An activating mutation in a *Caenorhabditis elegans* G_s protein induces neural degeneration

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Heterotrimeric guanine nucleotide-binding proteins (G proteins) act as signal-transducing molecules that connect serpentine-transmembrane receptors to a variety of intracellular effectors. We characterized a *Caenorhabditis elegans* G_s gene, *gsa-1*, which encodes a G_s α-subunit (Gα_s) that is expressed throughout the nervous system and in muscle cells. *gsa-1* is an essential gene; a loss-of-function mutation in *gsa-1* results in lethality at the first stage of larval development. Partial (mosaic) loss of Gα_s expression or overexpression of the protein results in reciprocal defects in movement and egg-laying, suggesting a role for Gα_s in the regulation of these behaviors. Expression of a constitutively active form of Gα_s from an inducible promoter results in hypercontraction of body-wall muscle cells and vacuolization and degeneration of neurons within hours of induction. Neurons that are susceptible to the degeneration induced by activated Gα_s are predominantly motoneurons located within the ventral nerve cord. Phenotypic analysis shows that the induced neural degeneration is not the result of programmed cell death but is probably caused by the activation of ion channels. A genetic suppressor of activated Gα_s was isolated that identifies a putative downstream target of G_s signaling.

[Key Words: G protein; Gα_s; loss-of-function mutation; gain-of-function mutation; neural degeneration; suppressor mutation]

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Signaling pathways using serpentine-transmembrane receptors and their associated heterotrimeric guanine nucleotide-binding proteins (G proteins) have a key role in triggering physiological responses to a wide variety of hormones, neurotransmitters, and sensory stimuli (Simon et al. 1991; Hepler and Gilman 1992). Heterotrimeric G proteins consist of a guanine nucleotide-binding Gα subunit and a Gβγ subunit (Kaziro et al. 1991; Wall et al. 1995; Lambright et al. 1996). Both subunits have signaling capabilities and are released on activation of the G protein by a ligand-bound receptor (Clapham and Neer 1993). Multiple serpentine receptors, G protein subunits, and downstream effectors have been identified in vertebrates, demonstrating the complexity of G protein-coupled signal transduction. Despite the extensive data on the biochemical properties of these components, it is still poorly understood how information from different G protein-coupled signal transduction pathways is integrated and influences complex behavioral phenotypes. Insight into the complexity of G protein-coupled signal transduction can be gained from genetic studies in a rela-

tively simple organism like the nematode *Caenorhabditis elegans*.

Several G protein subunit genes have been identified in *C. elegans*. These include homologs of mammalian Gα subunits; *C. elegans* Gα_o and Gα_q are >80% identical to their mammalian counterparts (Lochrie et al. 1991; Brundage et al. 1996). Gα_o is expressed abundantly in the nervous system and is involved in modulating behaviors such as locomotion and egg-laying, implicating this pathway in serotonin signaling (Mendel et al. 1995; Ségalat et al. 1995). *egl-10*, a regulator of Gα_o, encodes a member of a novel family of GTPase-activating proteins (Koelle and Horvitz 1996). Locomotion and egg-laying are also modulated by Gα_q, a gene identified previously genetically as *egl-30* (Brundage et al. 1996). In addition to these highly conserved Gα subunits, two novel subunits were cloned as well (Lochrie et al. 1991; Fino Silva and Plasterk 1990); GPA-2 and GPA-3 are involved in chemosensation of a dauer pheromone (Zwaal et al. 1997). *C. elegans* expresses a highly conserved Gβ subunit (van der Voorn et al. 1990). Loss of *gpb-1* results in embryonic lethality (Zwaal et al. 1996).

We studied a homolog of Gα_s in *C. elegans*. *gsa-1* encodes a protein that is 66% identical at the amino acid level to *Drosophila* and mammalian Gα_s (Park et al.

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1997). In vertebrates, $G\alpha_s$ was one of the first G proteins to be identified (Northup et al. 1980), functioning in signaling of hormones such as β -adrenergic agonists and glucagon. $G\alpha_s$ shows different alternatively spliced isoforms and is expressed ubiquitously in mammalian tissues (Kazirot et al. 1991). $G\alpha_s$ stimulates adenylyl cyclase, resulting in the generation of cAMP (Sunahara et al. 1996). This second messenger can subsequently mediate a wide range of responses through activating cAMP-dependent protein kinase A (Walsh and Van Patten 1994) and by modulating cyclic nucleotide-gated ion channels. In addition, $G\alpha_s$ may also directly regulate ion channels. L-type voltage-gated Ca^{2+} channels in skeletal muscle cells are activated by $G\alpha_s$, whereas $G\alpha_s$ inhibits cardiac Na^+ channels (Wickman and Clapham 1995). Mutations in $G\alpha_s$ have been described in both vertebrates and invertebrates. In *Drosophila*, lesions in a $G\alpha_s$ -coupled pathway affect learning and memory (Connolly et al. 1996). In humans, mutations in $G\alpha_s$ are implicated in a number of diseases. Reduction of $G\alpha_s$ function was found in Albright hereditary osteodystrophy (Schwindinger et al. 1994), whereas constitutively activating mutations in $G\alpha_s$ were found in pituitary and thyroid malignancies (Landis et al. 1989; Lyons et al. 1990) and endocrine disorders (Shenker et al. 1993; Iiri et al. 1994). Despite the description of $G\alpha_s$ mutations in human disease, $G\alpha_s$ has not been studied systematically in a genetic system.

