Analysis of diverse signal transduction pathways using the genetic model system *Caenorhabditis elegans*

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Analyse van verscheidene signaal transductie paden met behulp van het genetische model systeem *Caenorhabditis elegans*
(met een samenvatting in het Nederlands)

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In herinnering aan mijn vader.

Chapter 1

Introduction

Introduction

In a multicellular organism cells need to communicate with their environment and with one another for many aspects of life. The mechanism by which cells communicate is signal transduction. Defects in signal transducing molecules cause many diseases, including cancer, and signal transduction has therefore been one of the most studied biological processes over the past decades. The genetic model organism *Caenorhabditis elegans* shares many proteins involved in signal transduction with vertebrates and is thus very well suited to study signal transduction mechanisms in general.

The nematode *Caenorhabditis elegans* as a genetic model system

C. elegans is a simple and well-described organism

C. elegans is a small soil nematode that was chosen as a model organism to study animal development and behaviour (BRENNER 1973). It has great potential for genetic analysis for many reasons (reviewed in (BARGMANN and HODGKIN 2002; RIDDLE *et al.* 1997; WOOD 1988)). One of its most significant advantages is its simplicity, both in anatomy and in genomic organization. In addition, *C. elegans* has a short life cycle of 3 days, is only 1 mm long as an adult and is transparent throughout the life cycle, so that development can be easily followed. Large populations can be grown on agar plates or in liquid using *Escherichia coli* as the food source and strains can be kept as frozen stocks. Furthermore, the predominant sex in a *C. elegans* population is the self-fertilizing hermaphrodite, but males, which arise spontaneously at low frequency, exist as well and allow cross-fertilization. Finally, it is extremely easy to make and identify mutants using for instance chemical mutagens.

In the past, several studies have been performed that are invaluable for *C. elegans* geneticists nowadays. Studies on the cell lineage of *C. elegans* revealed that the pattern of cell divisions is essentially invariant among individuals (SULSTON and HORVITZ 1977). The total description of the invariant cell lineage from the single cell of the fertilized egg to the fully differentiated cells of the adult was completed in 1983 (SULSTON *et al.* 1983). The adult hermaphrodite has 959 somatic nuclei and fewer than two thousand germ cell nuclei. Adult males contain 1031 somatic nuclei.

The anatomy of *C. elegans* is also invariant among wild type individuals and has been completely reconstructed by electron microscopy of serial sections (reviewed in (WHITE 1988)). Muscle cells, nerves, epithelia, intestinal cells and other cell types were identified and revealed that the body plan is essentially the same as that of higher animals. The nervous system of *C. elegans* consists of only 302 cells in the hermaphrodite and 381 cells in the male. For all hermaphrodite neurons, the anatomy and connectivity has been elucidated (WHITE *et al.* 1986). In addition, the number and positions of chemical synapses (5000), gap junctions (600) and neuromuscular junctions (2000) that are made by these neurons were described. The study revealed that the *C. elegans* nervous system is slightly different from the nervous system of vertebrates: the synapses are simple and do not have complex postsynaptic specializations. However, despite its simple nervous system, the nematode has genes for most of the known molecular components of vertebrate brains, suggesting that the *C. elegans* nervous system provides a valid minimal model for that in animals (BARGMANN 1998).

C. elegans is the first multicellular organism for which the complete genomic sequence was reported (*C. ELEGANS* SEQUENCING CONSORTIUM 1998). *C.elegans* has a small genome, with a haploid DNA content of about 97 million base pairs, which is approximately one-thirtieth of the number in the human genome (LANDER *et al.* 2001; VENTER *et al.* 2001). It reveals more than 19,000 genes and over 40 percent of the predicted proteins have significant homology with proteins in other organisms (*C. ELEGANS* SEQUENCING CONSORTIUM

1998). The sequencing of the *C. elegans* genome was based on a clone-based physical map (*C. ELEGANS* SEQUENCING CONSORTIUM 1998; WATERSTON and SULSTON 1995). This physical map consists mainly of cosmids and YACs and has also been very useful to facilitate the positional cloning of chosen loci. Besides the genomic sequencing, sequencing of expressed sequence tags (EST) has been carried out, which was helpful in identifying and annotating genes in the genomic sequence (*C. ELEGANS* SEQUENCING CONSORTIUM 1998). The completion of the genome sequence made it possible to construct a gene expression map for all genes in *C. elegans* (KIM *et al.* 2001). This gene expression map is based on micro-array data and visualizes co-regulated genes. It can thus be used to identify genes that are regulated in a similar manner to a gene or group of genes of interest.

Although the advantages of *C. elegans* are numerous, it also has drawbacks. Cell-specific molecular studies are difficult to perform, because most somatic cells are small and because the cuticle around the animal hinders access to these cells. For instance, electrophysiological studies in *C. elegans* are technically extremely difficult. Patch clamp methods have been developed to study ion channel activity in a few neurons and body-wall muscles (GOODMAN *et al.* 1998; LOCKERY and GOODMAN 1998; PIERCE-SHIMOMURA *et al.* 2001; RICHMOND *et al.* 1999), but are not applicable to all muscles and neurons yet. In addition, it is not possible to isolate sufficient amounts of protein from a certain tissue to perform biochemical analyses. Another disadvantage is that, despite many attempts, no stable *C. elegans* cell lines exist. Recently, a primary culture system that allows culture of *C. elegans* embryonic cells has been described (CHRISTENSEN *et al.* 2002). These embryonic cells differentiate into the various cell types that comprise the newly hatched larva. The majority of cells differentiates into neuron or muscle cells, and these cells can be used for electrophysiological studies (CHRISTENSEN *et al.* 2002). However, analysis on other cell types remains difficult.

Forward genetics

One of the main advantages of *C. elegans* is that it is well suited to perform forward genetics: mutants with interesting phenotypes can be easily collected and subsequently the mutated gene can be identified by positional cloning. The spontaneous mutation rate is too low to be useful, but a variety of mutagenic agents (such as ethyl methanesulfate (EMS)) and treatments (such as ionizing radiation) can be used to obtain the desired mutations (reviewed in (ANDERSON 1995; JOHNSEN and BAILLIE 1997)). In addition, mutations can be obtained by insertional mutagenesis using transposons (GREENWALD 1985; MOERMAN *et al.* 1986). The wild type Bristol N2 strain cannot be used for transposon insertional mutagenesis, because transposons are not active in the germ line of these animals. Some strains, so-called "mutator" strains, do show germ line transposition, and it is these that are used for transposon insertional mutagenesis. The advantage of this method is that it is relatively simple to clone the causal insertion using a PCR-based method (WICKS *et al.* 2000).

Simple forward genetic screens

The first forward genetic screen performed in *C. elegans* was a simple F2 screen (BRENNER 1974). In this screen, a population of wild type hermaphrodites was exposed to a mutagen, resulting in randomly mutated genes in the germ cells. The mutagenized worms were grown for two generations to produce homozygous mutants (Figure 1A). In total 619 mutants with a visible phenotype were identified in this way (BRENNER 1974). Over two-thirds of the found mutants showed uncoordinated movement, indicating that the genes mutated in these uncoordinated (unc) animals are potentially required for the proper development or function of the nervous system. Indeed, cloning and molecular analysis of several of the *unc* genes revealed factors important for neuronal differentiation, axon guidance and outgrowth, for neurotransmitter synthesis and storage and for signal transduction (reviewed in (BARGMANN

1998; CHALFIE and JORGENSEN 1998)). Besides the *unc* mutants, Brenner isolated mutants with long, dumpy or small bodies, with blistered cuticle, twitching muscles or rolling locomotion and with forked or bent heads (BRENNER 1974).

After this first forward genetic screen, innumerable "simple" screens have been performed to find genes that function in a process of interest. For instance, to gain insight into the process of vulva differentiation, screens have been performed to identify mutants that either lack a vulva or have ectopic vulvae (FERGUSON and HORVITZ 1985; SULSTON and HORVITZ 1981). Subsequent molecular analyses of these mutants revealed that LET-60, which is a homologue of the well-known human oncogene RAS, is a key player in vulva differentiation (HAN and STERNBERG 1990; WANG and STERNBERG 2001). Besides mutants with clear visible phenotypes, more subtle mutants have been isolated as well that are affected in processes like apoptosis, lifespan, chemosensation or thermotaxis (BARGMANN and MORI 1997; ELLIS and HORVITZ 1986; KENYON 1997).

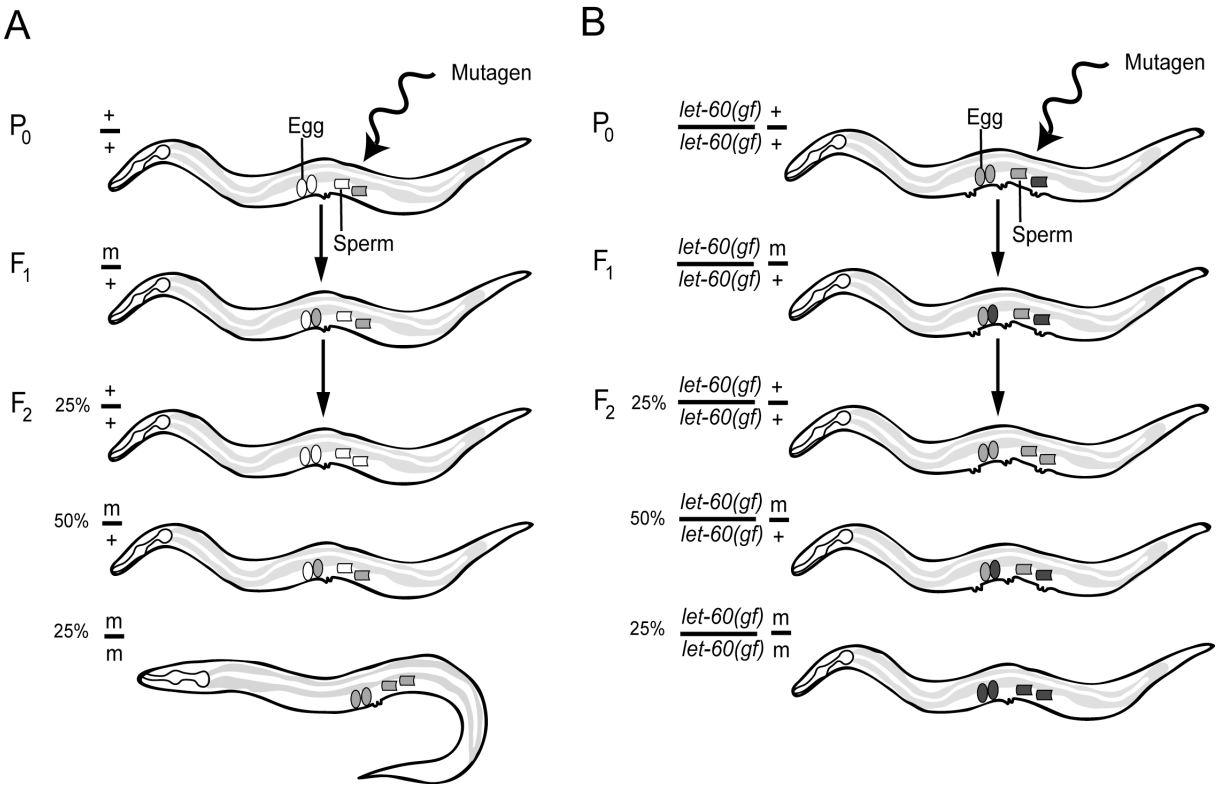

Figure 1. Forward genetic screens. (A) A simple F2 screen to identify uncoordinated animals. Wild type hermaphrodites (P_0) are exposed to a mutagen. Consequently, genes are randomly mutated in the germ cells. A certain sperm is mutated (indicated in grey), in this case in an *unc* gene. Fertilization of an oocyte by this mutated sperm will result in a heterozygous F1 hermaphrodite. One-quarter of its progeny will be homozygous for the mutation and have an uncoordinated phenotype. Progeny of this uncoordinated animal should all have an uncoordinated phenotype, as can be examined by transfer to a new plate. Adapted from (JORGENSEN and MANGO 2002). (B) A suppressor screen to identify downstream targets of LET-60. In this screen, hermaphrodites that have a multivulva phenotype caused by a dominant mutation in *let-60* (*let-60(gf)*; germ cells are indicated in grey) are used for mutagenesis. A sperm that obtains a second mutation in a suppressor gene (dark grey) will eventually result in animals with a wild type phenotype (i.e. one vulva) in the F2 generation.

Complicated forward genetics screens

Not all genes show a visible phenotype in the F2 generation when mutated. Therefore, various stategies have been used to increase the number of genes with a phenotype (reviewed in (HODGKIN and HERMAN 1998; JORGENSEN and MANGO 2002)). Maternal-effect lethal screens have been performed to identify genes that are essential for the earliest divisions of an embryo. The earliest divisions are performed by maternally supplied mRNA and protein, and animals mutated in these genes are therefore viable when derived from a heterozygous mother, but embryonic lethal when born from a homozygous mother (reviewed in (KEMPHUES and STROME 1997; SCHNABEL and PRIESS 1997)). The *par* genes, which are essential for embryonic asymmetry, were identified in this way (KEMPHUES *et al.* 1988). In addition to the maternal-effect lethal screens, maternal-effect viable screens were done to identify genes with a non-essential function relatively early in development (HEKIMI *et al.* 1995). Examples of such genes are the *clk* genes, which are involved in the temporal control of development and behaviour, including life span (LAKOWSKI and HEKIMI 1996). *clk-1* encodes for instance a highly conserved mitochondrial protein involved in ubiquinone biosynthesis, providing a link between the production of ubiquinone and aging (FELKAI *et al.* 1999; VAJO *et al.* 1999). To identify genes that are required for the immortality of the germ line, a screen was performed to isolate strains that become sterile after a couple of generations (AHMED and HODGKIN 2000). This screen identified a DNA-damage checkpoint protein, *mrt-2*, that is required to prevent telomere shortening (AHMED and HODGKIN 2000). Park and Horvitz screened for mutations that have dominant effects on the behaviour or morphology of *C. elegans* (PARK and HORVITZ 1986). For about half of the genes that give a phenotype with a dominant mutation loss of gene function did not result in a visible phenotype, indicating that loss-of-function alleles of these genes would not have been found using a simple F2 screen (PARK and HORVITZ 1986).

Suppressor screens

To identify genes involved in a particular process, a powerful method is to search for extragenic modifiers (either enhancers or suppressors) of a mutation that affects this process (reviewed in (HODGKIN and HERMAN 1998; JORGENSEN and MANGO 2002)). Suppressor screens in particular have frequently been performed in *C. elegans*. To study signal transduction, suppressor screens are very useful, because second-site mutations often identify genes that are involved in the same genetic pathway as the gene that is disrupted in the starting strain. For instance, to study RAS signalling, genetic screens for mutations that suppress the multivulva phenotype of *let-60 ras* gain-of-function mutations have been done (reviewed in (STERNBERG and HAN 1998); Figure 1B). Many genes that act downstream of *let-60 ras*, including *lin-45* (RAF), *mek-2* (MAP kinase kinase), *mpk-1* (MAP kinase) and *sur-2*, have been identified in this screen (HAN *et al.* 1993; KORNFELD *et al.* 1995; SINGH and HAN 1995; WU and HAN 1994). Subsequently, epistatic analyses determined the functional order of action of these genes. In this way, a complete genetic pathway for RAS signalling has been built (STERNBERG and HAN 1998). At the time of discovery of this RAS pathway in *C. elegans*, several components of this pathway were not known to play a role in mammalian RAS signalling. However, later studies in mammalian systems confirmed that they are also involved in the mammalian RAS pathway. This shows that suppressor screens in *C. elegans* are very potent in identifying (conserved) signal transduction pathways.

By screening for suppressors it should be kept in mind that not all mutants identified in such screens are involved in the same process as the original mutant (reviewed in (PRELICH 1999)). An example is the class of informational suppressors that were identified in early suppressor screens. These suppressors contained mutations in components of the translational machinery and were called informational because they change the passage of *information* from DNA to protein (reviewed in (MURGOLA 1990)). Most mutations altered tRNAs in such

a way that an amino acid is incorporated at a stop codon, thus suppressing nonsense or frameshift mutations. In *C. elegans*, several informational suppressors have been isolated and cloned (BOLTEN *et al.* 1984; WATERSTON 1981; WATERSTON and BRENNER 1978). Although these informational suppressors are useful to study the translational machinery, they do not give information about the process that is disrupted in the original strain. Another class of suppressors that frequently do not affect the process of interest alter the amount of mutant protein. The primary mutation often results in increased or decreased activity or amount of the protein, and second-site mutations that respectively decrease or increase the actual amount of protein are expected to suppress the phenotype of the primary mutation. Either specific regulators of the gene of interest or general components of gene expression pathways are identified. A general gene expression pathway that can be mutated in *C. elegans* to suppress a variety of phenotypes involves the *smg* genes (reviewed in (MANGO 2001)). These genes act in nonsense-mediated mRNA decay, and suppression is probably caused by increased stability of the mRNA encoded by the primary mutated gene (PULAK and ANDERSON 1993). Taken together, careful analysis of the obtained suppressors is necessary to reveal whether they act in the same or in an unrelated process as the original gene.

Caveats of forward genetics

Forward genetics cannot, unfortunately, unmask all components of a certain (signal transduction) pathway. One problem is that if two genes functionally substitute each other (i.e. they are functionally redundant), both genes have to be mutated to give a mutant phenotype. Because the frequency of mutating two genes at the same time is very low, it is difficult to identify redundant positions in a pathway. Another problem is that genes might be missed because they function in more than one pathway. The phenotype of a mutant in that gene might then be different from the specific phenotype that is being selected. An example is a gene that has also a function that is essential for viability. A knockout of such a gene will not be recovered from a forward genetic screen, because it is lethal. Finally, a gene can be difficult to target by mutagenesis. For instance, the size of a gene might be so small that the frequency with which it is mutated is too low to be isolated in a forward genetic screen.

Reverse genetics

With the completion of the sequencing project, reverse genetics has become more and more important in *C. elegans*. Using reverse genetics, the function of a gene with known sequence is determined (reviewed in (KUWABARA 1997; PLASTERK 1995)). It is even possible to study a complete gene family or the complete genome (JANSEN *et al.* 1999; KAMATH *et al.* 2003). The most common way to study a gene's function is by inactivating that gene. However, a gene's function can also be determined by overexpression or expression pattern studies. Most powerful is naturally a combination of these methods.

Stable gene knockouts

It is possible to obtain stable gene knockouts by several methods. Gene disruption by homologous recombination with transgenic DNA is feasible (BROVERMAN *et al.* 1993; E. Berezikov and R. Plasterk, personal communication), but is not yet used routinely. A standard method to knockout genes is target-selected gene inactivation: animals are randomly mutagenized and subsequently animals are searched that are mutated in the gene of interest. The first reports of target-selected gene inactivation in *C. elegans* made use of transposon insertional mutagenesis (RUSHFORTH *et al.* 1993; ZWAAL *et al.* 1993). Zwaal *et al.* constructed a frozen library of nematodes mutagenized by transposon insertion, from which insertion mutants in genes of interest can be found using PCR with one primer corresponding to the end of the transposon and another corresponding to the gene of interest. Since most transposon insertion do not (completely) knockout the gene of interest, because they are in an intron or

spliced out of the exon (RUSHFORTH and ANDERSON 1996), a deletion derivative, which occurs by imprecise excision, has to be obtained for a guaranteed null allele (ZWAAL *et al.* 1993).

A more direct approach for deleting a gene of interest makes use of a library of chemically mutagenized nematodes (JANSEN *et al.* 1997). A subset of the mutations introduced by the chemical mutagen are random deletions that remove a significant amount of DNA. A deletion in the gene of interest can be visualized with PCR. Recently, it has also become possible to search for specific mutations in a gene of interest using a library of chemically mutagenized nematodes (E. Cuppen and R. Plasterk, personal communication). The mutations in the gene of interest are detected by resequencing that gene. This method has already been used succesfully for knocking out genes in zebrafish (WIENHOLDS *et al.* 2002).

RNA interference

Another method to generate gene knockouts, although transient and not stable, uses RNA interference (RNAi) (FIRE *et al.* 1998). RNAi is the targeted experimental silencing of genes through the introduction of double-stranded RNA (dsRNA) of target genes. The dsRNA of the target gene triggers the sequence specific degradation of the mRNA of that target gene (reviewed in (GRISHOK and MELLO 2002; TIJSTERMAN *et al.* 2002)). RNAi was first described in *C. elegans*, but also works in other systems, such as plants and human cells (ELBASHIR *et al.* 2001; WATERHOUSE *et al.* 1998). In *C. elegans*, dsRNA of the gene of interest can be introduced by injection into the gonad or other tissues (FIRE *et al.* 1998), but also via the animal's food, either by feeding the animals bacteria that produce the dsRNA (TIMMONS *et al.* 2001; TIMMONS and FIRE 1998) or by soaking them in a dsRNA solution (TABARA *et al.* 1998). RNAi is particularly useful in performing genome-wide screens (reviewed in (BARSTEAD 2001)). Recently, a reusable library of bacterial clones that express double-stranded RNA of almost all predicted genes of *C. elegans* has been constructed (FRASER *et al.* 2000; KAMATH *et al.* 2003). This library is fed to wild type Bristol N2 animals to assign functions to as much genes as possible (KAMATH *et al.* 2003). To increase the number of genes with an RNAi phenotype, *rrf-3* mutants, which are hypersensitive to RNAi (SIMMER *et al.* 2002), have been fed to this library as well (SIMMER *et al.* in preparation). Disadvantages of RNAi are that the effect of RNAi is transient and variable, that a gene is often not completely knocked out and that a fraction of genes cannot be targeted at all by this method (FRASER *et al.* 2000).

Signal transduction pathways in *C. elegans*

The dissection of the RAS signalling pathway illustrates the usefulness of *C. elegans* genetics in studying signal transduction. Other conserved signal transduction pathways have been examined in *C. elegans* as well and these studies have provided new insights into the molecular components and mechanisms of these pathways. A few examples of signal transduction pathways extensively studied in *C. elegans* are given below.

Heterotrimeric G protein signalling

Heterotrimeric G proteins are signal-transducing molecules that are found in all eukaryotes. They consist of a Gα-subunit, which contains a guanine nucleotide binding site and intrinsic GTPase activity, and a tightly associated $\beta\gamma$-subunit complex (reviewed in (RENS-DOMIANO and HAMM 1995)). They act as molecular switches that alternate between active and inactive forms. In the inactive state, the Gα-subunit is associated with a GDP molecule and is in complex with the G$\beta\gamma$-subunit. Both the α- and the $\beta\gamma$-subunit are anchored to the plasma

membrane through lipid modifications (reviewed in (CHEN and MANNING 2001)). They connect G protein-coupled receptors, which have seven transmembrane-spanning domains, to intracellular signalling pathways. The seven-transmembrane receptors constitute the largest and most ubiquitous family of membrane receptors, and recently it has become clear that, besides coupling to the G proteins, they also couple to other signal transduction pathways (reviewed in (PIERCE *et al.* 2002)).

Binding of a specific ligand, such as a hormone or neurotransmitter, to the G protein-coupled receptor opens the guanine nucleotide-binding site of Gα and GDP is replaced by GTP, which is present at much higher concentrations in the cell. Subsequently, the Gα and the G$\beta\gamma$-subunit dissociate, and both have signalling functions and interact with specific downstream targets in the cell. Examples of downstream effectors are adenylyl cyclases, phospholipases, phosphodiesterases, tyrosine kinases and ion channels (reviewed in (NEVES *et al.* 2002; RADHIKA and DHANASEKARAN 2001)). The activation or deactivation of these downstream effectors results in alterations in the levels of intracellular second messengers, such as cAMP, inositol trisphosphate (IP_3), diacylglycerol (DAG) and Ca^{2+}, which in their turn transmit the signal by regulating the activities of other enzymes. The original signal is often enormously amplified, because one G protein-coupled receptor can activate multiple G proteins and every G protein can regulate several downstream effectors. In this way, many biological processes, including embryonic development, cell growth and differentiation, learning and memory, and neurotransmission, are being controlled (DHANASEKARAN *et al.* 1998; OFFERMANNS and SIMON 1998; RADHIKA and DHANASEKARAN 2001).

To end the activation state, the intrinsic GTPase activity of the Gα-subunit hydrolyses the bound GTP back to GDP. Following, the Gγ- and G$\beta\gamma$-subunit reassociate to the inactive heterotrimeric complex. Regulators of G protein signalling (RGS) proteins facilitate the inactivation of Gα-subunits by accelerating GTP hydrolysis, and probably provide an additional mechanism by which cells can integrate multiple incoming signals (reviewed in (ROSS and WILKIE 2000)). Signalling of the ligand-bound G protein-coupled receptor is terminated by phosphorylation and binding of arrestin (reviewed in (PIERCE and LEFKOWITZ 2001)).

In mammals, there are currently 20 Gα-, 6 Gβ- and 11 Gγ-subunits known (HAMM 1998). Based on sequence similarity, the Gα-subunits have been divided into four families, Gα_s, G$\alpha_{i/o}$, G$\alpha_{q/11}$ and G$\alpha_{12/13}$. Gα-subunits within a class usually couple to similar receptors and effectors. The Gα_s family of proteins signals via adenylyl cyclases, thereby producing the second messenger cAMP. cAMP is known to activate several targets, including protein kinase A (PKA), cyclic nucleotide-gated ion channels and the exchange factor for the small GTPase Rap1 (EPAC) (reviewed in (CHIN *et al.* 2002)). G$\alpha_{q/11}$ proteins activate phospholipase Cβ to produce IP_3 and DAG (reviewed in (RHEE 2001)). IP3 is involved in the release of calcium from intracellular stores and DAG activates protein kinase C (PKC) by recruiting it to the membrane. In the G$\alpha_{i/o}$ pathway, both Gα and G$\beta\gamma$ are known to signal to downstream effectors (reviewed in (ALBERT and ROBILLARD 2002)). An important role of G$\alpha_{i/o}$ is to inhibit adenylyl cyclase molecules. Recently, it has been demonstrated that G$\alpha_{i/o}$ is also involved in the direct stimulation of certain tyrosine kinases (MA *et al.* 2000). The G$\alpha_{12/13}$ pathway is the most poorly understood heterotrimeric G protein pathway. Some recent reports revealed a number of direct downstream targets of G$\alpha_{12/13}$, including a RasGAP, Bruton´s tyrosine kinase and cadherins, but it is not known in which physiological response these effectors are involved (JIANG *et al.* 1998; MEIGS *et al.* 2001).

In *C. elegans*, there are 21 Gα- (*gpa-1–17*, *egl-30*, *goa-1*, *gsa-1*, and *odr-3*), 2 Gβ- (*gpb-1* and *gpb-2*) and 2Gγ-subunit genes (*gpc-1* and *gpc-2*) (*C. ELEGANS* SEQUENCING CONSORTIUM 1998). Sequence alignment of the Gα-subunits showed that there is one clear homologue for each of the four mammalian classes of Gα-subunits: *goa-1* (G$\alpha_{i/o}$), *gsa-1*

($G\alpha_s$), *egl-30* ($G\alpha_{q/11}$) and *gpa-12* ($G\alpha_{12/13}$) (JANSEN *et al.* 1999). The other 17 Gα-subunits cannot be classified in one of the mammalian classes and are considered to be an outgroup of new Gα genes (JANSEN *et al.* 1999). The conserved Gα-subunits are broadly expressed and are involved in multiple behaviours, whereas most new Gα-subunits are only expressed in a few neurons in the head and specifically act in perception (reviewed in (WILKIE 1999). Below, a detailed description of signalling via conserved and new Gα-subunits of *C. elegans* is given. Gβ and Gγ signalling pathways are described as well.

The C. elegans homologue of Gα_s, GSA-1, signals via the adenylyl cyclases SGS-1 and ACY-2
The $G\alpha_s$ gene of *C. elegans*, *gsa-1*, encodes a protein that is 66% identical to mammalian $G\alpha_s$ (PARK *et al.* 1997). Expression pattern studies revealed that *gsa-1* is expressed throughout the nervous system and in muscle cells (KORSWAGEN *et al.* 1997). Loss of *gsa-1* results in lethality at the first stage of larval development, showing that *gsa-1* is an essential gene (KORSWAGEN *et al.* 1997). The lethal phenotype of *gsa-1* mutants is identical to the zygotic null phenotype of the Gβ-subunit gene *gpb-1* and to the lethal phenotype of animals in which the canal-associated neurons (CAN) have been cell-specifically ablated (ZWAAL *et al.* 1996; J. Sulston, personal communication). This suggests that GSA-1 together with GPB-1 performs an essential function in the CAN cells. Additional proof for this hypothesis came from studies on the adenylyl cyclase gene *acy-2*. Adenylyl cyclases convert ATP into the second messenger cAMP and are known to be downstream targets of $G\alpha_s$ proteins in mammals and several other organisms. *acy-2* is specifically expressed in the CAN cells and loss of *acy-2* results in an early larval lethal phenotype that is similar to the phenotype of *gsa-1* and *gpb-1* null mutants (KORSWAGEN *et al.* 1998). These results strongly suggest that the adenylyl cyclase ACY-2 is a downstream target of GSA-1 in the CAN cells.

Another adenylyl cyclase protein that acts downstream of GSA-1 is SGS-1 (or ACY-1). Like *acy-2*, *sgs-1* is an essential gene (MOORMAN and PLASTERK 2002, Chapter 2 of this thesis). However, the phenotype of *sgs-1* null mutants, larval arrest, does not resemble the null phenotype of *gsa-1* or *acy-2*. *sgs-1* is more broadly expressed than *acy-2*, with expression in motor neurons, vulva muscle cells and body-wall muscle cells (KORSWAGEN *et al.* 1998). Evidence for the activation of SGS-1 by GSA-1 came from studies using overexpression of constitutively active GSA-1. This constitutively active GSA-1 was constructed by changing a glutamine at position 208 (Q) to a leucine (L), a mutation that is analogous to the activating Q227L mutation in human $G\alpha_s$, which is found in certain pituitary and thyroid malignancies (LANDIS *et al.* 1989; LYONS *et al.* 1990) and endocrine disorders (IIRI *et al.* 1994; SHENKER *et al.* 1993). The amino-acid change disrupts the GTPase activity of $G\alpha_s$ and locks the protein in the active GTP-bound state. Overexpression of constitutively active GSA-1 results in hypercontraction of body-wall muscle cells and in swelling and vacuolization of ventral nerve cord motoneurons and neurons located in head and tail ganglia (BERGER *et al.* 1998; KORSWAGEN *et al.* 1997). Screens for suppressors of the activated GSA-1-induced neuronal degeneration identified mutations in the adenylyl cyclase gene *sgs-1*, thereby establishing a functional relationship between GSA-1 and SGS-1 (BERGER *et al.* 1998; KORSWAGEN *et al.* 1998). The mutations in *sgs-1* isolated in the suppressor screens were all reduction-of-function mutations; complete loss-of-function of *sgs-1* results in larval arrest (MOORMAN and PLASTERK 2002), and would therefore not be isolated in a suppressor screen. However, direct testing of *sgs-1* knockout animals revealed that these animals also suppress the neuronal degeneration phenotype.

