufacturer's instructions. Indirect immunofluorescence was done as described (Ruvkun and Giusto 1989), except 2× fixation buffer contained 50% MeOH and did not contain n-heptane, and Triton X-100 was used at 0.1%. Either a fluorescein-conjugated anti-rabbit antibody was used at 1:1000 (Vector Laboratories, Burlingame, CA) or the Vectastain Elite ABC avidin/biotin blocking kit was used with a biotinylated anti-rabbit antibody (Vector Laboratories). When the biotinylated antibody was used, an avidin/biotin blocking step was included after the collagenase digestion step. Animals were examined with a Zeiss axioskop under a ×100 objective. For examination of GFP fluorescence, animals were examined on a Leica DMIRBE confocal microscope.

Isolation of intragenic suppressor mutations: Ethyl methanesulfonate (EMS) mutagenesis of ~60,000 *egl-30(md186)* gametes was done as described (Brenner 1974) using 50 mM EMS. From this screen, we isolated two intragenic revertants and three strong extragenic suppressor mutations. F_2 progeny were selected on the basis of their ability to suppress egg-laying and/or movement defects of *egl-30(md186)* mutants. Intragenic suppressor mutants were outcrossed to N2 at least six times.

Sequencing of *egl-30* mutations: Suppressor mutations (*sup*) were picked as potential intragenic revertants on the basis of the inability to isolate *egl-30(md186)* homozygous F_1 progeny when *sup egl-30(md186)/++* males were mated to *egl-30 (md186)*. Fragments of the *egl-30* locus were amplified using Takara *LA-Taq* (PanVera) with the following primer sets: 5′-GCATCGAACTTCTATCCTC-3′/5′-GCTACGAGATATTT GTTGC-3′ (exons 1 and 2), 5′-GAGATTAAAATACTCAT TTCG-3′/5′-CGAAAAAAGCATCAGAGATTG-3′ (exons 3–5), and 5′-CTTTCTGTAAATTGTCAGC-3′/GTGGTGTCTCACTC GACC-3′ (exons 6–8). DNA was amplified in 10 50-μl reactions, gel purified using Qiaex II (QIAGEN), and directly sequenced on an automated sequencer (ABI). The DNA was compared with N2 and with the original *egl-30*(*md186*) strain. Mutations were confirmed by sequence from the reverse strand. Mutations were mapped onto known crystal structures using Protein Explorer (http://www.umass.edu/microbio/chime/explorer/index.htm). *egl-30(tg26)* was isolated in a screen for suppressors of *unc-31* by L. Khan (Doi and Iwasaki 2002) and obtained as a generous gift from the Iwasaki laboratory.

Sequencing of *egl-8* mutations: Primers flanking the *n488* deletion were used for PCR: 5′-CTA CTA CCC AAC AAG TGA GAG 3′/5′-CGG AAG TTT TGG GCT GTT TTG G-3′. The deletion was found to extend from 7905–9735 in the unspliced DNA sequence displayed in WormBase (WS62) such that the sequence 5′-GTGTGGGAAAAAATGTGT-3′ was fused to 5′-TGTTGATTATCATTTTCT-3′. Primers flanking the *md1971* mutation were used for PCR: 5′-CGA AGA CCC GAC AGA ACA TT-3′/5′-CCC GGG TAT TAC CTT CGT CT-3′. PCR products were amplified, purified, and sequenced as above.

Behavioral assays: To measure pharyngeal pumping rates, 1-day-old adults were placed on nematode growth media (NGM) plates seeded with OP50 and left undisturbed for at least 15 min before counting. Pharyngeal pumps were measured using a counter for a period of 3 min. Response was measured only from animals that remained in food throughout the period of observation.

To characterize egg-laying behavior, animals were examined 24–28 hr after selecting them as L4 larvae. Young adults were placed on fresh plates seeded with OP50 and newly laid eggs were examined every 5 min for their developmental stage using a Wild M420 macroscope and then removed from the plate. In all cases, at least two trials were performed on different days, and at least 10 different adults were assayed per trial. For mutants that never laid eggs past the gastrulation stage of development, at least three trials were performed.

For response to 5-hydroxytryptamine (5-HT; Sigma, St. Louis), levamisole (Sigma), and α-methyl 5-hydroxytryptamine (α-methyl 5-HT; Sigma), animals were tested singly (except as indicated) as 1-day-old adults in wells of a microtiter plate containing a solution of the indicated drug. Response was measured after 60 or 90 min, as indicated in figure legends. 5-HT was generally used at a concentration of 7.5 mM, levamisole at 6.25 μm, and α-methyl 5-HT at 1 mM. For 5-HT and levamisole, we used M9, but sodium phosphate was substituted for potassium phosphate. To test response to α-methyl 5-HT, assays were performed in water since M9 inhibited the response for all genotypes tested, except for strains that also contained *egl-1* mutations.

Phalloidin staining: Worms were stained with phalloidin using a modification of a previously described protocol (Myers *et al.* 1996). Worms were grown on NGM plates, harvested in S-basal medium, and washed once with S basal. Supernatant was removed with a Pasteur pipette and worms were frozen in liquid nitrogen. A few drops of ice-cold acetone were added to worms and worms were incubated on ice for 5 min. Acetone was removed and worms were air dried. A total of 10 μg of fluorescein-conjugated phalloidin was added to the worms in PBS buffer containing 1 mM magnesium chloride and 0.4% SDS. Worms were stained for 1 hr at room temperature, harvested, and washed three times in PBS containing 0.5% Tween-20. Animals were examined for fluorescence on a Zeiss axioscope.

Western analysis: Worms were harvested from NGM plates in M9 and washed one time with M9. Worm pellets were resuspended in 2× SDS sample buffer (Novex, San Diego) and heated at 70° for 10 min. Worm debris was pelleted at 14 K in a microcentrifuge for 10 min, and the supernatant was promptly loaded on an SDS/PAGE gel (Novex).

For Western analysis of EGL-30, the same number of *syIs36* mutants was first picked to separate plates for each time point. Four days later (prior to starvation) worms were heat-shocked for 30 min at 32°. At the indicated time points, worms from each plate were harvested and prepared as described above. Worm extracts were loaded onto a 10% gel.

For analysis of *egl-8* expression, worms were harvested in M9 as above. Extracts for analysis of *egl-8* expression were loaded onto a 4–12% gradient gel (Novex).

Electrophoretic transfer was carried out for 1.5 hr for EGL-30 Western analysis and overnight for EGL-8 analysis. Following transfer to nitrocellulose membranes, EGL-8 antibody (generously provided by Kenneth Miller) was used at a concentration of 1:1000 (Miller *et al.* 1999), and affinity-purified EGL-30 antibody generated against the peptide was used at a dilution of 1:50,000. HRP-linked anti-rabbit IgG (Amersham, Buckinghamshire, UK) was added at a concentration of 1:1000, and bands were visualized using enhanced chemiluminescence (Amersham).

RESULTS

Expression of *egl-30*: To clarify the site and mode of action for *egl-30* with respect to the behaviors that it regulates, to resolve differences in the inferred site-of-action from previous reports (Brundage *et al.* 1996; Lackner *et al.* 1999), and to examine the subcellular localization of *egl-30*, we sought to construct a rescuing GFP fusion transgene. This transgene contains all of the presumptive 5′-transcriptional regulatory sequences, introns, and presumptive 3′ regulatory sequences for *egl-30*, in addition to the coding sequences for GFP just 5′ of the *egl-30* initiating methionine. We expected that this transgene should be appropriately expressed as well as appropriately localized. This transgene was integrated into the genome of *egl-30(md186)* animals, and the integrated transgene, *syIs105*, was found to partially rescue *egl-30(md186)* with respect to egg laying, movement, pharyngeal pumping, and response to neurotransmitters in egg-laying assays (Tables 1 and 2). Expression was first observed in early embryos at cell peripheries and in a punctate pattern throughout embryos (Figure 1D). Later, expression was observed in larvae in the nerve ring and many neurons of the nerve ring ganglia, pharyngeal muscle, and ventral cord and many neurons in the tail ganglia and hypodermis (Figure 1). Highest expression levels were observed in L4 larvae and in adult animals. In the nervous system, expression was observed in cell bodies and in neural processes. However, the highest expression was observed in the axons of the nerve ring. Expression in larvae and in adults was also

TABLE 1

***egl-30(md186)* mutants exhibited defective egg-laying and pharyngeal-pumping phenotypes**

	Stages at which eggs were laid						
	5c-gastr. (%)	Comma (%)	2-fold (%)	Pretzel (%)	Hatched (%)	*N*	
N2	100					Many	
egl-30(md186)	1	5		48	46	100	
egl-30(md186)/+	79	9	9	3		43	
egl-30(md186); syls105	100					37	
	Pharyngeal pumps/min					*N*	SD
N2	277.9					5	9.3
egl-30(md186)	174.7					5	12.3
egl-30(md186)/+	223.8					5	9.4
egl-30(md186); syls105	217.7					5	8.4

egl-30(md186) is a reduction-of-function mutation and exhibits haplo-insufficiency, like other *egl-30* reduction-of-function alleles previously described (Brundage *et al.* 1996). GFP-tagged EGL-30 rescued *egl-30(md186)* with respect to egg-laying and pharyngeal-pumping defects. The integrated transgene, *syls105*, was used to analyze the expression pattern of *egl-30*. SD, standard deviation for the number of pumps per minute. 5c-gastr., from five-cell stage embryo to gastrulation; 2-fold, twofold stage embryo.

evident in the pharyngeal muscle. Expression was also observed in the dorsal nerve cord and in sublateral processes (Figure 1E). In addition, occasional expression was observed throughout the intestine (particularly in the posterior intestine), vulval muscle, head muscle, in sperm, tonofilaments, and in vulval cells. With the exception of the pharyngeal muscle, expression levels in muscle were generally extremely low such that only rare animals (10%) showed weak expression. The CAN cell bodies and associated canals were observed to express GFP-tagged EGL-30 in most animals (Figure 1C). The HSN cell bodies and the VC4 and VC5 neuronal cell bodies also occasionally expressed EGL-30 (10%), on the basis of proximity to the vulva. Other cell bodies in the ventral nerve cord were also observed to occasionally express GFP.