In this paper we describe the functional analysis of a *C. elegans* $G\alpha_s$ homolog. We show that *gsa-1::gfp* fusions are expressed in most neurons and muscle cells, that $G\alpha_s$ is essential for viability, that $G\alpha_s$ modulates behaviors such as locomotion and egg-laying, and that a constitutively active form of $G\alpha_s$ induces neuronal degeneration. The activated $G\alpha_s$ phenotype was used in further genetic analysis and an extragenic suppressor mutation identifying a putative downstream component of $G\alpha_s$ signaling was obtained.

Results

gsa-1 is expressed ubiquitously in neurons and muscle cells

Two fusions of *gsa-1* upstream control sequence with the gene encoding green fluorescent protein (GFP) (Chalfie et al. 1994) were used to analyze $G\alpha_s$ expression—a transcriptional fusion with *gfp* inserted at the 5'-untranslated region (UTR) of *gsa-1* and a translational fusion with *gfp* inserted at the fifth exon of *gsa-1* (Fig. 2A, below). Both reporter constructs showed similar expression patterns in the nervous system and in muscle cells. The transcriptional *gsa-1::gfp* fusion was expressed in the bilateral processes of the excretory cell (Fig. 1F) and at low levels in the intestine as well. Because different promoter and translational fusions resulted in similar expression patterns, it is likely that these patterns reflect the expression of the endogenous *gsa-1* gene. Animals transgenic for the *gsa-1::gfp* fusions showed reporter gene expression throughout development. *gsa-1*

was expressed extensively in embryos (data not shown). In the different larval stages and in adults, expression was restricted uniformly to neural and muscle cells. Virtually all neurons showed expression of *gsa-1*. These included neurons located in the head ganglia (Fig. 1A), the ventral nerve cord (Fig. 1B), and the tail ganglia (Fig. 1C). Also the hermaphrodite-specific neurons (HSNs), which control egg-laying and the canal cell-associated neurons (CANs) (Fig. 1E), showed expression of *gsa-1*. Most muscle cells expressed *gsa-1* as well. These included body-wall muscle cells used in locomotion (Fig. 1D). Body-wall muscle cells showed a punctate pattern of the translational *gsa-1::gfp* fusion, which may represent localization in dense bodies. Dense bodies function as attachment sites between the muscle cells and the cuticle and are flanked by membranes of the sarcoplasmic reticulum (Waterston 1988). In addition, *gsa-1* was expressed in the muscle cells of the pharynx (Fig. 1A) and in the uterine and vulva muscle cells that are used in egg-laying (Fig. 1B). In mammals, $G\alpha_s$ is expressed in all tissues (Kazirot et al. 1991). In *Drosophila*, however, the expression of $G\alpha_s$ is restricted to the nervous system and the eye (Quan et al. 1989; Wolfgang et al. 1991).

gsa-1 is essential for viability

We used a reverse-genetic approach to obtain a loss-of-function mutation of *gsa-1*. Using a transposon-based method (Zwaal et al. 1993), we isolated a deletion allele, *gsa-1(pk75)*, in which part of the *gsa-1*-coding sequence was removed. As is shown in Figure 2A, a deletion of genomic sequence between two 16-bp direct repeats (AAAAATGTGACGTCAG) in introns 6 and 7 removes 1828 bp of *gsa-1* sequence. Transposon-induced deletions occur frequently between such short direct repeats in the genomic sequence (Zwaal et al. 1993; Kurkulos et al. 1994). In addition to removing exon 7, splicing of exon 6 to exon 8 will result in a frameshift. Consequently, *pk75* removes about one-third of the *gsa-1*-coding sequence and is a probable null allele.

Animals homozygous for *pk75* arrest in larval development. At hatching, *pk75* homozygotes are morphologically normal but show little pharyngeal and body-wall muscle activity. In time, the internal tissues become shrunken (Fig. 2B, bottom) and the animals arrest at the first stage of larval development (L1), as indicated by the persistent presence of L1-specific alae. The lethal phenotype of *pk75* is identical to the zygotic null phenotype of the $G\beta$ subunit gene *gpb-1* (Zwaal et al. 1996) and resembles the rod-like larval lethal phenotype of Ras pathway mutants and *clr-1* (G. Garriga, pers. comm.). Animals heterozygous for *pk75* are wild type in development and behavior. The larval lethal phenotype of *pk75* was complemented with a transgene containing a wild-type *gsa-1* construct. Transgenes in the form of extra-chromosomal arrays are unstable at meiosis and mitosis (Stinchcomb et al. 1985; Mello and Fire 1995), resulting in mosaic expression of the transgene as it is lost in individual cells and lineages. Loss of the rescuing transgene in the germ line will result in broods that consist of

Figure 1. Expression of $G\alpha_s$ in *C. elegans*. The expression pattern of *gsa-1* was analyzed using GFP gene fusions. (A) Head region. Expression of *gsa-1* in the pharyngeal and body-wall muscle cells and in the nerve ring neuropil is shown. Note that this picture does not show the full extent of *gsa-1* expression in the head ganglia to better visualize the pharyngeal muscle cells. (B) Region surrounding the vulva. *gsa-1* is expressed in the ventral nerve cord and the vulva and uterine muscle cells. (C) Tail region. Expression of *gsa-1* in the ventral nerve cord and in the tail ganglia is shown. (D) Expression of a translational *gsa-1::GFP* fusion in body-wall muscle cells results in a punctate expression pattern that may represent localization in dense bodies. The fusion protein contains half of the $G\alpha_s$ amino-terminal sequence and may direct localization to these structures. (E) Expression of *gsa-1* in the CANs, the HSNs, and the posterior lateral ganglion (p). (F) Expression of *gsa-1* in one process of the H-shaped excretory cell (ec) is shown.

animals that lack functional *gsa-1* expression both in the form of the transgene and in the form of maternal inheritance of *gsa-1* mRNA or protein (Zwaal et al. 1996). Of rescued *pk75* transgenic animals, 1%–5% segregated broods that consisted only of arrested larvae. These are identical to the L1-arrested larvae segregated by *pk75* heterozygous animals. Consequently, the larval lethal phenotype is probably the result of an essential function of zygotic *gsa-1* during larval development and not the result of a limited supply of maternal *gsa-1* mRNA or protein that allows embryogenesis to be completed.