The neuronal degeneration induced by signalling of activated GSA-1 to SGS-1 is morphologically distinct from programmed cell death and is not dependent on genes involved in apoptosis (BERGER *et al.* 1998; KORSWAGEN *et al.* 1997). However, the neuronal degeneration is similar to the necrotic cell death observed in *deg-1*, *deg-3* and *mec-4* ion

channel mutants of *C. elegans* (CHALFIE and WOLINSKY 1990; DRISCOLL and CHALFIE 1991; TREININ and CHALFIE 1995). The degenerins *deg-1* and *mec-4* encode proteins that are similar to subunits of the amiloride-sensitive Na^+ channel (HONG and DRISCOLL 1994; HUANG and CHALFIE 1994). *deg-3* encodes an acetylcholine receptor channel subunit (TREININ and CHALFIE 1995). In the *deg-1*, *deg-3* and *mec-4* mutants, ion channels are thought to be hyperactivated, causing excess ion influx that is ultimately toxic. In humans, a similar mechanism is thought to be involved in the excitotoxic neuronal cell death observed in neurodegenerative disorders and stroke: hyperactivation of glutamate-gated ion channels results in increased ion influx and ultimately in cell death (COYLE and PUTTFARCKEN 1993). This suggests that ion channels might also be hyperactivated in activated GSA-1 animals. Recently, it has been shown that null mutations in calreticulin (CRT-1), which is a Ca^{2+} binding/storing protein predominantly found in the endoplasmic reticulum (ER), suppress the necrotic cell death induced in *deg-1* and *mec-4*, but not *deg-3*, mutants or by activated $G\alpha_s$ (XU *et al.* 2001). This suggests that DEG-1, MEC-4 and $G\alpha_s$ signal directly or indirectly to calreticulin to induce neuronal degeneration. In addition to calreticulin, three other genes involved in regulation of intracellular Ca^{2+} levels, *cnx-1*, *itr-1* and *unc-68*, are thought to function in necrotic cell death (XU *et al.* 2001). *itr-1* and *unc-68* encode ER Ca^{2+} release channels, and *cnx-1* encodes an ER calcium binding chaperone calnexin homolog (DAL SANTO *et al.* 1999; MARYON *et al.* 1996; XU *et al.* 2001). In *deg-1* and *mec-4* animals, elevated Na^+ concentrations caused by hyperactivation of Na^+ channels probably signal to calreticulin (XU *et al.* 2001). Possibly, GSA-1 also signals to calreticulin via hyperactivation of Na^+ channels, but GSA-1 might also activate calreticulin via a more direct mechanism. SGS-1 is likely to be the first downstream target of GSA-1 in both cases. The suppression of the activated $G\alpha_s$-induced neuronal degeneration by *crt-1* null mutation is partial (XU *et al.* 2001), in contrast to the complete suppression by *sgs-1* mutation (BERGER *et al.* 1998; KORSWAGEN *et al.* 1997). Therefore, it is likely that signalling of GSA-1 to SGS-1 also induces neuronal degeneration via other mechanisms.

Activation of calreticulin results in release of Ca^{2+} from the ER and thus in elevated Ca^{2+} levels in the cell (XU *et al.* 2001). A recent report demonstrated that specific aspartyl and calpain proteases are required for necrotic cell death in *C. elegans* (SYNTICHAKI *et al.* 2002). At least two calpain proteases, CLP-1 and TRA-3, and two aspartyl proteases, ASP-3 and ASP-4, are redundantly involved in the induction of neuronal degeneration caused by activated $G\alpha_s$ or mutations in *deg-1*, *deg-3* and *mec-4* (SYNTICHAKI *et al.* 2002). An increase in Ca^{2+} concentration in the cell probably activates CLP-1 and TRA-3, which in turn activate the aspartyl proteases ASP-3 and ASP-4 that execute cell death. Thus, an attractive model for induction of neuronal degeneration is that elevated Ca^{2+} concentrations induced by calreticulin in *deg-1*, *mec-4* and activated $G\alpha_s$ animals activate the calpain/aspartyl protease pathway to destroy the cell (Figure 2). In *deg-3* mutants, hyperactivation of the acetylcholine receptor channel subunit DEG-3 probably directly increases the Ca^{2+} concentrations in the cell via increased calcium influx (XU *et al.* 2001). In this way, the calpain/aspartyl protease pathway is activated in *deg-3* mutants as well.

Other components acting in the $G\alpha_s$–induced neuronal degeneration could be uncovered by cloning of additional suppressors of the neuronal degeneration phenotype. Unfortunately, besides *sgs-1*, only one other locus, *nxf-1* (or *sgs-2*), was found to suppress the $G\alpha_s$–induced neuronal degeneration when mutated in our suppressor screens (Chapter 3 of this thesis). *nxf-1* encodes a nuclear export factor that is required for the nuclear export of poly(A)-containing mRNAs (TAN *et al.* 2000). Mutations in *nxf-1* probably affect the export of activated $G\alpha_s$ mRNA, and are therefore examples of suppressor mutations that alter the amount of mutant protein, but do not affect the process of interest (i.e. $G\alpha_s$-induced signalling).

Overexpression of wild type GSA-1 results in hyperactive locomotion and egg-laying, whereas partial (mosaic) loss of GSA-1 results in reduced locomotion and egg-laying (KORSWAGEN *et al*. 1997). This indicates that Gα_s is involved in modulating these behaviours. Loss or reduction of SGS-1 function also results in reduced locomotion, and it was shown that Gα_s likely functions together with SGS-1 to regulate this behaviour (MOORMAN and PLASTERK 2002). It is not known whether the Gα_s–SGS-1 pathway acts in motor neurons, in body-wall muscle cells or in both.

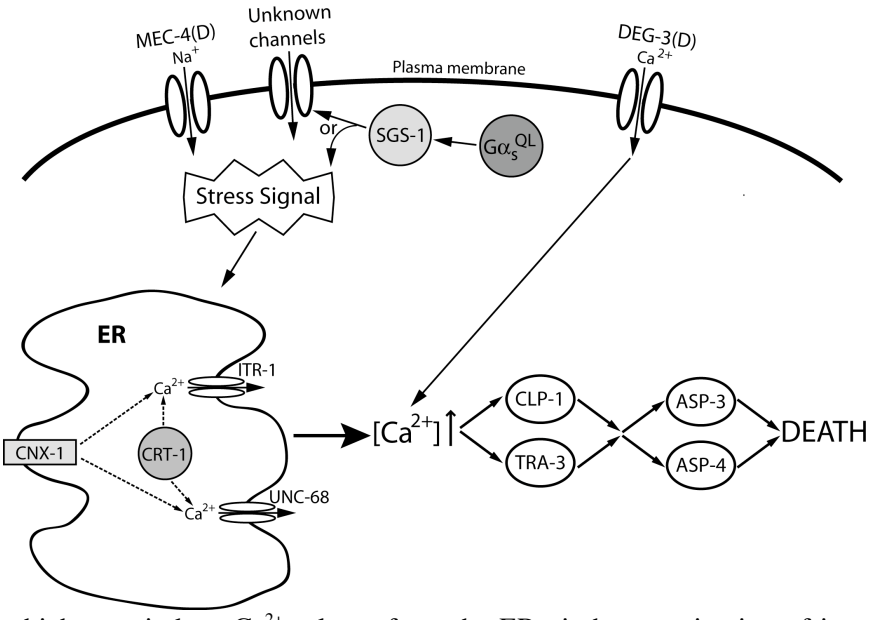

Figure 2. Model for induction of neuronal degeneration in *C. elegans*. Hyperactivation of MEC-4, DEG-3 or GSA-1 results in increase in intracellular Ca^{2+} levels. In the case of the hyperactivated DEG-3 (DEG-3(D)), Ca^{2+} levels are increased via elevated Ca^{2+} influx. In the case of the hyperactivated MEC-4 (MEC-4(D)) influx of Na^+ results in release of Ca^{2+} from stores in the endoplasmic reticulum (ER). Activated GSA-1 ($G\alpha_s^{QL}$) signals to SGS-1, which may induce Ca^{2+} release from the ER via hyperactivation of ion channels or by a more direct mechanism. The increase in intracellular Ca^{2+} levels is sensed by calpains, which activate a specific set of aspartyl proteases to execute cell death. Adapted from (SYNTICHAKI *et al*. 2002; XU *et al*. 2001).

Heterotrimeric G proteins control positioning of the mitotic spindle in C. elegans

Asymmetric cell division, the process producing two different daughter cells, is critically important in the generation of cell diversity (reviewed in (WODARZ 2002)). In this process, the cell must first set up an axis of polarity. Second, cytoplasmic components are asymmetrically distributed to one of the two poles. Finally, the mitotic spindle is oriented along the axis of polarity. In *C. elegans*, cell division planes follow an invariant pattern, thereby resulting in the fixed position of embryonic cells. One component that is involved in establishing different patterns of cell division is the Gβ-subunit GPB-1 (ZWAAL *et al*. 1996). As already notified above, a *gpb-1* knockout shows a larval lethal phenotype when maternal GPB-1 is present. In embryos without maternally supplied GPB-1, which are obtained from transgenically rescued homozygous mothers with little or no expression in the germline, spindle orientations in early cell divisions are randomized. This results in abnormal cell positions in these embryos and in embryonic lethality (ZWAAL *et al*. 1996). A practical method to study maternal genes is RNAi, because this technique degrades both maternally and zygotically supplied mRNAs. Indeed, in *gpb-1*(RNAi) embryos, spindle orientations are incorrect (GOTTA and AHRINGER 2001). It was shown that GPB-1 is important in regulating centrosome migration around the nucleus and thus in orienting the mitotic spindle. GPB-1 is not required for embryonic polarity of embryos. The Gγ-subunit that acts together with GPB-1 in controlling spindle orientation is GPC-2, because RNAi against GPC-2 but not GPC-1 results in spindle orientation defects that are identical to those in *gpb-1* mutants (GOTTA and AHRINGER 2001).

None of the single Gα-subunit knockouts showed fully penetrant spindle orientation defects (JANSEN *et al.* 1999), indicating that GPB-1 and GPC-2 might act together with redundant Gα-subunits. Both *goa-1*, which encodes a homologue of human Gα_o (MENDEL *et al.* 1995; SEGALAT *et al.* 1995), and *gpa-16*, which falls in the group of new Gα-subunits (JANSEN *et al.* 1999), mutants show a low percentage of embryonic lethality, indicating that these two Gα-subunits might have redundant functions (GOTTA and AHRINGER 2001; MILLER *et al.* 2000). Indeed, a *goa-1 gpa-16* double knockout shows 100% embryonic lethality and incorrect spindle orientations. RNAi against both GOA-1 and GPA-16 resulted in 100% embryonic lethality as well (GOTTA and AHRINGER 2001). This indicates that GOA-1 and GPA-16 are the functionally redundant partners of GPB-1 and GPC-2.

Inactivation of GOA-1 and GPA-16 does not only lead to loss of Gα signalling, but also to constitutive activation of G$\beta\gamma$. Comparison of *goa-1*(RNAi) *gpa-16*(RNAi) embryos, which have G$\beta\gamma$ signalling, with *goa-1*(RNAi) *gpa-16*(RNAi) *gpb-1*(RNAi) embryos, which have neither Gα nor G$\beta\gamma$ signalling, revealed that Gα and G$\beta\gamma$ are involved in separate processes (GOTTA and AHRINGER 2001). Whereas the G$\beta\gamma$-subunit is involved in regulating centrosome migration, GOA-1 and GPA-16 are required for correct behaviour and morphology of the mitotic spindle in the one-cell embryo. Without the Gα-subunits, the position of the mitotic spindle in the cell was symmetric instead of asymmetric (GOTTA and AHRINGER 2001).

A component that probably plays a role in the GOA-1/GPA-16 pathway that controls mitotic spindle orientation is the novel protein RIC-8 (MILLER and RAND 2000). RIC-8 is, like GOA-1, involved in a signalling network that regulates neurotransmitter release in *C. elegans* (MILLER *et al.* 2000). *ric-8* reduction-of-function mutants exhibit a low percentage of embryonic lethality that is dramatically increased in embryos derived from a *goa-1*/+; *ric-8*/*ric-8* parent (MILLER and RAND 2000). In these embryos, the position of the spindle was often more symmetric than in wild type embryos, a phenotype that is associated with loss of GOA-1/GPA-16 signalling.

What is the mechanism by which the heterotrimeric G proteins regulate spindle orientation in *C. elegans*? Recent experiments in *Drosophila* and *C. elegans* start to give insight in this process (SCHAEFER *et al.* 2001; M. Gotta and J. Ahringer, personal communication). Probably, the G proteins are activated by the PAR proteins, which are asymmetrically distributed in the cell, via proteins with GoLoco domains (PINS in *Drosophila* and AGS-3.2 and AGS-3.3 in *C. elegans*). Subsequently, the G proteins are thought to translate cell polarity into spindle position. In mammals, heterotrimeric G proteins are not known to be involved in asymmetric cell division yet, but a mammalian homologue of PINS is required for mitotic spindle organization (DU *et al.* 2001), suggesting that the role of heterotrimeric G proteins in asymmetric cell divisions is conserved.

A complex signalling network involving heterotrimeric G proteins controls locomotion in C. elegans

Recent genetic experiments show that a complex signalling network is involved in regulating the release of acetylcholine at the neuromuscular junction and thereby in modulating behaviours in *C. elegans* (Figure 3). Two key components in this signalling network are the homologue of mammalian Gα_o, *goa-1*, and the Gα_q homologue of *C. elegans*, *egl-30*. Activation of GOA-1 probably negatively regulates EGL-30 (HAJDU-CRONIN *et al.* 1999; MILLER *et al.* 1999). Both forward and reverse genetics have been used to elucidate this signalling network, as described below.

Animals lacking *goa-1* show hyperactive movement, premature egg-laying, inadequate male mating and hypersensitivity to the acetylcholinesterase inhibitor aldicarb (MENDEL *et al.* 1995; MILLER *et al.* 1999; SEGALAT *et al.* 1995). Aldicarb causes toxic accumulation of

secreted acetylcholine at the neuromuscular junction. The hypersensitivity of *goa-1* mutants to aldicarb suggests that GOA-1 is normally involved in decreasing acetylcholine release at the synapse, which is also consistent with the hyperactive locomotion of *goa-1* mutants. GOA-1 is activated by serotonin, since loss-of-function mutations in *goa-1* confer resistance to serotonin-induced inhibition of locomotion (NURRISH *et al.* 1999; SEGALAT *et al.* 1995). In addition, GOA-1 is thought to be activated by UNC-43, a Calcium/Calmodulin-dependent protein kinase II (CaMKII). Possibly, UNC-43 directly regulates GOA-1 by phosporylation (ROBATZEK and THOMAS 2000). A Calcium/Calmodulin-dependent protein phosphatase, calcineurin, probably antagonizes UNC-43 signalling to GOA-1 (BANDYOPADHYAY *et al.* 2002). Overexpression or constitutive activation of GOA-1 results in paralysis and defective egg-laying and feeding (MENDEL *et al.* 1995; SEGALAT *et al.* 1995). The expression pattern of *goa-1* is consistent with these phenotypes: *goa-1* is expressed in most neurons and in the muscles involved in egg-laying, pharyngeal pumping and male mating behaviour.

Mutations in *egl-30* were first identified in a screen for egg-laying defective mutants (TRENT *et al.* 1983). In addition to the egg-laying defect, *egl-30* mutants show reduced locomotion, decreased pharyngeal pumping rates and resistance to aldicarb (MILLER *et al.* 1996; TRENT *et al.* 1983). Brundage *et al.* showed that *egl-30* mutants have mutations in the

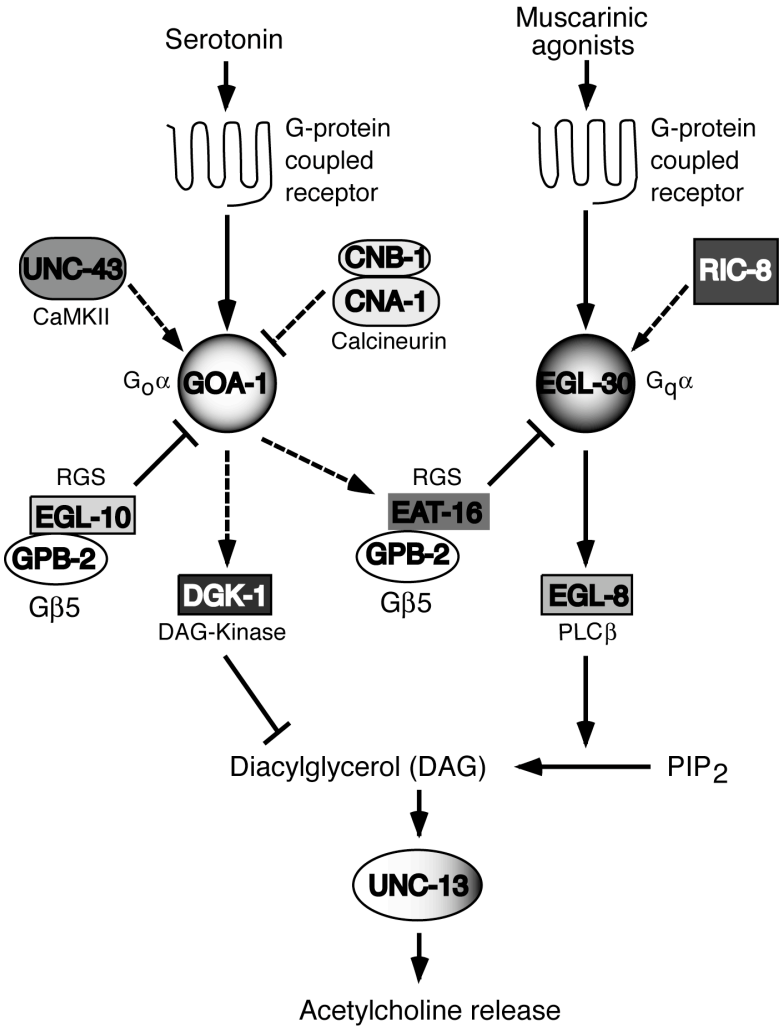

Figure 3. Model for acetylcholine release at the neuromuscular junction of *C. elegans* by a complex heterotrimeric G protein network. The molecules and steps in this signal transduction network are discussed in the text. Calcineurin functions as a heterodimeric protein, consisting of CNA-1 and CNB-1. Adapted from (VAN DER LINDEN *et al.* 2001).

$G\alpha_q$ homologue of *C. elegans* (BRUNDAGE *et al.* 1996). Strong *egl-30* alleles are lethal, because of severly reduced muscle activity (BRUNDAGE *et al.* 1996). *egl-30* is expressed in both the nervous system and in muscle cells (LACKNER *et al.* 1999). Overexpression of EGL-30 results in hyperactive locomotion and egg-laying (BRUNDAGE *et al.* 1996). Specific overexpression of EGL-30 in the motor neurons is sufficient to induce hyperactive locomotion, indicating that EGL-30 acts in motor neurons to regulate locomotion (LACKNER *et al.* 1999). Because *egl-30* mutants are resistant to aldicarb, EGL-30 is thought to increase acetylcholine release at the synapse (MILLER *et al.* 1996). *egl-30* mutations suppress hypersensitivity to muscarinic agonists, indicating that EGL-30 is activated by muscarinic agonists (BRUNDAGE *et al.* 1996). A forward genetic screen, which was performed to find genes with phenotypes similar to reduction-of-function mutants in *egl-30* when mutated, identified, among others, *egl-8* as a potential downstream target of *egl-30* in acetylcholine release (MILLER *et al.* 1999). *egl-8* encodes a phospholipase Cβ (PLCβ) homologue. Phospholipase Cβ is a known direct target of mammalian $G\alpha_q$, and thus it is likely that the *C. elegans* $G\alpha_q$ EGL-30 directly activates EGL-8. This hypothesis is further supported by biochemical experiments that showed that EGL-30 can activate phospholipase Cβ in cultured cells (BRUNDAGE *et al.* 1996). However, EGL-8 is probably not the only downstream target of EGL-30, since *egl-30* loss-of-function mutants have more severe defects than *egl-8* loss-of-function mutants (MILLER *et al.* 1999). Phospholipase Cβ cleaves phosphatidylinositol 4,5-biphospate (PIP_2) into the signalling molecules diacylglycerol (DAG) and inositol 1,4,5-triphsophate (IP_3). Treatment of wild type animals with analogs of DAG, phorbol esters, resulted in increased sensitivity to aldicarb, indicating that DAG positively regulates acetylcholine release at the neuromuscular junction (LACKNER *et al.* 1999; MILLER *et al.* 1999). The regulation of acetylcholine release by DAG is in part mediated by the DAG-binding protein UNC-13 (LACKNER *et al.* 1999; NURRISH *et al.* 1999). UNC-13 primes synaptic vesicles for fusion at the neuromuscular junction by promoting the open form of the SNARE complex protein syntaxin (RICHMOND *et al.* 2001). Thus, EGL-30 stimulates acetylcholine release by activating EGL-8, which stimulates UNC-13 via production of DAG. However, UNC-13 is probably not the only protein that is stimulated by EGL-30 to regulate acetylcholine release at the synapse (LACKNER *et al.* 1999).

Mutations in *egl-10* also confer phenotypes similar to *egl-30* mutants (KOELLE and HORVITZ 1996; MILLER *et al.* 1999). *egl-10* encodes a regulator of G protein signalling (RGS) protein (KOELLE and HORVITZ 1996). RGS proteins accelerate the slow intrinsic GTPase activity of Gα-subunits and are therefore able to inhibit Gα signalling. Epistatic analysis indicated that EGL-10 acts as a negative regulator of GOA-1 (KOELLE and HORVITZ 1996). In total, 13 RGS proteins are found in *C. elegans*, and two other RGS proteins, RGS-1 and RGS-2, act on GOA-1 (DONG *et al.* 2000). RGS-1 and RGS-2 appear to be redundantly involved in egg-laying in response to food, but not in locomotion (DONG *et al.* 2000).

Another RGS protein that is involved in the signalling network regulating acetylcholine release is EAT-16 (HAJDU-CRONIN *et al.* 1999). *eat-16* was originally isolated in a screen for defects in pharyngeal pumping (AVERY 1993). Screens for suppressors of the paralysis induced by overexpression of constitutively active GOA-1 revealed that mutations in *eat-16* restore locomotion (HAJDU-CRONIN *et al.* 1999). Without expression of activated GOA-1, *eat-16* mutations give phenotypes similar to that of *goa-1* loss-of-function mutations. Because the constitutively active GOA-1 that is used in the suppressor screen is locked in the GTP-bound state and thus insensitive to RGS proteins, it is not likely that GOA-1 is the target of EAT-16. Indeed, further genetic and biochemical experiments showed that EAT-16 does not regulate GOA-1, but rather acts downstream of GOA-1 and is involved in inhibiting the $G\alpha_q$ homologue EGL-30 (HAJDU-CRONIN *et al.* 1999). This is consistent with the epistatic

analysis of *goa-1 egl-30* double mutants that showed that EGL-30 functions downstream of or in parallel to GOA-1 (HAJDU-CRONIN *et al*. 1999; MILLER *et al*. 1999).

Both EGL-10 and EAT-16 have, besides an RGS domain, a G-like (GGL) domain and a conserved N-terminal region. It was shown that a divergent G-subunit in mammals, G5, can interact with GGL domains (SNOW *et al*. 1998). In *C. elegans*, GPB-2 is the closest homologue of G5. Loss of *gpb-2* results in animals that have delayed egg-laying and reduced pharyngeal pumping (CHASE *et al*. 2001; ROBATZEK *et al*. 2001; VAN DER LINDEN *et al*. 2001). This phenotype is similar to the phenotype of an *egl-10 eat-16* double knockout, indicating that GPB-2 may interact with both EAT-16 and EGL-10. Yeast-two hybrid analysis showed that indeed GPB-2, like GPB-1, could physically interact with the GGL domain of both EGL-10 and EAT-16 (VAN DER LINDEN *et al*. 2001). Recently, it has been proposed that GPB-2 cannot only bind to the GGL domain but also to the N-terminal region of EGL-10 and EAT-16. GPB-2 thereby makes a noncovalent interaction between the N-terminal region and the GGL/RGS domains of these RGS proteins (PATIKOGLOU and KOELLE 2002). The N-terminal region is involved in G-binding specificity and may serve as a membrane anchor for EGL-10 and EAT-16. Thus, GPB-2 directly interacts with the RGS proteins EGL-10 and EAT-16, and is in this way able to inhibit both GOA-1 and EGL-30 signalling.

Mutations in the diacylglycerol kinase *dgk-1* (or *sag-1*) suppress the paralysis induced by activated GOA-1, indicating that *dgk-1* acts downstream of or in parallel to *goa-1* (HAJDU-CRONIN *et al*. 1999; NURRISH *et al*. 1999). Diacylglycerol (DAG) kinases phosphorylate DAG to produce phosphatidic acid, thereby decreasing DAG levels. In a wild type background, *dgk-1* mutants show hyperactive locomotion, premature egg-laying and hypersensitivity to aldicarb (MILLER *et al*. 1999; NURRISH *et al*. 1999). In addition, *dgk-1* mutations suppress the inhibition of locomotion induced by serotonin (NURRISH *et al*. 1999). These data suggest that DGK-1 normally functions to decrease acetylcholine release at the synapse by reducing DAG levels. A *dgk-1 egl-30* double mutant exhibits phenotypes that were in between that of either single mutant (MILLER *et al*. 1999). This is different from a *goa-1 egl-30* double mutant that showed an *egl-30* phenotype (HAJDU-CRONIN *et al*. 1999; MILLER *et al*. 1999). Probably, EGL-30 and DGK-1 do not act in a linear pathway, but rather antagonistically.

Another gene that is thought to be involved in regulating acetylcholine release is *ric-8* (MILLER *et al*. 2000; MILLER *et al*. 1999). *ric-8* mutants have phenotypes similar to *egl-30* mutants. *ric-8* encodes a novel conserved protein that acts upstream of EGL-30 or in a parallel intersecting pathway (MILLER *et al*. 2000). However, it is not know what its exact function is in regulating acetylcholine release. Screens to identify mutations that suppress the paralyzed phenotype of *ric-8* mutants revealed that dominant mutations in *gsa-1* and *sgs-1* strongly suppress the *ric-8* mutant phenotype (N. Reynolds and K. Miller, personal communication). In addition, reduction-of-function mutations in a regulatory subunit of protein kinase A, *kin-2*, were found. These mutations probably constitutively activate KIN-1, which is the catalytic subunit of protein kinase A. In a wild type background, these mutations result in hyperactive locomotion and hypersensitivity to aldicarb, suggesting that GSA-1, SGS-1 and KIN-1 act presynaptically to increase acetylcholine release. Previously, it was already shown that G_s regulates locomotion via signalling to the adenylyl cyclase SGS-1 (MOORMAN and PLASTERK 2002). An *sgs-1 goa-1* double knockout mutant confers an *sgs-1* mutant phenotype (i.e. strongly reduced locomotion), indicating that the G_sSGS-1 pathway acts downstream or in parallel to the GOA-1 pathway (C. Moorman and R. Plasterk, unpublished observation). Further analyses should reveal where GSA-1, SGS-1 and KIN-1 exactly function in the acetylcholine release pathway.

Heterotrimeric G proteins are involved in chemosensation and adaptation in C. elegans

C. elegans normally lives in the soil, where it has to seek food on the one hand and avoid toxic compounds on the other hand. *C. elegans* receives most information about its environment through detection of chemicals, so-called chemosensation (reviewed in (BARGMANN and MORI 1997; MORI 1999)). *C. elegans* can detect both volatile chemicals and water-soluble chemicals. Certain chemicals are attractive to *C. elegans*, resulting in chemotaxis towards that chemical; other chemicals are repulsive, resulting in avoidance of that chemical. Thus, chemosensation allows *C. elegans* to respond to its environment by regulating its locomotory behaviour. Other behaviours that can be modulated via detection of chemicals are egg-laying, feeding and male-mating. In addition, chemosensation is implicated in the regulation of larval development. *C. elegans* can enter an alternative larval stage, the dauer stage, that is specialized to survive severe environmental conditions (CASSADA and RUSSELL 1975). High temperature, insufficient food and high nematode density are important regulators of dauer formation. Animals determine nematode density in an early larval stage by chemosensation of dauer-inducing pheromone, which is continuously excreted by nematodes, and enter the dauer stage if pheromone levels are high. When dauer larvae are returned to better circumstances, they can form normal fertile adults.

Chemosensory neurons are required to detect chemicals. In *C. elegans*, eleven classes of neurons are predicted to be chemosensory, because their endings are exposed to the environment through specialized sensory cilia. These chemosensory neurons are located in two bilaterally symmetric sensory organs in the head, called the amphids, in six inner labial sensory organs and in two bilaterally symmetric sensory organs in the tail, called the phasmids. Specific killing of neurons with laser ablation revealed functions for 11 pairs of sensory neurons in the amphids (reviewed in (TROEMEL 1999)). They are involved in chemosensation of volatile attractants (AWA, AWC) and repellents (ASH, AWB, ADL), water-soluble attractants (ASE, ADF, ASG, ASI, ASK) and repellents (ASH, ADL) and the dauer-inducing pheromone (ADF, ASG, ASI, ASJ). The ASH neurons also detect high osmolarity and nose touch. Recently, the phasmid neurons have been shown to function as chemosensory neurons as well; they respond to repellents (HILLIARD *et al.* 2002). Besides the chemosensory neurons, two neurons involved in thermosensation, the AFD neurons, are identified in the amphids.

Binding of the chemical to a receptor on the chemosensory neuron is the first step in chemosensation. Cloning of *odr-10*, which shows defective chemotaxis towards diacetyl but not other odorants when mutated, revealed that G protein-coupled seven transmembrane domain receptors are involved in chemosensation in *C. elegans* (SENGUPTA *et al.* 1996). In mammals, G protein-coupled receptors are also involved in recognition and transduction of chemicals (RESSLER *et al.* 1993). Only one or a few receptors are thought to be expressed in a mammalian olfactory neuron (reviewed in (RONNETT and MOON 2002). In *C. elegans*, the family of G protein-coupled receptors is the largest gene family (reviewed in (TROEMEL 1999)). In total, approximately a thousand genes encoding G protein-coupled receptors genes have been predicted, thus comprising about 5% of all *C. elegans* genes (BARGMANN 1998). They fall into four subfamilies based on sequence similarity: the *odr-10*-like, *sra*, *sro* and *srg* family. The *odr-10*-like family is subdivided into *str*, *stl*, *srd* and *srh* classes and the *sra* family into *sra*, *srb* and *sre* groups. Expression pattern studies on several G-protein coupled receptors revealed that all four subfamilies contain receptors that are expressed in chemosensory neurons (TROEMEL *et al.* 1995). They are localized to the ciliary endings of these neurons, probably to facilitate detection of chemicals in the environment. Based on the expression pattern studies, it is predicted that about half of the thousand G protein-coupled receptors in *C. elegans* are involved in detecting chemicals (TROEMEL 1999). Another class of candidate chemosensory receptors are the guanylyl cyclases (reviewed in (GIBSON and GARBERS 2000)). A number of guanylyl cyclase is expressed in cilia of chemosensory

neurons in *C. elegans*, and are thus potential chemoreceptors (YU *et al.* 1997). However, not all will act as chemoreceptors, since guanylyl cyclases can also be involved in signal transduction, as has been shown for ODR-1 (L'ETOILE and BARGMANN 2000). Given the expression pattern studies and the large number of (putative) chemosensory receptors, it is probable that, unlike in mammals, multiple receptors act in the same pair of neurons in *C. elegans*, which is in agreement with the observation that multiple odorants can be sensed by the same neuron in *C. elegans*.

