Molecular Basis of Loss-of-Function Mutations in the glp-1 Gene of Caenorhabitis elegans

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Molecular Basis of Loss-of-Function Mutations in the glp-1 Gene of Caenorhabditis elegans

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The glp-1 gene encodes a membrane protein required for inductive cell interactions during development of the nematode Caenorhabditis elegans. Here we report the molecular characterization of 15 loss-of-function (lf) mutations of glp-1. Two nonsense mutations appear to eliminate glp-1 activity; both truncate the glp-1 protein in its extracellular domain and have a strong loss-of-function phenotype. Twelve missense mutations and one in-frame deletion map to sites within the repeated motifs of the glp-1 protein (10 epidermal growth factor [EGF]-like and 3 LNG repeats extracellularly and 6 cdc10/SWI6, or ankyrin, repeats intracellularly). We find that all three types of repeated motifs are critical to glp-1 function, and two individual EGF-like repeats may have distinct functions. Intriguingly, all four missense mutations in one phenotypic class map to the N-terminal EGF-like repeats and all six missense mutations in a second phenotypic class reside in the intracellular cdc10/SWI6 repeats. These two clusters of mutations may identify functional domains within the glp-1 protein.

INTRODUCTION

Cell interactions regulate growth, differentiation, and pattern formation during the development of multicellular organisms. Yet the mechanisms by which cells communicate to regulate cell fates during development are poorly understood. The application of genetic tools to this question has uncovered numerous genes that mediate regulatory cell interactions (reviewed in Lambie and Kimble, 1991a; Greenwald and Rubin, 1992). Remarkably, similar proteins mediate regulatory cell interactions throughout phylogeny. For example, tyrosine kinase receptors of the epidermal growth factor (EGF) receptor class control vulval induction in Caenorhabditis elegans (Aroian et al., 1990), determine the dorsal/ventral axis in Drosophila (Price et al., 1989), and have apparently diverse roles during mammalian development (Adamson, 1990).

In this article, we focus on the glp-1 gene, which regulates several cell interactions during the development of the nematode C. elegans (Austin and Kimble, 1987; Priess et al., 1987). Figure 1 diagrams the major developmental defects of glp-1 loss-of-function (lf) mutants. Normally, germline proliferation is induced by a somatic regulatory cell called the distal tip cell (Kimble and White, 1981). If the distal tip cell is ablated, then germ cells that normally are mitotic instead enter meiosis and differentiate. Similarly, animals that lack zygotic glp-1 fail in germline proliferation; the few germ cells present at hatching enter meiosis prematurely and differentiate (Austin and Kimble, 1987; Priess et al., 1987). In hermaphrodites, this germline defect leads to sterility because only a few sperm and no oocytes are produced; in males, it drastically reduces fertility. Normally, development of the anterior portion of the pharynx is induced during early embryogenesis (Priess and Thomson, 1987). For a few glp-1 alleles, embryos that lack maternal glp-1 can undergo morphogenesis normally but fail in pharyngeal induction; these embryos hatch from their eggshell but die as young larvae because they are unable to feed (Priess et al., 1987). Embryos that lack maternal glp-1 die. Although these embryos generate hundreds of cells, neither pharyngeal induction nor morphogenesis to form an elongated worm occurs (Austin and Kimble, 1987; Priess et al., 1987). Based on these loss-of-function defects, it has been proposed that wild-type glp-1 activity is essential for germline induction, pharyngeal induction, and embryonic viability and morphogenesis.
Figure 1. The glp-1(If) mutant phenotypes. (A) Diagram of an adult wild-type hermaphrodite showing positions and relative sizes of germline (grey), pharynx (black), somatic gonad (striped), and intestine (stippled). (B) glp-1 loss-of-function mutants have three developmental defects. Left, development in wild-type animals; right, defects in glp-1 loss-of-function mutants. Germline induction, the wild-type germ line is organized into three regions (Kimble and White, 1981). Distally, germline nuclei are mitotic, more proximally, they enter meiosis, and most proximally, they undergo meiotgenesis. Both the postembryonic proliferation of the germ line, from 2 to 2500 cells, and its organization into three regions depend on the presence of the distal tip cell (DTC) (Kimble and White, 1981). In glp-1(If) mutants, germline mitoses arrest early during larval development and all germline precursor cells enter meiosis prematurely and differentiate as sperm (Austin and Kimble, 1987). Pharynx induction, the wild-type pharynx possesses anterior and posterior bulbs that are connected by an isthmus (Albertson and Thomsom, 1976). In embryos derived from glp-1 homozygous mothers, pharyngeal induction fails and the anterior bulb of the pharynx is missing (Priess et al., 1987). Embryonic viability, during the last half of embryogenesis, the wild-type embryo is transformed from a cluster of cells into a little worm. In embryos derived from glp-1 homozygous mother, the embryo dies as an amorphous cluster of cells and does not hatch (Austin and Kimble, 1987; Priess et al., 1987). Within the dying embryo, the posterior bulb of the pharynx can be detected, but the anterior bulb is missing.