Gα_s modulates behaviors such as locomotion and egg-laying

In rescued *pk75* transgenic lines, transgene mosaicism resulted in the segregation of arrested larvae, and in viable animals that showed a variety of behavioral defects

resulting from the absence of $G\alpha_s$ in specific cells. Notably, mosaic animals defective in locomotion (Fig. 3A), egg-laying (Fig. 3B), and defecation were observed. Animals mosaic for $G\alpha_s$ expression moved sluggishly or were paralyzed and locomotion was reduced to 5.0 ± 1.2 (mean $\pm$ S.E.M.) body bends per minute, compared with 19.0 ± 1.4 body bends per minute in wild-type animals. Mosaic animals were also defective in egg-laying; the number of unlaidd eggs per animal was increased from 10 ± 0.4 in wild-type animals to 23 ± 1.4 . Furthermore, the stage of newly laid eggs was significantly increased as well (Table 1). Overexpression of wild-type $G\alpha_s$ resulted in opposite behavioral defects. Transgenic animals overexpressing $G\alpha_s$ ($G\alpha_s$ XS) showed an increase in locomotion to 31 ± 2.0 body bends per minute (Fig. 3A). Moreover, $G\alpha_s$ XS animals showed a decrease in egg content (4 ± 0.4) and a reduction in the stage of newly laid eggs, with only 4.2 ± 0.2 cells per egg in the 1–8 cells per

Figure 2. Gene structure and loss-of-function phenotype of *gsa-1*. (A) Structure of the *gsa-1* gene. Black boxes show coding sequence. The region deleted in *gsa-1(pk75)* is indicated. (S) *SalI*; (F) *FspI*; (H) *HindIII*. (B) Larval lethal phenotype of *gsa-1(pk75)*. (Top) A wild-type L1 larva; (bottom) an L1 larva homozygous for *pk75*. The phenotype of *pk75* is characterized by the shrunk appearance of internal tissues.

egg category (Table 1). These defects are indicative of overactivity in the egg-laying system (Mendel et al. 1995; Ségalat et al. 1995). Egg-laying is regulated by the serotonergic HSNs. The HSNs synapse with the vulva muscle cells and stimulate these muscles to contract, resulting in the expulsion of an egg from the uterus (Trent et al. 1983). To determine whether the hyperactive egg-laying induced by $G\alpha_s$ overexpression is dependent on the HSN neurons, the phenotype of $G\alpha_sXS$ was examined in an *egl-1* background (Table 1). Mutations in *egl-1* induce programmed cell death of the HSN neurons, resulting in an egg-laying defective phenotype (Trent et al. 1983). In the absence of HSN neurons, overexpression of $G\alpha_s$ still resulted in hyperactive egg-laying, suggesting that $G\alpha_s$ acts in the vulva muscle cells. This is supported by the observation that *gsa-1(pk75)* mosaic animals that presumably lack $G\alpha_s$ expression in the egg-laying system are resistant to exogenous serotonin (1.2 ± 0.3 eggs/1.5 hr for *gsa-1(pk75)* mosaic animals, compared with 10 ± 0.9 eggs/1.5 hr for wild type), suggesting a defect in the vulva muscle cells in the absence of $G\alpha_s$ expression. Taken together, these data indicate that $G\alpha_s$ is involved in modulating behaviors such as locomotion and egg-laying in *C. elegans* and that it, at least for egg-laying, does so by virtue of its action in muscle cells.

An activating mutation in gsa-1 induces neural degeneration

A constitutively active *gsa-1* mutant ($G\alpha_sQL$) was constructed by changing a glutamine (Q) at position 208 to a leucine (L), a mutation analogous to the Q227L mutation found in mammalian-activated $G\alpha_s$ (Graziano and Gilman 1989; Masters et al. 1989). This residue is part of the guanine nucleotide-binding pocket and mutation decreases the GTPase activity and locks the protein in the active GTP-bound conformation. Expression of $G\alpha_sQL$

from the endogenous *gsa-1* promoter resulted in early L1 larval lethality, with animals showing large vacuoles at positions of neurons or neuronal precursors in the head ganglia, in the ventral nerve cord, and in the tail (Fig. 4A). This pattern of swollen and lysed neurons could be phenocopied by expression of $G\alpha_sQL$ from a heat shock promoter, enabling the generation of stable transgenic lines. Expression of $G\alpha_sQL$ from a heat shock promoter allowed precise timing of the vacuolization of neurons. Vacuolization sufficient to be detected by a high-powered dissection microscope was observed within 4 hr of $G\alpha_sQL$ induction. Expression of $G\alpha_sQL$ at different stages in larval development and in adults resulted in hypercontraction of body-wall muscle cells and vacuolization and degeneration of ventral nerve cord motoneurons. On average, 29 ± 4 ventral nerve cord motoneurons located between the posterior end of the pharynx and anterior of the tail ganglia were found to degenerate in animals ($n = 17$) older than the L2 stage (Fig. 4B,C), about half of the motoneurons present in the ventral nerve cord (White et al. 1986). Using an *unc-4::lacZ* marker (Miller and Niemeyer 1995), it was established that motoneurons that are susceptible to the $G\alpha_sQL$ -induced degeneration are derived from the postembryonic lineage of P neuroblasts, which generates the VAn, VBn, VCn, ASn, and VDn classes of ventral nerve cord motoneurons (Sulston and Horvitz 1977; data not shown). In addition to lysis of ventral nerve cord neurons, also a subset of neurons in the head and tail ganglia degenerated on $G\alpha_sQL$ expression (Fig. 4C). In some animals, neurons or epithelial cells in the terminal bulb of the pharynx were found to degenerate as well (Fig. 4B). Therefore, despite the ubiquitous expression of $G\alpha_sQL$ in the nervous system, degeneration was limited to a specific subset of neurons, suggesting that the targets of $G\alpha_sQL$ are restricted spatially in their expression or in their degenerating capacity. As a result of ventral nerve cord motoneuron degeneration, animals showed severe