After binding of the chemical to the G protein-coupled receptor, it is likely that heterotrimeric G proteins are activated and signal to downstream effectors in the chemosensory neuron. Indeed, several genetic and molecular analyses indicate that heterotrimeric G protein signalling is involved in chemosensation in a wide variety of organisms (KRIEGER *et al.* 1999; PRASAD and REED 1999; RONNETT and MOON 2002). In *C. elegans*, the Gα-subunit ODR-3, which falls in the group of new Gα-subunits, was found in a forward genetic screen for animals that fail to be attracted to the odorant benzaldehyde. In fact, ODR-3 is essential for responses to all odorants sensed by the AWA and AWC neurons (ROAYAIE *et al.* 1998). In addition, ODR-3 is required for responses to high osmolarity and nose touch, which are sensed by ASH. ODR-3 is expressed in AWA, AWC and ASH, and localizes to the ciliary endings. This suggests that ODR-3 acts directly downstream of chemosensory receptors in these cells. In addition to its role in chemosensation, *odr-3* is also involved in AWC cilium development (ROAYAIE *et al.* 1998).

Besides *odr-3*, 13 of the other new Gα-subunits are almost exclusively expressed in the (putative) chemosensory neurons: *gpa-1–6*; *gpa-8–11*; and *gpa-13–15* (JANSEN *et al.* 1999; ZWAAL *et al.* 1997). In most chemosensory neurons, multiple Gα-subunit genes are expressed. Loss-of-function alleles of five new Gα-subunits, *gpa-2*, *gpa-3*, *gpa-5*, *gpa-6* and *gpa-7*, show defects in chemosensation, indicating that they play a role in chemosensation. In addition, overexpression of four new Gα genes, *gpa-1*, *gpa-4*, *gpa-10* and *gpa-11*, resulted in chemosensation defects, indicating that these genes may also function in chemosensation (JANSEN *et al.* 1999). Other new Gα genes expressed in chemosensory neurons might be functionally redundant or might have negative regulatory roles. Of course, it is also possible that these Gα-subunits are defective in sensing chemicals that have not been tested. Thus, besides multiple G protein-coupled receptors, *C. elegans* also uses multiple Gα-subunits in chemosensation. The reason for this is probably that *C. elegans* needs to distinguish a large number of chemicals with only a few chemosensory neurons. Recently, genetic experiments showed that the new Gα-subunits can have both stimulatory and inhibitory functions, depending on the chemical, thereby further increasing the ability to discriminate between different chemicals (G. Jansen, personal communication). Taken together, these results suggest that multiple and distinct G protein signalling cascades mediate the sensing of and the discrimination between chemicals in *C. elegans*.

One other G protein subunit is exclusively expressed in chemosensory neurons, GPC-1 (JANSEN *et al.* 2002). Loss-of-function of this Gγ-subunit did not result in altered sensation of several chemicals tested nor in defects in the development of sensory neurons. In addition, overexpression of *gpc-1* had no effect on these processes. In mammals, it has been proposed that G$\beta\gamma$-subunits are involved in adaptation or desensitization of the G protein-coupled receptor to a stimulus (reviewed in (PITCHER *et al.* 1998). *C. elegans* shows adaptation to both volatile and water-soluble compounds, and assays have been developed to detect adaptation defects (COLBERT and BARGMANN 1995; JANSEN *et al.* 2002). These assays showed that loss of *gpc-1* results in defective adaptation to several water-soluble chemicals, but not to volatile chemicals (JANSEN *et al.* 2002). Thus, while *gpc-1* is not essential for sensing water-soluble chemicals, *gpc-1* is required for the subsequent desensitization of the signal transduction pathway used to detect these water-soluble chemicals.

Protein Kinase signalling

Protein phosphorylation is one of the most widely used mechanisms for signal transduction in eukaryotic cells. It is important for basic functions, including gene transcription, protein translation and DNA replication, but also for more advanced functions, such as cell differentiation, cell-cell communication and synaptic transmission. Protein kinases conduct phosporylation of proteins and thereby regulate the activity of these proteins. Some protein kinases transfer phosphate to serine or threonine residues, others phosphorylate tyrosine residues. About 1.7% of all human genes constitute protein kinases (MANNING *et al.* 2002b). Most protein kinases belong to a single superfamily that share a conserved kinase catalytic domain. This superfamily can be further classified in groups, families and subfamilies (HANKS and HUNTER 1995; MANNING *et al.* 2002b). Protein phosphatases can reverse the phosporylations performed by protein kinases.

C. elegans has 411 full length protein kinases and 21 partial kinase fragments (reviewed in (PLOWMAN *et al.* 1999)). The protein kinase gene family is thereby the second largest gene family of *C. elegans*, comprising 2-3% of all *C. elegans* genes. Like in humans, the *C. elegans* kinases can be further divided in groups, families and subfamilies (PLOWMAN *et al.* 1999). Most kinases have homologues in other organisms, although there are also some worm-specific protein kinase subfamilies (MANNING *et al.* 2002a; PLOWMAN *et al.* 1999). Below, two conserved kinase families of *C. elegans*, the PKC family and the DYRK family, are discussed in more detail.

The PKC family of protein kinases in C. elegans

The protein kinase C (PKC) family can be categorized into three subfamilies on the basis of structural similarities and cofactor requirements: conventional PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC) (reviewed in (NEWTON 2001)). The cPKCs are dependent on Ca^{2+} for their activation. In addition, they need diacylglycerol (DAG) to be activated. The nPKCs also need DAG to be activated, but are not dependent on Ca^{2+}. The aPKCs are dependent on neither Ca^{2+} nor DAG for their activation. When activated, PKCs catalyse the phosporylation of effector proteins at specific serine or threonine residues, thereby regulating their functions. In *C. elegans*, four *pkc* genes have been identified, *tpa-1*, *pkc-1*, *pkc-2* and *pkc-3* (reviewed in (TABUSE 2002)). TPA-1 and PKC-1 belong to the nPKCs, PKC-2 is a cPKC and PKC-3 is an aPKC.

TPA-1 was first identified as a target of the tumor-promoting phorbol ester PMA (phorbol 12-myristate 13-actetate; also known as 12-*O*-tetradecanoylphorbol-13-acetate, TPA) (TABUSE *et al.* 1989). In *C. elegans*, PMA induces a developmental growth arrest and uncoordinated locomotion. The developmental growth arrest is probably caused by a feeding defect (VAN DER LINDEN *et al.* 2003, Chapter 4 of this thesis). Mutations in *tpa-1* suppress the developmental growth arrest induced by PMA. *tpa-1* encodes two PKC isoforms, TPA-1A and TPA-1B, which are most similar to nPKCθ and η subunits (SANO *et al.* 1995). Both TPA-1 isoforms share the C-terminal region, which includes the pseudosubstrate region, two cysteine-rich C1 domains and the kinase domain. However, TPA-1B lacks an N-terminal extension of 137 amino acid residues. The expression of *tpa-1A* and *tpa-1B* is controlled by different promoters. *tpa-1A* is expressed in vulval muscle and epidermal cells and in a variety of neurons (VAN DER LINDEN *et al.* 2003; WAGGONER *et al.* 2000). *tpa-1B* is expressed in hypodermal cells and pharyngeal muscles (TABUSE 2002; VAN DER LINDEN *et al.* 2003). TPA-1A probably plays a role in long-term adaptation of the vulval cells to nicotine (WAGGONER *et al.* 2000). In addition, TPA-1A is required for the uncoordinated locomotion induced by PMA (A. van der Linden and R. Plasterk, personal communication). Both TPA-1A and TPA-1B are thought to be involved in the developmental growth arrest induced by PMA (VAN DER LINDEN *et al.* 2003). Interestingly, both TPA-1 isoforms are also involved in the developmental growth arrest induced by constitutively activated GPA-12 (VAN DER LINDEN *et al.* 2003).

GPA-12 is the *C. elegans* homologue of the $G\alpha_{12/13}$ class of heterotrimeric G proteins. Constitutively active mutations in mammalian $G\alpha_{12/13}$-subunits can lead to the oncogenic transformation of human cells (JIANG *et al.* 1993; XU *et al.* 1994). The finding that mutations in *tpa-1* suppress both the activated $G\alpha_{12}$- and PMA-induced growth arrest together with other genetic experiments suggests that in *C. elegans* activated $G\alpha_{12}$ signals through PKC to induce a developmental growth arrest, and that induction of this signaling pathway by $G\alpha_{12}$ can be bypassed by PMA (VAN DER LINDEN *et al.* 2003).

The *pkc-1* gene encodes two PKC isoforms, PKC-1A and PKC-1B (LAND *et al.* 1994b). PKC-1A contains an N-terminal extension of 56 amino acids compared to PKC-1B. The function of these PKC isoforms has not yet been elucidated, although the expression pattern of *pkc-1B* suggests that it has a neuronal function (LAND *et al.* 1994a). The *pkc-2* gene is predicted to encode six PKC isoforms (ISLAS-TREJO *et al.* 1997). The different isoforms probably perform distinct functions, since they have cell- and developmental-stage specific expression patterns.

The atypical PKC-3 protein plays an essential role in early embryogenesis (TABUSE *et al.* 1998; WU *et al.* 1998). PKC-3 interacts with one of the PAR proteins, PAR-3. The PAR proteins are involved in asymmetric cell division by establishing the anterior-posterior polarity of zygotes (KEMPHUES and STROME 1997), and it was shown that PKC-3 is required for establishing asymmetry in early embryos as well (TABUSE *et al.* 1998). Subsequently, studies in other systems showed that the role of atypical PKCs in asymmetric cell divisions is evolutionarily conserved (reviewed in (KNOBLICH 2001)). It is not yet known how atypical PKCs establish asymmetry, but in *Drosophila* an atypical PKC is required for the asymmetric localization of Inscuteable (Insc) (WODARZ *et al.* 2000). Insc can bind the GoLoco domain protein PINS, which can activate the G protein subunit $G\alpha_i$ (reviewed in (KNUST 2001)). This suggests that in *C. elegans* PKC-3 might also be involved in activation of heterotrimeric G proteins.

The DYRK family of protein kinases in C. elegans

The DYRK kinases are dual specificity protein kinases that catalyse autophosporylation on tyrosine residues as well as serine/threonine phosphorylation in other proteins (reviewed in (BECKER *et al.* 1998)). In *Drosophila*, a member of the DYRK family, *minibrain*, is involved in the generation of a proper number of neuronal cells in the central brain and optical lobe during postembryonic neurogenesis (TEJEDOR *et al.* 1995). The mammalian dual-specificity protein kinase DYRK1A is implicated in the cognitive defects of Down syndrome. Down syndrome is caused by trisomy of chromosome 21 and results in mental retardation. The DYRK1A locus maps to the Down syndrome candidate region and introduction of a 180-kb region of human chromosome 21, which contains the DYRK1A locus but probably no other genes, is sufficient to produce defects in learning and memory in transgenic mice (SHINDOH *et al.* 1996; SMITH *et al.* 1997).

In *C. elegans*, three DYRK family members have been described, *mbk-1*, *mbk-2* and *hpk-1*. MBK-1 is the orthologue of human DYRK1A and overexpression of this gene was shown to interfere with the acute function of olfactory neurons but not with the formation or differentiation of these neurons (RAICH *et al.* 2003, Chapter 5 of this thesis). This suggests that increased gene dosage of human DYRK1A in Down syndrome might disrupt the acute function of neurons as well. *mbk-1* knockout animals are viable and do not exhibit obvious morphological abnormalities. A similar result is found for *hpk-1* knockout animals. However, animals homozygous for an *mbk-2* knockout display 100% penetrant maternal effect embryonic lethality, indicating that *mbk-2* is essential for viability (RAICH *et al.* 2003). Further analysis of the DYRK family in *C. elegans* could provide additional insights into the role of this family in mental retardation.

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Chapter 2

Functional characterization of the adenylyl cyclase gene *sgs-1* by analysis of a mutational spectrum in *Caenorhabditis elegans*

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Functional Characterization of the Adenylyl Cyclase Gene *sgs-1* by Analysis of a Mutational Spectrum in *Caenorhabditis elegans*

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ABSTRACT

The *sgs-1* (*s*uppressor of activated $G\alpha_s$) gene encodes one of the four adenylyl cyclases in the nematode *C. elegans* and is most similar to mammalian adenylyl cyclase type IX. We isolated a complete loss-of-function mutation in *sgs-1* and found it to result in animals with retarded development that arrest in variable larval stages. *sgs-1* mutant animals exhibit lethargic movement and pharyngeal pumping and (while not reaching adulthood) have a mean life span that is >50% extended compared to wild type. An extensive set of reduction-of-function mutations in *sgs-1* was isolated in a screen for suppressors of a neuronal degeneration phenotype induced by the expression of a constitutively active version of the heterotrimeric $G\alpha_s$ subunit of *C. elegans*. Although most of these mutations change conserved residues within the catalytic domains of *sgs-1*, mutations in the less-conserved transmembrane domains are also found. The *sgs-1* reduction-of-function mutants are viable and have reduced locomotion rates, but do not show defects in pharyngeal pumping or life span.

ADENYLYL cyclases convert intracellular adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), a major second messenger in the cell that regulates many cellular processes. Mammalian membrane-bound adenylyl cyclases consist of a short cytoplasmic N-terminal sequence, a six-transmembrane-spanning region (M1), and a cytoplasmic catalytic domain (C1), followed by a second six-transmembrane-spanning region (M2) and a second cytoplasmic catalytic domain (C2). The C1 and C2 domains together form the catalytic core of the protein. The first 200–250 amino acids of each catalytic domain (the C1a and C2a regions) are similar to each other and to the catalytic domains of other adenylyl and guanylyl cyclases. In mammals, nine different types of adenylyl cyclase genes have been reported, all of which are directly activated by the heterotrimeric G-protein α-subunit $G\alpha_s$. In addition, certain adenylyl cyclases can be regulated by $G\alpha_i$, $G\beta\gamma$, calcineurin, calmodulin, protein kinase A, protein kinase C, and forskolin (reviewed in Hurley 1999; Simonds 1999; Patel *et al.* 2001).

We have previously reported that the *Caenorhabditis elegans* adenylyl cyclase SGS-1 (*s*uppressor of activated $G\alpha_s$) is a downstream target of the *C. elegans* $G\alpha_s$ subunit in motoneurons (Korswagen *et al.* 1998): mutations in SGS-1 suppress the neuronal degeneration induced by a constitutively activating mutation in $G\alpha_s$ (Berger *et al.* 1998; Korswagen *et al.* 1998). In humans, activating mutations in $G\alpha_s$ are implicated in pituitary and thyroid malignancies (Landis *et al.* 1989; Lyons *et al.* 1990) and endocrine disorders (Shenker *et al.* 1993; Iiri *et al.* 1994). SGS-1 has significant sequence similarity to mammalian membrane-bound adenylyl cyclases and is most similar to the divergent type IX mammalian adenylyl cyclase. Human adenylyl cyclase type IX is predominantly expressed in vital organs, including brain, heart, and pancreas (Paterson *et al.* 2000). The *sgs-1* gene is ubiquitously expressed in the nervous system and muscle cells of *C. elegans* (Berger *et al.* 1998; Korswagen *et al.* 1998). A second adenylyl cyclase gene in *C. elegans*, *acy-2*, shows a more restricted expression pattern (Korswagen *et al.* 1998). This gene is not involved in $G\alpha_s$-induced neuronal degeneration, but probably has an essential function together with $G\alpha_s$ in the canal-associated neurons of *C. elegans* (Korswagen *et al.* 1998).

Over the last decade, great effort has been undertaken to understand the catalytic mechanism of adenylyl cyclase and its regulation. Most studies have focused on the function of the highly conserved catalytic domains, and several residues that are important for catalytic activity (Tang *et al.* 1992, 1995; Liu *et al.* 1997; Yan *et al.* 1997b; Zimmermann *et al.* 1998b), $G\alpha_s$ binding (Yan *et al.* 1997a; Zimmermann *et al.* 1998a), $G\alpha_i$ binding (Dessauer *et al.* 1998), forskolin stimulation (Yan *et al.* 1998), and ATP binding (Dessauer *et al.* 1997) have been determined, mainly through *in vitro* studies. In addition, the determination of the crystal structure of the catalytic domains of adenylyl cyclase (Tesmer *et al.* 1997, 1999) has provided new insight into the catalytic mechanism and the key residues for catalytic activity and binding of its regulators. The function of the two transmembrane domains of adenylyl cyclase is less well

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understood. A recent study has now revealed that these domains interact with each other persistently and that this interaction plays a critical role in the targeting and functional assembly of adenylyl cyclase (Gu *et al.* 2001); thus, these domains are very important for the proper functioning of the protein.

In this study, we show that *sgs-1* is an essential gene and that *sgs-1* is involved in behaviors such as locomotion and pharyngeal pumping. To identify new residues in *sgs-1* that are important for normal response to $G\alpha_s$, we performed a genetic screen for suppressors of the activated $G\alpha_s$-induced neuronal degeneration. In total, we identified 14 residues in *sgs-1* that can be mutated to suppress the neuronal degeneration. Ten of these residues are located in the catalytic domains of *sgs-1*, but four mutations in the transmembrane domains were also found. Although none of the mutations are located in the proposed active site of the protein, we show here that catalytic activity of *sgs-1* is required for $G\alpha_s$-induced neuronal degeneration.

MATERIALS AND METHODS

Nematode strains and culturing: All strains were maintained as described by Lewis and Fleming (1995). DNA transformations by microinjection in *C. elegans* were as described by Mello and Fire (1995). Strains used in this study were Bristol N2, CB1282 (*dpy-20(e1282)* IV), NL545 (*dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) (Korswagen *et al.* 1997), NL585 (*sgs-1(pk301)* III, *dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) (Korswagen *et al.* 1998), NL586 (*sgs-1(pk310)* III, *dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) (Korswagen *et al.* 1998), NL597 (*sgs-1(pk384)* III, *dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) (Korswagen *et al.* 1998), NL1250 (*mut-2(r459)* I; *sgs-1(pk450::Tc1)* III) (Korswagen *et al.* 1998), NL3200 (*sgs-1(pk1279)/+* III; *dpy-20(e1282)* IV), and NL3224 (*sgs-1(pk1279) dpy-17(+)/sgs-1(+) dpy-17(e164)* III; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X). *sgs-1* alleles *pk484*, *pk487*, *pk862*, *pk866*, *pk867*, *pk871*, *pk875*, *pk880*, *pk884*, *pk886*, *pk904*, *pk907*, and *pk927* were generated in an NL545 background in this study.

Isolation of an *sgs-1* knockout animal: We used the outer primers delsgs1-9 GGCGGAAAAGTTGAAAATGA and delsgs1-10 TGCAATGCTTCTCACCTGTC and the nested primers delsgs1-11 CACGTGAAGGAGGTGGAAGT and delsgs1-12 CTGGTTTTTGTGCTGGGACT, spanning a genomic region of 4.1 kb, to screen for deletion derivatives of *pk450::Tc1* (Zwaal *et al.* 1993), a transposon insertion in the ninth exon of *sgs-1* (Korswagen *et al.* 1998). A 2.9-kb deletion derivative of *pk450::Tc1*, *sgs-1(pk1279)*, was isolated and could not be maintained as a homozygote. The *sgs-1(pk1279)* PCR product was isolated from gel and sequenced to determine the deletion end points. The following sequence in capitals was found around the deletion site: (5′-TGGAAGTAatacagat-gtgac cacTATCTTTA-3′). The *pk1279* allele was backcrossed four times to an N2 background and then crossed with CB164 (*dpy-17(e164)* III), resulting in strain NL1999 *sgs-1(pk1279)/dpy-17(e164)*. One-fourth of the progeny of NL1999 *sgs-1 (pk1279)/dpy-17(e164)* arrested in larval development, and we confirmed by PCR that these are homozygous for *sgs-1(pk1279)* (data not shown). The homozygous *pk1279* phenotype was rescued with a wild-type *sgs-1* genomic construct, pRP1522 (Korswagen *et al.* 1998). This construct was injected at a concentration of 50 μg/ml together with 100 μg/ml pMH86 (Han and Sternberg 1991) in CB1282, and the transgenic array was crossed in NL3200 (*sgs-1(pk1279)/+* III; *dpy-20 (e1282)* IV). A rescuing strain, NL3212 (*sgs-1(pk1279)* III; *dpy-20(e1282)* IV; *pkEx1288*[*sgs-1(+) dpy-20(+)*]), which does not segregate viable Dpy-20 worms, was obtained. Single worm PCR confirmed homozygosity for the *pk1279* allele (data not shown).

Isolation of suppressors of the activated $G\alpha_s$-induced neuronal degeneration: The screen that was used to identify suppressors of the activated $G\alpha_s$ phenotype was a modified version of an earlier described screen (Korswagen *et al.* 1998). L4 NL545 animals were treated with 50 mM EMS in M9 buffer for 4 hr, washed, and seeded at 40–60 animals per 9-cm nematode growth medium (NGM) agar plate. The F_2 generation was synchronized using NaOCl bleaching, and L1 larvae were heat-shocked for 2 hr at 33° to induce expression of *pkIs296*. The animals that grew out to adults were again synchronized using NaOCl bleaching, and L1 larvae were heat-shocked for 2 hr at 33° to induce expression of *pkIs296*. Animals that did not show neuronal degeneration were collected. *sgs-1* alleles were identified by complementation testing.

Activated $G\alpha_s$-induced neuronal degeneration: The activated $G\alpha_s$-induced pattern of neuronal cell death was measured as described in Korswagen *et al.* (1997). Synchronized L1-L2 larvae were heat-shocked for 2 hr at 33° and after 24 hr the neuronal degeneration pattern was examined in at least 20 animals using a high-powered dissection microscope (Wild M3C). Animals that did not show 100% suppression of the neuronal degeneration were further examined using a Nomarski microscope (Zeiss Axioskop 2): the total number of degenerated neurons was determined in 20 animals. The percentage suppression was calculated by comparing this number with the total number of degenerated neurons in NL545 (*dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) (100% degeneration) and NL586 (*sgs-1(pk310)* III; *dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) (100% suppression). Statistics were calculated using directed Student's *t*-tests.

***sgs-1(pk1279) trans* experiments:** NL1999 *sgs-1(pk1279)/dpy-17(e164)* males were crossed with Bristol N2 hermaphrodites and with homozygous *sgs-1* mutants. 40 L1 progeny were picked and their growth was followed. After 1-week of growth at 20°, we checked for the presence of the *sgs-1(pk1279)* allele by PCR.

Construction and expression of a catalytically inactive *sgs-1* mutant: SGS-1 R1187A was constructed by PCR using primers R1187-1 TGCTAGCGCAATGTACAGCAC and R1187-2 GCTG TACATTGCGCTAGCAATG containing the mutation. The PCR fragment was cloned into a 15.6-kb *Nhe*I-*Bsp*EI fragment of pRP1522 (Korswagen *et al.* 1998), giving rise to SGS-1 R1187A. The cloned PCR fragment was sequenced completely and found to be free of errors. SGS-1 R1187A was injected at the same concentration as the wild-type SGS-1 construct pRP1522 (50 μg/ml; Korswagen *et al.* 1998) together with the markers pRF6 (100 μg/ml; Kramer *et al.* 1990) and pRP2017 (*gpb-2::gfp*; 50 μg/ml; van der Linden *et al.* 2001) in N2 animals. Three independent SGS-1 R1187 transgenic lines and two independent wild-type SGS-1 transgenic lines were crossed in NL3224 and examined for their rescue of the larval lethality and suppression of the neuronal degeneration.

Behavioral assays: Life span was determined by transferring L1-L2 larvae individually to plates 24 hr after synchronization using NaOCl bleaching. The animals were incubated at 20° and monitored once daily until death. Day of synchronization was used as the first time point. The animals were transferred to fresh plates once in 2 days while producing eggs to keep them separate from their progeny. Animals were scored as dead when they stopped moving and no longer responded to

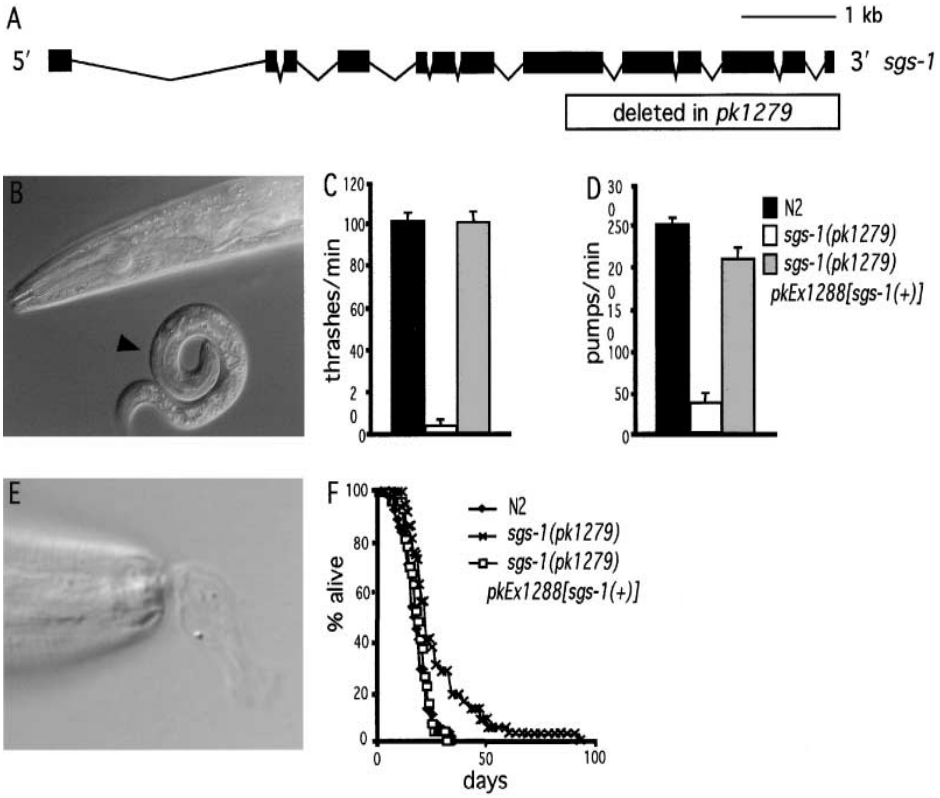

FIGURE 1.—Phenotypes of *sgs-1(pk1279)* animals. (A) Genomic representation of the *sgs-1* gene showing the deleted region in *pk1279*. GENEFINDER prediction of the *sgs-1* coding sequence was confirmed by sequencing of random and oligo(dT) primed cDNA amplified by reverse transcriptase PCR (KORSWAGEN *et al.* 1998). (B) The development of the *sgs-1(pk1279)* animal (arrowhead) is compared with similarly aged Bristol N2 animal. (C) Movement levels of N2, *sgs-1 (pk1279)* animals and *sgs-1 (pk1279) pkEx1228*[*sgs-1*(+)] animals were assayed 96 hr after synchronization. Each bar represents the average of 10 animals. The error bars represent the standard error of the mean (SEM). Differences between N2 and *sgs-1(pk1279)* were analyzed using Student's *t*-test. *sgs-1 (pk1279)* animals are significantly different for movement when compared with N2 animals ($P < 0.001$). (D) Pharyngeal pumping levels of N2, *sgs-1(pk1279)* animals and *sgs-1(pk1279) pkEx1288*[*sgs-1*(+)] animals were assayed 96 hr after synchronization. Each bar represents the average of 20 determinations. The error bars represent the SEM. Differences between N2 and *sgs-1(pk1279)* were analyzed using Student's *t*-test. *sgs-1(pk1279)* animals are significantly different with respect to pharyngeal pumping when compared with N2 animals ($P < 0.001$). (E) In many *sgs-1(pk1279)* animals, a cuticular plug is attached to the mouth opening. (F) The percentage of worms alive on a given day after synchronization: N2, *sgs-1(pk1279)* animals and *sgs-1(pk1279) pkEx1288*[*sgs-1*(+)] animals. Mean life spans ± SEM, with sample size in parentheses, are 18.0 ± 0.8 (56), 27.9 ± 2.8 (32), and 19.0 ± 1.0 (27), respectively. Significance levels were calculated using Student's *t*-test. *sgs-1(pk1279)* lived significantly longer than the wild-type strain ($P < 0.001$), whereas there was no significant difference between N2 and *sgs-1(pk1279) pkEx1288*[*sgs-1*(+)] animals ($P = 0.42$).

prodding the head (KENYON *et al.* 1993). Mean life spans were compared by using Student's *t*-test assuming unequal variances.

Pharyngeal pumping rates were determined by measuring the number of pumps per minute. Animals that had been raised for 96 hr after synchronization using NaOCl bleaching were placed on NGM agar plates seeded with OP50. Ten animals for each genotype were scored for 2 min and each animal was scored twice. Statistics were calculated by using directed Student's *t*-tests.

Movement levels were determined by measuring thrashing levels. One thrash is defined as one change in direction of bending at the mid-body (MILLER *et al.* 1996). Animals were synchronized using NaOCl bleaching and were raised at 20° for 96 hr after synchronization using NaOCl. An animal was placed in microtiter plates with 50 μl M9 and allowed to recover for 2 min. Subsequently, thrashes were counted for 2 min. Ten animals were examined for each genotype. Statistics were calculated by using directed Student's *t*-tests.

RESULTS

***sgs-1* is essential for viability:** A transposon-based method (ZWAAL *et al.* 1993) was used to isolate a deletion allele of *sgs-1*, *sgs-1(pk1279)*. As shown in Figure 1A, 2.9 kb of *sgs-1* sequence is deleted in this allele, thereby removing exon 8 up to exon 13. Nearly one-half of the *sgs-1* coding sequence is removed, including the complete second transmembrane and second catalytic domain. Therefore, it is highly likely that *pk1279* is a null allele. Animals homozygous for *pk1279* undergo severely retarded development (Figure 1B). All animals passed the first larval stage, but arrested and died in one of the later larval stages. The length of embryogenesis in *pk1279* homozygotes was comparable to Bristol N2 animals, and *pk1279* homozygotes were morphologically normal at hatching (results not shown). The *pk1279* homozygotes showed reduced body-wall muscle activity (Figure 1C) and pharyngeal muscle activity (Figure 1D). We also observed difficulties with molting (Figure 1E). In spite of these developmental abnormalities, *pk1279* animals had an extended life span: they lived >50% longer than the wild type (Figure 1F).

Rescue of the larval lethality was obtained with an extrachromosomal array containing pRP1522, a 14-kb subclone containing the entire *sgs-1* gene (KORSWAGEN *et al.* 1998). The rescued *sgs-1(pk1279)* transgenic ani-

mals were used to address whether maternally provided *sgs-1* mRNA or protein is needed for development. Extrachromosomal arrays are unstable and can be lost during meiosis and mitosis (Stinchcomb *et al.* 1985; Mello and Fire 1995). This results in mosaic expression of the transgene as it is lost in certain cells and cell lineages. Rescued *sgs-1(pk1279)* transgenic animals that lost the rescuing transgene in the germ line will have progeny that lack both *sgs-1* transgene expression and *sgs-1* expression in the form of maternal inheritance of *sgs-1* mRNA or protein (Zwaal *et al.* 1996). Of the rescued *pk1279* animals, 1–2% segregated only arrested larvae that were identical to the arrested larvae segregated by heterozygous *pk1279* animals. We did not observe any brood consisting of animals with a phenotype more severe than that of the arrested larvae. This result suggests that zygotic *sgs-1* performs an essential function during development and that the ability to develop into larva is not the result of the presence of maternal *sgs-1* mRNA or protein.