The glp-1 gene is a member of a small gene family that includes lin-12 in C. elegans (Yochem et al., 1988; Austin and Kimble, 1989; Yochem and Greenwald, 1989); Notch in Drosophila (Wharton et al., 1985; Kidd et al., 1986); and homologues in Xenopus (Coffman et al., 1990), rat (Weinmaster et al., 1991), and humans (Ellisen et al., 1991). We call this the LNG gene family for its founding members, lin-12, Notch, and glp-1. The overall organization and amino acid sequences of the LNG proteins are strikingly similar. All are predicted to be membrane proteins that contain three types of repeated motifs (Wharton et al., 1985; Yochem et al., 1988; Austin and Kimble, 1989; Yochem and Greenwald, 1989; Coffman et al., 1990; Ellisen et al., 1991; Weinmaster et al., 1991). Biochemical studies confirm that the Notch product is indeed a membrane protein and that its amino-terminus is extracellular and its carboxy-terminus intracellular (Johansen et al., 1989; Kidd et al., 1989). By analogy, other LNG proteins are likely to be membrane proteins oriented in similar fashion. Extracellularly, the LNG proteins possess a tandem array of EGF-like repeats, first found in the protein precursor of EGF (Gray et al., 1983; Scott et al., 1983). In addition, a cysteine-rich motif, which is unique to LNG proteins, is found in three copies in the region between the EGF-like repeats and the transmembrane domain. We call this cysteine-rich sequence the LNG motif; others have called it the Notch repeat (Wharton et al., 1985), the B-type cysteine-rich repeat (Kidd et al., 1989), or the LNR (lin-12/Notch repeat) motif (Yochem and Greenwald, 1989). Intracellularly, the LNG proteins have six copies of the cdc10/SWI6 repeat, a motif originally discovered in two yeast genes: cdc10 is required in Schizosaccharomyces pombe for initiation of the cell cycle (Aves et al., 1985) and SWI6 is required in Saccharomyces cerevisiae for HO gene transcription during G1 (Breeden and Nasmyth, 1987,a,b). More recently, cdc10/SWI6 repeats have been found in the protein ankyrin (as well as numerous other proteins; see DISCUSSION) and dubbed ankyrin repeats (Lux et al., 1990). The conservation of structural motifs among these proteins suggests that they may function by a similar molecular mechanism.

The lin-12, Notch, and glp-1 genes are all required for cell interactions that control cell fates (Greenwald et al., 1983; Lehmann et al., 1983; Austin and Kimble, 1987; Priess et al., 1987). The C. elegans genes, glp-1 and lin-12, control two superficially distinct types of cell–cell interactions: glp-1, as described above, mediates inductive interactions (Austin and Kimble, 1987; Priess et al., 1987), whereas lin-12 regulates interactions between cells of equivalent developmental potential so that they adopt distinct fates (Greenwald et al., 1983). However, recent evidence makes it clear that glp-1 and lin-12 are interchangeable and therefore function by a similar mechanism (Lambie and Kimble, 1991b; Mango et al., 1991; Seydoux et al., 1990). The Drosophila gene, Notch, is required for an interaction among neuroepithelial cells that allows some cells to differentiate as epidermal cells and others to develop as neurons (Lehmann et al., 1983). Genetic mosaic analyses of the tissues in which these genes function show that all three are required in the receiving rather than the signaling cell (Hoppe and Greenspan, 1986, 1990; Austin and Kimble, 1987; Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991), suggesting that these presumptive membrane proteins may act as receptors.