Immunolocalization analysis with a polyclonal antibody generated against the carboxyl terminus of EGL-30 generally revealed the areas with highest expression levels. Using an HRP-conjugated secondary antibody and a FITC-conjugated secondary antibody, we observed high expression in axons of the nerve ring. The FITC-conjugated antibody also indicated expression in pharyngeal muscle. Both secondary antibodies revealed expression in sperm, and the FITC-conjugated antibody consistently showed expression in the anal sphincter muscle (Figure 1H). Nonspecific antibody staining was observed in intestinal nuclei using both secondary anti-

TABLE 2

Egg-laying response of 1-day-old adult hermaphrodites to pharmacological agonists

	5-HT			Levamisole + 5-HT		
Genotype	Response	*N*	SD	Response	*N*	SD
N2	5.0	23	2.7	3.7	15	0.6
egl-30(md186)	1.9	10	2.6	0.5	15	0.1
egl-8(md1971)	9.3	12	4.1			
egl-8(n488)	0	19	0	0.3	15	0
egl-8(sa47)	9.3	10	4.4			
unc-13(e51)	4.4	11	3.7			
egl-30(md186); syls105	5.5	22	3.1	8.2	15	0.3

Controls in M9 were performed on each strain. Only *egl-30(md186); syls105*, which contained the integrated *egl-30::GFP* transgene, responded by laying an average of 1.8 eggs per worm in M9. To measure response to 7.5 mM 5-HT, single worms were assayed in a total volume of 150 μl. Eggs were counted after a 90-min incubation at room temperature. SD, standard deviation per worm. To measure response to 7.5 mM 5-HT and 6.25 μM levamisole, five worms were assayed in a total volume of 150 μl. Eggs were counted after a 1-hr incubation at room temperature. SD, standard deviation per five worms.

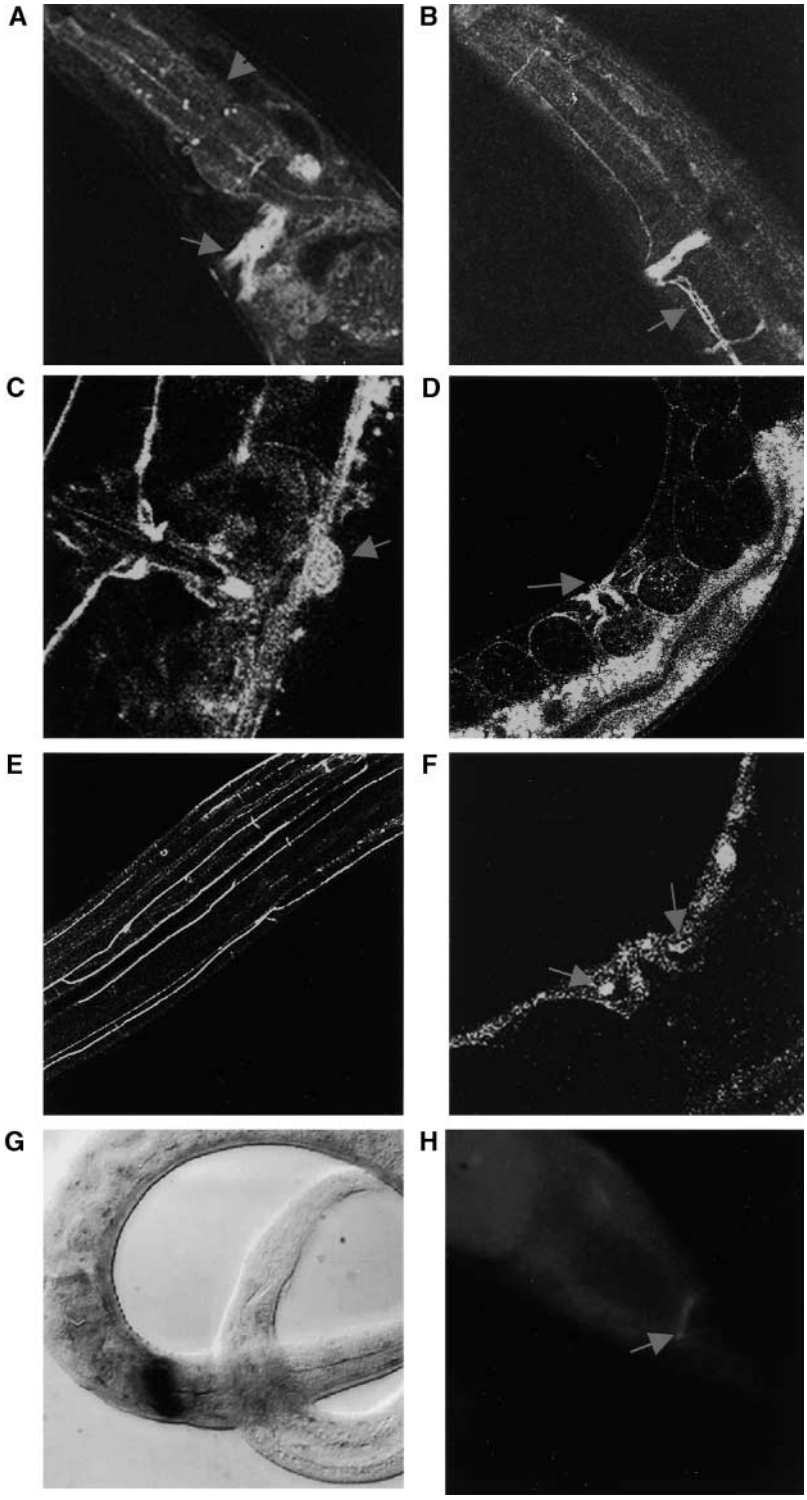

Figure 1.—Expression of *egl-30*, based on an integrated GFP fusion transgene (A–F) and indirect immunofluorescence (G and H). (A) The arrow indicates expression in the nerve ring. The arrowhead indicates expression in the pharyngeal muscle. (B) Arrow indicates expression in the ventral nerve cord. (C) Arrow indicates expression in the CAN neuron. (D) Arrow points to expression in the vulval muscle. (E) Expression in nerve tracts. (F) Expression in the VC4 and VC5 motor neurons, based on their positions, is indicated by the arrow. (G) Expression in the nerve ring, as detected using a biotinylated secondary antibody and HRP-avidin. (H) Expression in the anal sphincter muscle, as visualized with a FITC-labeled secondary antibody. Images in A–F were obtained with a Leica DMIRBE microscope and images in G and H were obtained on a Zeiss axioscope.

bodies and is most likely due to cross-reactivity with an unrelated protein. Western analysis supports this hypothesis since the band that migrates at the position where EGL-30 migrates on an SDS/PAGE gel is present at almost equivalent intensities in protein extracts prepared from *egl-30(ad805)* mutants compared to wild-type extracts (data not shown), but the levels of this band increase significantly after heat-shock induction of an *egl-30* transgene (Figure 2A). When *egl-30* was overexpressed from an integrated array (*syIs36*), an increase in the intensity of staining was observed in the nerve ring and in pharyngeal muscle, but not in intestinal nuclei.

The broad expression pattern of *egl-30* is consistent with its pleiotropic and essential function. Expression levels of *egl-30* were clearly higher in the nervous system

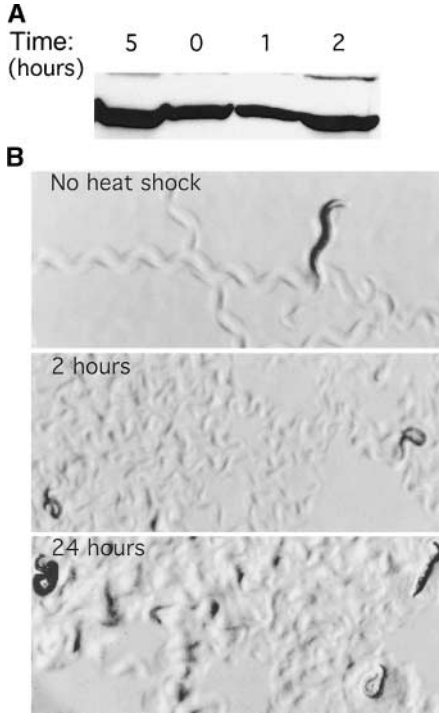

FIGURE 2.—Heat-shock induction of EGL-30(Q205L) under the control of the *hsp-16-2* promoter element (integrant *syIs38*). (A) Protein levels following a 30-min heat shock at 32°. Times indicated represent the amount of time following the end of the heat-shock regimen. Protein levels did not appear to increase more than threefold above basal levels. (B) Phenotypic effects on adults following heat shock. After 2 hr worms exhibited a hyperactive phenotype, laid early eggs, and moved with exaggerated body bends. Within 4 hr, animals were completely paralyzed. Twenty-four hours later, adults displayed a hypercontracted posture, constitutive pumping of many muscle types, and exhibited vacuolization of many cells.

and in pharyngeal muscle, suggesting that EGL-30 exerts a primary role in these tissues. Weak or occasional expression in other muscle-cell types, sperm, intestine, tonofilaments, and vulval cells indicates the possibility that EGL-30 might have an almost ubiquitous expression pattern.