***sgs-1* mutants are suppressors of activated $G\alpha_s$-induced neuronal degeneration:** *sgs-1* mutants were initially selected as suppressors of activated $G\alpha_s$-induced neuronal degeneration (Berger *et al.* 1998; Korswagen *et al.* 1998). The neuronal degeneration phenotype is already visible in the first larval stage, and it is therefore possible to test the *sgs-1* knockout animals for suppression of this neuronal degeneration phenotype. Animals homozygous for the null allele *pk1279* that express the activated $G\alpha_s$ from a heat-shock promoter did not show neuronal degeneration. Thus, *sgs-1(pk1279)* is a suppressor of the activated $G\alpha_s$-induced neuronal degeneration (Table 1). In screens for suppressors of the activated $G\alpha_s$ phenotype, we have now obtained a set of 23 *sgs-1* alleles (Table 1), of which 7 were described earlier (Korswagen *et al.* 1998). The *sgs-1* alleles were isolated with a frequency of ~1 in 50,000 mutagenized genomes; they are all homozygous viable and recessive for the suppression of the activated $G\alpha_s$ phenotype. We conclude from the viability of these *sgs-1* alleles that they are not complete loss-of-function alleles, but partial reduction-of-function alleles. All 23 *sgs-1* alleles were sequenced and mutations were found in all alleles, except allele *pk310*, which was not studied further (Table 1). In three independent alleles (*pk311*, *pk474*, and *pk871*), an identical splice donor site mutation in the first catalytic domain was found. One allele (*pk862*) contained a mutation 39 bp upstream of the ATG. In 18 independent alleles, mutations were found that change 14 distinct (mostly conserved) amino acids. Most mutations that were found to suppress the activated $G\alpha_s$ phenotype were located in one of the conserved regions of the catalytic domains of *sgs-1*, suggesting that catalytic activity of SGS-1 is needed for the activated $G\alpha_s$ phenotype. The mutations that did not map in the conserved regions of the catalytic domains were located in the first transmembrane domain (*pk393*, *pk884*, *pk363*, *pk863*, and *pk866*) or at the border of the second transmembrane domain and the nonconserved region of the second catalytic domain (*pk907*).

SGS-1 mutations are not located in the active site: The availability of the crystal structure of the catalytic domains of adenylyl cyclase (Tesmer *et al.* 1997, 1999) has made it possible to map the mutations in the catalytic domains of *sgs-1* to the crystal structure. This analysis revealed that none of the 10 residues mutated in the catalytic domains of *sgs-1* was located in the proposed active site of the protein (Figure 2). Two of the mutated amino acids (G1068 and R1074) were located at the interaction interface with $G\alpha_s$. It has been shown for mammalian adenylyl cyclases that mutations in the amino acid analogous to R1074 result in decreased binding to $G\alpha_s$, with a consequent decrease in activation (Yan *et al.* 1997a; Zimmermann *et al.* 1998a). This suggests that in the mutants G1068R and R1074K suppression of the neuronal degeneration is the result of decreased binding to $G\alpha_s$. The remaining eight mutated amino acids were distributed over the structure and were changed to larger residues (L314F, L347F, A374V, H455Y, and S457F) or to residues that no longer make contact with the backbone or side chain of other amino acids (M411I, S1109N, and R1216H). Biochemical analysis of mammalian adenylyl cyclase type II with a mutation analogous to R1216H has shown that mutation of this amino acid does not result in gross structural alterations (Dessauer *et al.* 1997). This suggests that R1216H has a specific effect on adenylyl cyclase activity or regulation.

Catalytic activity of *sgs-1* is necessary for induction of neuronal degeneration: Induction of activated $G\alpha_s$ in an *sgs-1* null background did not result in neuronal degeneration, showing that *sgs-1* is essential for this process. Indeed, overexpression of the wild-type *sgs-1* allele in *sgs-1(pk1279)* animals both rescued the lethal phenotype and restored the neuronal degeneration phenotype upon induction of activated $G\alpha_s$ (Figure 3A). Since none of the *sgs-1* reduction-of-function mutations directly affected the active site of the SGS-1 protein, we asked whether catalytic activity of SGS-1 is required for the activated $G\alpha_s$-induced neuronal degeneration. We constructed a point mutation in the active site of SGS-1 (SGS-1 R1187A). R1187 is the residue analogous to R1029 of mammalian type II adenylyl cyclase. R1029 of type II adenylyl cyclase is an important residue for catalysis, and it was shown that changing this residue into an alanine reduces catalysis by 30-fold, but does not cause gross structural alterations (Yan *et al.* 1997b). To our surprise, overexpression of the SGS-1 R1187 mutant into *sgs-1(pk1279)* animals rescued the lethality of these animals, most probably due to residual catalytic activity (Yan *et al.* 1997b). However, we found that this residual activity was not sufficient for the induction of neuronal degeneration, as these animals looked completely wild type upon induction of activated $G\alpha_s$ expression (Figure 3B). We conclude from this result that a

TABLE 1

Properties of the *sgs-1* mutants

Allele	Mutation	Change	Domain	Suppression of neural degeneration (%)	Growth *in trans* to *pk1279*
pk862	caacgtc → caatgtc		Upstream of ATG	100	+
pk393[a]	gga → aga	G69R	M1	100	ND
pk884	cca → tca	P152A	M1	72	+
pk363[a]	gga → gaa	G181E	M1	100	ND
pk863	gga → gaa	G181E	M1	ND	ND
pk866	gga → gaa	G181E	M1	100	+
pk487	ctc → ttc	L314F	C1a	100	+
pk485	ctt → ttt	L347F	C1a	ND	ND
pk886	ctt → ttt	L347F	C1a	100	+
pk880	gct → gtt	A374V	C1a	100	+
pk867	atg → ata	M411I	C1a	87	+
pk927	cac → tac	H455Y	C1a	100	+
pk301[a]	tcc → ttc	S457F	C1a	100	+/−
pk311[a]	gtaactt → ataactt	Splice donor, site intron 7	C1a	100	ND
pk474[a]	gtaactt → ataactt	Splice donor, site intron 7	C1a	100	ND
pk871	gtaactt → ataactt	Splice donor, site intron 7	C1a	100	+
pk907	gaa → aaa	E992K	M2-C2a	100	−
pk384[a]	ggg → agg	G1068R	C2a	93	+
pk904	aga → aaa	R1074K	C2a	100	+
pk484	agt → aat	S1109N	C2a	100	−
pk874	agt → aat	S1109N	C2a	ND	ND
pk875	cgc → cac	R1216H	C2a	100	+
pk310[a]	Unknown			100	ND
pk1279	Deletion		C1b-M2-C2	100	−−
pk1279/+	Deletion/wild type			9	+

Neuronal degeneration induced by activated $G\alpha_s$ under heat-shock promoter control as described in MATERIALS AND METHODS. Phenotype was scored in at least 20 L1-L2 larvae for each genotype 24 hr after a 2-hr heat shock at 33°. Mean total numbers of degenerated neurons ±SEM, with sample size in parentheses, are 23.2 ± 0.3 (60) for NL545 *pkIs296*[*hsp::gsa-1QL*]; 0.15 ± 0.1 (20) for *sgs-1(pk310)*; 21.1 ± 0.4 (20) for *sgs-1(pk1279)/+*; 1.9 ± 0.4 (20) for *sgs-1(pk384)*; 3.3 ± 0.5 (40) for *sgs-1(pk867)*; and 6.5 ± 0.6 (60) for *sgs-1(pk884)*. The mean total number of degenerated neurons was not determined for the other *sgs-1* alleles, but examination using a high-powered dissection microscope showed a level of neuronal degeneration comparable to *sgs-1(pk310)*. Differences between genotypes were calculated using Student's *t*-test. There were significant differences between *sgs-1(pk310)* and *sgs-1(pk384)* or *sgs-1(pk867)* or *sgs-1(pk884)* (for all $P < 0.001$). There was also a significant difference between NL545 and *sgs-1(pk1279)/+* ($P < 0.004$). The growth *in trans* to *pk1279* was determined as described in MATERIALS AND METHODS. +, none of the animals tested has a growth delay; +/−, ~80% of the animals have a growth delay, but all eventually become adults; −, 100% of the animals have a growth delay, and ~50% are still in larval stage after 1 week of growth at 20°; −−, all animals have a growth delay, and none become adults. ND, not determined; C1b, nonconserved part of first catalytic domain.

[a] *sgs-1* alleles that were identified in a previous study (KORSWAGEN *et al.* 1998).

mutation in the active site suppresses the activated $G\alpha_s$-induced neuronal degeneration and that catalytic activity of *sgs-1* is required for the neuronal degeneration phenotype. Probably, mutations in the active site are not found in our screen for suppressors of the neuronal degeneration because these mutations are lethal. However, we cannot exclude other explanations for the fact that we have not found these mutations in our screen.

***sgs-1* alleles form an allelic series:** Most *sgs-1* alleles, including the null allele *pk1279*, showed 100% suppression of the activated $G\alpha_s$-induced neuronal degeneration. However, three alleles, *pk384* (G1068R), *pk867* (M411I), and *pk884* (P152S), showed <100% suppression: a few neurons were degenerated in most animals (Table 1). This suggests that the SGS-1 protein is less disturbed in these alleles than in the other alleles. These alleles had mutations in different domains of the SGS-1 protein, and thus there was no correlation between the activity of the mutant proteins and the position of their mutations. Animals heterozygous for *pk1279* suppressed the activated $G\alpha_s$-induced neuronal degeneration weakly: they showed a slight reduction in the number of degenerated neurons induced by activated $G\alpha_s$ expression compared to homozygous *sgs-1(+)* animals (Table 1). A possible explanation for this observation is that in these animals, *sgs-1* activity is reduced due to the

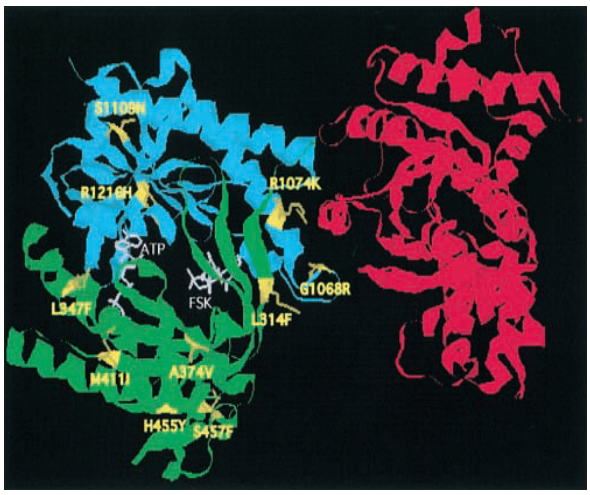

FIGURE 2.—Structural representation of the residues that are changed in *sgs-1* mutants. Complex of $G\alpha_s$ (red) with the catalytic domains of mammalian adenylyl (TESMER *et al.* 1997, 1999). Green, the C1a domain from adenylyl cyclase V; cyan, the C2 domain from adenylyl cyclase II; yellow, the residues that are changed in *sgs-1* mutants mapped on their analogous residues in the crystal structure. Forskolin (FSK) and adenosine 5′-(α thio)-triphosphate (ATP) are located in the active site of the enzyme. Modeling was done using RasMol.

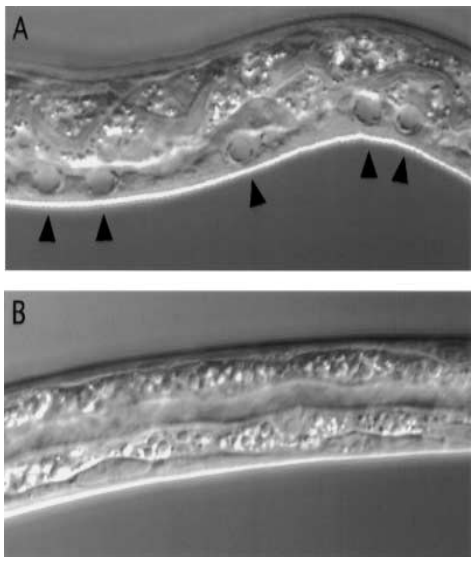

FIGURE 3.—Catalytically inactive SGS-1 does not rescue the suppression of activated $G\alpha_s$-induced neuronal degeneration. (A) *sgs-1(pk1279) pkIs296*[*hsp::gsa-1QL*] animal expressing *pkEx1353*[*sgs-1(+)*] shows neuronal degeneration (arrowheads) after induction of activated $G\alpha_s$ by 2 hr of heat shock at 33°. (B) *sgs-1(pk1279) pkIs296*[*hsp::gsa-1QL*] animal expressing *pkEx1348*[*sgs-1R1187A*] does not show neuronal degeneration after induction of activated $G\alpha_s$ by 2 hr of heat shock at 33°.

presence of only one functional copy and that in a small percentage of neurons the *sgs-1* activity is reduced to a level that is too low to induce neuronal degeneration.

We were also able to detect variation in SGS-1 activity among the different *sgs-1* alleles when we placed all alleles *in trans* to *pk1279* (Table 1). Most heterozygous animals grew normally. However, animals that had allele *pk301* (S457F), *pk484* (S1109N), or *pk907* (E992K) *in trans* to *pk1279* did not show normal growth: their phenotype was an intermediate form between wild-type and homozygous *pk1279* animals. These results suggest that the SGS-1 protein was more affected in *pk301*, *pk484*, and *pk907* than in the other alleles. The growth defect of *pk484/pk1279* and *pk907/pk1279* animals is more severe than the growth defect in *pk301/pk1279* animals, indicating that *pk484* and *pk907* are the most severe alleles of *sgs-1*. Both these alleles have mutations in the part of *sgs-1* that is deleted in *sgs-1(pk1279)*. We cannot exclude that certain alleles with a mutation in the part of *sgs-1* that is not deleted in *sgs-1(pk1279)* experience intragenic complementation when placed *in trans* to *sgs-1(pk1279)* and therefore have a less severe phenotype than *pk484* and *pk907*. When we placed *sgs-1(pk484)* (S1109N), which has a point mutation in the second catalytic domain, *in trans* to *sgs-1(pk301)* (S457F), *sgs-1 (pk487)* (L314F), *sgs-1(pk867)* (M411I), or *sgs-1(pk880)* (A374V), which are all mutated in the first catalytic domain, we did not observe an enhancement of the neuronal degeneration phenotype after expression of the activated $G\alpha_s$ from heat shock. This indicates that there is no intragenic complementation between the different *sgs-1* reduction-of-function mutants.

***sgs-1* reduction-of-function mutants have a normal life span and pharyngeal pumping rates, but reduced locomotion rates:** Observation of the *sgs-1(pk1279)* allele suggests that SGS-1 is involved in locomotion and pharyngeal pumping and life span determination. We measured these three phenotypes in two additional *sgs-1* reduction-of-function alleles, one strong *sgs-1* allele (*pk484*) and one weak *sgs-1* allele (*pk867*). We found that there was no difference compared to the background strain for either life span (Figure 4A) or pharyngeal pumping (Figure 4B). This indicates that in these mutants the remaining SGS-1 activity is sufficient for normal function in these processes. Since *sgs-1(pk484)* is the strongest *sgs-1* reduction-of-function allele, it is not likely that any of the other *sgs-1* reduction-of-function alleles will show effects on either life span or pharyngeal pumping. The locomotion rate, however, was reduced in the two *sgs-1* reduction-of-function mutants tested (Figure 4C), indicating that in these *sgs-1* reduction-of-function mutants, SGS-1 activity is not sufficient for wild-type locomotion. The locomotion rate of *sgs-1(pk484)* was significantly less than the locomotion rate of *sgs-1(pk867)*. This confirms that the allelic series showed that *sgs-1(pk484)* is a more severe allele than *sgs-1(pk867)*. Since *sgs-1 (pk867)* is one of the weakest alleles we have, probably all *sgs-1* reduction-of-function alleles will show reduced locomotion rates. Indeed, *sgs-1(pk904)* (R1074K) animals also showed significantly reduced locomotion rates compared to the background strain (results not shown). In mammalian adenylyl cyclases, mutation of the amino acid analogous to R1074 was shown to result in de-

creased binding of $G\alpha_s$, but in normal regulation by $G\alpha_i$ or the adenylyl cyclase activator forskolin (YAN *et al.* 1997a; ZIMMERMANN *et al.* 1998a). This suggests that the reduced locomotion of *sgs-1(pk904)* animals is the result of decreased response to $G\alpha_s$ and thus that $G\alpha_s$ functions together with SGS-1 in locomotion. We did not observe any other obvious defects in development or behavior in the *sgs-1* reduction-of-function alleles.

DISCUSSION

***sgs-1* is an essential gene:** Adenylyl cyclases have complex functions and integrate and respond to diverse extracellular and intracellular signals. Genetic and biochemical evidence indicates that both catalytic domains (C1 and C2) are essential for high enzymatic activity (TANG and GILMAN 1995). In this study, we have isolated a deletion allele of an adenylyl cyclase of *C. elegans*, *sgs-1(pk1279)*, in which part of the nonconserved first catalytic domain (C1b) and the complete second transmembrane (M2) and second catalytic domain (C2) of the SGS-1 protein are removed. Therefore, *pk1279* is likely to be a null allele. Deletion of *sgs-1* results in retarded development and eventually in larval lethality. This larval lethal phenotype is different from the larval lethal phenotype of knockout animals for *gsa-1* (KORSWAGEN *et al.* 1997), the heterotrimeric $G\alpha_s$ subunit that acts directly upstream of SGS-1 (KORSWAGEN *et al.* 1998). This suggests that SGS-1 is not the only downstream target of $G\alpha_s$. Presumably, ACY-2, a second adenylyl cyclase protein in *C. elegans* that shows a more limited expression pattern than *sgs-1*, is one of the other targets of $G\alpha_s$ (KORSWAGEN *et al.* 1998), since *acy-2* null mutant animals have a larval lethal phenotype similar to *gsa-1* null mutants. Two additional genes encode adenylyl cyclases in the *C. elegans* genome, *acy-3* (located on chromosome V, our unpublished observation) and *acy-4* (located on chromosome III, C44F1.5; *C. ELEGANS* SEQUENCING CONSORTIUM 1998). Little is known about the role played by these molecules in $G\alpha_s$ signaling.

***sgs-1* is required for pharyngeal pumping and affects life span:** Pharyngeal pumping is disturbed in homozygous *sgs-1(pk1279)* animals, indicating that SGS-1 is required for proper functioning of the pharynx. SGS-1 is a downstream target of $G\alpha_s$, but it is not known whether

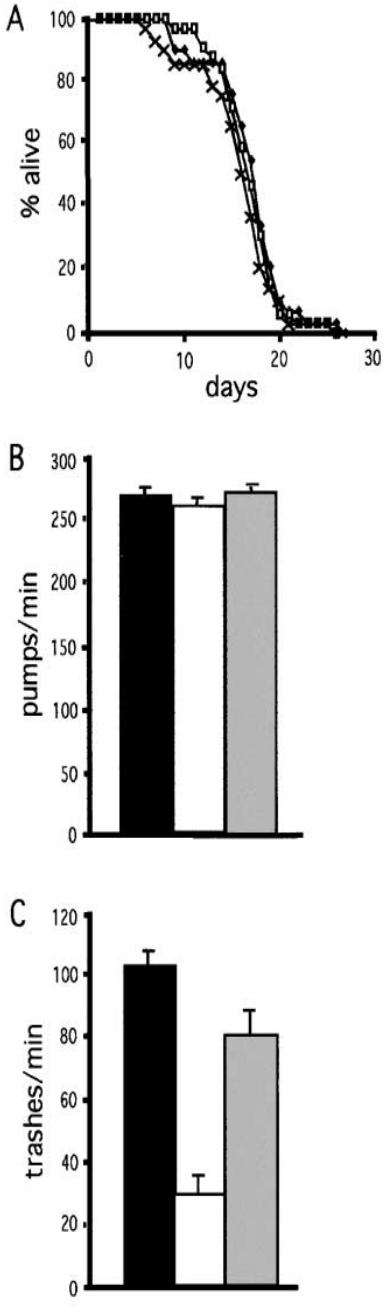

FIGURE 4.—Phenotypes of *sgs-1* reduction-of-function animals. *sgs-1(pk484)* and *sgs-1(pk867)* were backcrossed two times to NL545 (*dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20 (+)*]X), the background strain in which these alleles were generated. (A) The percentage of worms alive on a given day after synchronization: (◆) NL545, (□) *sgs-1(pk484)* animals, and (**X**) *sgs-1(pk867)* animals. Mean life spans ± SEM, with sample size in parentheses, are 17.1 ± 0.7 (29), 17.0 ± 0.6 (32), and 16.0 ± 0.8 (29), respectively. Significance levels were calculated using Student's *t*-test. There was no significant difference between NL545 and *sgs-1(pk484)* ($P = 0.91$) or *sgs-1(pk867)* ($P = 0.14$). (B) Pharnygeal pumping levels of (■) NL545, (□) *sgs-1 (pk484)* animals, and (■) *sgs-1(pk867)* animals were assayed 96 hr after synchronization. Each bar represents the average of 20 determinations. The error bars represent the SEM. Significance levels were calculated using Student's *t*-test. There was no significant difference between NL545 and *sgs-1(pk484)* ($P = 0.076$) or *sgs-1(pk867)* ($P = 0.80$). (C) Movement levels of (■) NL545, (□) *sgs-1(pk484)* animals, and (■) *sgs-1(pk867)* animals were assayed 96 hr after synchronization. Each bar represents the average of 10 animals. The error bars represent the SEM. Differences between the different genotypes for the movement levels were analyzed using Student's *t*-test. NL545 animals are significantly different with respect to movement levels when compared to *sgs-1(pk484)* ($P < 0.001$) or *sgs-1(pk867)* ($P = 0.048$). In addition, *sgs-1(pk484)* animals are significantly different with respect to movement when compared to *sgs-1(pk867)* ($P < 0.001$).

$G\alpha_s$ is also involved in pharyngeal pumping. It is therefore possible that in the pharynx, SGS-1 is regulated by a protein other than $G\alpha_s$. In several homozygous *sgs-1 (pk1279)* animals the molting process was not completed, since a cuticular plug remains attached to the mouth opening. The pharynx plays an important role in the molting process (Singh and Sulston 1978), suggesting that the pharyngeal defects seen in *sgs-1* deletion animals account for the incomplete molting. *sgs-1* reduction-of-function mutants do not have pharyngeal defects, and we do not observe difficulties with molting in these mutants.

We observed that loss of *sgs-1* significantly lengthens life span: *sgs-1(pk1279)* mutants live >50% longer than wild-type animals. Lakowski and co-workers showed that *eat* mutants, which have defects in pharyngeal function, have significantly increased mean and maximum life span because of food restriction (Lakowski and Hekimi 1998). Furthermore, it has been shown that signals from the reproductive system influence the life span of an animal (Hsin and Kenyon 1999). *sgs-1* mutants have both pharyngeal defects and a defective reproductive system, and we therefore suggest that the extended life span is the result of these phenotypes. This is further supported by the fact that *sgs-1* reduction-of-function mutants do not show pharyngeal or reproductive system defects and that their life span is comparable with the background strain. However, it is also possible that *sgs-1* has a direct effect on life span.

SGS-1 and $G\alpha_s$ function together in locomotion: Recent studies have shown that two G-protein α-subunit genes in *C. elegans*, *goa-1* ($G\alpha_o$) and *egl-30* ($G\alpha_q$), are involved in a complex signaling network that regulates locomotion (Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999; Chase *et al.* 2001; Robatzek *et al.* 2001; van der Linden *et al.* 2001). Loss of function of *goa-1* results in increased locomotion rates (Mendel *et al.* 1995; Segalat *et al.* 1995), whereas reduction of function of *egl-30* results in decreased locomotion rates (Brundage *et al.* 1996). These G-protein pathways regulate diacylglycerol levels in an antagonistic manner (Lackner *et al.* 1999; Miller *et al.* 1999), either stimulating or inhibiting acetylcholine release through the diacylglycerol-binding protein, UNC-13. Our results show that there is yet another G-protein signaling cascade involved in locomotion: $G\alpha_s$ signals to the adenylyl cyclase SGS-1 to regulate locomotion. The GOA-1-GL-30 network is shown to act in ventral nerve cord motoneurons (Lackner *et al.* 1999; Miller *et al.* 1999). *sgs-1* and *gsa-1* ($G\alpha_s$) are both expressed in ventral nerve cord motor neurons and in body-wall muscle cells. It is therefore possible that the $G\alpha_s$–SGS-1 pathway acts in parallel to the GOA-1−EGL-30 network in motor neurons, but it is also possible that the $G\alpha_s$–SGS-1 pathway acts downstream of the GOA-1–EGL-30 network in the body-wall muscles. In the latter hypothesis, acetylcholine release at the neuromuscular junction regulated by EGL-30 and GOA-1 possibly results in regulation of $G\alpha_s$ in muscle cells.

***sgs-1* reduction-of-function mutants reveal important residues in the adenylyl cyclase gene:** Several studies have been performed to identify residues that are involved in the catalytic mechanism of adenylyl cyclase and its regulation. However, none of these studies was performed in a complex organism, and in only one study, using the *Dictyostelium discoideum* adenylyl cyclase ACA, were mutations in the transmembrane domains described (Parent and Devreotes 1995). Here, we have used a genetic selection system in a complex organism, *C. elegans*, that identified mutations in both the catalytic and transmembrane domains of the adenylyl cyclase gene. *sgs-1* is an essential gene, and therefore only mutations that do not completely disrupt *sgs-1* function were isolated. Out of the 14 residues that were mutated, 10 residues were located in the catalytic domains. All, except L314, are conserved residues between different adenylyl cyclases. Only 2 of the conserved residues, R1074 and R1216H, were described in other studies, and there it was shown by biochemical analysis that mutation of these residues does not result in gross structural alterations (Dessauer *et al.* 1997; Yan *et al.* 1997a; Zimmermann *et al.* 1998a). Further studies should reveal whether, in the other *sgs-1* reduction-of-function alleles, the reduced *sgs-1* activity is the result of a defect in a specific function of the protein or the result of improper folding or instability of the protein.

Four mutations revealed residues that are important for a normal response to $G\alpha_s$, but that are not located in one of the catalytic domains. Three of these residues (G68, P152, and G181) are located in the first transmembrane domain, and one (E992) is located at the border of the second transmembrane domain and the nonconserved part of the second catalytic domain. Recently, it was shown that the transmembrane domains interact persistently and are involved in two important processes, namely, the proper localization of the protein in the membrane and functional assembly of the two catalytic domains (Gu *et al.* 2001). It is possible that our transmembrane mutants are disturbed in the localization of the adenylyl cyclase gene, as was shown for the Dictyostelium ACA mutants in the transmembrane domain (Parent and Devreotes 1995), but it is also possible that in these mutants the two catalytic domains are not correctly assembled and therefore result in reduced adenylyl cyclase activity. Further studies should reveal, for each of these transmembrane domain mutants, which of these two possibilities is correct. In summary, our study shows not only that *C. elegans* can be used to study gene function, but also that it can be a powerful tool to study protein domain function.

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Chapter 3

Mutations in the nuclear export factor NXF-1 suppress heat-shock promoter-induced phenotypes in *Caenorhabditis elegans*

Mutations in the nuclear export factor NXF-1 suppress heat-shock promoter-induced phenotypes in *Caenorhabditis elegans*

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ABSTRACT

C. elegans NXF-1 is an essential protein that is required for the nuclear export of poly(A)-containing mRNAs. In a screen for suppressors of the neuronal degeneration induced by activated $G\alpha_s$ under the control of a heat-shock promoter, two alleles of *nxf-1* were isolated. In addition to suppressing the heat-shock-induced activated $G\alpha_s$ phenotype, the *nxf-1* mutations also suppressed other phenotypes under heat-shock promoter control. *nxf-1* tagged with green fluorescent protein (GFP) is found in the nucleus of all cells of *C. elegans*. Mutant *nxf-1* tagged with GFP shows a similar expression pattern to wild type *nxf-1* at 20°C, but is differently expressed at 33°C, suggesting that the *nxf-1* alleles are temperature-sensitive. We propose that mutant NXF-1 is in an inactive conformation during heat-shock, and therefore not able to transport the activated $G\alpha_s$ mRNA (or more generally any mRNA) out of the nucleus, resulting in absence of the activated $G\alpha_s$ phenotype after heat-shock.

In the nucleus, newly synthesized precursor mRNAs undergo several processing steps, such as 5' capping, splicing, polyadenylation and 3' end cleavage. They become associated with a multitude of RNA binding proteins, resulting in the formation of an export-competent ribonucleoprotein (mRNP) complex. Transport of mRNP from the nucleus to the cytoplasm occurs through the nuclear pore complex (NPC). Recent studies suggest that interactions between mRNP and NPC are mediated by homologues of the NXF (nuclear export factor) family of putative mRNA export receptors (reviewed in (CONTI and IZAURRALDE 2001; LEI and SILVER 2002; ZENKLUSEN and STUTZ 2001)).

The first evidence for the highly conserved NXF protein family being involved in mRNA export came from a study in *Saccharomyces cerevisiae*. A family member, Mex67, was identified through its genetic interaction with the nuclear pore complex protein nucleoporin Nup85 (SEGREF *et al.* 1997). In the temperature-sensitive *mex67-5* mutant, poly(A)$^+$ RNA accumulates in the nucleus after shifting to the restrictive temperature (SEGREF *et al.* 1997). Mex67 is a nucleocytoplasmic shuttling protein that forms a heterodimer with another protein, Mtr2 (SANTOS-ROSA *et al.* 1998). The Mex67/Mtr2 mRNA export complex can bind to RNA via Mex67 and interacts with NPC via Mtr2 (SANTOS-ROSA *et al.* 1998), and it is thought that this complex drives the mRNP through the nuclear pore.

NXF1 (or TAP) is the human homologue of Mex67 and was first implicated in the nuclear export of the unspliced genomic RNA of simian type D retroviruses (GRÜTER *et al.* 1998; KANG and CULLEN 1999). It was shown that NXF1 interacts directly with the cis-acting constitutive transport element of this retrovirus. In addition to transporting viral RNAs, human NXF1 is now also known to be involved in cellular mRNA export (BRAUN *et al.* 2001; KATAHIRA *et al.* 1999). NXF1 needs to bind a 15 kDa protein (p15, also known as NXT1), which has homology to the nuclear transport factor NTF2, for its activity (BRAUN *et al.* 2001; GUZIK *et al.* 2001; KATAHIRA *et al.* 1999; LEVESQUE *et al.* 2001; WIEGAND *et al.* 2002). p15 performs a similar function to Mtr2 in yeast, although these proteins do not share significant

sequence similarity. Besides NXF1, there are four other NXF proteins (NXF2-5) in humans, and there is one homologue of p15 (NXT2) (HEROLD *et al*. 2000).

In *Drosophila*, four NXF1 members and one p15 homolog were identified (HEROLD *et al*. 2000). NXF1 and p15 are required for mRNA export, whereas NXF2-NXF4 are probably not (HEROLD *et al*. 2000; WILKIE *et al*. 2001). A temperature-sensitive allele of *nxf1* (or *small bristles* (*sbr*)) was identified in a screen for mutants defective in mRNA export (WILKIE *et al*. 2001). Furthermore, it was shown that NXF1 is required for the morphogenesis of several tissues during development (KOREY *et al*. 2001).

In *C. elegans*, two NXF homologues were identified, NXF-1 and NXF-2 (HEROLD *et al*. 2000; SEGREF *et al*. 1997). Using RNA interference experiments, it was shown that NXF-1 is an essential protein that is required for the nuclear export of mRNA in *C. elegans*, but that NXF-2 is not involved in this process (TAN *et al*. 2000). NXF-1 has 42% homology with human NXF-1, and binds directly to RNA and nucleoporins. Furthermore, NXF-1 is able to shuttle between the nucleus and the cytoplasm (TAN *et al*. 2000). Transient expression of NXF-1 in human HeLa cells showed that this protein accumulates in the nucleoplasm and at the nuclear rim (TAN *et al*. 2000). In *C. elegans*, one p15 homolog is identified, NXT-1, that binds to NXF-1 (BLACK *et al*. 1999; HEROLD *et al*. 2000; WIEGAND *et al*. 2002). *C. elegans* NXF-1 could only mediate mRNA export in human cells when *C. elegans* NXT-1 was also expressed (WIEGAND *et al*. 2002).