In this article, we report the identification of molecular lesions in 15 recessive loss-of-function (If) mutations of the glp-1 gene. Our characterization of these glp-1(If) mutations provides molecular evidence for the glp-1 null phenotype and suggests that all three motif types, EGF-like, LNG, and cdc10/SWI6, are critical to glp-1 function. Two phenotypic classes of glp-1(If) mutants carry molecular defects that are clustered to specific regions of
the glp-1 protein. We discuss the implications of these results for identifying functional domains within the glp-1 protein. In addition to providing insight into the functional domains of the glp-1 protein, mutants with known molecular lesions provide a battery of reagents that can be used for further genetic and molecular analyses of glp-1 function. A detailed understanding of how glp-1 regulates cell fates in C. elegans will shed light on how similar proteins regulate cell interactions, not only in worms and flies, but also in higher vertebrates, including humans.

**MATERIALS AND METHODS**

**Worm Strains**

All C. elegans strains described in this paper were derived from the wild-type parent C. elegans var. Bristol strain N2 (Brenner, 1974). The mutations used are LGL, smg(1)(r681), LGIII, glp-1(q46, q50, q158, q172, q175, q224, q231, q415, bn18, e2072, e2141, e2142, e2144, e225 and sy56), dpy-17(e164), dpy-19(e259), unc-32(e189), unc-69(e587), and LGV, him-5(e490). All glp-1 alleles (except qD2) were obtained after ethyl methane sulfonate mutagenesis (EMS). qD2 is a γ-ray induced allele (Austin and Kimble, 1989). Most glp-1 mutations are described in Austin and Kimble (1987) and Priess et al. (1987). glp-1(q415) is a new allele isolated by P. Balandry in our lab, bn18 was provided by S. Strome (Indiana University), e225 by T. Schedl (Washington University), and sy56 by W. Boorstein and P. Sternberg (Cal. Tech.). Other mutations are described in Hodgkin et al. (1988). In addition, we used the translolation elit(I; III; V) (Rosenbluth and Baillie, 1981), the free duplication qDp3(III; IV) (Austin and Kimble, 1987), and the lethal mutation qD2 (Austin and Kimble, 1989).

**Cloning of Mutant glp-1 Genes**

Genomic DNA was prepared by the method of Emmons and Yesner (1984). For the six glp-1 (ts) alleles, genomic DNA was isolated from homozygous stocks grown at permissive temperature. For alleles that could not be grown as homozygous strains, genomic DNA was prepared from hand-picked glp-1(ts) homozygotes identified by one of the closely linked markers unc-32 or dpy-19.

For glp-1 alleles, q224, q231, bn18, q158, q46, q172, e2142, qD2, genomic DNA was digested with Xho I and cloned into the Sal I site of EMBL3 a phage vector. Recombinant phage clones containing the 9.3 kilobase (kb) glp-1 insert were identified by screening the resulting libraries with 32P-labeled glp-1 genomic probes. This 9.3-kb Xho I glp-1 genomic insert includes 1.7 kb of 5' flanking sequence, the 3' UTR (360 bases), and 110 bases of 3' flanking sequence. The 5' end of the glp-1 coding region is known by primer extension; glp-1 mRNA is trans-spliced at a splice acceptor site (TTCCAG) 72 nucleotides upstream of the translation initiation codon (Kodoyianni, unpublished data). In addition, the glp-1 polyadenylation site and 3' untranslated region (3'UTR) were defined by cDNA clones (Kodoyianni, unpublished data; Yochem and Greenwald, 1989).