Figure 3. Gain- and loss-of-function mutations in $G\alpha_s$ affect locomotion and egg-laying. (A) Locomotion rate of transgenic animals overexpressing $G\alpha_s$ (GsXS) ($n = 20$), wild-type animals ($n = 20$), and animals mosaically expressing $G\alpha_s$ ($n = 10$). Overexpression of $G\alpha_s$ increases movement. (B) Egg-laying was assayed as the number of eggs contained within the uterus of animals overexpressing $G\alpha_s$, wild-type animals and animals mosaically expressing $G\alpha_s$ (in each case, $n = 25$). Overexpression of $G\alpha_s$ results in fewer eggs that are retained in the uterus. Note that mosaic animals were selected for a paralyzed or egg-laying-defective phenotype.

movement defects, ranging from twitching movement to completely uncoordinated movement.

Genetic analysis of the activated $G\alpha_s$ phenotype

The $G\alpha_s$ QL-induced neural degeneration is morphologically different from programmed cell death; cells that are subject to apoptosis show a refractile and condensed appearance when viewed with Nomarski optics (Sulston and Horvitz 1977). To investigate whether the $G\alpha_s$ QL-induced degeneration is also genetically distinct from programmed cell death, we investigated if an intact

apoptotic pathway is required for the $G\alpha_s$ QL-induced degeneration. The neurodegenerative phenotype of $G\alpha_s$ QL was analyzed in *ced-3* and *ced-4* mutant backgrounds (Table 2). CED-3 and CED-4 are essential, cell-autonomous components of the cell-death machinery and mutation of these components prevents death by apoptosis (Yuan and Horvitz 1990, 1992; Yuan et al. 1993). We found that the $G\alpha_s$ QL-induced neural degeneration was not affected by loss of the cell-death pathway, demonstrating that $G\alpha_s$ QL induces neural degeneration through a different mechanism. The $G\alpha_s$ QL-induced swelling and lysis of neurons is, however, similar to the vacuolar cell deaths observed in degenerin ion channel mutants. Dominant mutations in homologs of mammalian amiloride-sensitive epithelial Na^+ channels induce vacuolization and degeneration of a specific subset of neurons, probably as a result of an osmotic imbalance or abnormal influx of Ca^{2+} caused by aberrant functioning of these mutated channels (Hong and Driscoll 1994). Given the similarity in phenotype of $G\alpha_s$ QL and degenerin mutants, it is likely that $G\alpha_s$ QL acts by deregulating ion channels as well. We tested whether mutations in *C. elegans* ion-channel genes could suppress the $G\alpha_s$ QL-induced neural degeneration (Table 2). The $G\alpha_s$ QL phenotype was analyzed in a *mec-6* mutant background. MEC-6 is postulated to be an essential component of degenerin ion channels and loss-of-function mutations in *mec-6* suppress the degenerative phenotype of degenerin channel mutants (Chalfie and Wolinsky 1990; Huang and Chalfie 1994), including the motor neuron-specific degenerin *unc-8* (Shreffler et al. 1995). *mec-6* did not suppress the $G\alpha_s$ QL-induced neural degeneration, indicating that $G\alpha_s$ QL does not act specifically through degenerin ion channels. Similar results were obtained when $G\alpha_s$ QL activity was analyzed in combination with loss-of-function mutations in three different Ca^{2+} channel genes, *unc-2* (Schafer and Kenyon 1995), *egl-19*, and *unc-36* (L. Lobel, pers. comm.), suggesting that $G\alpha_s$ QL

Table 1. Gain- and loss-of-function mutations in $G\alpha_s$ affect egg-laying

Genotype	Developmental stage of newly laid eggs ^a			N
	1 to 8 cell	9-cell comma	post-comma	
Wild type	3	97	0	303
$G\alpha_s$ XS	53	47	0	149
<i>gsa-1(pk75)</i> mosaic ^b	0	1	99	156
<i>egl-1</i> ^c	0	3	97	75
<i>egl-1; G\alpha_s</i> XS ^c	20	65	15	69

^aAnimals were placed on separate *E. coli* OP50-seeded NGM agar plates, and based on the developmental stage, newly laid eggs were divided into three categories. Numbers indicate the percentage of laid eggs (N).

^bNote that mosaically rescued *gsa-1(pk75)* animals were selected for an egg-laying defective phenotype.

^cBoth *egl-1* and *egl-1; G\alpha_s*XS strains were in a *rol-6(su1006)* transgenic background.