In this study, we identified *C. elegans* NXF-1 mutants that suppress the phenotype induced by expression of a constitutively active *gsa-1* mutant from a heat-shock promoter. *gsa-1* encodes a heterotrimeric $G_s\alpha$-subunit, and expression of the constitutively active form of *gsa-1* from its own or heat-shock promoter results in vacuolization and degeneration of ventral nerve cord neurons (KORSWAGEN *et al*. 1997). We show that other phenotypes under heat-shock promoter control are also suppressed by the mutations in *nxf-1*. A full length *nxf-1::gfp* fusion that rescues the suppression of the neuronal degeneration is expressed in nuclei of all cells in *C. elegans*, indicating that NXF-1 acts in all cells. Our results suggest that the *nxf-1* mutations are temperature-sensitive, thereby resulting in normal mRNA export at the permissive temperature, but strongly reduced mRNA export at the restrictive temperature (so during heat-shock).

MATERIALS AND METHODS

Nematode strains and culturing: General methods for culture, manipulation and genetics of *C. elegans* were as described (LEWIS and FLEMING 1995). Strains were cultured at 20°C, unless indicated otherwise. DNA transformations by microinjection in *C. elegans* were as described (MELLO and FIRE 1995). Strains used in this study were Bristol N2, CB4856, CB1282 (*dpy-20(e1282)* IV), NL545 (*dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) (KORSWAGEN *et al*. 1997), and NL3178 (*pkIs1392*[*hsp::gfp rol-6(su1006)*]). *nxf-1(pk386)* was generated in an NL545 background as described (KORSWAGEN *et al*. 1998). *nxf-1(pk864)* was isolated in this study. Both *nxf-1(pk386)* and *nxf-1(pk864)* were backcrossed twice to NL545, resulting in respectively NL4004 and NL4003. The following strains were used for mapping: DR102 (*dpy-5(e61) unc-29(e193)* I), CB187 (*rol-6(e187)* II), MT4306 (*lin-39(n1190) unc-36(e251)* III), MT2405 (*ced-3(n17) unc-26(e205)* IV), CB224 (*dpy-11(e224)* V), NL1257 (*dpy-11(e224) unc-42(e270)*; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) and NL1916 (*rol-4(sc8) lin-25(n545) him-5(e1467) unc-76(e911)* V; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X).

Isolation of suppressors of the activated $G\alpha_s$-induced neuronal degeneration: The screen that was used to identify suppressors of the activated $G\alpha_s$ ($G\alpha_s^{QL}$) phenotype was as described (MOORMAN and PLASTERK 2002). In short, L4 NL545 animals were treated with 50 mM EMS in M9 buffer for 4 hr, washed and seeded at 40-60 animals per 9-cm nematode growth medium (NGM) agar plate. The F2 generation was synchronized, and L1 larvae were heat-shocked for 2 hours at 33°C to induce expression of *pkIs296*[*hsp::gsa-1QL*]. The animals that grew out to adults were again

synchronized, and heat-shocked for 2 hours at 33°C as L1 larvae. Animals that did not show neuronal degeneration were collected. *nxf-1(pk386)* and *nxf-1(pk864)* were identified by complementation testing.

Positional cloning of *nxf-1(pk386)*: *nxf-1(pk386)* was mapped to linkage group V. Using three-factor crosses with NL1257 (*dpy-11(e224) unc-42(e270)*; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X), *nxf-1(pk386)* was placed to the right of *unc-42* (13/13 were Sgs(suppressor of activated Gs)Dpy, 0/5 were SgsUnc). Crosses with NL1916 (*rol-4(sc8) lin-25(n545) him-5(e1467) unc-76(e911)* V; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) placed *nxf-1(pk386)* in between *him-5(e1467)* and *unc-76(e911)* (20/20 were RolSgs and 4/18 were RolHimSgs). Subsequent crosses with NL1994 (*pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) that has CB4856 DNA between *him-5(e1467)* and *unc-76(e911)* revealed that *nxf-1(pk386)* is located right of the single nucleotide polymorphism (SNP) pkP1538 at position 23,876 on cosmid F56A12 and left of SNP pkP1539 at position 13,555 on cosmid F23B12. This region contains 13 predicted open reading frames (ORFs). Sequencing of these ORFs in *nxf-1(pk386)* and *nxf-1(pk864)* revealed that both alleles have a mutation in the same ORF, C15H11.3, which encodes NXF-1. The mutation in *nxf-1(pk864)* destroys an *Ava*II restriction site, and can therefore be visualized using restriction digestion: a PCR with primers SNPsgs-2a TTCAGAGTAAGTTCCTGCGG and SNPsgs-2b AACCCACTCATTCTCTCTCG digested with *Ava*II gives one band (~600 bp) when performed on *nxf-1(pk864)* animals, but two bands (~140 bp and ~460 bp) when performed on Bristol N2 wild type animals. Two *nxf-1* genomic constructs, pRP2110 and pRP2112, were able to rescue the suppression of the neuronal degeneration (Table 1). pRP2110 contains a 6 kb *Xho*I/*Sst*I fragment of C15H11 and was injected at 20 μg/ml together with the markers pRF4 (100 μg/ml) (KRAMER *et al*. 1990) and pRP2017 (*gpb-2::gfp*; 50 μg/ml) (VAN DER LINDEN *et al*. 2001) in NL4003 (*nxf-1(pk864)* V; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X) animals, resulting in NL4021 (*nxf-1(pk864)* V; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X; *pkEx1581*[*nxf-1(+) gpb-2::gfp rol-6(su1006)*]). pRP2112, a full length GFP construct (see below), was injected in the same strain at the same concentration (20 μg/ml) together with pRF4 (150 μg/ml), resulting in NL4020 (*nxf-1(pk864)* V; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X; *pkEx1638*[*nxf-1::gfp rol-6(su1006)*]).

Activated $G\alpha_s$-induced neuronal degeneration: The activated $G\alpha_s$-induced pattern of neuronal cell death was measured as described in Korswagen *et al*. (1997). Synchronized L1-L2 larvae were heat-shocked for 2 hours at 33°C and after 24 hours the neuronal degeneration pattern was examined in at least 20 animals using a Nomarski microscope (Zeiss Axioskop 2). The percentage neuronal degeneration was calculated by comparing this number with the total number of degenerated neurons in NL545 (*dpy-20(e1362)* IV; *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] X).

Construction and expression of wild type and mutant full length *nxf-1::gfp*: A 3.9 kb *Xho*I/*Msc*I fragment from C15H11 was cloned in *Sal*I and *Sma*I sites of pPD95.77 (A. Fire, personal communication), resulting in pRP2111. A full length GFP fusion (pRP2112) was constructed by cloning a *Bgl*II/*Sma*I PCR fragment, generated using primers sgs-2gfp2 AAGTTGATGCCAAGTGGCTG and sgs-2gfp1a CTCC**CCCGGG**CATGAGCAAACGCTTCTG CTG (*Sma*I site in bold), into *Bgl*II-*Msc*I fragment of pRP2111. The cloned PCR fragment was sequenced completely and found to be free of errors. pRP2112 (20 μg/ml) was injected together with pMH86 (150 μg/ml) (HAN and STERNBERG 1991) in CB1282 (*dpy-20(e1282)* IV) animals, resulting in two independent transgenic lines, NL4007 (*dpy-20(e1282)* IV; *pkEx1578*[*nxf-1::gfp; dpy-20(+)*]) and NL4008 (*dpy-20(e1282)* IV; *pkEx1579*[*nxf-1::gfp; dpy-20(+)*]). Animals expressing GFP were examined using a Nomarski microscope (Zeiss Axioskop 2). A full length GFP fusion containing the mutation present in *pk864* (pRP2113) or *pk386* (pRP2114) was constructed by cloning a *Bgl*II-*Msc*I PCR fragment, generated using primers seqsgs-2a CAATCGCATCCGTCATTTGG and snpsgs-2b AACCCACTCATTCTCTCTCG on respectively *nxf-1(pk864)* and *nxf-1(pk386)* animals, into *Bgl*II-*Msc*I fragment of pRP2112. The cloned PCR fragments were sequenced completely and found to be free of errors. pRP2113 and pRP2114 were injected at the same concentration as pRP2112 (20 μg/ml) together with pMH86 (150 μg/ml) (HAN and STERNBERG 1991) in CB1282 (*dpy-20(e1282)* IV) animals, resulting in respectively NL4025(*dpy-20(e1282)* IV; *pkEx1583*[*nxf-1(pk864) ::gfp; dpy-20(+)*]) and NL4034 (*dpy-20(e1282)* IV; *pkEx1639*[*nxf-1(pk386) ::gfp; dpy-20(+)*]).

Temperature-sensitivity experiments: Temperature-sensitivity of *nxf-1(pk386)* and *nxf-1(pk864)* animals at 25°C and 27°C was determined as follows. *nxf-1(pk386) pkIs296*[*hsp::gsa-1QL dpy-20(+)*] and *nxf-1(pk864) pkIs296*[*hsp::gsa-1QL dpy-20(+)*] animals were crossed

respectively 2 and 4 times to wild type Bristol N2 animals. Animals without the *pkIs296*[*hsp::gsa-1QL dpy-20(+)*] transgene were selected, resulting in NL3223 (*nxf-1(pk386)* V) and NL4005 (*nxf-1(pk864)* V). L1 larvae of these two strains were placed at 25°C or 27°C, and were followed for phenotypes.

The temperature-sensitivity assay using mutant and wild type full length GFP constructs was done as follows. NL4008 (*dpy-20(e1282)* IV; *pkEx1579*[*nxf-1::gfp; dpy-20(+)*]), NL4034 (*dpy-20(e1282)* IV; *pkEx1639*[*pk386::gfp; dpy-20(+)*]) and NL4025 (*dpy-20(e1282)* IV; *pkEx1583*[*nxf-1(pk864)::gfp; dpy-20(+)*]) L4 animals were picked and examined using a confocal microscope (Leica TCS NT). Subsequently, the animals were heat-shocked for 2 hr at 33°C and examined immediately with a confocal microscope.

RESULTS

nxf-1(pk386) and *nxf-1(pk864)* suppress heat-shock promoter-induced phenotypes

Expression of a constitutively active *gsa-1* mutant from its own or heat-shock promoter results in vacuolization and degeneration of ventral nerve cord neurons (KORSWAGEN *et al.* 1997). *gsa-1* encodes a heterotrimeric $G_s\alpha$-subunit, and previously it was shown that mutations in an adenylyl cyclase gene, *sgs-1*, suppress the neuronal degeneration phenotype (KORSWAGEN *et al.* 1998). One other locus (previously called *sgs-2* (KORSWAGEN *et al.* 1998)) was identified that can be mutated to suppress the neuronal degeneration phenotype induced from heat-shock promoter. The two alleles that have mutations in this locus, *pk386* and *pk864*, were isolated at a very low frequency of ~1 in 500,000 mutagenized genomes, and both alleles are recessive for the suppression of the activated $G\alpha_s$ phenotype. The mutant animals are homozygous viable and do not show obvious defects in behavior or development. In addition to suppressing the heat-shock promoter-induced activated $G\alpha_s$ phenotype (Figure 1A and B), the alleles also suppress heat-shock promoter-induced GFP expression (Figure 1C and D) and partially suppress the lethargic movement induced by expression of activated $G\alpha_o$ from a heat-shock promoter (results not shown) (MENDEL *et al.* 1995). This suggests that this locus is not a specific downstream target of $G\alpha_s$, but rather a general suppressor of heat-shock promoter-induced phenotypes.

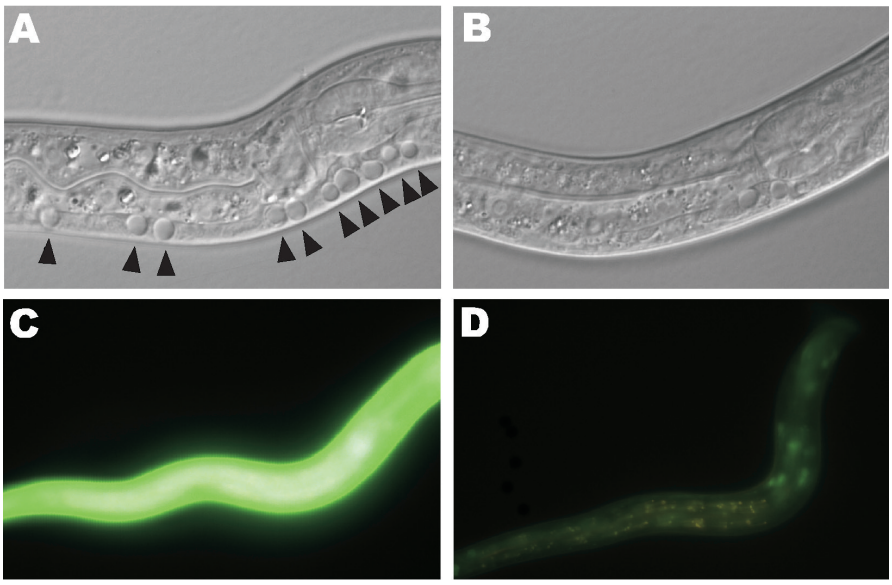

Figure 1. *pk864* animals suppress heat-shock promoter-induced phenotypes. (A) *pkIs296* [*hsp::gsa-1QL*] animal shows neuronal degeneration (arrowheads) after induction of activated $G\alpha_s$ by 2 hours heat-shock at 33°C. (B) *pk864 pkIs296* [*hsp::gsa-1QL*] animal does not show neuronal degeneration after 2 hours heat-shock at 33°C. (C) *pkIs1392* [*hsp::gfp*] animal shows strong GFP expression directly after a 2 hr heat-shock at 33°C. (D) *pk864 pkIs1392*[*hsp::gfp*] animal shows hardly any GFP expression directly after a 2 hr heat-shock at 33°C. Picture C and D are taken with 2300 millisecond (ms) exposure time.

The mutation in *pk386* was mapped to chromosome V, and placed between markers *him-5* and *unc-76* (Figure 2A). Further fine-mapping was performed by using the polymorphic strain CB4856, placing the mutation right of the single nucleotide polymorphism (SNP) pkP1538 at position 23,876 on cosmid F56A12 and left of SNP pkP1539 at position 13,555 on cosmid F23B12. Sequencing of all 13 ORFs in this region revealed that both *pk386* and *pk864* contained a mutation in the same gene, *nxf-1*. *nxf-1* belongs to a family of nuclear export factors that are involved in mRNA transport out of the nucleus (HEROLD *et al*. 2000; SEGREF *et al*. 1997; TAN *et al*. 2000). Two genomic subclones, pRP2110 and pRP2112, containing wild type *nxf-1* (Fig. 2B), were able to rescue *nxf-1(pk864)* (Table 1). This indicates that indeed the *nxf-1* mutations are responsible for the suppression of the heat-shock promoter-induced neuronal degeneration.

NXF-1 is a 628 amino acid protein, that has a ribonucleoprotein (RNP) domain, two leucine-rich repeats (LRR), an NTF2-like domain and a nuclear pore complex (NPC) binding domain (Figure 2C). In *nxf-1(pk386)*, a conserved glutamic acid at position 287 is changed to lysine. This amino acid is located in the second LRR of NXF-1 (Figure 2C). In *nxf-1(pk864)*, a nonconserved glycine at position 379 is changed to arginine. This amino acid is located in the NTF2-like domain of NXF-1 (Figure 2C). Since NXF-1 is known to be involved in mRNA transport out of the nucleus (TAN *et al*. 2000; WIEGAND *et al*. 2002), it is likely that the *nxf-1* mutant animals are disturbed in this process. Thus, *nxf-1* animals probably do not show heat-shock promoter-induced phenotypes because mRNAs produced during heat-shock are not (or less efficiently) transported out of the nucleus.

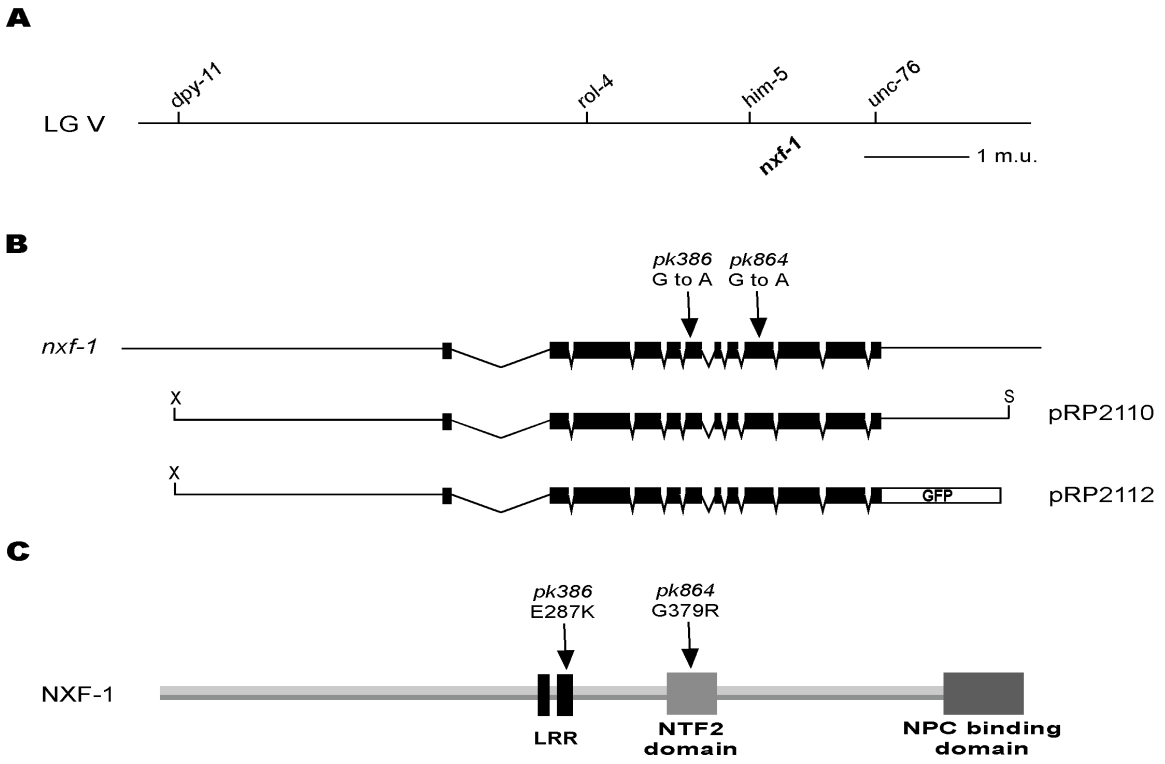

Figure 2. *pk386* and *pk864* have mutations in *nxf-1*. (A) Genetic map of linkage group V, showing the location of *pk386* and *nxf-1* and the markers used to map. (B) Structure of *nxf-1* gene. Arrows show the location of the mutated bases found in *nxf-1(pk386)* and *nxf-1(pk864)*. Two rescuing constructs, pRP2110 and pRP2112, are indicated. X, *Xho*I; S, *Sst*I; GFP, Green Fluorescent Protein. (C) Structure of NXF-1 protein. The leucine rich repeats (LRR), the NTF2-like domain and the nuclear pore complex (NPC) binding domain are shown. Arrows indicate the location of the changed amino acids in the alleles *nxf-1(pk386)* and *nxf-1(pk864)*.

TABLE 1

***nxf-1* mutations suppress activated Gα_s-induced neural degeneration**

genotype	transgene	mutation	neural degeneration (%)
N2 (wild type)	-		0
pkIs296[*hsp-gsa1QL*]	-		100
nxf-1(pk386); *pkIs296*[*hsp-gsa1QL*]	-	E287K	14
nxf-1(pk864); *pkIs296*[*hsp-gsa1QL*]	-	G379R	15
nxf-1(pk864); *pkIs296*[*hsp-gsa1QL*]	*pkEx1581*[*nxf-1(+)*]		94
nxf-1(pk864); *pkIs296*[*hsp-gsa1QL*]	*pkEx1638*[*nxf-1::gfp*]		96

Neuronal degeneration induced by activated Gα_s under heat-shock promoter control as described in Material and Methods. Phenotype was scored in 20 L1-L2 larvae for each genotype 24 hr after a 2 hr heat-shock at 33°C. Mean total numbers of degenerated neurons ± SEM, with sample size in parentheses, are 23.2 ± 0.7 (20) for NL545 *pkIs296*[*hsp::gsa-1QL*]; 3.4 ± 0.5 (20) for *nxf-1(pk386)*; 3.5 ± 0.6 (20) for *nxf-1(pk864)*; 21.8 ± 0.7 (20) for *nxf-1(pk864) pkEx1581*[*nxf-1(+)*]; and 22.3 ± 1.0 (20) for *nxf-1(pk864) pkEx1638*[*nxf-1::gfp*].

nxf-1 shows a ubiquitous and nuclear expression pattern

A fusion of full length *nxf-1*, including 1.6 kb promoter sequence, with GFP was constructed to analyze *nxf-1* expression (Figure 2B). Because this full length GFP fusion is able to rescue the suppression of the neuronal degeneration (Table 1), it is likely that the observed expression pattern reflects the expression of the endogenous *nxf-1* gene. Animals transgenic for this *nxf-1::gfp* fusion showed GFP expression in virtually every cell and tissue of *C. elegans* (Figure 3A). Expression begins during embryogenesis (Figure 3B), and continues during all larval stages and adulthood. As is shown in Figure 3C, *nxf-1* is expressed in ventral nerve cord neurons, which are susceptible to the activated Gα_s-induced degeneration. Furthermore, we always observe high expression in intestinal cells (Figure 3C). The intestinal cells undergo endoreduplication during larval growth to reach a DNA content of 32n and they are responsible for the production of all yolk protein (HEDGECOCK and WHITE 1985; KIMBLE and SHARROCK 1983; WOOD *et al.* 1985). Consequently, a high amount of mRNA has to be transported out of the nucleus in these cells, which may explain the high levels of NXF-1. Previously, transient expression of an *nxf-1* full length GFP fusion in human HeLa cells showed that NXF-1 was found predominantly in the nucleus, where it accumulated in the nucleoplasm but not in the nucleoli (TAN *et al.* 2000). In addition, Tan *et al.* observed that NXF-1 was localized at the nuclear rim. In *C. elegans*, NXF-1 accumulates in the nucleus as well (Figure 3), and we also observed localization at the nuclear rim. This nuclear rim expression is most clear in the large intestinal nuclei (Figure 3D and 4A), but is also seen in nuclei of other tissue. We did not observe exclusion of NXF-1 from the nucleolus (Figure 3D). However, we cannot exclude that the nucleolar localization is the result of overexpression of the NXF-1::GFP fusion protein.

nxf-1 mutations are temperature-sensitive

Previously, it was shown that functional knockdown of *nxf-1* by using RNAi was lethal for both embryos and adult animals (TAN *et al.* 2000). *pk386* and *pk864* animals are viable and do not show any obvious defects in development or morphogenesis. This strongly suggests that the mutations in *pk386* and *pk864* are not complete loss-of-function mutations. So, what is the nature of these mutations? One possibility is that the mutations are reduction-of-function mutations, thereby reducing (but not abolishing) the total level of exported mRNAs in these

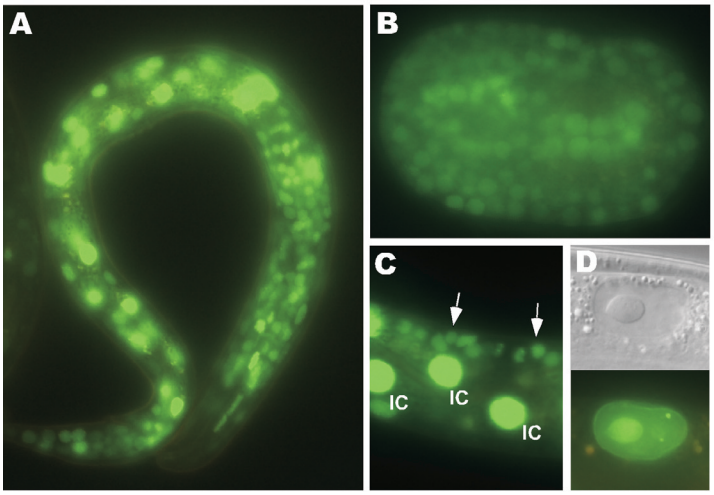

Figure 3. Expression pattern of *nxf-1* as analyzed using a full length GFP gene fusion. (A) Overview of *nxf-1* expression in an L2 larva. *nxf-1* shows nuclear expression in all cells and tissues of *C. elegans*. Similar expression is seen in other stages. (B) *nxf-1* is expressed in the early embryo. (C) Expression of *nxf-1* in ventral nerve cord (VNC) neurons (arrows show group of VNC neurons), and intestinal cells (IC). (D) NXF-1 accumulates in the nucleus, and is also located at the nuclear rim. Shown is a Nomarski image (upper panel) and fluorescence image (lower panel) of the same intestinal cell. Other cell types show similar localization.

animals. It is also possible that the mutations only affect export of heat-shock mRNAs. However, the fact that the two mutations are located in different domains of the protein makes this latter possibility less likely. Another possibility is that *pk386* and *pk864* are temperature-sensitive alleles, so that during the heat-shock at 33°C (the restrictive temperature) the NXF-1 protein is in an inactive conformation, and cannot export mRNAs (including the newly produced heat-shock promoter mRNAs). In both *S. cerevisiae* and *D. melanogaster*, temperature-sensitive alleles of *nxf* homologues were identified (SEGREF *et al.* 1997; WILKIE *et al.* 2001).

One method to test whether the *nxf-1* mutants are temperature-sensitive is to raise these mutants at the restrictive temperature and examine whether their phenotype is different from wild type animals raised at the restrictive temperature and/or from *nxf-1* mutant animals raised at normal temperature (20°C). Unfortunately, it is not possible to grow *C. elegans* at 33°C, the temperature that is used for heat-shock and that is expected to be the restrictive temperature, for longer periods of time. Therefore, we cannot directly test temperature-sensitivity at 33°C. When raised at 25°C, *nxf-1(pk386)* and *nxf-1(pk864)* animals did not show a temperature-sensitive phenotype. *nxf-1(pk386)* and *nxf-1(pk864)* animals that were placed at 27°C as L1 larvae showed a temperature-sensitive phenotype: they became completely sterile, whereas wild type Bristol N2 animals still produced progeny at that temperature (data not shown). However, we were not able to rescue this phenotype by introduction of wild type *nxf-1*, and thus cannot exclude that this phenotype is caused by other (linked) mutations.

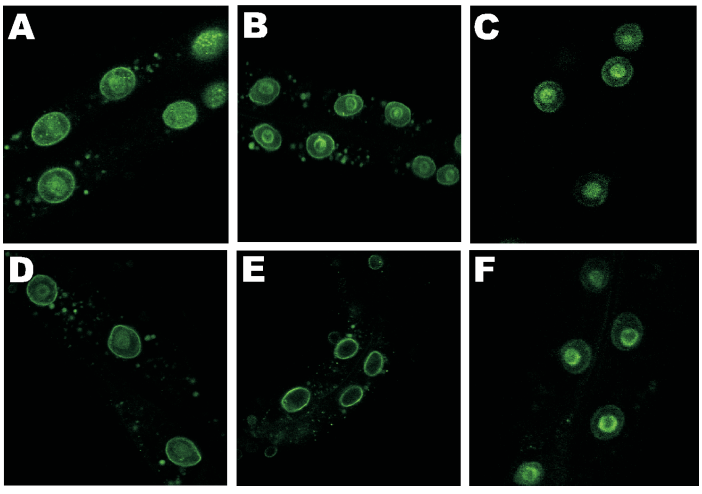

Figure 4. Localization of mutant and wild type NXF-1 at different temperatures. (A) Wild type *nxf-1* shows a general nuclear expression pattern at 20°C. (B) *nxf-1(pk386)::gfp* and (C) *nxf-1(pk864)::gfp* are similarly expressed to wild type *nxf-1::gfp* at 20°C. (D) Wild type *nxf-1* is similarly expressed after 2 hr at 33°C compared to at 20°C. (E) *nxf-1(pk386)::gfp* shows predominantly expression at the nuclear rim after 2 hr heat-shock at 33°C. (F) *nxf-1(pk864)::gfp* shows a similar expression pattern at 33°C to wild type *nxf-1* at 33°C. All pictures show intestinal cells, but other cell types show similar expression.

Segref *et al.* showed that a temperature-sensitive mutation in the yeast homologue of NXF-1, Mex67, results in misexpression of this protein at the restrictive temperature, but not at the permissive temperature (SEGREF *et al.* 1997). To address the possible temperature-sensitivity of *nxf-1(pk386)* and *nxf-1(pk864)*, we constructed fusions of full length *nxf-1* containing the *pk386* or *pk864* mutation with GFP. Animals that overexpress *nxf-1(pk386)::gfp* show a similar expression pattern to wild type *nxf-1::gfp* at 20°C (Figure 4A and B). However, directly after a 2 hr heat-shock at 33°C, *nxf-1(pk386)::gfp* shows a different expression pattern than wild type *nxf-1::gfp*: all *nxf-1(pk386)::gfp* is localized at the nuclear rim, whereas wild type *nxf-1::gfp* is localized at the nuclear rim, in the nucleoplasm and around the nucleolus, as at 20°C (Figure 4D and E). This result suggests that the mutant NXF-1 protein is temperature-sensitive and has a different (inactive) conformation to wild type NXF-1 at 33°C. *nxf-1(pk864)::gfp* shows a similar expression pattern to wild type *nxf-1::gfp* at 20°C and 33°C (Figure 4E and F), although the expression at the nuclear rim might be slightly reduced at 33°C. Since *nxf-1(pk386)* and *nxf-1(pk864)* are similar with respect to the suppression of the neuronal degeneration induced by activated Gα_s, it is likely that *nxf-1(pk864)* is also a temperature-sensitive allele. We propose that mRNAs that are produced during heat-shock are not exported out of the nucleus in *nxf-1(pk386)* and *nxf-1(pk864)* animals due to a conformational change in the mutant NXF-1.

DISCUSSION

In this study we report the identification of two mutations in the nuclear export factor NXF-1 of *C. elegans*. The first mutation changes a conserved glutamic acid at position 287 in the Leucine-rich repeat (LRR) into a lysine. LRR domains are short sequence motifs that are thought to be involved in protein-protein interactions (KOBE and DEISENHOFER 1994). The LRR domain of human NXF1 is essential for binding the CTE viral RNA, but not for general RNA binding affinity, binding to nucleoporins and localization to the nuclear rim (BRAUN *et al.* 2001; BRAUN *et al.* 1999; HO *et al.* 2002). Deletion of the LRR domain completely abolishes mRNA export, suggesting that this domain binds to one or more (unidentified) cellular proteins, and that this binding is essential for export of cellular mRNA (BRAUN *et al.* 2001). Recently, it has been shown that NXF members interact with several proteins, including REF/Aly and Y14, that are part of a complex that is deposited by the spliceosome at exon-exon junctions (KATAOKA *et al.* 2000; RODRIGUES *et al.* 2001; STRASSER and HURT 2000, STUTZ *et al.* 2000). It is thought that these proteins recruit NXF to cellular mRNA and that this is mediated by the N-terminal domain of NXF including the LRR domain (STRASSER and HURT 2000, STUTZ *et al.* 2000). In *C. elegans*, homologues of the REF family are present (STUTZ *et al.* 2000), and these might mediate binding to mRNA via the N-terminal domain of NXF-1 as well. This raises the possibility that the mutation in the LRR of *C. elegans* NXF-1 interferes with recruitment to cellular mRNA by these *C. elegans* homologues. The observation that the *C. elegans* NXF-1 E287K is predominantly localized at the nuclear rim at the restrictive temperature, whereas wild type NXF-1 is not, is in agreement with this hypothesis. The NXF-1 E287K protein is expected to bind normally to NPC components, since the LRR is not important for this process, but, when bound, may no longer be able to be released. This would result in localization of the mutant protein at the nuclear rim and in a strong reduction in mRNA export.