Dose sensitivity of *egl-30*: Haplo-insufficiency of the *egl-30* locus, together with phenotypic analysis of gain- and reduction-of-function mutations in *egl-30*, demonstrates that perturbations in activity levels of *egl-30* result in a clear phenotypic readout. Heterozygous reduction-of-function mutants exhibit semidominance with respect to lethargic locomotion and egg-laying defects (BRUNDAGE *et al.* 1996). *egl-30(md186)* mutants behaved like other reduction-of-function mutations previously described and lay >94% of their eggs after the gastrulation phase of development (Table 1). Wild-type hermaphrodites lay eggs ~2–3 hr after fertilization during the gastrulation phase (beginning at the 28-cell stage) of development and always before morphogenesis begins (comma stage; SULSTON *et al.* 1983). For our purposes, late-stage eggs are defined as eggs laid at or after the comma stage of development. *egl-30(md186)* mutants also exhibited semidominance with respect to pharyngeal-pumping and egg-laying defects (Table 1). Consequently, it is clear that reducing activity levels of EGL-30 by <50% has significant phenotypic consequences.

Similarly, raising both expression or activity levels of EGL-30 has drastic effects on behavior (BRUNDAGE *et al.* 1996). A transgenic strain that overexpresses wild-type *egl-30* under its own regulatory regions, *syIs36*, exhibited hyperactive movement, moved with exaggerated body bends, and laid eggs at an early stage of development (data not shown and Table 3). These animals also exhibited a pale, starved appearance. Heat-shock induction of *syIs38*, an integrated transgene that contains a gain-of-function cDNA of *egl-30* fused to the *hsp-16-2* enhancer-promoter element, demonstrated the progressive effects caused by increasing EGL-30 expression and activity levels over time. Approximately 2 hr after a heat-shock induction of 32° for 30 min, worms became hyperactive, started to move with exaggerated body bends, and had laid all of the eggs contained within their uteri (Figure 2B). After 4 hr, worms became paralyzed and hypercontracted. After 24 hr, the hypercontracted phenotype was still apparent, and animals exhibited weak but constitutive pumping of all muscle types and displayed vacuolization of many cell types (Figure 2B). Eventually, these worms died. These effects are evident in spite of the fact that expression levels of *egl-30* increased only at most threefold above endogenous levels (Figure 2A). This low induced level of expression is in marked contrast to the high induced levels of expression that were obtained with an analogous GOA-1 construct (C. BASTIANI, W. CHEN, M. I. SIMON and P. W. STERNBERG, unpublished results). The same differences in expression levels are observed when levels of GOA-1 and EGL-30 are compared after transfection of cDNA expression plasmids into HEK293T cell lines (C. BASTIANI, W. CHEN, M. I. SIMON and P. W. STERNBERG, unpublished results).

Gain-of-function mutants of *egl-30:* Mutations that do not affect expression levels of *egl-30*, on the basis of Western blot analysis (data not shown), similarly demonstrate the effects of altered activity levels of EGL-30. Two intragenic revertants of a reduction-of-function allele, *egl-30(md186)*, were obtained in screens for mutations for suppression of the allele's egg-laying-defective and lethargic phenotypes (Tables 3 and 4 and data not shown). *egl-30(md186)* encodes a D201E change immediately adjacent to the switch 2 region, suggesting that association with guanine nucleotides is probably altered. The two intragenic revertants differ in their strengths.

The stronger intragenic suppressor mutant, *sy676*, exhibited semidominant hyperactive movement, movement with exaggerated body bends, and constitutive egg-laying phenotypes (heterozygotes lay 19% of eggs at or before the four-cell stage; $N = 224$), similar to phenotypes caused by overproduction of wild-type EGL-

TABLE 3

Genetic interactions with respect to egg laying of *egl-30* mutations with mutations in genes that encode candidate downstream-signaling molecules

		Stages at which eggs were laid						
	Behavior	1–4c (%)	5c-gastr. (%)	Comma (%)	2-fold (%)	Pretzel (%)	Hatched (%)	*N*
N2	WT		100					Many
syIs36	Egl-C	88	12					12
egl-30(md186sy676)	Egl-C	42	58					224
egl-30(md186sy676); egl-8(sa47)	Egl-C	24	76					71
egl-30(md186sy676); egl-8(n488)	Egl-C	36	64					52
egl-30(md186sy676); egl-8(md1971)	Egl-C	37	63					71
egl-30(md186sy676); tpa-1(k501)	Egl-C	59	41					63
egl-30(md186sy676); itr-1(sa73)	Egl-C	25	75					91
egl-30(md186sy676); unc-13(e51)	Egl-D		93	2		2	3	61
egl-30(tg26)	Egl-C	91	9					51
egl-30(tg26); egl-8(sa47)	Egl-C	78	22					78
egl-30(tg26); egl-8(n488)	Egl-C	79	21					47
egl-30(md186sy679)	Egl-D		71	15	2	12		93
egl-30(md186sy679); egl-8(sa47)	Egl-D		33	29	2	36		45
egl-30(md186sy679); unc-13(e51)	Egl-D		36	11	7	39	7	61
egl-30(md186)	Egl-D		1	5		48	46	100
egl-30(md186); egl-8(sa47)	Egl-D		3	31	3	63		32
egl-30(md186); unc-13(e51)	Egl-D		50	19	3	27	4	119
egl-8(sa47)	Egl-D		62	16		18	4	45
egl-8(n488)	Egl-D		8	21	14	51	6	47
egl-8(md1971)	WT		100					40
tpa-1(k501)	WT		100					55
itr-1(sa73)	WT		100					86
unc-13(e51)	Egl-D		29	21		48	2	58

Assays were performed on 1-day-old adults after transferring 10 worms to fresh plates. Trials were performed on at least two separate days. The differences between the percentage of eggs laid at the one- to four-cell (1c–4c) stage between *egl-30(md186sy676)* single and double mutants with *egl-8*, *tpa-1*, and *itr-1* mutations were not significant on the basis of the Kruskal-Wallis nonparametric ANOVA test (two-tailed P value > 0.2). The differences were also not significant between the *egl-30(tg26)* single and double mutants (two-tailed Pvalue < 0.2). *unc-13* mutations suppressed hyperactive egg laying conferred by *egl-30(md186sy676)* mutations, but *egl-30(676)* mutations partially suppressed the Egl-D phenotype conferred by *unc-13* mutations. WT, wild type. 5c-gastr., five cell to gastrulation.

30 (Brundage *et al.* 1996). This intragenic revertant encodes a R334W change within the α5 helix. This amino acid change results in a string of eight consecutive hydrophobic residues, of which W334 is the second. Mutations of residues within the β6/α5 loop promote dissociation of GTP and GTP/GDP exchange on several Gα subunits (Thomas *et al.* 1993; Iiri *et al.* 1994; Posner *et al.* 1998). This region also lies very close to the carboxyl terminus of EGL-30, which in Gα subunits is implicated in interactions with membrane receptors (Hamm *et al.* 1988; Garcia *et al.* 1995; Osawa and Weiss 1995; Kisselev *et al.* 1998). On the basis of comparative analysis with the crystal structure of $G\alpha_i$ in a complex with RGS4 (Tesmer *et al.* 1997), it is likely that the mutated amino acid does not directly interact with the site of the original *egl-30(md186)* mutation (Figure 3). Also on the basis of comparative analysis with the crystal structure of $G\alpha_i$ in a complex with β/γ (Wall *et al.* 1995), we know that this mutation lies in a region that likely does not affect interaction with β/γ. In addition, alignments with mammalian $G\alpha_i$ indicate that this mutation does not lie in a region important for activation of PLCβ (Venkatakrishnan and Exton 1996). Consequently, we speculate that GTP dissociation, GTP/GDP exchange, or interaction with seven-pass membrane receptors is affected by this mutation.

The weaker intragenic suppressor mutation, *sy679*, encodes an E277K change, a change from an acidic residue to a basic residue, within the αG helix. On the crystal structure of $G\alpha_i$ (Raw *et al.* 1997), this mutation is in close proximity to the residues involved in guanine ring binding and is diametrically opposite from the original *egl-30(md186)* mutation (Figure 3). It is possible that this mutation partially compensates for any alterations

TABLE 4

Interactions with respect to pharyngeal-pumping behavior of *egl-30(gf)* and *egl-30(rf)* mutations with mutations in genes that encode candidate downstream signaling molecules

	Pumps/min	SD
N2	277.9	9.3
egl-30(md186sy676)	>300	NA
egl-8(sa47)	186.2	3.3
egl-30(md186sy676); egl-8(sa47)	206.4	11.0
itr-1(sa73)	79.8	32.7
egl-30(md186sy676); itr-1(sa73)	134.2	20.8
unc-13(e51)	84.1	18.7
egl-30(md186sy676); unc-13(e51)	186.6	4.9
egl-30(md186)	174.7	12.3
egl-30(md186); egl-8(sa47)	106.7	27.0
egl-30(md186); unc-13(e51)	2.4	1.1

Pumping assays were performed on 1-day-old adults as described in MATERIALS AND METHODS. SD, standard deviation per worm. NA, not applicable.

in guanine nucleotide interactions modified by the original *egl-30(md186)* mutation. However, E277K did not completely suppress the original D201E change in *egl-30(md186)* with respect to pharyngeal pumping (data not shown) and egg laying (Table 3). Again, on the basis of comparative crystal structure analysis, β/γ interactions are probably not affected.