For glp-1 alleles q50, q175, q415, and e2072, four overlapping DNA fragments containing the entire glp-1 genomic region (as described above but with only 60 bases of 3' flanking sequence) were generated by polymerase chain reaction (PCR) (Saiki et al., 1988) using Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). For glp-1 alleles e225, e2141, e2144, and sy56, only specific regions of the glp-1 gene were cloned by PCR (see below). The conditions used for amplification were as follows: 1–3 μg of genomic DNA was amplified for 35 cycles of 1 min at 94°C, 40 s at 55°C, and 3.5 min at 72°C for each cycle. The pairs of 5' and 3' primers used and their nucleotide coordinates (from Yochem and Greenwald, 1989) are shown below. Each pair of primers flanks restriction sites that were used to clone the PCR fragments.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>VK22</td>
<td>5' GCCATTAGGGACCTTATAAGGT</td>
<td>505–525</td>
</tr>
<tr>
<td>VK21</td>
<td>5' TAGAGGGAATATGGACAGTG</td>
<td>2850–2830</td>
</tr>
<tr>
<td>EM7</td>
<td>5' CACCAAGAGCTGCTTAAACA</td>
<td>2456–2475</td>
</tr>
<tr>
<td>VK3</td>
<td>5' CAGGTCACAGACAGAG</td>
<td>5586–5570</td>
</tr>
<tr>
<td>VK4</td>
<td>5' GGTACTCTGGCCATAGA</td>
<td>5381–5397</td>
</tr>
<tr>
<td>VK1</td>
<td>5' GTGACAACAGCTGCGG</td>
<td>7230–7214</td>
</tr>
<tr>
<td>EM4</td>
<td>5' CTGTTTTTACGTGCGG</td>
<td>6935–6952</td>
</tr>
<tr>
<td>EM1</td>
<td>5' TCCGATCGAATAGGAG</td>
<td>9986–9969</td>
</tr>
</tbody>
</table>

**Identification of glp-1(lf) Mutations**

For most glp-1 alleles, the approximate site of the lesion was first identified by one of two methods for mismatch detection, using either PCR amplified or cloned glp-1 genomic DNA (see above); the exact base change was then determined by sequencing. For a few alleles, the lesion was identified directly by sequencing.

For glp-1(q50, q158, q172, q175, q415, e2072, and e2142) were examined by the chemical modification method of Cotton et al. (1988) with minor modifications. Cloned wild-type glp-1 genomic regions were used as probes: probe A, Xho I (804) to HindIII (2781); probe B, HindIII (2781) to EcoRI (5481); probe C, EcoRI (5481) to EcoRI (7021); and probe D, EcoRI (7021) to EcoRI (9964) (nucleotide numbering from Yochem and Greenwald, 1989). The following nucleotide numbers are given for the probe: glp-1 trans-splice acceptor site TTCCAG, 2440; translation initiation codon AUG 2513; translation termination codon TAG 9530 or 9539; polyadenylation site 9899. Fragments were used as probes were first gel purified and then digested with restriction enzyme(s) to yield a ladder of fragments with sizes between 100 and 1000 base pair (bp) and finally labeled with T4 kinase and 32P-ATP. End-labeled wild-type probe was then hybridized to a 100- to 200-fold excess of unlabeled mutant DNA, which had also been digested with the same restriction enzyme(s). The mutant DNA was either prepared from a recombinant phage or a PCR fragment (see above). The DNA hybrids (wild-type probe + mutant) were treated first with hydroxylamine (5M, pH 6.5), which modifies all mismatched cytosines, and then with piperidine, which cleaves at the modified cytosines. Analysis of the products of a 4% polyacrylamide gel revealed extra bands when the hybrids contained mismatched cytosines. Negative controls (i.e., wild-type probe hybridized to wild-type DNA) were run with every set of reactions. To rule out PCR artifacts, two independently amplified PCR fragments were examined in each experiment. To locate the site of a specific base substitution more accurately, the mismatch was usually repeated twice for each region, each time using different restriction enzymes to generate the fragment ladder.