The second mutation in NXF-1 changes glycine 379 to an arginine. Glycine 379 is located in the NTF2-like domain of NXF-1. The NTF2-like domain in NXF proteins is known to mediate heterodimerization with NXTs and is essential for the export of cellular mRNA (BACHI *et al.* 2000; SUYAMA *et al.* 2000). Recently, it has been demonstrated that this heterodimer has a single nucleoporin-binding site located at the NXF side of the heterodimer

(FRIBOURG *et al.* 2001) and that the sole function of this heterodimer is to bind to nucleoporins (BRAUN *et al.* 2002). Probably, the temperature-sensitive mutation in the NTF2-like domain results in decreased affinity of NXF-1 for NXT-1 at the restrictive temperature, and thereby in strongly reduced binding to nucleoporins and diminished mRNA export. This hypothesis is in agreement with the observation that NXF-1 G379R might have reduced localization at the nuclear rim at the restrictive temperature. *C. elegans* NXT-1 is, like NXF-1, an essential protein, since RNAi against NXT-1 results in 100% embryonic lethality (FRASER *et al.* 2000). It is therefore not possible to test whether NXT-1 knockdown is able to suppress the activated $G\alpha_s$-induced neuronal degeneration.

The *C. elegans* genome contains one additional NXF member, NXF-2 (HEROLD *et al.* 2000; SEGREF *et al.* 1997; TAN *et al.* 2000). NXF-2 misses the NPC-binding domain, which is essential for mRNA export (HEROLD *et al.* 2000). RNAi against NXF-2 did not result in nuclear accumulation of mRNA or any other phenotype (TAN *et al.* 2000), indicating that NXF-2 is probably not involved in mRNA export. We showed that *nxf-1* is expressed in all tissues and cells of *C. elegans*, and therefore it is likely that NXF-1 is responsible for all mRNA export in *C. elegans*. The identification of mutations in NXF-1 that suppress heat-shock promoter-induced phenotypes provides a mechanism to conditionally overexpress a certain protein in a specific tissue. NXF-1 mutant animals that express wild type NXF-1 in a specific tissue (by cloning wild type NXF-1 under a tissue-specific promoter) can only export mRNA in the tissue with wild type NXF-1 during heat-shock. So, if a transgene with a gene of interest under heat-shock promoter control is present, then mRNA of this gene (produced during heat-shock) will only be exported from the nucleus in the tissue with wild type NXF-1. In this way, a gene is conditionally (e.g. after heat-shock) expressed in a specific tissue.

In conclusion, we have identified mutations in the nuclear export factor NXF-1 that is essential for mRNA export out of the nucleus in *C. elegans*. These mutations in NXF-1 are likely to be temperature-sensitive and to suppress heat-shock promoter-induced phenotypes by preventing the export of mRNAs that are produced during heat-shock.

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Chapter 4

Hyperactivation of the G_{12}-mediated signaling pathway in *Caenorhabditis elegans* induces a developmental growth arrest via protein kinase C

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Hyperactivation of the G_{12}-Mediated Signaling Pathway in *Caenorhabditis elegans* Induces a Developmental Growth Arrest via Protein Kinase C

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Summary

The G_{12} type of heterotrimeric G-proteins play an important role in development and behave as potent oncogenes in cultured cells [1–5]. However, little is known about the molecular nature of the components that act in the G_{12}-signaling pathway in an organism. We characterized a *C. elegans* Gα subunit gene, *gpa-12*, which is a homolog of mammalian $G_{12}/G_{13}\alpha$, and found that animals defective in *gpa-12* are viable. Expression of activated GPA-12 (G_{12}QL) results in a developmental growth arrest caused by a feeding behavior defect that is due to a dramatic reduction in pharyngeal pumping. To elucidate the molecular nature of the signaling pathways in which G_{12} participates, we screened for suppressors of the G_{12}QL phenotype. We isolated 50 suppressors that contain mutations in *tpa-1*, which encodes two protein kinase C isoforms, TPA-1A and TPA-1B, most similar to PKCθ/δ. TPA-1 mediates the action of the tumor promoter PMA [6]. Expression of G_{12}QL and treatment of wild-type animals with PMA induce an identical growth arrest caused by inhibition of larval feeding, which is dependent on TPA-1A and TPA-1B function. These results suggest that TPA-1 is a downstream target of both G_{12} signaling and PMA in modulating feeding and growth in *C. elegans*. Taken together, our findings provide a potential molecular mechanism for the transforming capability of G_{12} proteins.

Results and Discussion

The *C. elegans* homolog of mammalian $G_{12}/G_{13}\alpha$, GPA-12, is expressed throughout development in pharyngeal muscle cells that control pumping of the pharynx (Figure 1A) and in hypodermal cells (Figure 1B). We isolated a loss-of-function *gpa-12(pk322)* mutant [7] in which 2 kb of the *gpa-12* coding sequence is deleted. Since the deletion includes the regions needed for GTP binding and hydrolysis, *pk322* is likely a null allele. Unlike the $G_{12}/G_{13}\alpha$ homolog Concertina in *Drosophila* [4] and $G_{13}\alpha$ in mice [5], the inactivation of *gpa-12* does not cause embryonic lethality. This raises the possibility that GPA-12 is redundant with other Gα subunits of *C. elegans*, as demonstrated for $G_{12}\alpha$ and $G_q\alpha$ in mice [8]. Since GPA-12 is highly expressed in the pharynx, we quantified the pharyngeal pumping rate in *gpa-12* mutants. Mutations that affect pumping cause feeding behavior defects and a delay in growth [9]. However, we observed no significant difference in the rate of pumping (Figure 2A) or growth rate (data not shown) of *gpa-12* mutants compared to wild-type. Furthermore, no disorganization of the hypodermis is seen in *gpa-12* mutants, as demonstrated by normal expression of a *jam-1::gfp* reporter construct in hypodermal cells [10] (data not shown).

Because no phenotypic defects are observed in *gpa-12* mutants, we analyzed the effect of *gpa-12* gene dosage on behavior. Therefore, we generated transgenic animals that overexpress either wild-type or activated GPA-12. To construct the activated form of GPA-12, we changed a glutamine (Q) at position 205 to a leucine (L), which is analogous to the Q229L mutation in mammalian activated $G_{12}\alpha$. Overexpression was accomplished by using transgenes that carry a multicopy array of the *gpa-12* wild-type gene or the constitutively active *gpa-12* mutant gene under control of its endogenous promoter or a heat shock promoter (*hsp*). Interestingly, when we heat shocked animals that carry a chromosomal integrated transgene overexpressing activated GPA-12 (*pkIs1330*[*hsp::gpa-12^{QL}*]) for 2 hr at an early larval stage, we found that all animals arrest their developmental growth (Figure 2B) but eventually recover. Similar results were found for another independently integrated transgene, *pkIs1355*[*hsp::myc:gpa-12^{QL}*], which contains a Myc-tag fused to the active form of GPA-12 (data not shown). Also, animals carrying an extrachromosomal array of the constitutively activated *gpa-12* gene under control of its endogenous promoter, *pkEx1467*, arrest their growth (see the Supplemental Experimental Procedures available with this article online). In contrast to *pkIs1330* and *pkIs1355* animals, these developmentally arrested larvae could not recover to adulthood and ultimately died. No growth arrest or reduced pumping (see below; Figure 2C) was observed in animals overexpressing wild-type GPA-12 from its endogenous promoter (*gpa-12::gpa-12^{WT}*) or a heat shock promoter (*hsp::gpa-12^{WT}*). Thus, while GPA-12 is not essential, overexpression of activated GPA-12 causes an arrest of larval growth.

Based on the expression of GPA-12 in the pharynx, we determined whether reduced pharyngeal pumping causes the growth arrest induced by activated GPA-12 (G_{12}QL). As shown in Figure 2C, heat-shocked *pkIs1330* [*hsp::gpa-12^{QL}*] transgenic larvae exhibit slow and irregular pumping, whereas wild-type larvae pump normally after heat shock. Slow pumping is not caused by structurally defective pharyngeal muscle, because these *pkIs1330* animals completely recover from the growth arrest and do not show any pharyngeal defects. As a result of slow pumping, almost no food is taken up by the pharynx in *pkIs1330* larvae, as can be visualized by staining bacteria in the gut of these animals with the lipophilic fluorescent dye (DiO) [11] (Figure S1). Taken together, these data indicate that the arrest of larval growth is caused by a feeding defect.

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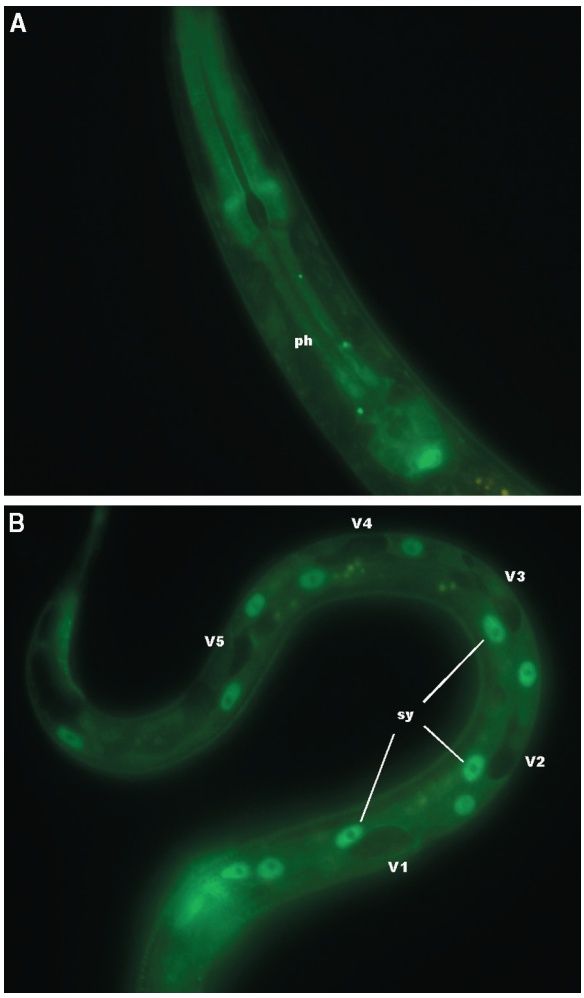

Figure 1. Expression of *gpa-12* in *C. elegans* as Detected by Using a Translational GFP Gene Fusion

(A and B) pRP2018 contains the full-length wild-type *gpa-12* sequence fused to *gfp*. (A) The head region of an L2-staged larva showing expression of GPA-12 in the pharyngeal muscle cells (ph). (B) L1 larva showing expression in the cytoplasm and nuclei of the hyp7 syncytium (sy), which comprises the main body hypodermis, and in additional cells, which are presumably the P cells (precursors of motorneurons in the ventral nerve cord). No expression is detected in hypodermal seam cells (V1–V6 cells). The nuclear localization of the *gpa-12::gfp* reporter construct (pRP2018) may reflect the endogenous GPA-12 expression, since the *gfp*-reporter vector used does not contain a nuclear localization signal. We observed an identical expression pattern of GPA-12 by using another *gpa-12::gfp* reporter construct (pRP2015; see the Supplemental Experimental Procedures), including nuclear localization.

To study the molecular nature of the components involved in G_{12}-mediated signaling, we performed genetic screens to isolate extragenic mutations that suppress the activated GPA-12 (G_{12}QL)-induced growth arrest. In total, 50 independent mutations were isolated that suppress the G_{12}QL-induced growth arrest after heat shock treatment (2 hr at 33°C); 44 mutations were caused by the use of ethylmethane-sulfonate (EMS) as a mutagen, and 6 were caused by using a transposon insertion method [12]. By means of the latter approach, we found that four suppressors contained a Tc1 transposon insertion in the coding region of the *tpa-1* gene. Sequence analyses revealed that all EMS-derived suppressors also contain a mutation in the *tpa-1* gene; most mutations change conserved amino acid residues or result in premature stops (Figure 3A; Table S1). *tpa-1* encodes two isoforms of protein kinase C (PKC), TPA-1A and TPA-1B, which are most similar to the "novel" PKCθ/δ [13]. All isolated mutations disrupt both isoforms of TPA-1, whereas none are located in the first four exons of *tpa-1A*. In a separate mutagenesis screen in which shorter heat shock treatment (5 min) was used, we isolated two alleles of *tpa-1*, *pk1584* and *pk1585*, that have a stop in *tpa-1A*, thereby disrupting TPA-1A, but not TPA-1B (Figure 3A). These mutations only moderately suppress the growth arrest induced by 2 hr of heat shock (Table S1). Introduction of *tpa-1* wild-type genomic sequence rescued *tpa-1(pk1401)* mutants, resulting again in developmentally arrested animals after the heat shock-induced G_{12}QL expression of *pkIs1330*. Moreover, reduced pumping induced by G_{12}QL is completely suppressed by *tpa-1(pk1401)* mutants (Figure 2C). Importantly, *tpa-1* mutations do not affect GPA-12 expression, but rather act on GPA-12 signaling (see the Supplemental Experimental Procedures). Together, these data indicate that both TPA-1A and TPA-1B are involved in the growth arrest induced by G_{12}QL.

Mutations in *tpa-1* were first identified as suppressors of the developmental growth arrest induced by the tumor-promoting phorbol ester PMA [6]. To further investigate whether the *tpa-1* mutations isolated in this study are able to suppress the growth arrest induced by PMA, we analyzed all suppressors of the G_{12}QL phenotype in the presence of 0.1–1 μg/ml PMA, a concentration range commonly used to activate PKCs. Most homozygous mutations in *tpa-1* that disrupt both isoforms of TPA-1 cause resistance to the PMA-induced growth arrest compared to wild-type and non-heat-shocked *pkIs1330* [*hsp::gpa-12*^{QL}] control animals (Figure 4; Table S1). Only 2 *tpa-1* alleles, *pk1478* and *pk1517*, of the 48 isolated *tpa-1* suppressor mutations are sensitive to 1 μg/ml PMA but are resistant to lower PMA concentrations compared to non-heat-shocked *pkIs1330* control animals (Figure 4). Animals with mutations in *tpa-1* that disrupt TPA-1A, but not TPA-1B (*pk1584* and *pk1585*), are moderately resistant to the developmental growth arrest induced by 1 μg/ml PMA (Table S1). These data suggest that both TPA-1A and TPA-1B are required for the growth arrest induced by PMA.

We observed that both PMA and activated GPA-12 (G_{12}QL) induce a growth arrest that is mediated by TPA-1A and TPA-1B. After treatment with PMA for 6 hr, wild-type larvae pump slowly (94 ± 8 pumps/min), as they do following heat shock-induced expression of G_{12}QL, and this is suppressed by *tpa-1(pk1401)* (206 ± 9 pumps/min). This suggests that both PMA and GPA-12 act via TPA-1 in the pharynx to induce a growth arrest. To test this hypothesis, we cloned *tpa-1A* and *tpa-1B* cDNA under the control of the *myo-2* promoter; *myo-2* encodes a myosin heavy chain that is specifically expressed in the pharyngeal muscle. Indeed, specific expression of wild-type TPA-1A and TPA-1B in the pharynx in a *tpa-1* background results in the inhibition of larval growth after heat shock-induced G_{12}QL expression of *pkIs1330*[*hsp::gpa-12*^{QL}] animals or treatment with PMA (Table 1). Transgenic animals carrying *tpa-1B* cDNA un-

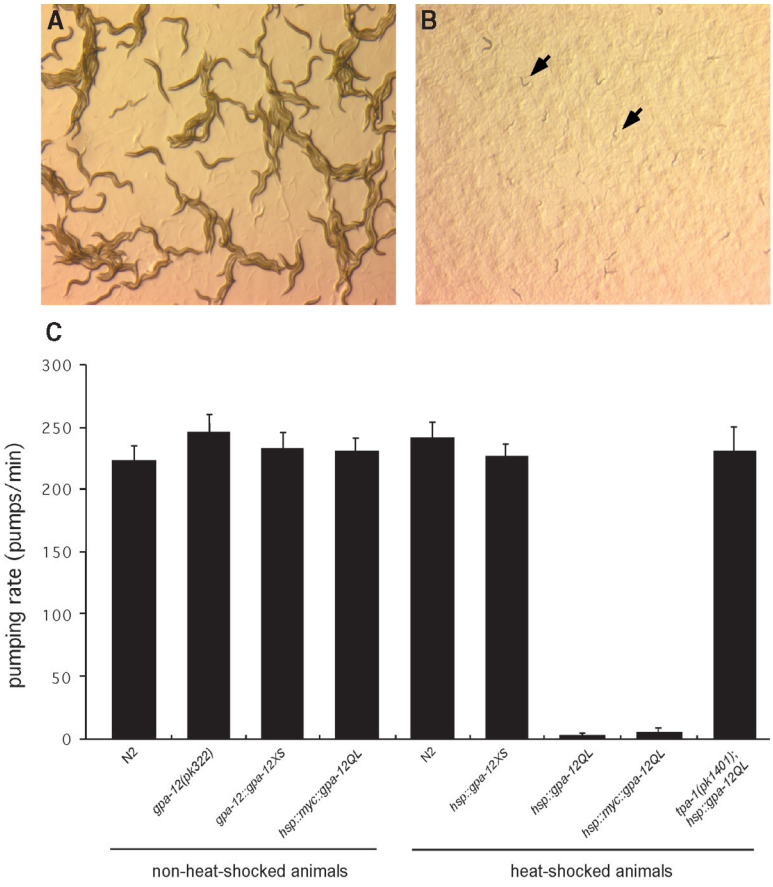

Figure 2. Transgenic Overexpression of Activated GPA-12 Induces Severely Reduced Pumping that Causes a Developmental Growth Arrest

(A and B) (A) In the presence of a heat shock treatment, growth of N2 (wild-type) animals is not inhibited and all larvae reach adulthood, (B) whereas *pkIs1330* transgenic animals that overexpress G_{12}QL show a developmental growth arrest, as indicated by the young larvae (arrows). Animals were synchronized, heat shocked for 2 hr at the L1 larval stage, and photographed 3 days later. Pictures were taken at the same magnification.

(C) The pharyngeal pumping rate is decreased to almost zero in animals after heat shock-induced expression of G_{12}QL using two independently integrated transgenes: *pkIs1330* [*hsp::gpa-12*^{QL}] and *pkIs1355*[*hsp::myc::gpa-12*^{QL}]. Both *pkIs1330* and *pkIs1355* contain multiple copies of the constitutive active *gpa-12* gene under control of a heat shock promoter (*hsp*); *pkIs1355* also contains a Myc-tag fused to the active form of GPA-12. The pumping activity is not affected in *gpa-12(pk322)* mutants compared to N2 (wild-type) animals. Normal pumping activity is also observed in multiple transgenic lines that overexpress wild-type GPA-12 from a transgene carrying a multicopy array, either extrachromosomal or integrated, of the wild-type *gpa-12* gene under control of its endogenous (*gpa-12::gpa-12*^{WT}) or heat shock promoter (*hsp:::gpa-12*^{WT}); in each case, data is shown for one transgenic line. Mutations in *tpa-1* suppress the pharyngeal pumping defect after heat shock treatment of *pkIs1330* animals. The rate of pumps is quantified in at least 20 larvae with or without heat shock treatment for each strain (see the Supplemental Experimental Procedures). The error bars indicate the 95% confidence interval of the mean.

der control of a hypodermal promoter (*dpy-7*) did not show a growth arrest, but resulted in cuticle defects (data not shown). Thus, the growth arrest is caused by activation of TPA-1 in the pharynx. This is further confirmed by the fact that expression of G_{12}QL from the *myo-2* promoter (*myo-2::gpa-12*^{QL}) is also able to induce a developmental growth arrest (see the Supplemental Experimental Procedures). The growth arrest of *myo-2::gpa-12*^{QL} transgenic animals is restored in a *tpa-1* suppressor background. We found one homozygous wild-type animal of the progeny of heterozygous *tpa-1* suppressor animals that contained the *myo-2::gpa-12*^{QL} transgenic array and survived to adulthood. Interestingly, this mosaic transgenic animal did not express the coinjected *gpb-2::gfp* reporter construct in the pharynx, where it is normally expressed [14], indicating that the *myo-2::gpa-12*^{QL} array has been lost in this lineage. Altogether, these data support a role for PMA and activated GPA-12 in the pharynx in regulating feeding behavior and therefore indirectly the growth of *C. elegans*.

Since the phorbol ester PMA directly interacts with and mediates the action of TPA-1, we examined whether the effects of PMA require the function of GPA-12. However, unlike *tpa-1* mutants, *gpa-12* mutants are sensitive to the PMA-induced developmental growth arrest like wild-type animals (Figure 4), suggesting that wild-type GPA-12 function is not required to cause PMA sensitivity. Therefore, we examined whether PMA sensitivity is affected in animals overexpressing activated GPA-12 (G_{12}QL). In the absence of heat shock-induced expression of G_{12}QL, two independent transgenic lines, *pkIs1330* and *pkIs1355*, are hypersensitive to PMA compared to wild-type (Figure 4). This result can be explained by a leaky expression of the heat shock promoter used. After heat shock-induced expression of G_{12}QL, the sensitivity of both transgenic lines toward PMA becomes even higher. As a consequence, these animals died at an early larval stage (100%). However, when we tested several transgenic lines that overexpress wild-type GPA-12 from its endogenous promoter or a heat shock promoter, no hypersensitivity toward PMA is observed (data not shown). These findings suggest that excessive activated GPA-12, and not loss or overexpression of wild-type GPA-12 function, affects the PMA-induced developmental sensitivity.

The genetic experiments presented here support a model in which TPA-1, most similar to nPKCθ or δ subunits, functions as a direct or indirect downstream effector of activated GPA-12. The two other subclasses of PKC isoforms, the "conventional" and "atypical" PKCs, as well as another member of the nPKC class, appear not to be involved in the activated GPA-12- and PMA-induced growth arrest (Table S2). Our experiments do not conclusively demonstrate that wild-type GPA-12 regulates TPA-1 activity, since we were unable to detect any phenotype (in particular a growth arrest) for either

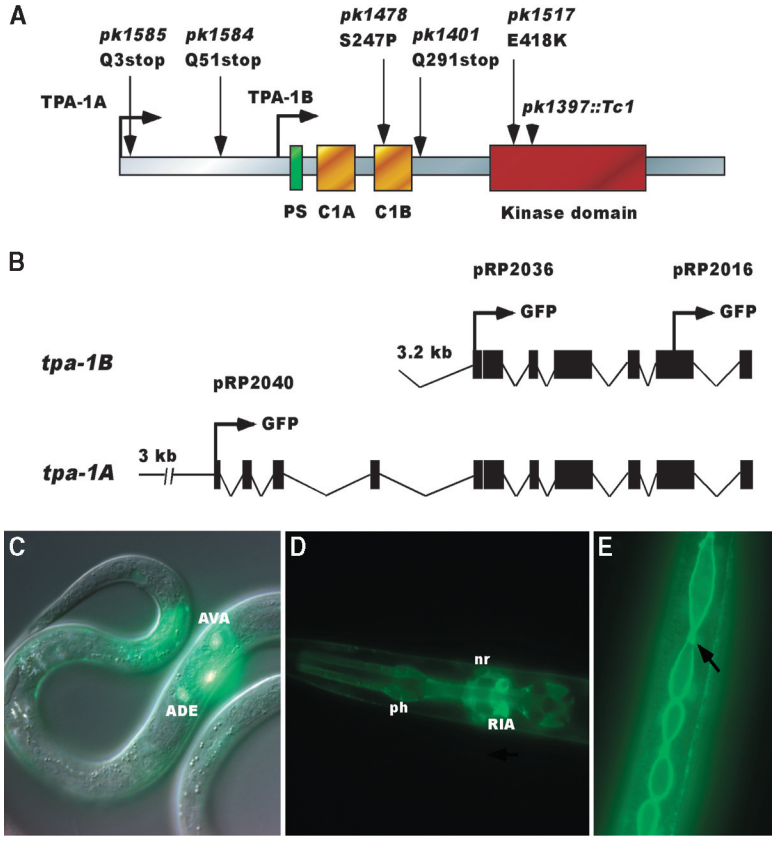

Figure 3. The Gene Structure of *tpa-1* and the Expression Pattern of *tpa-1A* and *tpa-1B* in *C. elegans*

(A) Structure of TPA-1 containing the pseudosubstrate region (PS), the cysteine-rich domains (C1A and C1B), and the kinase domain. Shown are the different mutations in *tpa-1*A and *tpa-1B* mentioned in the results section. We found 15 independent mutations in *tpa-1* that cause a premature termination of TPA-1, including 3 that contain an identical mutation (see Table S1 for the properties of all 50 *tpa-1* alleles).

(B) Genomic organization of the *tpa-1A* and *tpa-1B* gene. Solid boxes represent the coding sequence. *gfp*-reporter constructs of *tpa-1B* that only show expression of TPA-1B are made by using the intronic promoter present in the fourth intron of *tpa-1*. pRP2016 includes 3.2 kb of the upstream *tpa-1B* sequence and contains most of the coding sequence of *tpa-1B*, pRP2036 includes 3.2 kb of the upstream sequence of *tpa-1B*, and pRP2040 includes 3 kb of the upstream sequence of *tpa-1A*.

(C) Expression of *tpa-1A* in various neurons of the head, including AVA and ADE of an L1-staged larva using the *gfp*-reporter construct pRP2036.

(D and E) Animals that carry an integrated *tpa-1B::gfp* transgene (pRP2016) show expression of *tpa-1B* in the pharyngeal muscle cells (ph) (D), in neurons in the head (nr), including RIA (D), and in the membrane surrounding the hypodermal seam cells (E). More intense expression of *tpa-1B* is seen at the boundary between two seam cells ([E], arrow).

animals that overexpress wild-type GPA-12 or lack GPA-12 function. This is, however, in agreement with data on TPA-1 isoforms, because animals overexpressing TPA-1A or TPA-1B also do not show a discernible phenotype (data not shown). In *tpa-1* null mutants, the only detectable phenotypes relate to tissues in which GPA-12 is not expressed [15, 16]. Furthermore, the finding that activated GPA-12 and PMA induce an identical arrest of larval growth caused by their action in the pharynx suggests that the apparent activation of TPA-1 by activated GPA-12 reflects the actual signaling pathway normally used by wild-type GPA-12. In this pathway, PMA can bypass GPA-12 function by directly interacting with TPA-1, since animals lacking GPA-12 are not resistant to the developmental defects induced by PMA. The data do not support a model in which GPA-12 serves as a substrate for TPA-1, such that GPA-12 is inactivated by TPA-1. This is emphasized by the finding that animals overexpressing activated GPA-12 in the presence of PMA show a more severe phenotype (lethality at an early larval stage), which is probably due to an excessive activation and recruitment of TPA-1. We also do not believe that TPA-1 acts as positive regulator of GPA-12 in a feedback loop, since animals overexpressing wild-type GPA-12 are not hypersensitive to PMA (data not shown).

The discovery that PKCs function as receptors for phorbol ester tumor promoters established a key role for PKCs in tumorigenesis. In addition, constitutively active mutants of members of the G_{12} family are capable of malignant transformation of human cells. Therefore, it is an attractive model that the transforming capability of activated G_{12} is due (at least in part) to their ability to activate PKCs. Several biochemical studies performed in mammalian cells have shown that PKCs are activated by diacylglycerol (DAG) or other lipids (for review, see [17, 18]). In line with these data, GPA-12 may activate TPA-1 via lipid intermediates that can be generated by, for instance, phospholipases. Mutations in *egl-8* encoding a phospholipase Cβ isoform (PLCβ) do not suppress the growth arrest induced by G_{12}QL or PMA (data not shown). This finding suggests that there should be other lipid-mediated signal transducing proteins, like phospholipase D or A2, that activate TPA-1 through the production of DAG. All 44 EMS-mutagenized mutants that were identified as suppressors of the G_{12}QL-induced growth arrest by using 2 hr of heat shock have mutations in *tpa-1*. Components that act between GPA-12 and TPA-1 may not be identified in our suppressor screen, because they are either redundant or essential. Initial studies on suppressors isolated after 5 min of heat shock suggest that loci other than *tpa-1* are involved in the G_{12}QL-induced phenotype. Further characterization of these loci should uncover more components in the G_{12} pathway and give insight in the molecular mechanism with which activated G_{12} and TPA-1 induce a growth arrest.

Supplemental Data

Supplemental Data including the Experimental Procedures as well as Figure S1 and Tables S1 and S2 are available at http://images.cellpress.com/supmat/supmatin.htm.

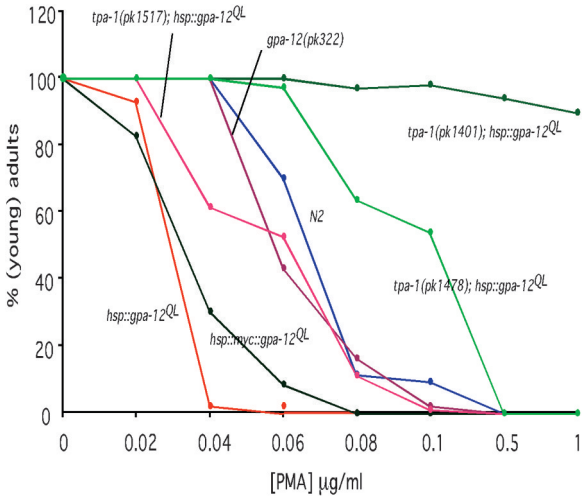

Figure 4. Developmental Growth Sensitivity in the Presence of the Phorbol Ester PMA

Loss-of-function *gpa-12(pk322)* animals are sensitive to PMA like N2 (wild-type) animals. In the absence of a heat shock treatment, *pkIs1330*[*hsp::gpa-12*^{QL}] and *pkIs1355*[*hsp::myc::gpa-12*^{QL}] transgenic animals are hypersensitive to PMA, whereas they die when a heat shock is applied (larvae showed pharyngeal defects and vacuoles in their hypodermis). Both *pkIs1330* and *pkIs1355* animals bear multiple copies of the constitutive active *gpa-12* gene under control of a heat shock promoter (*hsp*); *pkIs1355* also contains a Myc-tag fused to the active form of GPA-12. Three *tpa-1* mutants (*pk1401*, *pk1487*, and *pk1517*) in a *pkIs1330* background suppress the PMA-induced developmental sensitivity compared to non-heat-shocked *pkIs1330* control animals. *tpa-1(pk1401)* is a putative null allele. *tpa-1 (pk1478*) contains a S247P amino acid substitution in the first C1A region, which is responsible for binding of phorbol esters [19], indicating that this mutation in *tpa-1* may lower its affinity for PMA. In *tpa-1(pk1517)*, a conserved glutamic acid (E) at position 418 in the kinase domain is changed into a lysine (K), which presumably lowers the catalytic activity of TPA-1. Inhibition of larval growth induced by PMA is quantified by measuring the number of young adults at different concentrations of PMA after 5 days (see the Supplemental Experimental Procedures).