Another gain-of-function mutant, *egl-30(tg26)*, was obtained in a screen for suppressors of *unc-31* (DOI and IWASAKI 2002). *tg26* encodes an R243Q change (DOI and IWASAKI 2002), and this mutation is contained within the switch III region of the α3 helix, indicating that R243Q probably alters guanine nucleotide binding (Figure 3). *tg26* is also semidominant (heterozygotes lay 23% of eggs at or before the four-cell stage; $N = 44$) and confers the most severe gain-of-function phenotypes compared with the two other mutants. Mutants moved with exaggerated body bends and laid eggs at an earlier stage of development than wild type did (Table 3). In addition, brood sizes were significantly lower than those in *egl-30(md186sy676)* mutants (77 ± 44 *vs.* 175 ± 32). Unlike the *egl-30(md186sy676)* mutant, but like *syIs36* that overexpresses wild-type *egl-30*, the pharyngeal pumping exhibited by these mutants would alternate from a very fast rate to no visibly evident pumping, possibly due to tonic contraction of these muscles.

In summary, haplo-insufficiency of the *egl-30* locus, the semidominance of the gain-of-function mutants, and the progressive phenotypes caused by heat-shock-induced overexpression of a gain-of-function transgene demonstrated a clear phenotypic readout when levels of *egl-30* expression or activity were altered. Given this result, if there is only one primary downstream effector for *egl-30*, a knockout or near knockout in a primary downstream effector should strongly suppress *egl-30* gain-of-function mutations.

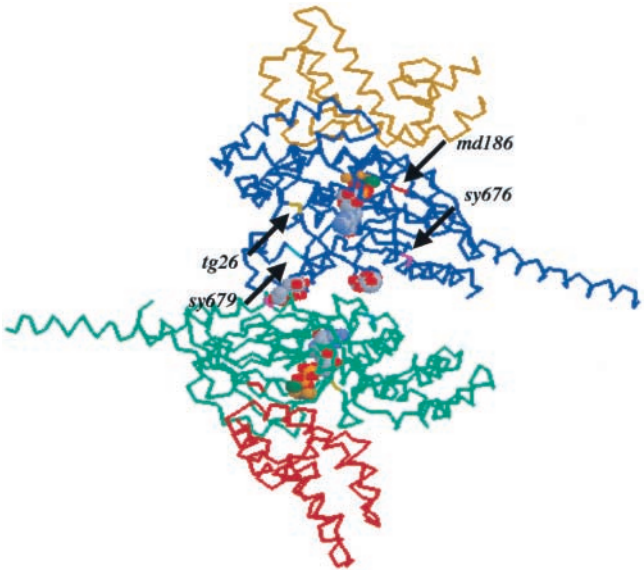

FIGURE 3.—Mutations in *egl-30* positioned on the crystal structure of rat $G\alpha_{i1}$ bound to GDP, AlF_4^-, and Mg^{2+} in a complex with RGS4 (TESMER *et al.* 1997). Blue and green chains show $G\alpha_{i1}$ α-, β-, and γ-subunits. RGS4 chains are shown in red and yellow. Two citrate ions are positioned within the $G\alpha_{i1}$ dimer interface.

The PLCβ-signaling pathway is not the predominant pathway downstream of EGL-30 with respect to egg laying: Mammalian $G\alpha_q$ family members are believed to principally activate isoforms of PLCβ (LEE *et al.* 1992). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into the signaling molecules diacylglycerol (DAG) and IP_3. DAG activates PKC while IP_3 activates release of calcium from intracellular stores via activation of the IP_3 receptor, a calcium channel. *egl-8* encodes the only recognizable PLCβ isoform in the worm and is most closely related to vertebrate PLCβ4. *egl-8* reduction-of-function mutants exhibit lethargic locomotion and a reduced rate of pharyngeal pumping, and they lay late-stage eggs (TRENT *et al.* 1983; MILLER *et al.* 1999; Tables 3 and 4), as do *egl-30* reduction-of-function mutants. However, a candidate null allele in *egl-8* (LACKNER *et al.* 1999) is not as severely impaired as *egl-30* reduction-of-function mutants with respect to egg laying and locomotion (Table 3 and data not shown). In addition, *egl-8* mutants are fully viable, unlike *egl-30* severe loss-of-function alleles (BRUNDAGE *et al.* 1996), suggesting that EGL-8 is not the only downstream effector for EGL-30.

Three alleles of *egl-8* have been previously described with respect to their molecular lesions. *egl-8(sa47)* encodes an early termination codon and is presumed to be a null allele (LACKNER *et al.* 1999). *egl-8(md1971)* encodes a premature termination codon 3′ of the C2 domain (although the mutation was predicted to be contained within the catalytic Y domain as reported; MILLER *et al.* 1999). *egl-8(n488)* deletes 1.8 kb of sequence including exons 10 and 11 (MILLER *et al.* 1999).

Unlike *egl-30* reduction-of-function mutants, *sa47* and *md1971* exhibit a strong induction of egg laying in response to 5-HT (serotonin; Table 2). On the basis of phenotypic similarities with *egl-8(sa47)*, *egl-8(md1971)* is likely a partial reduction-of-function allele (Table 2). Like *egl-30* reduction-of-function mutations, but unlike *sa47* and *md1971*, *egl-8(n488)* mutants are completely resistant to serotonin in egg-laying assays (Trent *et al.* 1983). *egl-8(n488)* is the canonical *egl-8* allele and has been the only allele previously tested for pharmacological response in egg-laying assays.

To investigate the interaction between *C. elegans* $G\alpha_q$ and PLCβ, we constructed strains defective in *egl-30* as well as in genes that encode components of the PLCβ-signaling pathway. Interactions were examined with the three alleles of *egl-8* described above: *egl-8(sa47)*, *egl-8 (md1971)*, and *egl-8(n488)* (Table 3). We also examined interactions with *itr-1*, *tpa-1*, and *unc-13* mutations. *itr-1* encodes the only IP_3 receptor homolog in the worm (Clandinin *et al.* 1998; Baylis *et al.* 1999; Dal Santo *et al.* 1999) and loss-of-function mutations were originally isolated because of their defecation defects (Iwasaki *et al.* 1995). *tpa-1* encodes a protein most similar to vertebrate PKCδ and was identified since mutations in the gene that encode TPA-1 confer resistance to TPA (Tabuse and Miwa 1983; Tabuse *et al.* 1989). UNC-13 is also implicated in a pathway downstream of DAG since the discovery that its mammalian ortholog, mUNC-13, is a receptor for DAG and enhances neurotransmitter release (Betz *et al.* 1998). Later, UNC-13 was found to regulate acetylcholine and GABA release from neuromuscular junctions in *C. elegans* (Lackner *et al.* 1999; Richmond *et al.* 1999) and to be essential for neurotransmitter release in *Drosophila melanogaster* (Aravamudan *et al.* 1999).

To analyze epistasis, we constructed all double mutants using *egl-30(md186sy676)* and confirmed selected interactions using *egl-30(tg26)*, because the *egl-30(md186 sy676)* strain does not exhibit the pale, scrawny, starved appearance of *egl-30(tg26)* and has a brood size comparable to wild type. *egl-30(gf)* mutants exhibited a constitutive egg-laying phenotype, defined in these assays as worms that lay eggs at or before the four-cell stage. *egl-8 (sa47)* and *egl-8(n488)* mutants exhibited an egg-laying-defective phenotype as single mutants; however, these mutations did not significantly suppress *egl-30(gf)* mutations with respect to egg-laying behavior (Table 3). Some level of mutual suppression appeared to be generally true with respect to movement since *egl-30(gf)*; *egl-8* double mutants still moved with exaggerated body bends, but activity levels appeared slightly reduced (data not shown). *egl-30(md186sy679)*; *egl-8(sa47)* double mutants did display a further reduction in egg-laying behavior, which is expected if these genes act in parallel. The apparent increase in egg-laying activity exhibited by *egl-30(md186); egl-8(sa47)* mutants, on the basis of hatched larvae observed on the plate, is not absolutely understood at this point. The most likely possibility is that the *egl-30(md186); egl-8(sa47)* larvae are more seriously impaired with respect to movement and are therefore not as competent to crawl out of the mother. These results indicate either that *egl-8* is a minor effector for *egl-30* or that *egl-8* functions completely in parallel with *egl-30* with respect to egg laying and possibly movement.

unc-13 single mutants displayed a severe egg-laying defect, and *unc-13(e51)* mutations did suppress constitutive egg laying conferred by *egl-30(gf)* mutants (Table 3). However, double mutants were not nearly as impaired with respect to egg laying as *unc-13* single mutants were. The observation that *egl-30(md186); unc-13* double mutants are apparently less defective in egg laying than either single mutant may reflect the fact that acetylcholine must exert both a positive and a negative regulatory role in egg laying; *cha-1* mutants lay early embryos and are hypersensitive to serotonin (Schafer *et al.* 1996).