Figure 4. Expression of activated $G\alpha_s$ induces neural degeneration. (A) Early L1 larva. Activated $G\alpha_s$ induces swelling and lysis (visible as large vacuoles) of neurons or neuronal precursors in the areas of the head ganglia, the ventral nerve cord and the tail. (B) Degenerated ventral nerve cord motoneurons in an L3 larva. The arrow indicates a pharyngeal neuron or epithelial cell that has degenerated. (C) Overview of neural degeneration induced by activated $G\alpha_s$. Vacuolized and degenerated neurons are mainly present in the ventral nerve cord. Also some neurons in the head and tail ganglia degenerate. Pictures were taken 24 hr after induction of activated $G\alpha_s$.

may act through an ion channel distinct from the channels tested in this study or, alternatively, through a combination of ion channels.

Another approach to identify downstream components acting in $G\alpha_s$ signaling is to isolate and clone extragenic mutations that suppress the $G\alpha_s$ QL phenotype. Using ethylmethane sulfonate (EMS) mutagenesis, we obtained several mutations that suppress the $G\alpha_s$ QL-induced neural degeneration and identify putative downstream genes functioning in $G\alpha_s$ -coupled signal transduction. These mutations were isolated at a frequency of ~ 1 in 5000 mutagenized genomes, were found to be recessive, and fell into one complementation group, *sgs-1* (suppressor of activated $G\alpha_s$). In addition, one other suppressor mutant was isolated that proved to be a complex locus. *sgs-1* suppressed completely the $G\alpha_s$ QL-induced neural degeneration [expressed ubiquitously from a heat-shock promoter or specifically from the *gsa-1* promoter],

but did not suppress the activated $G\alpha_s$ -induced body-wall muscle hypercontraction (Table 2) or the hyperactive egg-laying induced by wild-type $G\alpha_s$ overexpression (data not shown). In the absence of $G\alpha_s$ QL expression, *sgs-1* did not show an obvious developmental or behavioral phenotype. *sgs-1* was mapped to chromosome III, in an interval between *unc-32* and *vab-7*, ~ 1 map unit to the right of *unc-32* (see Materials and Methods).

Discussion

The strong evolutionary conservation of key components in signal transduction has allowed detailed genetic analysis of signal transduction pathways in model organisms like *Drosophila* and *C. elegans* (Sternberg 1993; Wassarman et al. 1995). The powerful genetic techniques available for these organisms have led to important insights in signal transduction networks and have in many

Table 2. Genetic interactions between activated $G\alpha_s$ and loss-of-function mutations in cell death and ion channel genes

Gene	Neural degeneration ^a	Body-wall muscle hypercontraction ^a	Reference
Cell death genes			
<i>ced-3</i>	+	N.D.	Yuan et al. (1993)
<i>ced-4</i>	+	N.D.	Yuan and Horvitz (1992)
Ion channel genes			
<i>mec-6</i>	+	+	Chalfie and Wolinsky (1990)
<i>unc-2</i>	+	+	Schafer and Kenyon (1995)
<i>egl-19</i>	+	+	L. Lobel (pers. comm.)
<i>unc-36</i>	+	±	L. Lobel (pers. comm.)
Suppressor			
<i>sgs-1</i>	–	+	this paper

Combinations of *gsa-1(pkIs296)* with *ced-3(n717)*, *ced-4(n1162)*, *mec-6(e1342)*, *unc-2(e55)*, *egl-19(n582)*, *unc-36(e251)*, *sgs-1(pk301)*, or *sgs-2(pk325)* were assayed for degeneration of neurons and body-wall muscle hypercontraction 12 hr after induction of activated $G\alpha_s$.

^a(+) *pkIs296*-induced neural degeneration and muscle hypercontraction; (±) incomplete suppression of *pkIs296* phenotype; (–) suppression of *pkIs296* phenotype.

cases complemented biochemical studies in mammals. We describe a genetic analysis of $G\alpha_s$ function in *C. elegans*. We show that $G\alpha_s$ is expressed ubiquitously in the nervous system and in muscle cells, that $G\alpha_s$ is essential for viability, and that $G\alpha_s$ modulates behaviors such as locomotion and egg-laying. Moreover, we show that an activating mutation in $G\alpha_s$ results in the degeneration of neurons.

Inactivation of *gsa-1* results in larval lethality. The predicted protein generated by *gsa-1(pk75)* is truncated at position arginine 246 in the amino-acid sequence (Park et al. 1997) and lacks a third of the $G\alpha_s$ carboxy-terminal sequence. The deleted region includes conserved sequences involved in interactions with adenylyl cyclase and receptors. Deletion of the carboxy-terminal part of $G\alpha_s$ probably affects other functions of the protein as well and it is likely that *pk75* is a null allele. Animals heterozygous for *pk75* are wild type. This suggests that there are no dominant neomorphic effects associated with expression of a truncated $G\alpha_s$ protein. Animals homozygous for *pk75* hatch normally, but arrest during the first stage of larval development. The internal tissues of mutant animals become progressively shrunken and they acquire a starved appearance. This phenotype is similar to the zygotic null phenotype of animals mutant for the $G\beta$ subunit gene *gpb-1* (Zwaal et al. 1996) and suggests that the larval lethal phenotype of *gsa-1* and *gpb-1* mutant animals results from the absence of an essential function of G_s -coupled signal transduction during larval development. The larval lethal phenotype of *gsa-1* is also similar to the rod-like larval lethal phenotype of *clr-1* and mutations in components of the Ras pathway. This lethal phenotype is associated with defects in the CANs, which have a role in osmoregulation (G. Garriga, pers. comm.). Cell-specific ablation of the CANs results in a rod-like larval lethal phenotype (J. Sulston, pers. comm.). Furthermore, it was shown for the epidermal growth factor (EGF) receptor homolog *let-23* that lethality in mosaic animals correlated with absence of functional *let-23* in the excretory system (Koga and Ohshima 1995). Consequently, because *gsa-1* is expressed in the CANs, $G\alpha_s$ may perform an essential function in these specialized cells as well.