Acknowledgments

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Table 1. Expression of *tpa-1A* and *tpa-1B* in the Pharyngeal Muscle Rescues the Activated GPA-12- and PMA-Induced Developmental Growth Arrest

Genotype	Transgene	Noninduced Animals (Percentage of the Total Number of Young Adults)	Growth after Expression of G_{12}QL (Percentage of the Total Number of Young Adults)	Growth in the Presence of PMA (Percentage of the Total Number of Young Adults)
tpa-1(pk1397)	none	99 (n = 368)	98 (n = 328)	94 (n = 317)
tpa-1(pk1397); hsp::gpa-12^{QL}	none	95 (n = 433)	96 (n = 384)	95 (n = 459)
tpa-1(pk1397)	*myo-2::tpa-1A*	97 (n = 413)	96 (n = 392)	7 (n = 229)
tpa-1(pk1397); hsp::gpa-12^{QL}	*myo-2::tpa-1A*	91 (n = 280)	5 (n = 193)	3 (n = 177)
tpa-1(pk1397)	*myo-2::tpa-1B*	98 (n = 358)	95 (n = 368)	8 (n = 296)
tpa-1(pk1397); hsp::gpa-12^{QL}	*myo-2::tpa-1B*	82 (n = 136)	2 (n = 144)	4 (n = 97)

Assays are described in the Supplemental Experimental Procedures. For each injected construct, we generated at least two extrachromosomal transgenic lines and crossed these lines in a *pkIs1330*[*hsp::gpa12*^{QL}] background. *pkIs1330* contains multiple copies of the constitutive active *gpa-12* gene under control of a heat shock promoter (*hsp*). Growth after expression of G_{12}QL (2 hr at 33°C) or in the presence of PMA (1.0 µg/ml) was determined by counting the number of animals (n) after 4 days and calculating the percentage of young adults.

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Chapter 5

Characterization of *Caenorhabditis elegans* homologs of the Down syndrome candidate gene DYRK1A

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Characterization of *Caenorhabditis elegans* Homologs of the Down Syndrome Candidate Gene DYRK1A

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ABSTRACT

The pathology of trisomy 21/Down syndrome includes cognitive and memory deficits. Increased expression of the dual-specificity protein kinase DYRK1A kinase (DYRK1A) appears to play a significant role in the neuropathology of Down syndrome. To shed light on the cellular role of DYRK1A and related genes we identified three *DYRK/minibrain*-like genes in the genome sequence of *Caenorhabditis elegans*, termed *mbk-1*, *mbk-2*, and *hpk-1*. We found these genes to be widely expressed and to localize to distinct subcellular compartments. We isolated deletion alleles in all three genes and show that loss of *mbk-1*, the gene most closely related to DYRK1A, causes no obvious defects, while another gene, *mbk-2*, is essential for viability. The overexpression of DYRK1A in Down syndrome led us to examine the effects of overexpression of its *C. elegans* ortholog *mbk-1*. We found that animals containing additional copies of the *mbk-1* gene display behavioral defects in chemotaxis toward volatile chemoattractants and that the extent of these defects correlates with *mbk-1* gene dosage. Using tissue-specific and inducible promoters, we show that additional copies of *mbk-1* can impair olfaction cell-autonomously in mature, fully differentiated neurons and that this impairment is reversible. Our results suggest that increased gene dosage of human DYRK1A in trisomy 21 may disrupt the function of fully differentiated neurons and that this disruption is reversible.

TRISOMY of chromosome 21, or Down syndrome, is the most frequent chromosomal abnormality in human infants that come to term. Besides manifesting a characteristic set of facial and physical features, heart defects, and abnormalities in the immune and endocrine systems, patients with Down syndrome have deficits in spatial memory and difficulty in converting short-term to long-term memories (Johannsen *et al.* 1996; Takashima 1997). Although the cognitive defects of Down syndrome are likely to arise from increased dosage of many genes, several lines of evidence suggest that increased expression of the dual-specificity protein kinase DYRK1A plays a significant role. First, the DYRK1A locus maps to the Down syndrome candidate region (DSCR), a region of 70–100 genes (Shindoh *et al.* 1996). Rare patients with a partial trisomy of the DSCR display defects in cognition, suggesting that the genes in this region are sufficient to produce the cognitive defects of Down syndrome. Second, a 180-kb region on human chromosome 21 containing the DYRK1A locus is sufficient to produce defects in learning and memory in transgenic mice (Smith *et al.* 1997). Third, transgenic mice overexpressing the full-length *Dyrk1A* cDNA exhibit impairment in spatial learning and cognitive flexibility (Altafaj *et al.* 2001). The cellular and molecular consequences of these DYRK1A perturbations are unknown.

The first vertebrate member of the DYRK family was originally identified in a PCR screen for protein kinases (Kentrup *et al.* 1996) and subsequently shown to belong to a larger family of related dual-specificity kinases (Becker and Joost 1999) to which we refer here as the *DYRK/minibrain* family. Members of this family are localized to distinct subcellular compartments and phosphorylate various substrates *in vitro* (Becker and Joost 1999). They share the unusual property of tyrosine-directed autophosphorylation as well as phosphorylation of serine/threonine residues in exogenous substrates (Becker and Joost 1999). Their precise cellular role, however, remains elusive. Insights into their physiological relevance were provided by the finding that the Drosophila homolog of DYRK1A, termed *minibrain*, is involved in neuroblast proliferation in the fly brain (Tejedor *et al.* 1995). A similar reduction in brain size has been recently observed in DYRK1A knockout mice (Fotaki *et al.* 2002). The sole *Saccharomyces cerevisiae* representative of the *DYRK/minibrain* family, Yak1p, acts in parallel to a protein kinase A pathway to negatively regulate cell-cycle progression (Garrett and Broach 1989; Garrett *et al.* 1991).

In metazoan animals, the only *DYRK/minibrain* family

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member for which mutant alleles exist is the Drosophila *minibrain* gene. Here, we describe the expression pattern and loss-of-function alleles of all three *DYRK/minibrain* family members in the nematode *Caenorhabditis elegans.* In contrast to Drosophila *minibrain*, we observe no obvious morphological defects in *mbk-1* mutants, but find that another *DYRK/minibrain* family member, *mbk-2*, is required for viability. In an attempt to model the cellular consequences of DYRK1A overexpression that are observed in Down syndrome patients, we analyzed the effects of providing extra copies of the worm ortholog of DYRK1A and describe dosage-sensitive and reversible defects in the processing of acute sensory information.

MATERIALS AND METHODS

cDNAs: A full-length *mbk-1* cDNA was amplified with SL1 and gene-specific primers. The structure of the cDNA is similar to the predicted T04C10.1 gene, with the exception of an incorrect first exon predicted in T04C10.1. The *mbk-2* locus generates two messages, a long form (*mbk-2L*) and a short form (*mbk-2S*) that uses an internal start site from an alternatively spliced exon. Both splice forms are represented in expressed sequence tag (EST) clones (a gift from Y. Kohara) that have been completely sequenced. Similarly, *hpk-1* full-length cDNA clones are represented in Y. Kohara's EST collection.

DNA constructs: Constructs are shown schematically in Figure 1.

mbk-1: To build *mbk-1::gfp*, the *mbk-1* genomic locus, including 7 kb of 5′ noncoding sequence and all exons and introns, was amplified by Expand long-template PCR (Boehringer Mannheim, Indianapolis). The PCR product was cloned in frame with *gfp* in the promoterless vector pPD95.75 (from A. Fire), generating pBR104. To build *mbk-1(YA)::gfp*, the QuickChange kit (Stratagene, La Jolla, CA) was used to modify pBR104 with primers YAs (5′-ctggacaccgaatcgcccaggccattcagt cgagattctatcg) and YAas (5′-cgatagaatctcgactgaatggcctgggcgat tcggtgtccag), generating pBR113. To build *mbk-1(pk1389)::gfp*, the *mbk-1* locus was amplified from homozygous *mbk-1(pk1389)* animals by Expand long-template PCR (Boehringer Mannheim). Following the cloning and sequencing of this PCR product, a 4.0-kb *Bgl*II-*Asp*718 fragment was subcloned into pBR104, generating pBR177. To build *pgcy-10::mbk-1*, the *gcy-10* promoter (Yu *et al.* 1997) was amplified from N2 genomic DNA by Expand long-template PCR (Boehringer Mannheim) and cloned in front of the full-length *mbk-1* cDNA, generating pBR145. To build *pgcy-10::mbk-1(YA)*, the QuickChange kit (Stratagene) was used to modify pBR145 with primers YAs and YAas, generating pBR147. To build *phsp16-2::mbk-1::gfp*, the full-length *mbk-1* cDNA was cloned into the heat-shock vector pPD49.78 (from A. Fire), generating pBR144. To generate a fusion with *gfp*, the promoter and cDNA were subsequently fused in frame with pBR104, generating pBR144.

mbk-2: To build *pmbk-2L::gfp*, 8 kb of 5′ noncoding sequence was amplified by Expand long-template PCR (Boehringer Mannheim) and cloned into pPD95.77 (from A. Fire), generating pBR126. To build *mbk-2S::gfp*, Expand long-template PCR (Boehringer Mannheim) was used to amplify exons 7–11 of the *mbk-2* locus and fused in frame with *gfp* in pPD95.77 (from A. Fire), generating pBR138. This construct uses intron 6 as the 5′ noncoding sequence. *pmbk-2L::gfp* and *mbk-2S* do not contain the full *mbk-2* genomic locus, and this may result in an artifactual or incomplete expression pattern. To build *phsp16-2::mbk-2L::gfp*, full-length *mbk-2L* cDNA was fused with *gfp* and inserted into the heat-shock vector pPD49.79 (from A. Fire), generating pBR169.

hpk-1: To build a translational fusion between HPK-1 and green fluorescent protein (GFP), the full-length *hpk-1* genomic locus, including 4 kb of 5′ noncoding sequence and all exons and introns, was amplified by Expand long-template PCR (Boehringer Mannheim) and cloned into the promoterless vector pPD95.77 (from A. Fire), generating pBR132.

Transgenic and mutant strains: The strains are as follows:

EK224 *cmIs6* [pBR104, pNC4.21] I; 6× outcrossed
EK270 *cmIs8* [pBR113, pNC4.21]; 2× outcrossed
EK176 *cmEx20* [pBR145, *unc-122::gfp*]
EK179 *cmEx21* [pBR147, *unc-122::gfp*]
EK173 *unc-4(e120)* II; *cmEx19* [pBR144, pNC4.21]
EK234 *unc-4(e120)* II; *cmEx31* [pBR169, pNC4.21]
EK123 *cmEx6* [pBR126, pRF4]
EK251 *cmEx16* [pBR138, pRF4]
EK135 *cmEx11* [pBR132, pRF4]
EK228 *mbk-1(pk1389)* X; 6× outcrossed
EK264 *cmEx36* [pBR177, pRF4]
EK273 *hpk-1(pk1393)* X; 6× outcrossed
EK275 *mbk-2(pk1427)/mgIs18* IV; 3× outcrossed

All expression constructs were injected at 50 ng/μl. pNC4.21 [*unc-4(+)*] and *unc-122::gfp* were injected at 50 ng/μl. pRF4 [*rol-6(d)*] was injected at 100 ng/μl. Integrated lines were obtained using a protocol described at http://cpmcnet.columbia.edu/dept/gsas/biochem/labs/hobert/integration_protocol.html.

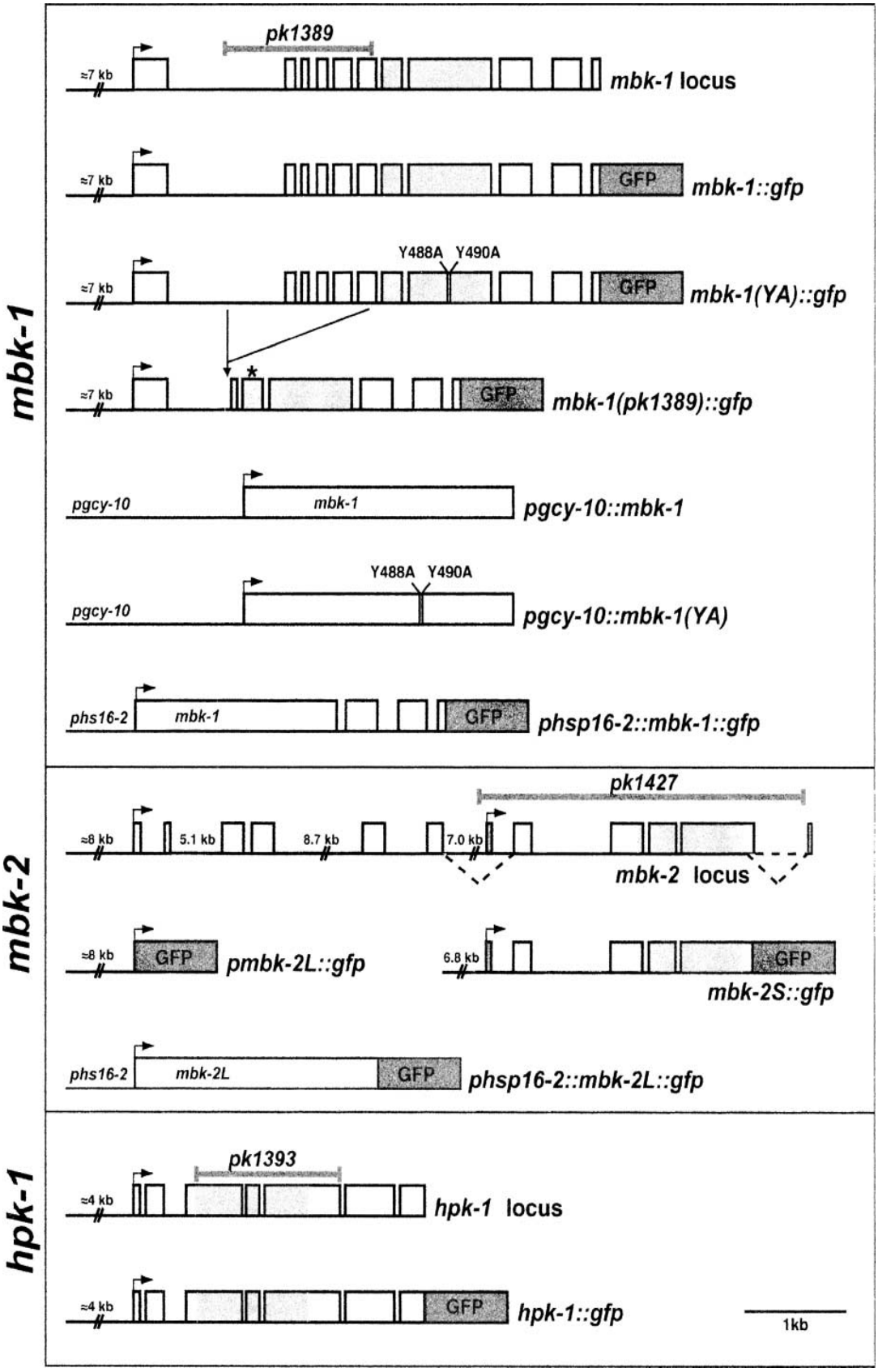
Isolation of deletions in the *mbk-1*, *mbk-2*, and *hpk-1* loci: PCR screening of a chemically induced deletion library was done as previously described (Jansen *et al.* 1997). The relative position within the genomic loci of all three mutant alleles is schematically indicated in Figure 1. A 1658-bp deletion mutant of *mbk-1*, *pk1389*, was isolated using primers MBK1A (5′-gca gacgtgcctgacaatcttc) and MBK1D (5′-tgtaggtatggcggtatccgtc) and nested primers MBK1B (5′-ctcaaatacccagcaacactcac) and MBK1C (5′-caatagtagatcccatcctcag). The deleted PCR product from *pk1389* was sequenced and the deletion was confirmed by Southern analysis. The following sequence in capitals is deleted by *pk1389*: 5′-ataaaGCTTT-TTACGactat. The deletion extends from the first intron into the sixth exon. If in this context exon 1 is spliced to exon 7 (the beginning of the kinase domain), a frameshift and a premature stop would result; splicing of exon 1 to more downstream exons would also create frameshifts and/or delete the kinase domain. The *mbk-2* deletion, *pk1427*, spans 3398 bp in the genomic region represented in cosmid F49E11 and deletes the capitalized sequence 5′-tcgtcGTCCG-AAAACttgta. The *hpk-1* deletion, *pk1393*, spans 1457 bp in the genomic region represented in

Figure 1.—Reporter gene constructs and deletion alleles used to study *minibrain*-like genes. Boxes denote exons of the respective genes. Dark shaded bars above the genomic locus denote the location of the deletion in the respective mutant alleles (see materials and methods for precise location). Kinase domains are lightly shaded. The kinase domain was predicted using SMART (http://smart.embl-heidelberg.de/). The large size of the *mbk-2* genomic locus prevented us from constructing a full-length, translational *gfp* fusion construct. To monitor subcellular localization of *mbk-2* we thus fused the cDNA of the *mbk-2L* splice form to a heterologous promoter, *phsp16-2* (Fire *et al.* 1990). The two alternatively spliced *mbk-2* isoforms produced by the *mbk-2* locus were deduced by cDNA analysis (see materials and methods).

cosmid F20B6 and deletes the capitalized sequence 5′-caca cATGCC-TGACAtaatg. All deletion alleles lead to a disruption of the majority of the respective kinase domains and are predicted to act as null alleles.

RNAi: *mbk-2* dsRNA was delivered by bacterial feeding as previously described (TIMMONS *et al.* 2001). The effectiveness of RNAi could be assessed by monitoring the decrease in fluorescence of transgenic animals that expressed *gfp*-tagged *mbk-2*.

Behavioral assays: Chemotaxis assays toward volatile odor-



ants were performed as described (Bargmann *et al.* 1993). Chemotaxis index = (the number of animals attracted to the odorant) − (the number of animals at the opposite end of the plate)/(the total number of animals at both ends of the plate). When transgenic animals carrying extrachromsomal arrays were scored, only those animals that carry the injection marker were scored.

Scoring neuroanatomy: AWC morphology was observed by crossing *kyIs140,* a chromosomally integrated *str-2::gfp* construct (Troemel *et al.* 1999), into the respective mutant background. The generation, proliferation, and anatomy of several sensory neuron classes (ASK, ADL, ASI, AWB, ASH, ASJ, PHA, and PHB) were visualized by filling exposed sensory neurons with the lipophilic dye DiI, as previously described (Hedgecock *et al.* 1985). Briefly, a mixed population of well-fed worms was washed with M9 buffer, incubated at room temperature with 10 μg/ml DiI for 1 hr, washed several times with M9, and then mounted on a compound fluorescence microscope.

DAPI staining: 4′,6-Diamidino-2-phenylindole (DAPI) staining on *gfp*-expressing transgenic lines was done by placing animals in a drop of water on a coverslip, letting the water evaporate, adding a drop of acetone, letting the drop evaporate, drying for 20 min, and then adding 1 μg/ml DAPI in M9 medium.

RESULTS

Identification of *DYRK/minibrain*-like genes in *C. elegans*: Through sequence homology searching of the complete *C. elegans* genome sequence, we identified two *C. elegans* genes with close homology to the *DYRK/minibrain* family, termed *mbk-1* and *mbk-2* (for ***mini*brain-*k*inase**), and one gene, *hpk-1* (named after its vertebrate homologs, ***h*omeodomain-*i*nteracting *p*rotein *k*inase *1-3***), with a more distant homology (Figure 2A). *mbk-1* and *mbk-2* both carry the characteristic DYRK family signature motifs in their kinase domain, as well as a DH-box, a conserved sequence motif preceding the catalytic domain (Becker and Joost 1999). *mbk-1* is unique among the *C. elegans DYRK/minibrain* family members in clustering in the DYRK1A subgroup (Figure 2A). In addition to highest overall sequence similarity, MBK-1 and DYRK1A can also be distinguished from other DYRK family members by their sharing of an N-terminal nuclear localization sequence, a leucine zipper in the kinase domain, and stretches of homopolymeric amino acids at their C termini (Figure 2B). We thus consider MBK-1 the *C. elegans* ortholog of human DYRK1A.

Expression and subcellular localization of *DYRK/minibrain*-like genes in *C. elegans*: To determine the sites of expression and subcellular localization of *DYRK/minibrain*-like genes, we fused *gfp* in frame with the respective genomic loci (Figure 1). The *mbk-1::gfp* construct included 7 kb of 5′ noncoding sequence and all of the exons and introns present in the endogenous *mbk-1* locus. The *mbk-1::gfp* gene product is expressed in all somatic cells and primarily localizes to nuclei (Figure 3), similar to the reported expression and subcellular localization of human DYRK1A (Song *et al.* 1997; Becker *et al.* 1998). During development, *mbk-1::gfp* expression can first be observed around the 300-min stage, when

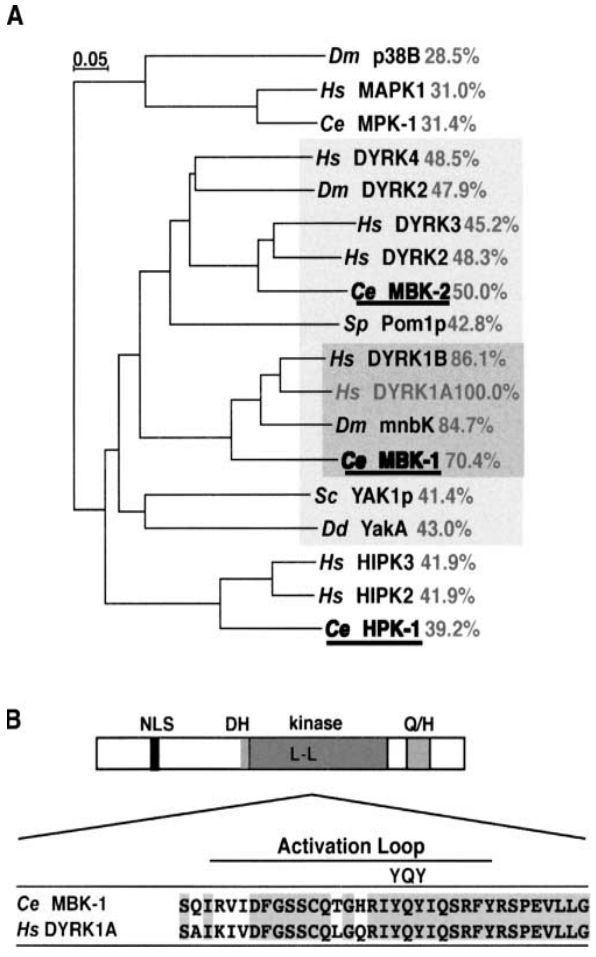


Figure 2.—The *DYRK/minibrain*-like gene family in *C. elegans.* (A) Classification of the DYRK subfamily of protein kinases. On the basis of BLAST- and PFAM-based similarity searches of the complete genome sequence, *C. elegans* has two members of the DYRK subfamily of protein kinases, MBK-1 (T04C10.1; LGX; GenBank accession no. AY064464) and MBK-2 (F49E11.1; LGIV; GenBank accession no. AY090019), as well as a related kinase called HPK-1 (F20B6.8; LGX). The gene structures of *mbk-1* and *mbk-2* were confirmed by sequence analysis of EST clones. The alignment and dendrogram were generated from the predicted kinase domains using ClustalX and Align with default parameters. The DYRK1A group is boxed by dark shading, the DYRK subfamily is boxed by light shading, *C. elegans* proteins are in boldface type and underlined, and the percentage identity between each kinase domain and that of DYRK1A is listed to the right. (B) MBK-1 is the closest homolog to DYRK1A, whose overexpression is implicated in Down syndrome. The relative positions of the N-terminal nuclear localization sequence [NLS; amino acids (aa) 120–127], the DH box, the kinase domain (aa 317–649), leucine zipper (L-L; aa 443–464; contains four leucines each spaced by 6 aa), and C-terminal homo-polymeric stretch of amino acids (histidines in DYRK1A, glutamines in MBK-1) sites are shown. DYRK kinases are regulated through the phosphorylation of two DYRK-specific tyrosines within the activation loop (Y-Y; aa 488 and 490). For a more detailed alignment and more sequence features of DYRK kinases, see Becker and Joost (1999).

cell division ceases and morphogenesis begins (Figure 3A). Expression levels increase during later embryonic stages and remain at comparable levels throughout larval and adult stages (Figure 3).

In contrast to *mbk-1*, *mbk-2::gfp* reporter constructs were expressed in subsets of tissues, including the nervous system, body wall muscle, and the pharynx (Figure 4, A–C). To determine the subcellular localization of MBK-2 protein, we fused a cDNA—encoding the longer splice form of *mbk-2* (see Figure 1 and MATERIALS AND METHODS)—to *gfp* and expressed it under control of a ubiquitously expressed promoter (Figure 1). Unlike MBK-1::GFP, but like its vertebrate ortholog DYRK2 (BECKER *et al.* 1998), MBK-2::GFP was excluded from the nucleus (Figure 4D).

A translational HPK-1::GFP reporter construct (Figure 1) was broadly expressed during embryogenesis and localized to moving nuclear puncta (Figure 4, E–H). In adult animals, the number, intensity, and movement of HPK-1-GFP puncta are greatly reduced. Although we have not further examined the identity of these puncta, the observation that the three vertebrate homologs of HPK-1, HIPK1–3, stably bind and phosphorylate nuclear transcription factors (KIM *et al.* 1998) suggests that these dots may represent sites of active transcription.

Taken together, the distinctive subcellular localization patterns described here for the individual *C. elegans DYRK/minibrain* family members reveal a striking similarity to their presumptive vertebrate orthologs and, together with the primary sequence similarity, suggests that vertebrate and *C. elegans DYRK/minibrain* proteins fulfill similar functions.

Providing extra copies of *mbk-1* causes dosage-sensitive olfactory defects: Because human DYRK1A overexpression has been implicated in the behavioral defects of Down syndrome, we wanted to examine the consequences of overexpression of the DYRK1A ortholog *mbk-1* on *C. elegans* behavior. Toward this end, a multicopy array derived from a subcloned amplicon of the *mbk-1* genomic locus was integrated into the genome of wild-type worms, giving rise to multiple independent lines that harbor additional copies of *mbk-1* (see Figure 1 for constructs). An appended *gfp* tag allowed us to monitor ectopic *mbk-1* expression, which we observed broadly throughout the whole animal (Figure 3). In light of the multicopy nature of stably transmitted DNA arrays in *C. elegans* (MELLO *et al.* 1991), the level of ectopic *mbk-1* expression is possibly significantly greater than the 1.5-fold DYRK1A overexpression resulting from trisomy 21; however, as we have no means of assessing expression of endogenous MBK-1 protein we cannot conclusively state that we "overexpress" *mbk-1*, but we can clearly state, on the basis of the appended *gfp* tag, that we provided extra copies of the *mbk-1* gene.

All of the transgenic lines that contain extra copies of

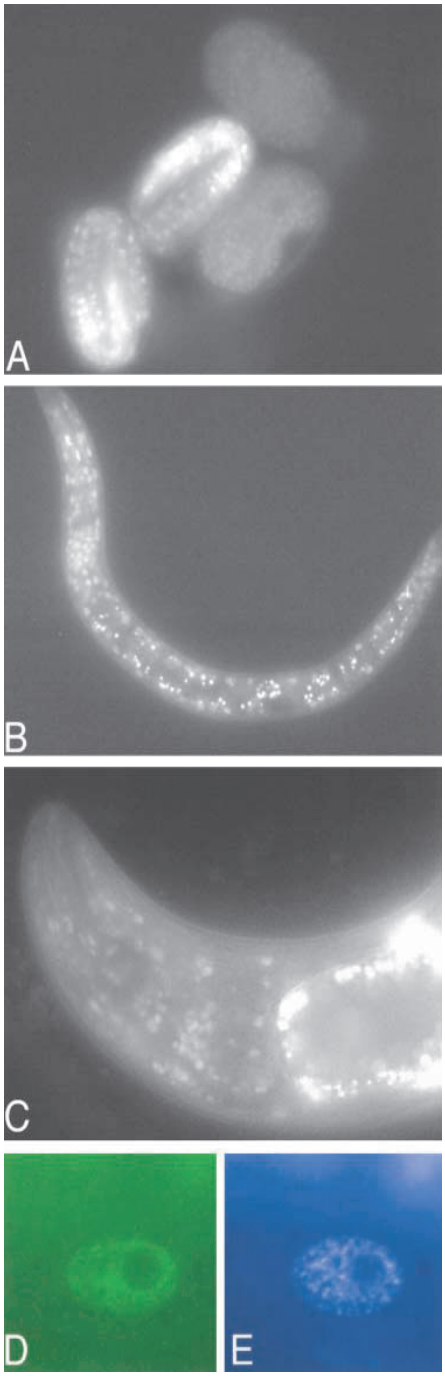


FIGURE 3.—Expression of the *DYRK1A/minibrain* ortholog *mbk-1* gene as assayed by reporter gene analysis. MBK-1::GFP is expressed in all somatic tissues and primarily localizes to nuclei in embryos (A), larvae (B), and adults (C). Nuclear localization can be observed in all cells and is particularly obvious in the enlarged nuclei of hypodermal cells (D, MBK-1::GFP; E, DAPI staining; the dark center is the nucleolus). Expression is monitored from a chromosomally integrated array, *cmIs6*. (A) Embryos at different stages; the embryo at the top is roughly at 300 min of development entering morphogenesis and showing the first signs of *mbk-1::gfp* expression; the embryo on the lower left is at the comma stage with slightly increased *mbk-1::gfp* expression. Maximal levels of *mbk-1::gfp* expression are then observed around threefold stages (embryos on left).

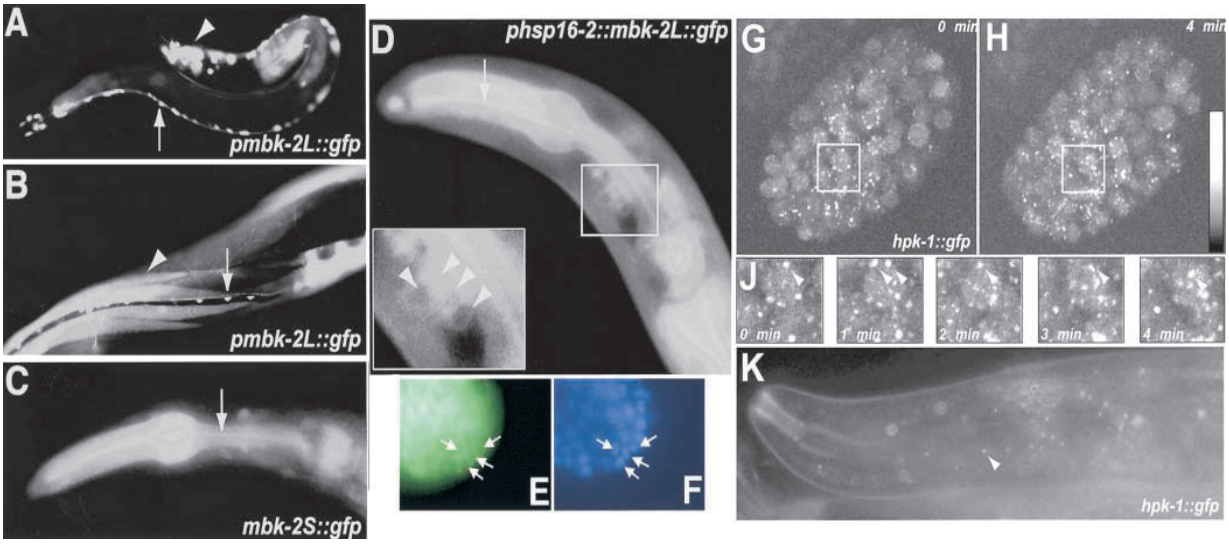


FIGURE 4.—Expression of other DYRK1A/minibrain family members. (A–F) MBK-2::GFP is excluded from the nucleus and is expressed in the nervous system. Expression constructs are shown in Figure 1. (A) In L1 larvae, the 5′ noncoding sequence of *mbk-2L* drives *gfp* expression widely in the nervous system, including head neurons (arrowhead) and the ventral nerve cord (arrow). (B) In adult animals, *gfp* continues to be expressed in the nervous system (arrow) and is also expressed in body wall muscle (arrowhead). (C) A translational fusion between *mbk-2S* and *gfp* is expressed at the periphery of the pharynx (arrow). (D) Induced expression of a translational fusion protein between *mbk-2L* and *gfp* also localizes to the periphery of the pharynx (arrow). In addition, the protein is clearly excluded from the nuclei of neurons in the head (inset, arrowheads); nuclear exclusion was further corroborated by DAPI staining of an embryo (F) that expresses *mbk-2::gfp* (E). (G–K) HPK-1::GFP expression. The expression construct is shown in Figure 1. To monitor GFP expression in living embryos, two-photon laser scanning microscopy was used on mounted embryos. (G) A translational fusion protein between *hpk-1* and *gfp* localizes to nuclei and is widely expressed in cleavage stage embryos. (H) After 4 min, the number and location of HPK-1-GFP puncta within the embryo has dramatically changed. (J) The boxed region is shown at 1-min intervals. The puncta within the highlighted nucleus rapidly rearrange (arrowheads). (K) A fluorescence micrograph of HPK-1-GFP at postembryonic stages. In adult animals, the number, intensity, and movement of HPK-1-GFP puncta (arrowhead) are greatly reduced.

mbk-1, here on referred to as *mbk-1(gf)* animals, appeared normal in respect to overall morphology, life span, dauer formation, thermotaxis, mechanosensation, and chemotaxis toward water-soluble attractants (data not shown). *mbk-1(gf)* animals display no defects in locomotory rate or reversal behavior (data not shown). However, olfaction toward several volatile chemoattractants was markedly disrupted in *mbk-1(gf)* animals (Figure 5). Comparable defects were observed with chromosomally integrated lines that contain extra copies of either wild-type *mbk-1* or a mutated version of *mbk-1* in which the kinase activity was potentially hyperactivated (Y-to-E change in kinase domain; data not shown). *mbk-1(gf)* defects are apparent throughout a wide range of odor concentrations.