C. elegans UNC-13 is known to affect evoked neurotransmitter release at both GABAergic and cholinergic synapses, but it has not been shown to affect serotonin release (Richmond *et al.* 1999). The *unc-13(e51)* allele appears to almost fully compromise the function of UNC-13, as assessed by measuring evoked responses in muscle by whole-cell patch clamp recordings in response to stimulation of the ventral nerve cord (Richmond *et al.* 1999). Acetylcholine is a known positive regulator of egg-laying behavior (Waggoner *et al.* 1998), and a role for EGL-30 in the regulation of the release of acetylcholine from motor neurons has previously been reported (Lackner *et al.* 1999). Our analysis of egg-laying behavior suggests either that the regulation of acetylcholine release is not the only neuronal pathway downstream of EGL-30 or that EGL-30 function in the vulval muscle can partially bypass the requirement for acetylcholine.

tpa-1 is the only PKC in the worm for which mutations have been identified (Tabuse *et al.* 1995). *tpa-1* encodes a protein with similarity to PKCγ. Loss-of-function *tpa-1* (*k501*) mutants lay eggs at significantly lower rates than wild type do, and mutants are resistant to the stimulation of egg laying by serotonin (Waggoner *et al.* 1998). TPA-1 acts in and is expressed in vulval muscle and in the nervous system (Waggoner *et al.* 2000). Although egg-laying defects are evident in single-worm tracking assays over long periods, the developmental stage of eggs laid by *tpa-1* mutants did not differ from wild-type worms (Table 3). Mutations also do not suppress *egl-30 (gf)* mutations with respect to the developmental stage of eggs laid (Table 3). We conclude from these results that *tpa-1* either is not downstream of *egl-30* or functions redundantly with another gene downstream of *egl-30*.

egl-30(md186sy676); *itr-1* double mutants also did not display significant differences with respect to egg laying compared with *egl-30(gf)* single mutants. *itr-1(sa73)* mutants have reduced brood sizes, but the stage of eggs laid by these worms is similar to wild type (Table 3).

itr-1(sa73) encodes a C1525Y change in the central modulatory domain (Dal Santo *et al.* 1999). We did not use a null allele for our analysis since null alleles exhibit a sterile phenotype (Dal Santo *et al.* 1999). These results indicate that *egl-30(gf)* can also bypass the requirement for *itr-1* with respect to the regulation of egg-laying behavior.

Mutations in the PLCβ gene and in genes encoding components described as downstream of PLCβ conferred a weak, but insignificant, suppression of the hyperactive egg laying conferred by *egl-30(md186sy676)* mutations. We conclude that this pathway either is a minor component downstream of EGL-30 or functions completely in parallel to EGL-30 with respect to egg laying.

PLCβ is likely a major component downstream of EGL-30 with respect to pharyngeal pumping behavior: On the basis of defects in pumping exhibited by *egl-30(rf)* mutants and of expression of *egl-30* in the pharyngeal muscle, EGL-30 may act in pharyngeal muscle to regulate pharyngeal pumping behavior (Brundage *et al.* 1996; Figure 1). Genetic interactions among *egl-30* mutations and *egl-8(sa47)*, *itr-1(sa73)*, and *unc-13(e51)* mutations were also examined with respect to pharyngeal pumping behavior (Table 4). In contrast to the insignificant effect of these mutations on the constitutive egg-laying behavior conferred by *egl-30(gf)* mutations, the interaction of these mutations on the hyperactive pumping exhibited by *egl-30(md186 sy676)* mutations is clear.

egl-30(md186sy676); egl-8(sa47) double mutants exhibited a pharyngeal-pumping rate that is similar to *egl-8 (sa47)* single mutants, suggesting that EGL-8 could be a primary effector for EGL-30. *unc-13(e51)* suppressed pharyngeal pumping to a similar extent as *egl-8(sa47)*, suggesting that these genes function in a common pathway. *itr-1(sa73)* exhibits the most severe pumping defect and accordingly exhibits the strongest suppression of *egl-30(md186sy676)*. The stronger effect of the *itr-1* mutation on pumping rate, compared with *egl-8* mutations, may reflect the fact that ITR-1 responds to other receptor pathways, such as the EGF receptor, LET-23 (Clandinin *et al.* 1998; Bui and Sternberg 2002). We also investigated the genetic interactions between *egl-30 (md186)* and *egl-8(sa47)* or *unc-13(e51)* and found that double mutants exhibited a roughly additive decrease in overall pharyngeal pumping rate compared with single mutants. If *egl-8(sa47)* completely lacks EGL-8 activity, and EGL-8 is the only direct effector for *egl-30*, then double mutants are expected to exhibit a phenotype not more severe than that of *egl-8* single mutants. Consequently, we believe that although EGL-8 could represent the primary effector for EGL-30 with respect to the regulation of pharyngeal pumping, it is likely that there are also other minor downstream effector molecules involved with the regulation of this behavior.

Analysis of *egl-8* alleles: *egl-8(sa47)* is believed to represent the null allele of *egl-8* since it encodes a Q85Term change in exon 2 (Lackner *et al.* 1999). However, the egg-laying defect exhibited by *egl-8(sa7)* mutants is not as severe as the defect exhibited by *egl-8(n488)* mutants (Table 3). Furthermore, *egl-8(sa47)* responds to the stimulation of egg-laying behavior by 5-HT and *egl-8(n488)* does not (Table 2). The weaker defects exhibited by *egl-8(sa47)* mutants might be explained by the presence of alternative downstream transcriptional initiation sites or by readthrough past the termination codon. In this case, *egl-8(n488)* mutants might better represent the null phenotype of *egl-8*. The endpoints of the deletion in *egl-8(n488)* (see Figure 4) predict exons 10 and 11 are deleted, thus disrupting the reading frame (Miller *et al.* 1999). Exons 10 and 11 correspond to a portion of the region between the catalytic X and Y domains, and it is possible that a protein of altered function is produced. To examine the molecular nature of the *egl-8* alleles, we performed Western analysis using the three mutant alleles of EGL-8 that were used in this study.

Consistent with the hypothesis that *egl-8(sa47)* represents a null allele, Western analysis using a carboxy-terminal antibody produced against residues 1041–1382 (Miller *et al.* 1999) indicated that the extracts obtained from the *egl-8(sa47)* mutants did not contain EGL-8 or contained undetectable amounts of the complete protein (Figure 5). The faint band present in *egl-8(sa47)* extracts that migrates slightly faster than the EGL-8 band is also present in *egl-8(n488)* extracts. It is also present, but partially obscured, in N2 and *unc-13* extracts. Furthermore, extracts from *egl-8(sa47)* did not contain any additional smaller bands compared with N2, *egl-8(n488)*, or *unc-13* mutant extracts, indicating that *egl-8(sa47)* mutants do not produce protein from an alternative downstream start site.

An extra band also was not present in *egl-8(n488)*, consistent with antibody staining of whole worms described previously (Miller *et al.* 1999). The differences in response of *egl-8(n488)* and *sa47* and *md1971* mutants to 5-HT in egg-laying assays suggested that the *egl-8 (n488)* allele is neomorphic, so we determined the molecular lesion of n488 and inferred the effect on the reading frame in *egl-8(n488)* mutants on the basis of deletion endpoints. We found that fusion of exons 9–12 would produce a termination codon at amino acid residue 681, just 44 amino acids after the last amino acid encoded in exon 9. Therefore, the hypothesis that *egl-8 (n488)* encodes the truncated protein of altered function, that it contains an X domain but not a Y domain, and that it is not recognized by the EGL-8 antibody to the carboxyl terminus is possible.

Like *egl-8* null mutants, *unc-13* mutants also exhibit a response to serotonin in egg-laying assays that is comparable to wild type (Table 2). This result lends support to a hypothesis that *egl-8* functions in a linear pathway with *unc-13* to regulate egg laying. Unlike loss of EGL-30-regulated response pathways, loss of EGL-8- or UNC-

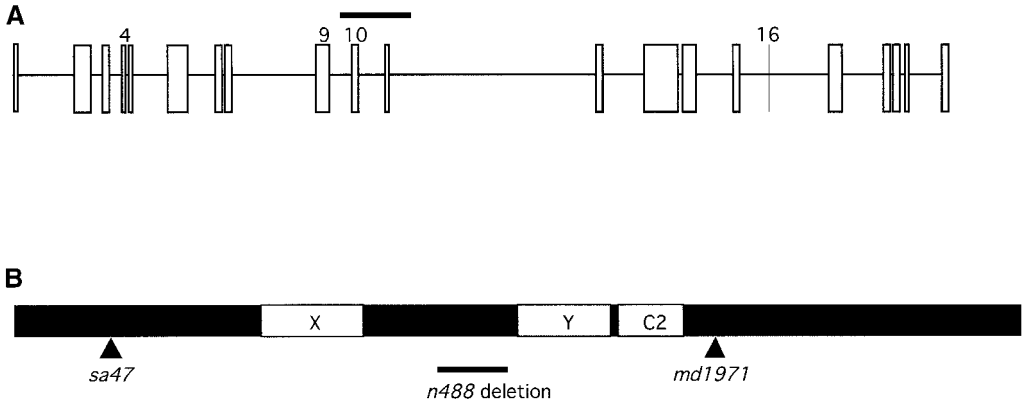

FIGURE 4.—Gene and protein domain structure of EGL-8. (A) Exon/intron structure, starting with the first ATG and ending with the stop codon within a coding region that extends >22,164 bp. Numbered exons are exons that have been shown to be alternatively spliced (MILLER *et al.* 1999). The solid line indicates the region deleted in *egl-8 (n488)*. The *egl-8(n488)* deletion extends from an intron 212 bp after exon 9 to an intron 434 bp after exon 11 (MILLER *et al.* 1999; and see MATERIALS AND METHODS). The catalytic X domain is contained within exons 6–8, and the catalytic Y domain is contained within exons 12–13. (B) Domain structure of EGL-8, encoded by 1431-amino-acid residues. The *egl-8(n488)* deletion extends between the X and Y catalytic domains, and the deleted region is indicated by the solid line. (▲) The position of mutations encoding early termination codons in *sa47* (LACKNER *et al.* 1999) and *md1971* (MILLER *et al.* 1999). The position of the *md1971* mutation is slightly different within the context of the protein domain structure from what was previously predicted.