Overexpression and mosaic rescue experiments revealed functions of $G\alpha_s$ later in development. Transgenic animals that overexpress $G\alpha_s$ showed an increase in locomotory activity and egg-laying, whereas animals that mosaically express $G\alpha_s$ showed a reduction in these behaviors. These phenotypes correlate with the expression pattern of $G\alpha_s$. *gsa-1::gfp* fusions are expressed in the ventral nerve cord motoneurons and body-wall muscle cells that control locomotion, and the HSNs and vulva and uterine muscle cells that control egg-laying. The locomotory and egg-laying phenotypes are associated with enhanced or reduced activities of the muscle cells that are required for these behaviors. In the egg-laying system, overexpression of $G\alpha_s$ stimulated egg-laying in the absence of the HSNs. This suggests that $G\alpha_s$ acts directly in the vulva muscles. This conclusion is supported by the observation that the egg-laying defect of *gsa-1(pk75)*

mosaic animals is not rescued by adding exogenous serotonin, the neurotransmitter that is released by the HSNs.

Two other strongly conserved *C. elegans* $G\alpha$ proteins also modulate locomotion and egg-laying—the $G\alpha_q$ homolog EGL-30 (Brundage et al. 1996) and $G\alpha_o$ (Mendel et al. 1995; Ségalat et al. 1995). Mutations in $G\alpha_q$ result in similar behavioral phenotypes as described for $G\alpha_s$, but mutations in $G\alpha_o$, a member of the inhibitory class of $G\alpha$ subunits, result in opposite phenotypes. Loss of $G\alpha_o$ results in hyperactivity in locomotion and egg-laying, whereas expression of a constitutively active $G\alpha_o$ allele induces paralysis and egg-laying defects. The significance of this convergence on a limited set of behavioral phenotypes of the three different $G\alpha$ subunits is not known. It is not clear whether the three G protein pathways modulate these behaviors specifically through the nervous system, through the muscle cells that control locomotion and egg-laying, or through both. Furthermore, mutations in the three different $G\alpha$ subunits could also modulate locomotion and egg-laying by changing the levels of $G\beta\gamma$ subunits. Overexpression of $G\beta$ results in phenotypes that are similar to $G\alpha_o$, but opposite to $G\alpha_s$ and $G\alpha_q$ (Zwaal et al. 1996).

Mutations that disrupt the GTPase activity of $G\alpha_s$ and lock the protein in the active GTP-bound conformation are found in human malignancies and endocrine disorders (Landis et al. 1989; Lyons et al. 1990; Shenker et al. 1993; Iiri et al. 1994). We constructed a similar mutation in *C. elegans* $G\alpha_s$ and found that expression of this activated form of the protein results in hypercontraction of body-wall muscle cells and the degeneration of a specific subset of neurons. Epistatic analysis showed that this degenerating capacity of $G\alpha_s^{QL}$ is not dependent on an intact apoptotic pathway. A similar neurodegenerative phenotype as induced by $G\alpha_s^{QL}$ has, however, been described for degenerin ion channel genes. Dominant mutations in *deg-1*, *mec-4*, and *mec-10* (Chalfie and Wolinsky 1990; Driscoll and Chalfie 1991; Huang and Chalfie 1994), which are homologs of mammalian epithelial Na^+ channels, result in the degeneration of a specific subset of touch receptor neurons, whereas mutations of *unc-8* result in degeneration of ventral nerve cord motoneurons (Shreffler et al. 1995). Also, rare dominant mutations in *deg-3*, a nicotinic acetylcholine receptor subunit gene, induce neural degeneration (Treinin and Chalfie 1995). These mutations presumably disturb the regulation of these channels, resulting in an ionic imbalance, vacuolization and degenerative cell death (Hong and Driscoll 1994). Dominant mutation of degenerin channels can also induce muscle hypercontraction; constitutive activation of the degenerin channel *unc-105* results in body-wall muscle hypercontraction (Liu et al. 1996). Given the similarity in phenotype, it is likely that $G\alpha_s^{QL}$ acts by deregulating ion channels as well. This can be a direct activation of ion channels by $G\alpha_s$, or can be indirect by deregulation of cAMP-gated ion channels or by deregulation of ion channels through cAMP-dependent protein kinase A (Walsh and Van Patten 1994; Wickman and Clapham 1995). Epistatic analysis with *C. elegans* ion-

channel genes demonstrated that $G\alpha_s$ QL does not act specifically through degenerin channels or Ca^{2+} channels like UNC-2, UNC-36, or EGL-19. It is, however, not excluded that activated $G\alpha_s$ acts through a combination of these channels. Screens for revertants of the activated $G\alpha_s$ phenotype resulted in a locus, *sgs-1*, that suppresses the neural degeneration phenotype of $G\alpha_s$ QL. Mutation of *sgs-1* does not, however, suppress the body-wall muscle hypercontraction induced by activated $G\alpha_s$. In addition, *sgs-1* does not suppress the hyperactive egg-laying in animals overexpressing $G\alpha_s$, which is mediated through the vulva muscles. Therefore, it is likely that $G\alpha_s$ acts in at least two roles, in neurons and in muscle cells, and that the action of *sgs-1* is restricted to the neuronal pathway.