The olfactory-defective phenotype was sensitive to gene dosage, since animals heterozygous for the integrated *mbk-1* array display intermediate odortaxis defects. As a control, we provided extra copies of *mbk-1*, again under the control of its own promoter and tagged with *gfp* to monitor expression, but containing an inactivated kinase domain (Figure 1). We found that worms containing integrated copies of the *mbk-1(YA)* genomic construct showed no defects in odortaxis (Figure 5A). Additional copies of *mbk-2* also produced no olfaction defect (Figure 6B). All ectopic expression constructs yielded comparable levels of protein expression as assessed by the added GFP tag. The olfaction phenotype thus requires the kinase activity of *mbk-1*, is specific to *mbk-1*, and is sensitive to gene dosage.

Attractive olfactory responses in *C. elegans* are largely mediated by two bilaterally symmetric pairs of ciliated sensory neurons, called AWA and AWC (BARGMANN *et al.* 1993). These neurons are capable of sensing a wide variety of discrete odorants. We found that extra copies of *mbk-1* strongly inhibited odortaxis toward AWC-sensed odorants such as benzaldehyde, isoamylalcohol, and 2-butanone across a wide range of concentrations (Figure 5B). In contrast, extra copies of *mbk-1* caused only marginal defects in the response to AWA-sensed odorants such as diacetyl and pyrazine (Figure 5B).

The cellular specificity of odortaxis defects induced by extra copies of *mbk-1* agrees with our observation that *mbk-1(gf)* animals are indistinguishable from wild-type animals in all other behaviors tested and suggests that *mbk-1* acts selectively within specific cellular contexts rather than affecting neuronal signaling in a broad and unspecific way.

***mbk-1* can act autonomously in sensory neurons:** To elucidate the cellular context in which extra copies of *mbk-1* can affect olfactory behavior, we added extra copies of *mbk-1* specifically in olfactory neurons using the

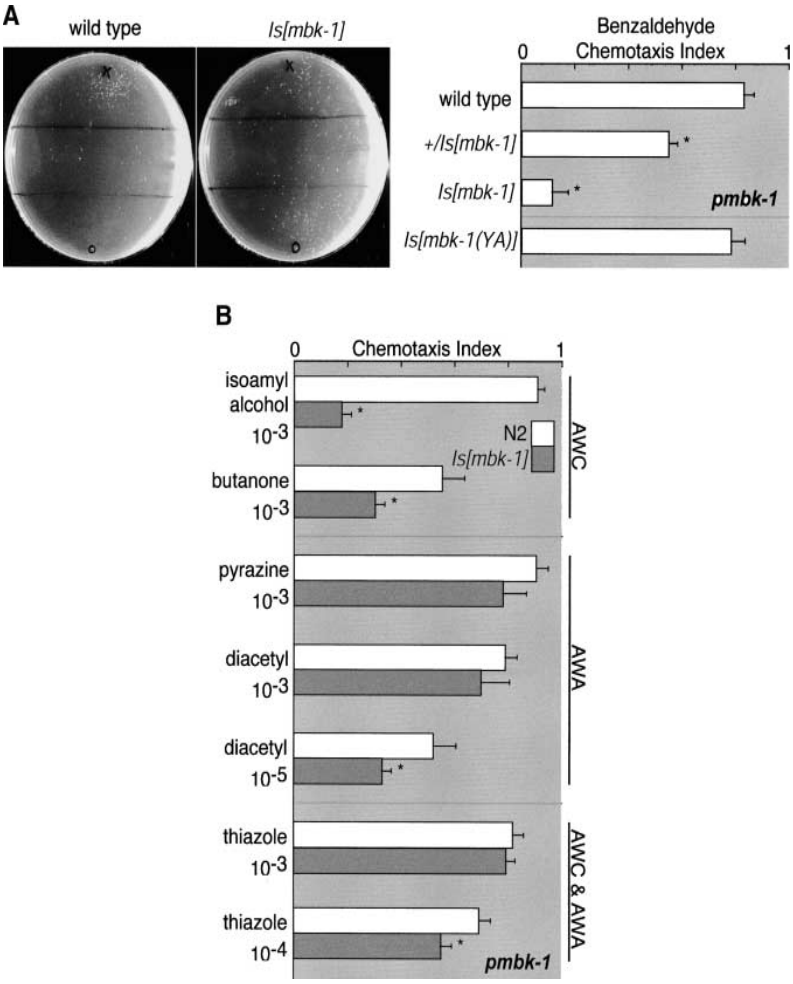


FIGURE 5.—Extra copies of *mbk-1* produce defects in chemotaxis to volatile attractants. (A) Odortaxis to benzaldehyde, which is sensed mainly by AWC chemosensory neurons. Representative odortaxis plates are shown for wild type (N2) and animals that are homozygous for an integrated multicopy *mbk-1* array (*Is[mbk-1]* = *cmIs6*). Odortaxis results with wild-type animals and animals that are homozygous (*Is[mbk-1]*) and heterozygous (+/*Is[mbk-1]*) for extra copies of wild-type *mbk-1* arrays and homozygous for kinase-inactivated *mbk-1* arrays (*Is[mbk-1(YA)]* = *cmIs8*) are shown. Tyrosines 488 and 490 were mutated to alanine in kinase-inactivated MBK-1, since equivalent tyrosines in DYRK1A are essential for kinase activity (KENTRUP *et al.* 1996; HIMPEL *et al.* 2001). The *mbk-1(YA)* construct was also tagged with *gfp* and expression levels were found to be indistinguishable from those of animals that express extra copies of wild-type *mbk-1*. For a summary of expression constructs see Figure 1. (B) The response to odorants sensed by the AWC chemosensory neurons (isoamylalcohol, 2-butanone) is affected more strongly by providing extra copies of *mbk-1* than the response to odorants sensed by the AWA chemosensory neurons (pyrazine, diacetyl) or by both AWC and AWA (2,4,5-trimethylthiazole). N2 (open bars) and *Is[mbk-1]* (shaded bars) are shown. Each bar represents at least five independent assays; error bars show SEM and an asterisk indicates that the values differ significantly ($P < 0.05$) from controls.

gcy-10 promoter, which is active in the AWC and AWB chemosensory neurons, as well as in the pharyngeal interneuron I1 (YU *et al.* 1997; Figure 1). As a control, kinase-inactivated *mbk-1* was also expressed under control of the *gcy-10* promoter (Figure 6A). Transgenic lines expressing wild-type *mbk-1* in AWB/C/I1 showed impaired odortaxis toward benzaldehyde, sensed by AWC (Figure 6A), but normal odortaxis toward diacetyl, sensed by AWA (data not shown). In contrast, transgenic lines expressing kinase-inactivated *mbk-1* in AWB/C/I1 displayed normal odortaxis toward both benzaldehyde and diacetyl. Because AWB is dispensable for chemotaxis toward volatile attractants (BARGMANN *et al.* 1993), and because the pharyngeal neuron I1 is primarily isolated from the somatic nervous system (ALBERTSON and THOMSON 1976), these results suggest that *mbk-1* overexpression can act cell-autonomously in AWC to disrupt neuronal function. The disruption in odortaxis is less severe in animals expressing *mbk-1* under control of the *gcy-10* promoter than in animals expressing *mbk-1* under control of its own promoter or of the heat-shock promoter. This may arise because *gcy-10* is a relatively weak promoter or because additional copies of *mbk-1* provided under control of its own ubiquitous promoter disrupt the function not only of AWC but also of other cells (such as, for example, in downstream interneurons).

***mbk-1* can act reversibly and acutely in differentiated neurons:** Given that odortaxis defects arise from increasing *mbk-1* gene dosage, an important issue is determining the temporal window of *mbk-1* function: Is the increased gene dosage altering development or the function of already differentiated cells? To address this issue, we followed several approaches: First, we tested whether AWC development is affected in *mbk-1(gf)* mutants by assessing AWC generation, proliferation, or anatomy using *str-2::gfp* (TROEMEL *et al.* 1999) as an anatomical marker (Figure 7). We observed no obvious abnormalities. Moreover, the characteristic left/right asymmetric expression of the *str-2* marker, a sensitive indicator of function and development of the left/right AWC neuron pair (TROEMEL *et al.* 1999), is unaffected in *mbk-1(gf)* animals. As a second approach to delineate *mbk-1* function, we used the inducible heat-shock promoter *hsp 16-2* (FIRE *et al.* 1990) to drive expression of an *mbk-1* cDNA fused to *gfp*. Adult animals that received a dose of *mbk-1* expression during embryogenesis and the first larval stage, steps in which the cells and structures essential for chemotaxis differ-

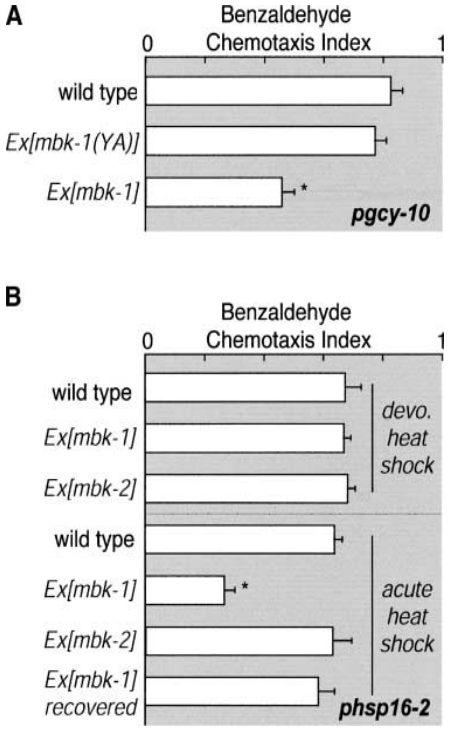


FIGURE 6.—*mbk-1(gf)* acts reversibly and acutely in differentiated neurons. (A) Odortaxis to benzaldehyde of wild-type animals (N2) and animals that express *mbk-1* (*Ex[mbk-1]*) and kinase-inactivated *mbk-1* (*Ex[mbk-1(YA)]*) under control of the *gcy-10* promoter. (B) Odortaxis to benzaldehyde. *mbk-1* or *mbk-2* was fused with *gfp* and expressed using the inducible promoter *hsp 16-2* (FIRE *et al.* 1990). To developmentally stage the heat shocks, gravid hermaphrodites were collected and treated with alkaline hypochlorite to physically separate the embryos. For the developmental heat shocks (*devo. heat shock*), these embryos were immediately placed at 33° for 1 hr, returned to 20° for 12 hr, heat-shocked again for 1 hr, and allowed to grow to adulthood at 20° prior to the assay. For adult expression, young adult animals were heat-shocked at 33° for 1 hr, returned to 20° for 2 hr, and assayed either immediately (*acute heat shock*) or following 24 hr (*recovered*). On the basis of GFP fluorescence, MBK-1::GFP or MBK-2::GFP were expressed for >12 hr but <24 hr following 1 hr at 33° and both were expressed at approximately similar levels. Each bar represents at least five independent assays; error bars show SEM and an asterisk indicates that the values differ significantly ($P < 0.05$) from controls.

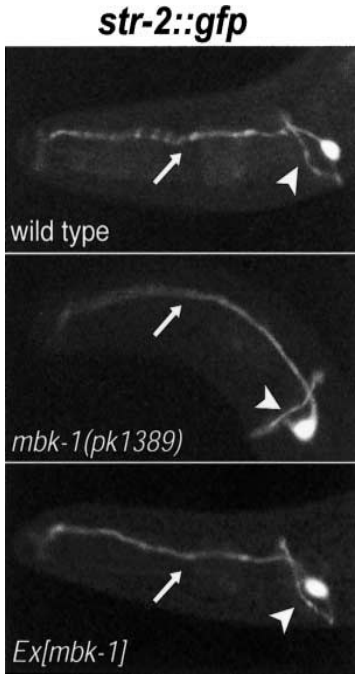


FIGURE 7.—Decreases or increases in *mbk-1* gene function do not affect olfactory neuron proliferation or differentiation. *str-2::gfp(kyIs140)* was used to examine the morphology of the AWC neurons in *mbk-1(pk1389)* and *mbk-1(gf)* animals. The dendrites (arrows) and axons (arrowheads) showed no gross morphological abnormalities following *mbk-1* perturbation. In addition, the left/right asymmetrical expression of *str-2::gfp* was preserved. Other neurons also show no proliferation defects (data not shown).

entiate, showed a normal response to the AWC-sensed odorant benzaldehyde (Figure 6B and data not shown). In contrast, adult animals in which *mbk-1* expression was induced 2 hr before the assay showed a significant decrease in odortaxis toward benzaldehyde. Extra copies of *mbk-2*, whose expression was also monitored with an attached *gfp* tag (Figure 1), produced no defects (Figure 6B).

Intriguingly, the effects of providing extra copies of *mbk-1* overexpression were reversible, since adult animals tested 24 hr after *mbk-1* induction displayed normal olfaction (Figure 6B; note that decrease in *mbk-1* expression could be monitored with the attached *gfp* tag). These results indicate that extra copies of *mbk-1* interfere with the acute function, but not with the formation or differentiation, of the olfactory neurons and that the phenotypes resulting from overexpressed *mbk-1* can be reversed by reducing *mbk-1* levels.

Loss-of-function analysis of *mbk-1*: The defects caused by providing additional copies of *mbk-1* prompted us to elucidate the *mbk-1* loss-of-function phenotype. We isolated a *mbk-1* loss-of-function allele, *pk1389*, through PCR screening of a *C. elegans* deletion library. *pk1389* is a likely null allele since it eliminates exons 2–6 of *mbk-1*. Exon splicing around this deletion would lead to a frameshift and to a premature stop in the message or to a deletion of the entire kinase domain (Figure 1 and MATERIALS AND METHODS). To confirm this notion, we introduced the *pk1389* deletion into the context of the translational *mbk-1::gfp* construct (Figure 1) and found that transgenic animals expressing this construct show no *gfp* expression (data not shown).

mbk-1(pk1389) homozygous mutant animals are viable and show no obvious morphological abnormalities. We examined several aspects of nervous system function in *mbk-1* mutants, including the behavior of *mbk-1* mutants in response to various sensory inputs (mechano- and chemosensory), and detailed aspects of its locomotory behavior. We observed no obvious defects under standard conditions (data not shown). We observed slight

but significant defects in attraction to low doses of olfactory cues, but since we were not able to rescue this phenotype through expression of *mbk-1* from extrachromosomal arrays, we could not conclude that these defects are indeed due to loss of *mbk-1* (data not shown; these rescue experiments may have failed because of an inability to supply precisely the right amount of *mbk-1*).

We examined nervous system architecture of *mbk-1* mutant animals in more detail. We visualized a total of 18 individual head and tail sensory neurons and their axonal anatomy in the main head ganglia of *C. elegans* both with *gfp* reporter genes that label individual cells and with the fluorescent dye DiI (Hedgecock *et al.* 1985) and found no morphological defects or aberrant cell numbers in *mbk-1(lf)* mutants (Figure 7 and data not shown). Since most of these 18 visualized neurons derive nonclonally from different embryonic precursors (ABalp, ABpla, ABplp, Abpra, and ABprp), their visualization allowed us to ascertain the integrity of various lineage branches in *mbk-1* mutants. The absence of any defects in cell generation and number is significant in light of the widespread neuroblast proliferation defects that lead to a significantly reduced brain size observed in Drosophila *minibrain* mutants (Tejedor *et al.* 1995).

Isolation of loss-of-function alleles in other *DYRK1A/minibrain* family members: Since *mbk-1* may act redundantly with other *DYRK1A/minibrain* family members to affect *C. elegans* nervous system development or function, we isolated mutations in the only other two *DYRK1A/minibrain*-related genes in the *C. elegans* genome, namely *mbk-2* and *hpk-1* (Figures 1 and 2). Due to their deletion of the respective kinase domains, the *mbk-2* and *hpk-1* mutant alleles constitute likely null alleles (Figure 1).

While *hpk-1* null mutant animals are viable and appear indistinguishable from wild type (data not shown), *mbk-2(pk1427)* homozygous animals display 100% penetrant maternal-effect embryonic lethality. The maternal-effect lethality is caused by defects in spindle positioning and cytokinesis in the early embryo (J. Pellettieri and G. Seydoux, personal communication). In addition, *mbk-2* homozygous animals derived from a heterozygous parent appear sick and grow poorly. To independently confirm the linkage of the lethality with the *mbk-2* locus, we inactivated *mbk-2* by RNAi (Fire *et al.* 1998) and observed 100% embryonic lethality in the progeny of 7 out of 10 adult *mbk-2(RNAi)* animals and >80% embryonic lethality in the progeny of the remaining 3 animals. The survivors display vulval defects at low penetrance (11%; data not shown). The embryonic lethality and overall sickness of maternally rescued *mbk-2* mutant animals did not allow us to test whether *mbk-1* and *mbk-2* have redundant functions in nervous system function.

DISCUSSION

Our analysis of the *C. elegans* homologs of the *DYRK/minibrain* family of protein kinases reveals strong patterns of similarity in expression and subcellular localization of these proteins across phylogeny. Like their vertebrate orthologs, all three *DYRK/minibrain*-like genes are broadly expressed. MBK-1, like its vertebrate ortholog DYRK1A, localizes to the nucleus while the closely related MBK-2, like its vertebrate ortholog DYRK2, is predominantly cytoplasmic and excluded from the nucleus. HPK-1, like its vertebrate homolog HIPK2, is localized to subnuclear puncta. However, interspecies comparisons in mutant phenotypes reveal a strong dissimilarity. While the Drosophila *minibrain* locus affects neuroblast proliferation and hence brain size (Fischbach and Heisenberg 1984; Tejedor *et al.* 1995), a physiological function apparently conserved in mice (Fotaki *et al.* 2002), we observed no apparent cellular proliferation defects in the nervous system of *mbk-1* mutant animals. Our mutant analysis of the other two *DYRK/minibrain*-like genes, none of which have been knocked out in any other species, revealed that one of them, *mbk-2*, is essential for viability, due to a function of the gene in cytokinesis in the early embryo (J. Pellettieri and G. Seydoux, personal communication); perhaps this defect relates to the neuroblast proliferation defects in Drosophila *minibrain* mutants. However, *mbk-1*, and not *mbk-2*, represents the Drosophila sequence ortholog of *minibrain* and Drosophila has a separate *mbk-2*-orthologous gene (Figure 2).

The most important conclusions from our functional analysis of *DYRK1/minibrain* family genes derive from providing extra copies of the *mbk-1* gene. First, increases in *mbk-1* expression produce functional olfactory defects without apparently interfering with either neuronal proliferation or neuronal differentiation. Second, the phenotypes resulting from providing extra copies of *mbk-1* can be specifically induced in adult animals and are thus acute rather than developmental defects. Third, the defects can be reversed by restoring *mbk-1* to normal levels of expression. Fourth, extra copies of the ubiquitously expressed *mbk-1* gene specifically disrupt olfactory behavior but no other behaviors tested. This apparent specificity suggests that additional copies of *mbk-1* do not cause an unspecific disruption of signaling or sickening of a neuron, but act in a cellular-context-dependent manner.

We conclude that aberrant expression levels of *mbk-1* interfere with acute sensory processing in olfactory neurons. This finding is relevant since it is the additional copy of the vertebrate *mbk-1* ortholog DYRK1A that causes neurological defects either in humans or in mouse models. Our experiments with the temporal regulation and reversibility of *mbk-1* expression phenotypes may be taken as an indication that human DYRK1A overexpression disrupts the function of terminally differentiated neurons. Moreover, the reversibility of defects observed upon the reversion of *mbk-1* expression levels back to normal suggests that therapies aimed at the reduction of DYRK1A expression or activity may provide a viable

therapeutic approach for the neurological defects of Down syndrome.

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Summary

Signal transduction allows cells to respond to signals from their environment and is therefore important for most biological processes. The binding of an extracellular signalling molecule to a cell-surface receptor is the first step in most signal transduction pathways. Cell-surface receptors transduce the extracellular signals by generating a cascade of intracellular signals that alter the behaviour of the cell. The proteins involved in signal transduction include, besides the receptors, GTP-binding proteins (or G proteins), protein kinases, phospolipases and ion channels, and these proteins are conserved between multicellular organisms. In this study, we used the nematode *Caenorhabditis elegans*, which is a simple and well-described organism that is very well suited to perform genetic experiments, to analyse conserved signal transduction pathways.

Chapter two of this thesis describes the genetic analysis of an important signal-transducing molecule, the adenylyl cyclase SGS-1. Adenylyl cyclases act downstream of certain heterotrimeric G proteins and convert ATP into the second messenger cyclic AMP. In *C. elegans*, SGS-1 can be activated by the homologue of mammalian $G\alpha_s$, GSA-1, since mutations in *sgs-1* suppress the neuronal degeneration induced by expression of constitutively active GSA-1 from its own or heat-shock promoter. We show that SGS-1 is essential for viability and that it is involved in behaviours as diverse as pharyngeal pumping and locomotion. To regulate this latter behaviour SGS-1 needs to be activated by GSA-1.

In chapter three, *nxf-1*, another suppressor of the neuronal degeneration induced by expression of constitutively active GSA-1 from a heat-shock promoter, is characterized. *nxf-1* encodes a nuclear export factor that is involved in transport of mRNA out of the nucleus. Mutations in *nxf-1* also suppress other heat-shock promoter-induced phenotypes, indicating that this locus is not a specific downstream target of $G\alpha_s$, but rather a general suppressor of heat-shock promoter-induced phenotypes. We postulate that the mutations in *nxf-1* make the protein inactive during heat-shock, resulting in reduced transport of the activated $G\alpha_s$ and other mRNAs out of the nucleus and thus the absence of the activated $G\alpha_s$ phenotype after heat-shock.

Chapter four reports the analysis of the *C. elegans* homologue of $G\alpha_{12/13}$, GPA-12. Loss of GPA-12 does not result in any obvious defect in development or behaviour of the animal. However, overexpression of constitutively active GPA-12 from its own or heat-shock promoter results in a developmental growth arrest that is caused by a feeding defect. Mutations in *tpa-1*, which encodes two PKC isoforms, suppress the developmental growth arrest induced by activated GPA-12, indicating that TPA-1 acts downstream of GPA-12. Activation of TPA-1 by the tumour-promoting phorbol ester TPA (or PMA) results in an identical developmental growth arrest, showing that activated GPA-12 and PMA use the same PKC signalling pathway.

In chapter five of this thesis, the DYRK family of protein kinases is described. A human DYRK kinase is implicated in the cognitive defects caused by trisomy of chromosome 21/Down syndrome, and we demonstrate that in *Caenorhabditis elegans* overexpression of a DYRK family member, *mbk-1*, results in signal transduction defects in olfactory neurons. *mbk-1* knockout animals do not show any obvious defects. Loss of *hpk-1*, another member of the DYRK family in *C. elegans*, does also not result in any obvious phenotype. However, a third member, *mbk-2*, is essential for viability.

Samenvatting

Signaal transductie is een belangrijk mechanisme, gebruikt in verschillende biologische processen, waarmee cellen kunnen reageren op signalen vanuit hun omgeving. Defecten in signaal transductie moleculen leiden tot het ontstaan van verschillende ziektes, zoals bijvoorbeeld kanker, en daarom worden signaal transductie paden veelvuldig bestudeerd. De eerste stap in veel signaal transductie paden is het binden van een signaalstof, bijvoorbeeld een hormoon, aan een receptor op de cel. Receptoren kunnen vervolgens in de cel een cascade van moleculen activeren die uiteindelijk zorgen voor een respons. De meeste eiwitten die betrokken zijn bij signaal transductie zijn geconserveerd gedurende de evolutie en hebben dezelfde functies in verschillende organismes. Hierdoor kan signaal transductie uitstekend bestudeerd worden in modelorganismes, zoals het rondwormpje *Caenorhabditis elegans*. Dit rondwormpje is een simpel en goed beschreven organisme, dat vooral geschikt is voor genetisch onderzoek. Zo is het mogelijk om genen gericht uit te schakelen of te muteren in *C. elegans*. Door een gen uit te schakelen dat codeert voor een signaal transductie eiwit kan de functie van zo´n eiwit in een signaal transductie pad worden achterhaald. Daarnaast is het mogelijk om andere genen die een rol spelen in dat signaal transductie pad op te sporen in *C. elegans*.

In hoofdstuk twee van dit proefschrift wordt een belangrijk signaal transductie eiwit bestudeerd, namelijk de adenylyl cyclase SGS-1. Adenylyl cyclases worden geactiveerd door bepaalde heterotrimere G eiwitten en produceren het signaal molecuul cyclisch AMP. SGS-1 wordt geactiveerd door het $G\alpha_s$ eiwit van *C. elegans*, GSA-1. Overexpressie van een actieve versie van GSA-1 leidt tot de dood van specifieke zenuwcellen, zowel bij expressie geïnduceerd door zijn eigen promoter als bij expressie geïnduceerd door de heat-shock promoter. Deze zogenaamde neuronale degeneratie wordt veroorzaakt doordat GSA-1 te veel signaleert naar SGS-1, want mutaties die de activiteit van SGS-1 verminderen onderdrukken de neuronale degeneratie. We laten zien dat SGS-1 ook wordt geactiveerd door GSA-1 om beweging te reguleren. Daarnaast is SGS-1 essentieel voor de levensvatbaarheid van *C. elegans*, omdat bij complete uitschakeling van dit eiwit wormen in een larvaal stadium blijven en niet kunnen uitgroeien tot volwassenen.

Hoofdstuk drie beschrijft een ander gen dat, als het wordt gemuteerd, de neuronale degeneratie geïnduceerd door expressie van actief GSA-1 vanaf de heat-shock promoter onderdrukt, namelijk *nxf-1*. *nxf-1* codeert voor een eiwit dat messenger RNA (mRNA) uit de kern transporteert. Mutaties in *nxf-1* onderdrukken ook phenotypes die geïnduceerd worden door expressie van andere eiwitten vanaf de heat-shock promoter, en dus is het waarschijnlijk dat NXF-1 niet specifiek betrokken is bij signaal transductie via GSA-1. Waarschijnlijk maken de mutaties in *nxf-1* het eiwit inactief gedurende de heat-shock, waardoor het transport van geactiveerd $G\alpha_s$ en andere mRNAs uit de kern sterk gereduceerd is, wat resulteert in een verwaarloosbare hoeveelheid geactiveerd GSA-1 in de cel en dus in afwezigheid van neuronale degeneratie.

In hoofdstuk vier wordt de genetische analyse van het $G\alpha_{12/13}$ eiwit van *C. elegans*, GPA-12, beschreven. Verlies van GPA-12 leidt niet tot een zichtbaar defect in de ontwikkeling of het gedrag van *C. elegans*. Echter, overexpressie van actief GPA-12 resulteert in een groei stilstand die veroorzaakt wordt door een gereduceerde opname van voedsel. Mutaties in het gen *tpa-1*, dat codeert voor twee verschillende protein kinase C (PKC) eiwitten, onderdrukken deze groei stilstand, wat suggereert dat TPA-1 wordt geactiveerd door actief GPA-12. Activatie van TPA-1 door de tumor-inducerende stof TPA, oftewel PMA, resulteert in een identieke groei stilstand, zodat het waarschijnlijk is dat actief GPA-12 en PMA hetzelfde PKC signaal transductie pad gebruiken.

In hoofdstuk vijf van dit proefschrift wordt de DYRK familie van protein kinases besproken. Een humaan DYRK kinase, DYRK1A, is betrokken bij de cognitieve defecten in Down syndroom dat veroorzaakt wordt door de aanwezigheid van een extra kopie van chromosoom 21. Expressie van extra kopiën van een DYRK familielid, *mbk-1*, in *C. elegans* resulteert in defecten in zenuwcellen die betrokken zijn bij reuk. Verlies van MBK-1 heeft geen duidelijk effect op het phenotype van de worm, evenals verlies van een ander lid van de DYRK familie, HPK-1. Het derde lid van de familie, MBK-2, is echter nodig voor levensvatbaarheid.

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Curriculum vitae

Celine Moorman is geboren op 30 juli 1975 te Nijmegen. In 1993 behaalde zij het gymnasium diploma aan het St. Ignatiusgymnasium te Amsterdam en begon zij met de studie Scheikunde aan de Rijksuniversiteit Leiden. Haar stages volgde zij bij de werkgroep Moleculaire Genetica aan de Rijksuniversiteit Leiden, onder begeleiding van G. Moolenaar en Dr. N. Goosen, en bij de Target Discovery unit van NV Organon te Oss, onder begeleiding van Dr. G. Zaman. Zij ontving voor haar studie-resultaten de Unilever Research Prijs 1997. In april 1998 werd het doctoraal examen Scheikunde *cum laude* afgelegd. Vanaf mei 1998 is het in dit proefschrift beschreven onderzoek verricht bij de afdeling Moleculaire Biologie van het Nederlands Kanker Instituut te Amsterdam en vanaf maart 2000 bij de afdeling Functional Genomics van het Hubrecht Laboratorium te Utrecht onder begeleiding van Prof. Dr. R. H. A. Plasterk.

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* authors contributed equally

Abbreviations

ACY	adenylyl cyclase
aPKC	atypical protein kinase C
ATP	adenosine triphosphate
cAMP	cyclic-3',5'-adenosine monophosphate
CAN	canal-associated neuron
cPKC	conventional protein kinase C
DAG	diacylglycerol
GFP	green fluorescent protein
GDP	guanosine diphosphate
GGL	Gγ-like
GTP	guanosine triphosphate
HPK	homeodomain interacting protein kinase
IP_3	inositol 1,4,5-triphosphate
MBK	minibrain kinase
nPKC	novel protein kinase C
NXF	nuclear export factor
PAR	partitioning abnormal
PINS	partner of inscuteable
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate13-acetate
RGS	regulator of G protein signalling
RNAi	RNA interference
SGS	suppressor of activated Gα_s
TPA	12-*O*-tetradecanoylphorbol-13-acetate
YAC	yeast artificial chromosome