13-mediated regulation of egg laying does not interfere with serotonin response. In fact, *cha-1* mutants also exhibit increased sensitivity to serotonin (SCHAFER *et al.* 1996), lending support to the notion that acetylcholine response pathways are directly coupled to *egl-8* and *unc-13* with respect to egg laying.

***egl-30* function in vulval muscle:** Our expression analysis revealed only rare expression of *egl-30* in the vulval muscles (evident in 10% of adult animals). We sought to determine whether the low expression levels reflected a true site-of-action for *egl-30* in the vulval muscles. Vulval muscle expression of *egl-8* has not been previously reported and to facilitate the interpretation of our double-mutant analysis, we wanted to determine whether EGL-8 might have a site-of-action in the vulval muscles. The egg-laying system of *C. elegans* consists of the vulval muscles with direct innervation from two classes of motor neurons, the VCs and the HSNs (WHITE *et al.* 1986; DESAI *et al.* 1988). Dominant mutations in *egl-1* cause the cells that would normally be destined to become the HSN motor neurons to undergo programmed cell death (ELLIS and HORVITZ 1986). In wild-type hermaphrodites, genetic ablation of the HSNs, via *egl-1* mutations, causes worms to lay eggs at a very late stage of development (TRENT *et al.* 1983; ELLIS and HORVITZ 1986; DESAI and HORVITZ 1989). Ablation of the VC4 and VC5 motor neurons alone does not have a drastic effect on egg-laying behavior (GARRIGA *et al.* 1993; WAGGONER *et al.* 1998). However, laser ablation of the VC4 and VC5 motor neurons of *egl-1* hermaphrodites during the L4 larval stage further compromises egg-laying behavior and animals do not respond to the neurotransmitter 5-HT (WAGGONER *et al.* 1998), normally a strong stimulator of egg-laying behavior (HORVITZ *et al.* 1982; TRENT *et al.* 1983). Egg laying can be rescued in *egl-1* animals that have ablated VC4 and VC5 motor neurons with the exogenous addition of both serotonin and levamisole (WAGGONER *et al.* 1998).

egl-30 reduction-of-function mutants exhibit a reduced response to serotonin in egg-laying assays (TRENT *et al.* 1983; BRUNDAGE *et al.* 1996). Like other *egl-30* reduction-of-function mutants, *egl-30(md186)* displayed a reduced response to serotonin (Table 2). On the basis of the neuron ablations described above, if nervous system but not muscle function is compromised in *egl-30* mutants, then reduction-of-function mutants should respond to serotonin and levamisole in egg-laying assays. In contrast to animals that lack the motor neurons that innervate the vulval muscle, egg laying was not restored in *egl-30(md186)* mutants or in the stronger reduction-of-function mutant, *egl-30(ad805)* (data not shown)

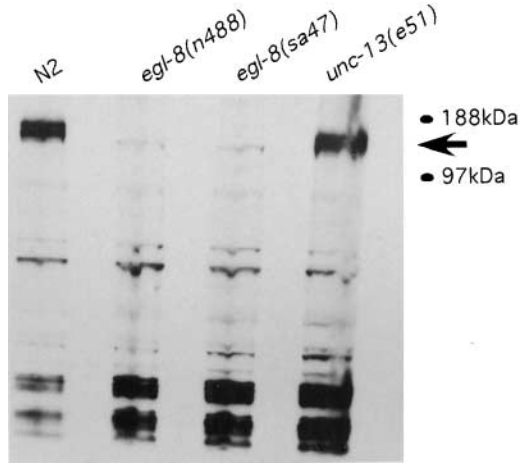

FIGURE 5.—Western analysis of *egl-8* mutant protein extracts. Arrow indicates the band present from N2 and *unc-13* mutant extracts but not from *egl-8(n488)* or *egl-8(sa47)* mutant extracts.

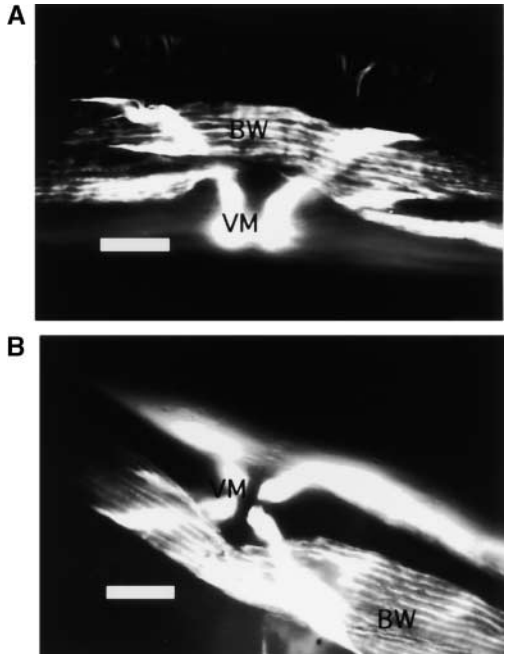

FIGURE 6.—Tetramethyl rhodamine isothiocyanate-phalloidin staining of N2 and *egl-30 (ad805)* adults to visualize actin filaments. Staining was performed as described in MATERIALS AND METHODS. BM, body-wall muscle; VM, vulval muscle. Bars, 10 μm. (A) The vulval muscle structure of a wild-type hermaphrodite, lateral view. (B) The vulval muscle structure of an *egl-30(ad805)* mutant, ventral view.

when treated with exogenous serotonin and levamisole (Table 2). Like *egl-30* mutants, egg laying was not rescued in *egl-8(n488)* mutants with serotonin and levamisole (Table 2), also indicating a previously unsuspected role for EGL-8 in vulval muscle (Table 2).

Two simple possibilities could explain the reduced response of *egl-30* and *egl-8* mutants to serotonin and levamisole. First, *egl-30* and *egl-8* might have a cell-autonomous site of action in the vulval muscle. Second, when function of the protein is reduced throughout development, muscle development or function might be compromised. However, the actin structure of *egl-30* mutants appears to be intact on the basis of phalloidin staining (Figure 6). Also, the contractile apparatus of the pharyngeal muscle in the putative null mutant, *egl-30(ad810)*, is still functional, on the basis of the ability of the pharyngeal muscle to contract in response to laser stimulation of the plasma membrane (BRUNDAGE *et al.* 1996). We conclude that the function of the muscle is more likely compromised with respect to the ability to transduce a signal.

***egl-30(md186sy676)* cannot bypass the requirement for serotonin with respect to egg laying:** Serotonin is the primary neurotransmitter that potentiates egg laying (DESAI and HORVITZ 1989) and *egl-30(rf)* mutants are resistant to the effects of serotonin in egg-laying assays (Table 2). Thus, *egl-30(rf)* mutants might be resistant to the effects of serotonin either because EGL-30 couples to a serotonin receptor or because EGL-30 is involved with response or release of serotonin from the motor neurons that innervate the vulval muscles. In mammals, α-methyl 5-HT stimulates 5-HT_2 receptor subtypes (RICHARDSON *et al.* 1985; ISMAIEL *et al.* 1990) that can couple to mammalian $G\alpha_q$ (SEUWEN *et al.* 1988; LAUNAY *et al.* 1996). On the basis of stimulation by α-methyl 5-HT, 5-HT_2 receptors have been shown to mediate tonic firing in pyramidal neurons of the rat prefrontal cortex (ZHANG 2003).

To investigate whether a receptor that exhibits a similar pharmacological response exists in *C. elegans*, we tested the response of worms to α-methyl 5-HT in egg-laying assays. We found that wild-type worms exhibited a robust response to α-methyl 5-HT (Table 5B). In contrast, *egl-30(md186)* mutants did not respond to this agonist (Table 5B). *egl-30(md186sy676)* mutants cannot be accurately tested since they immediately laid eggs when placed in water, and the activity of this agonist is inhibited in M9, similar to dopamine and dopamine antagonists (WEINSHENKER *et al.* 1995). However, *egl-30(md186 sy676); egl-1* double mutants exhibited a similar or higher response to α-methyl 5-HT compared with *egl-1* single mutants (Table 5B). We then compared the response to 5-HT and α-methyl 5-HT of *syIs36*, a strain that overexpresses wild-type *egl-30*. As shown in Table 5, A and C, *syIs36* exhibited similar responses to both α-methyl 5-HT and 5-HT as *egl-30(md186sy676)* mutants. These results suggest that EGL-30 mediates response to a 5-HT_2-type receptor to regulate egg laying. Finally, on the basis of the observation that a response was elicited from *egl-1* mutants, we conclude that an α-methyl 5-HT-responsive receptor functions either in a VC-dependent neuronal pathway or in the vulval muscle.