Materials and Methods

Nematode strains and culturing

General methods for culture, manipulation, and genetics of *C. elegans* were as described (Lewis and Fleming 1995). Unless indicated, strains were cultured at 20°C. Strains used in this study were Bristol N2 and LGI: CB1472 [*mec-6(e1342)*], DR102 [*dpy-5(e61)unc-29(e193)*], KR236 [*dpy-5(e61)unc-13(e51);sDp2(l,f)*], KR1787 [*unc-13(e51)*], MT3126 [*mut-2(r459)*], NL511 [*gsa-1(pk27::Tc1)mut-2(r459)*], NL550 [*gsa-1(pk75);sDp2(l,f)*], LGII: CB3241 [*clr-1(e1745)*], LGIII: GE24 [*pha-1(e2123)*], GE182 5 [*tDf1/unc-32(e189)dpy-18(e499)*], MT2551 [*ced-4(n1162)dpy-17(e164)*], MT4306 [*lin-39(n1190)unc-36(e251)*]; LGIV: CB1362 [*dpy-20(e1362)*], MT1212 [*egl-19(n582)*], MT2405 [*ced-3(n717)unc-26(e205)*]; LGV: MT1082 [*egl-1(n487)*]; LGX: CB55 [*unc-2(e55)*].

GFP reporter constructs

A 3.8-kb *FspI* promoter fragment of *gsa-1* was introduced into the GFP modular vector pPD95.77 (A. Fire, pers. comm.), resulting in pRP1511. A translational fusion with GFP (pRP1518) was constructed by inserting a 2.0-kb *SalI-SpeI* fragment of pPD95.77 containing the GFP-coding sequence and the 3' UTR of *unc-54* downstream of a *SalI* site located within exon 5 of *gsa-1* in construct pRP1505 (see below). Each fusion DNA was introduced into *pha-1(e2123)* hermaphrodites together with *pha-1(+)* containing plasmid pC1 (Mello et al. 1991; Granato et al. 1994; Mello and Fire 1995) or in *dpy-20(e1362)* together with *dpy-20(+)* containing plasmid pMH86 (Han and Sternberg 1991). Each construct was injected at 100 ng/μl. Transformed animals were identified by survival at 25°C or a Dpy(+) phenotype. Multiple transgenic lines were obtained that showed identical expression patterns. Cells were identified in reference to Sulston and Horvitz (1977) and White (White et al. 1986).

Generation of a *gsa-1* loss of function mutation

A deletion mutation of *gsa-1* was isolated as described (Zwaal et al. 1993). A Tc1 insertion mutant *gsa-1::Tc1(pk27);mut-2(r459)*I was isolated using a library of Tc1 insertion mutants. This insertion, located at a TA dinucleotide in the seventh intron of *gsa-1*, did not produce a mutant phenotype. The primers 3831 (5'-GCAATGGACGAGATCGTGCC) and 1917 (5'-ACCCCTTCGGATGTTCTCTGTGTCGAC) and nested primers 3832 (5'-GGAAGATAACTACCCCTTCAC) and 1916 (5'-CGTAGTGTCGTAGATGAATGC) located in the third and eighth exons of

gsa-1, respectively, and spanning a genomic region of 3.9 kb, were used in a screen for deletion derivatives of *pk27::Tc1*. A 1.8-kb deletion allele, *gsa-1(pk75)*, was isolated (Fig. 2A). The PCR product detecting the deletion was directly sequenced using linear amplification sequencing (Craxton 1993). The *pk75* allele was backcrossed four times to an N2 background, including a double crossover using a *dpy-5(e61)unc-29(e193)*-marked chromosome I to ensure removal of the *mut-2(r459)*I locus. The *pk75* allele was balanced with the free duplication sDp2 (Howell et al. 1987) and the deletion was confirmed by Southern analysis (data not shown). The larval lethality of *pk75* homozygotes was confirmed by single worm PCR using primers spanning the deletion to detect *pk75* and primers inside the deleted region to detect the wild-type allele as described (Zwaal et al. 1993). Arrested larvae showed only the *pk75* deletion allele; viable progeny of *pk75* heterozygotes were either heterozygous for *pk75* or homozygous for the *gsa-1* wild-type allele (data not shown). The larval-lethal phenotype of *pk75* was complemented with a wild-type *gsa-1* genomic construct, pRP1505, which contained a 13-kb *HindIII* fragment including the *gsa-1*-coding sequence (Park et al., 1997) and the 3' UTR and polyadenylation signal of *unc-54*. The rescuing construct was injected at a concentration of 5 ng/μl together with 150 ng/μl of the marker plasmid pRF4 (Kramer et al. 1990). Rescue was scored as the generation of obligatory Rol lines that segregated viable Rol animals and arrested larvae, resulting in strain NL542 [*gsa-1(pk75);pkEx270[gsa-1(+)]rol-6(su1006)*]. Homozygosity for the *pk75* allele was confirmed by single worm PCR.