If, as we propose, serotonin is required for EGL-30-induced egg-laying behavior, then *egl-30(md186sy676)* mutants that lack endogenous serotonin should not exhibit constitutive egg laying. To further test this hypothesis, we analyzed epistasis between *egl-30(gf)* and *tph-1(lf)* mutants. *tph-1(mg280)* mutants lack detectable serotonin because of a defect in tryptophan hydroxylase, an enzyme involved in serotonin biosynthesis (SZE *et al.* 2000). *tph-1* mutants lay late-stage eggs (SZE *et al.* 2000), but *tph-1* mutants were not as defective in egg laying as *egl-1* mutants (Table 6). *egl-30(gf); tph-1* double mutants displayed an egg-laying defect that is similar to *tph-1* single mutants. This result suggests a direct linear pathway between serotonin response and EGL-30 function and indicates that *egl-30(md186sy676)* cannot bypass the requirement for serotonin with respect to egg-laying behavior. However, *tph-1* mutants were not as defective in egg laying as *egl-30* mutants (Tables 1 and 6B). Consistent with the partial suppression of *egl-30(md186sy676)* by *unc-13* (Table 3), acetylcholine may represent another minor regulatory pathway associated with *egl-30* (see DISCUSSION).

TABLE 5

Response of mutants to serotonin agonists

	5-HT	*N*	SD			
A. Response to 7.5 mM serotonin (5-HT)						
N2	5	23	2.7			
egl-30(md186sy676)	1.6	20	1.5			
egl-1	19.2	15	8.7			
egl-30(md186sy676); egl-1	12.5	14	5.9			
syls36	1.4	12	1.1			
	α-Methyl	*N*	SD	ddH_2O	*N*	SD
B. Response to α-methyl 5-HT, a 5-HT_2 receptor agonist						
N2	15	10	3.8	1.3	15	1.2
egl-30(md186)	1	8	2	NA	8	
egl-30(md186sy676)[a]	5.3	15	2.5	5.4	10	3.8
egl-1	15	8	4.2	1.1	8	2.4
egl-30(md186sy676); egl-1[a]	19.3	10	3.2	3.6	10	2.4
C. Response of *syls36* to α-methyl 5-HT						
N2	19.5[a]**	8	8.2	6.5	8	5.1
syls36	3.4[b]**	14	2.4	0.4	12	0.7

(A) Individual worms were assayed in M9 solution at room temperature. Eggs were counted after 90 min.

(B) Individual worms were assayed in a solution of 1 mM α-methyl 5-HT in water at room temperature. Eggs were counted after 1 hr.

(C) Single worms were assayed in a total volume of 200 μl. Eggs were counted after a 90-min incubation at room temperature. Approximately 2 out of 10 animals from each genotype exploded in water over this longer time period, and these animals were not included in the assay. Two-tailed *P* values (Mann-Whitney *U*-test nonpaired comparisons of experimental to water controls) are indicated for each value due to high responses in water: $*P = 0.008$, very significant; $**P < 0.0001$, extremely significant. NA, not applicable; this strain explodes in ddH_2O. SD, standard deviation per worm.

[a] Hermaphrodites laid more eggs than wild type in water. The response to *egl-30(md186sy676)* mutants is not significant, and this is due to its high response in water.

[b] Two of 10 animals exploded when placed in water.

The HSNs are the primary serotonergic motor neurons that regulate egg laying. Accordingly, *egl-1* mutants exhibit a severe egg-laying defect. However, *egl-1* single mutants exhibit a more severe egg-laying defect than do *egl-30(gf)*; *egl-1* double mutants (Table 6A), indicating that EGL-30 can partially bypass the requirement for the HSNs. This result is evident even with the stronger gain-of-function allele, *egl-30(tg26)*, that does not contain the *egl-30(md186)* mutation in the background. Epistasis with *tph-1*, but not with *egl-1*, is likely due to the fact that the VC motor neurons are also known to be weakly serotonergic (Duerr *et al.* 1999). We infer that *egl-30* does function in or via the HSN motor neurons. However, since EGL-30 can partially bypass the HSN neurons, a role for *egl-30* either in the VC motor neurons or in the muscle is indicated.

DISCUSSION

The expression pattern reported here indicates that *egl-30* is broadly expressed, consistent with its pleiotropic function. Similarly, vertebrate $G\alpha_q$ and $G\alpha_{11}$, two members of the $G\alpha_q$ family that share 88% sequence identity with one another, are coexpressed in almost every cell type (Wilkie *et al.* 1991). The other $G\alpha_q$ subunits are expressed in a more restricted pattern (Amatruda *et al.* 1991; Wilkie *et al.* 1991). Like *C. elegans* EGL-30, the vertebrate $G\alpha_q$ family is required for viability since targeted disruption of both $G\alpha_q$ and $G\alpha_{11}$ results in embryonic lethality at embryonic day 11 (Offermanns and Simon 1998).

The expression pattern of *egl-30* suggests that EGL-30 functions in many cell types and likely has a role throughout development. As larval development begins and progresses, we found that expression levels of *egl-30* generally increased with the highest expression in L4 larvae and adult animals. Its earliest role in development was previously presumed to be during larval development since mutations that should abort translation at Trp212 or Trp259 (*ad810* and *ad813*) arrest at various stages during larval development (Brundage *et al.* 1996). However, the rolling movement of embryos during embryonic elongation was found to be indistinguishable from wild type, and some of these animals were

TABLE 6

Interaction of *egl-30(gf)* mutations with *egl-1* mutations and with mutations in *tph-1*

A. Genotype	HSN?	2c–4c	5c-gastr.	Comma	2-fold	Pretzel	Hatched	*N*
N2	+		100					Many
egl-30(md186sy676)	+	52	48					199
egl-30(tg26)	+	91	9					38
egl-1	—			6	3	66	25	61
egl-30(md186sy676); egl-1	—		18	12	5	48	17	60
egl-30(tg26); egl-1	—		86	14				49

B. Genotype	2–4C	5c-gastr.	Comma	2-fold	Pretzel	*N*
egl-30(sy676)	52	48				199
tph-1; him-5		57	26		16	135
egl-30(md186sy676); tph-1; him-5		63	7	3	27	59

egl-1 mutations result in genetic ablation of the HSN motor neuron, the primary serotonergic motor neuron that regulates egg laying. *tph-1* mutations eliminate detectable levels of serotonin. 2c–4c, two- to four-cell stage. Egg-laying assays were performed as described in MATERIALS AND METHODS. 5c-gastr., five-cell stage embryo to gastrulating embryo. (A) Genetic ablation of the HSN motor neurons suppressed constitutive egg laying of *egl-30(gf)* mutations; *egl-30(gf)* mutations partially suppressed the Egl-D phenotype exhibited by *egl-1* mutants. (B) *tph-1* was epistatic to *egl-30(sy676)* with respect to egg-laying behavior.

also able to develop to semifertile adults (BRUNDAGE *et al.* 1996). We speculate that since embryos express only low levels of *egl-30*, low levels of translational readthrough (as indicated by the ability of some animals to develop into adults) or maternal rescue from heterozygous mothers is sufficient to rescue embryonic development. We also speculate that the general increase in expression levels of *egl-30* during larval development, revealed by the GFP fusion transgene, reflects a requirement for increased activity levels as animals develop. This hypothesis is supported by the larval arrest phenotype exhibited by *egl-30(ad810* and *ad813)* mutants.

Expression and site of action of *egl-30*: In L4 larvae and in adult animals, *egl-30* is expressed at highest levels in the nervous system and then in the pharyngeal muscle. In spite of the rare expression observed in the vulval muscle, the pharmacological response of *egl-30* mutants in egg-laying assays indicates that EGL-30 also functions in the vulval muscle. Antibody staining also revealed consistent expression in the anal sphincter muscle, which is consistent with a weak defect that *egl-30* reduction-of-function mutants exhibit with respect to defecation (MILLER *et al.* 1996). Rare expression was also observed in the head muscles and in body wall muscle, and the role for *egl-30* in these tissues remains to be explored.

Initial analysis regarding the site of action for EGL-30 indicated that EGL-30 has a general site of action in the muscle, *i.e.*, pharyngeal, vulval, or uterine, and in the body (BRUNDAGE *et al.* 1996). Specifically, ablation of the pharyngeal neurons, with the exception of the essential M4 neuron, suggests that *egl-30* functions in the muscle with respect to the suppression of *gpb-2*-induced arecoline hypersensitivity (BRUNDAGE *et al.* 1996). LACKNER *et al.* (1999) later found that overexpression of constitutively active EGL-30, EGL-30(QL), in the motor neurons of the ventral cord is sufficient to alter the locomotion of wild-type animals and results in enhanced sensitivity to the acetylcholinesterase inhibitor, aldicarb (LACKNER *et al.* 1999). On the basis of expression pattern, HSN ablations, and pharmacological analysis with *egl-30* mutants, we found that *egl-30* likely acts in or via neurons and in muscle during egg laying.

However, our unpublished experiments suggest that *C. elegans* is not tolerant to overproduction of *egl-30* in muscle cells, on the basis of our attempts using muscle-specific heterologous promoter elements such as *myo-3* (body wall), *myo-2* (pharyngeal muscle), and *hlh-8* (M-cell lineage specific; HARFE *et al.* 1998). We infer that these transgenes confer lethality on the basis of the inability to obtain stably inherited transgenic lines. On the basis of GFP reporter analysis, the *myo-2* enhancer confers very high levels of expression in the pharyngeal muscle. We conclude that, especially for this expression construct, it is difficult to titrate levels low enough to obtain viable stable lines. Accordingly, arrested eggs were often observed to express the *myo-2*::GFP expression construct that was coinjected with the *myo-2::egl-30* expression construct. A few worms that we examined exhibited slow pharyngeal pumping, which might have mimicked what would be expected from high levels of overexpression since *egl-30(tg26)* and *syIs36* exhibit irregular pumping (data not shown). A minimal artificial construct derived from the *ceh-24* promoter element that contained only a GAGA box and two NdE boxes is expected to confer

only vulval-muscle-specific expression (Harfe and Fire 1998). When we fused this element to the minimal *myo-2* promoter element to drive expression of *egl-30*, stable lines were obtained only at a concentration of 0.05 ng/μl and the lines obtained did not exhibit a phenotype and transmitted the array at a low frequency (see also Moghal *et al.* 2003). The low level of *egl-30* expression that we observed in muscle is consistent with the possibility that high levels of expression are lethal. It may also be consistent with the possibility that *egl-30* does not actually function in muscle cells, which would indicate that the reduced function of the nervous system in *egl-30* mutants affects the function of the vulval muscle in a non-cell-autonomous fashion.

EGL-30- and EGL-8-signaling pathways: EGL-8 is genetically downstream of EGL-30 with respect to acetylcholine release at neuromuscular junctions (Lackner *et al.* 1999). However, we believe that EGL-8 is not the only effector for EGL-30. Aside from the observation that *egl-30* and *egl-8* expression do not overlap in pharyngeal muscle (Miller *et al.* 1999), slight differences in the subcellular localization of EGL-30 and EGL-8 indicate that EGL-30 probably interacts with effectors other than EGL-8. Like EGL-30, EGL-8 is broadly expressed in the nervous system, with strong expression in neuronal cell bodies (Miller *et al.* 1999). In contrast, the strongest expression of EGL-30 was evident in neuronal cell processes (Figure 1).

Genetic interactions of *egl-30(gf)* mutations with mutations in *egl-8* and with the genes that act downstream of *egl-8* also support the conclusion that PLCβ is at most a minor component downstream of $G\alpha_q$ signaling in *C. elegans* with respect to egg-laying behavior. However, the genetic data do not eliminate the possibility that EGL-8 is the primary effector for EGL-30 in the nervous system, if *egl-30* functions in the vulval muscle and *egl-8* does not, since the final behavioral readout is ultimately dependent on muscle activity. We do not believe this for the following reasons. Pharmacological data indicate that both *egl-30* and *egl-8* might function in vulval muscle. More importantly, *C. elegans* are extremely sensitive to EGL-30 activity levels. If EGL-8 is a primary effector for EGL-30 in the nervous system, then loss-of-function mutations in *egl-8* would be expected to exert strong suppression of nervous system function. Double-mutant analysis of *egl-30(gf)* mutations with mutations in *egl-1* and with *tph-1* indicate that if *egl-30* functions in the vulval muscle, muscle function is not sufficient to confer constitutive egg laying.

Since *egl-8* mutations are clearly epistatic to *egl-30(gf)* mutations with respect to pharyngeal pumping, we do not believe that the gain-of-function EGL-30 mutant proteins promiscuously activate downstream effector molecules that do not normally interact with wild-type EGL-30. We also believe that we have demonstrated that the *egl-8(sa47)* allele represents a null allele, and so the weaker phenotype exhibited by *egl-8* null mutants compared to *egl-30* mutants accurately reflects the nonoverlapping roles of these two genes. Clear epistasis was observed with *tph-1* mutations with respect to egg laying. Since *tph-1* mutants exhibited an egg-laying defect that is similar to *egl-8(sa47)* mutants, and since *tph-1* mutants are less impaired with respect to egg laying than are *egl-8(n488)* mutants, these gain-of-function alleles do retain specificity with respect to their response pathways.

There are no other clear PLCβ homologs in *C. elegans*. The next most closely related predicted protein is Y75B12b.6 (Miller *et al.* 1999). Y75B12b.6 lacks a C2 domain on the basis of SMART and Pfam analysis of the predicted protein. The C2 domains of PLCβ1 and PLCβ2 have been shown to mediate an interaction with $G\alpha_q$ (Wang *et al.* 1999). Deletion analyses have revealed that the C-terminal ~400 amino acids of PLCβ1 (Park *et al.* 1993; Wu *et al.* 1993) and PLCβ2 (Lee *et al.* 1993) are critical for their interaction with $G\alpha_q$, but not critical for their interaction with βγ. Further studies have pinpointed specific amino acid residues of PLCβ1 critical for interaction with $G\alpha_q$ (Kim *et al.* 1996) and for dimerization of the PLCβ C-terminal domain (Singer *et al.* 2002). Dimerization has also been proposed to be important for regulation by $G\alpha_q$ (Singer *et al.* 2002). These regions contain a high level of similarity with other PLCβs, but the C-terminal region of Y75B12b.6 does not contain similarity within these critical determinants for interaction with $G\alpha_q$.

We conducted a BLAST search of the genome sequence available in WS84 using the C-terminal 400 amino acids of PLCβ1, PLCβ2, PLCβ3, and PLCβ4. On the basis of sequence alignments, the C-terminal 400 amino acids contain the regions in PLCβ1 and PLCβ2 that are required for interaction with $G\alpha_q$ and the corresponding regions in PLCβ3 and PLCβ4. Using a probability cutoff of 10^{-5}, none of the hits obtained, with the exception of EGL-8, were putative PLC homologs. In fact, some of the proteins with significant similarity were related to muscle component proteins. As shown in Figure 6, the actin structure of *egl-30(ad805)* mutants appears normal compared to wild type. We believe that it is likely that other effectors for EGL-30 interact with a domain that is distinct from the PLCβ interaction domain.

EGL-30 and serotonin signaling: While it is possible that the acetylcholine/EGL-8 pathway represents one of the minor pathways downstream of *egl-30* with respect to egg laying, it is likely that this pathway would act in parallel to modulate the more significant serotonin-responsive and *egl-8*-independent pathway downstream of *egl-30*. For example, *egl-8* and *unc-13* reduction-of-function mutants, implicated in acetylcholine release, are sensitive to serotonin, like *cha-1* mutants (Schafer *et al.* 1996) and unlike *egl-30* mutants. However, *egl-30* mutants are essentially resistant to the stimulation of egg laying by serotonin in egg-laying assays. Moreover, mutations in *tph-1* that result in a loss of detectable

levels of serotonin are epistatic to *egl-30* gain-of-function mutants with respect to egg laying. Furthermore, pharmacological data support the possibility that EGL-30 is coupled to a serotonin receptor. One such candidate receptor is SER-1, encoded by F59C12.2, which can mediate increases in intracellular calcium in COS-7 cells (Hamdan *et al.* 1999).

Our observations are consistent with the roles for serotonin and acetylcholine previously proposed in which serotonin is hypothesized to regulate the transformation from an inactive to an active state of egg laying, and acetylcholine is implicated in the regulation of individual egg-laying events within the active state (Waggoner *et al.* 1998). This hypothesis would explain the epistasis observed with *tph-1* mutations over *egl-30(gf)* mutations. If acetylcholine release represents a more minor response pathway associated with EGL-30 signaling that acts in parallel to serotonin, as suggested by *egl-30(gf); unc-13* double mutants, this would explain why *tph-1* mutants were not as defective as *egl-30* mutations with respect to egg laying. Our data implicate *egl-30* as a primary candidate for the regulator of the interaction between the acetylcholine and serotonin response pathways with respect to egg-laying behavior.

There are likely multiple effector pathways downstream of EGL-30 in *C. elegans*, and unique signaling pathways appear to dominate in different cell types. The PLCβ signaling pathway may predominate with respect to pharyngeal pumping, but does not predominate with respect to egg-laying behavior. The results of our suppressor screens are also consistent with this hypothesis. In screens in which we suppressed overproduction of a constitutively active allele of *egl-30* expressed under the control of the *hsp-16* heat-shock promoter element, we isolated only mutations that affected heat-shock-induced levels of the integrated transgene. We expect that this might reflect the fact that heat-shock induction of *egl-30* expression activates multiple downstream pathways in a variety of cell types and so cannot be suppressed by a single mutation. We also isolated extragenic suppressor mutations that suppress the egg-laying defects and lethargic locomotion exhibited by *egl-30(md186)* mutants (C. A. Bastiani, S. Gharib and P. W. Sternberg, unpublished results). We expect these mutations to define additional regulators/downstream components of $G\alpha_q$-mediated signaling in worms and to provide more insight into $G\alpha_q$-signaling pathways in vertebrates.

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