Behavioral analysis of mosaic and $G\alpha_s$ overexpression phenotypes

Adult animals of the rescued *gsa-1(pk75)* transgenic line NL542 showing defects in movement or egg-laying (<1% of the population) were selected for locomotion and egg-laying assays as described (Zwaal et al. 1996). Locomotion was assayed by placing animals on *Escherichia coli* OP50-seeded NGM agar plates 30 min before measuring locomotion by counting body bends in 3-min intervals. A body bend was defined as movement of one-quarter of a body length in a forward or backward direction (Brundage et al. 1996; Koelle and Horvitz 1996), a definition that could also be applied in a Rol background. Egg-laying was assayed by placing adult animals on OP50-seeded NGM agar plates and counting eggs laid in a 2.5-hr interval. The number of eggs present in the uterus and the stage of newly laid eggs were analyzed by counting eggs or cells using a high power dissection microscope (Wild M3C). Data were analyzed for statistical significance using an unpaired t-test (SPSS v. 7.0, SPSS, Inc., Chicago, IL) and were stated as mean ± S.E.M.. Assays for egg-laying in serotonin (5 mg/ml) were as described (Trent et al. 1983). NL556 [*dpy-20(e1362);pkEx331[gsa-1(+)]dpy-20(+)*], a transgenic line overexpressing $G\alpha_s$, was used in behavioral assays as described above. NL556 was generated by injecting a mixture of pRP1505, *dpy-20(+)* containing plasmid pMH86 (Han and Sternberg 1991) and pGEM5 carrier DNA (all at a concentration of 50 ng/μl) in *dpy-20(e1362)* animals (Mello et al. 1991; Mello and Fire 1995). NL592 [*egl-1(n487);pkEx343[gsa-1(+)]rol-6(su1006)*], a transgenic line overexpressing $G\alpha_s$ in an *egl-1* mutant background, was made by injecting pRP1505 at 50 μg/ml and pRF4 (Kramer et al. 1990) at 100 μg/ml in *egl-1* animals. About half of the NL592 animals showed reversion of the *egl-1* egg-laying defect and were used in egg-laying assays as described above. NL594 [*egl-1(n487);pkEx343[rol-6(su1006)]*] served as a control.

Construction of a constitutively active *gsa-1* mutant

A substitution of glutamine (Q) 208 to a leucine (L) that results

in constitutive activation of G_{α_s} (Graziano and Gilman 1989; Masters et al. 1989) was constructed by PCR using primer GsQL (5'-ATGTTTGACGTCGGAGGTCTACGTGAC) containing the mutation. The region of the PCR fragment used for further cloning was sequenced completely and found to be free of errors. This fragment was subcloned in a 2.8-kb *SalI* *gsa-1* genomic fragment, which was cloned into pRP1505, giving rise to pRP1510. A construct with *gsa-1* expression under the control of a heat-shock promotor (hsp) was generated by cloning a 0.25-kb *HindIII/BamHI* hsp16.41-containing fragment of pPC16.41-51 (Stringham et al. 1992) in front of a *FspI-HindIII* fragment of *gsa-1*. Addition of an *unc-54* 3' UTR and polyadenylation signal resulted in pRP1506. Exchange of a Q208L-containing *SmaI-HindIII* fragment between pRP1510 and pRP1506 resulted in pRP1509. Injection of pRP1510 at 10 ng/ μ l did not result in transgenic lines, presumably attributable to lethality of the transformants. pRP1509 was injected at 5 ng/ μ l, together with 20 ng/ μ l of pMH86 (Han and Sternberg 1991) and 100 ng/ μ l of pGEM5 carrier DNA into *dpy-20(e1362)*, resulting in NL512 (*dpy-20(e1362);pkEx332[gsa-1(Q208L)dpy-20(+)]*). The transgenic array was integrated by irradiating NL512 animals with 40 Gy of γ radiation from a ^{137}Cs source (Way et al. 1991), resulting in NL520 (*dpy-20(e1362);pkIs296[gsa-1(Q208L)dpy-20(+)]X*). *pkIs296* was outcrossed twice, resulting in strain NL545. Optimal heat-shock conditions were 2 hr at 33°C (Stringham et al. 1992). The phenotype of *pkIs296* was completely penetrant in L1 and L2 larvae, but less so at later stages and in adults. This is presumably the result of incomplete hsp-induced expression in late stage larvae and in adults (Stringham et al. 1992).

Genetic analysis of the activated G_{α_s} phenotype

Double mutant strains of *pkIs296* with *ced-3* or *ced-4* were made using *ced-3(n717)unc-26(e205)-* and *ced-4(n1162)dpy-17(e164)*-marked strains, respectively. A double mutant strain of *mec-6(e1342)* and *pkIs296* was constructed using *unc-13(e51)* and *dpy-5(e61)unc-29(e193)* as markers in *trans*. Double mutant strains of *pkIs296* with *unc-2(e55)*, *unc-36(e251)*, and *egl-19(n582)* were made using the visible phenotype of these mutations in crosses. Homozygosity for *pkIs296* was determined by heat shock or single-worm PCR using primers 994 (5'-GCATTCATCTACGACACTACG) and UNC54B (5'-CTC-TCTAAGCTTCGCCGACTAGTAGGAAAAG) as described (Zwaal et al. 1993).

Revertants of the *pkIs296* phenotype were isolated after EMS treatment as described (Anderson 1995). Briefly, L4 NL545 animals were incubated in M9 buffer containing 50 mM EMS for 4 hr, washed and seeded at 20–40 animals per plate on 9-cm NGM agar plates. The F_2 generation was synchronized using NaOCl bleaching and L1 larvae were heat-shocked at 33°C to induce expression of *pkIs296*. Animals that did not arrest in L1 and did not show vacuolized neurons were collected. Four alleles were isolated that suppressed the neural degeneration phenotype of *pkIs296*. These fell into one complementation group: *sgs-1(pk301, pk310, pk311, pk327)*. *sgs-1* also suppressed the neural degeneration induced by pRP1510. *sgs-1* was found to be recessive and was mapped to linkage group III. *sgs-1* was further localized using a three-factor cross with an *unc-32(e189)dpy-18(e346)*- and an *unc-32(e189)vab-7(e1562)*-marked chromosome and was mapped to a position ~1 map unit to the right of *unc-32* (5/34 were SgsUnc, 25/25 were SgsDpy, and 1/8 was SgsUnc, 10/10 were SgsVab).

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