**Detection by DNA:RNA Mismatch.** Mutations in glp-1(q46, q172, bn18, q224, q231, and qD2) were mapped by the DNA:RNA mismatch detection method of Myers et al. (1985) with the following modifications. For this procedure, we used genomic DNA cloned in λ phage EMBL3 as described above. One hundred to 200 ng of DNA in 1 ml of Tris (pH 8.0), EDTA (TE) was mixed with 1 ml of 200 mM NaOH and incubated at 37°C for 10–15 min. The DNA solution was neutralized by addition of 30 μl of hybridization solution, and the 32P-labeled RNA probe was immediately added. Hybridizations were carried out at 47°C for 1 h. RNAse A was typically used at a concentration of 1–4 μg/ml.

RNA probes were synthesized (Melton et al., 1984) from a series of clones containing DNA spanning the wild-type glp-1 gene (probes A to D above except that two smaller probes were used to subdivide the D region: probe D-1, EcoRI (7021) to Pst I [8288] and probe D-2, Pst I [8288] to EcoRI [9964]). A wild-type DNA control was included in each experiment; in addition, each probe (without prior DNA hybridization) was treated with RNAse A to ensure that digestion was complete and did not produce artifactual bands.
Detection by Direct Sequencing. Mutations in glp-1 (q50, oz25, e2141, e2144, and q56) were identified directly by DNA sequencing of specific genomic regions.

1) glp-1 (q50). For q50, the chemical modification mismatch method, which detects G → A,C,T and C → A,G,T nucleotide changes, yielded no aberrant fragments. A “reversed labeling” mismatch procedure (i.e., 32P-labeled mutant DNA used as probe and wild-type DNA used as target DNA) that should detect A → C,G and T → C,G transitions, similarly yielded no changes. To find the T → A change in glp-1(q50), the glp-1 coding sequence plus flanking regions were subcloned in duplicate from PCR fragments and the entire coding region sequenced.

2) glp-1 (oz25). The oz25 mutation was identified within the 1.5-kb EcoRI fragment (5481-7021) containing EGFL-7 to 10 and the 3′ LNF repeats. This fragment was cloned from two separate PCR reactions and sequenced in duplicate.

3) glp-1 (e2141, e2144, q56). Because we had mapped three ts alleles to the cdc10/SW16 repeats, we subcloned the 2.9-kb EcoRI fragment (7021–9964) containing these repeats from each of the three remaining ts alleles. Direct sequencing of clones from duplicate PCR reactions revealed the lesion sites.

Nucleic Acid Manipulations

Standard procedures were used as described (Sambrook et al., 1989). PCR fragments were cut with restriction enzymes to generate cloning sites, purified on 0.8% agarose gels (Geneel, BIO101 Inc., La Jolla, CA) and subcloned into pGEM-7z (+) (Promega, Madison, WI) or PBSKII (+) (Stratagene, La Jolla, CA). Plasmid DNA was isolated by alkaline lysis and purified either on a QiaGen column or by cesium chloride density gradient centrifugation. Templates were sequenced by the dideoxy chain termination method (Sanger et al., 1977) with Sequenase 2.0 (United States Biochemical, Cleveland, OH).

Phenotypic Characterization

We have characterized the phenotypes of several glp-1 homoyzgotes (glp-1(x)/glp-1(x)); Table 1) and transheterozygotes (glp-1(x)/glp-1(y)); Table 3). Homoyzgotes characterized include four new alleles, oz25 (isolated by T. Schedel), q56 (isolated by W. Boorstein), br18 (isolated by S. Strome), q415 (isolated by P. Balandyk), and four previously reported alleles (e2072, e2141, e2142, and e2144). The new alleles were obtained after EMS mutagenesis, backcrossed, mapped to chromosome III, and shown to fail to complement glp-1(q46) (Schedel, Boorstein, and Strome, personal communication; Kodyianni, Maine, and Kimble, unpublished data).

The effect of each mutant on germline proliferation was assayed in two ways. First, when germline proliferation fails, hermaphrodites are sterile. Therefore, fertility was scored by counting the number of embryos produced. Second, when germline proliferation fails, the number of germ cells is drastically reduced. Therefore, the total number of germ cells produced was determined by one of two methods. For some mutants, we first counted sperm in 4′-diamidino-2-phenylindole-2-HCl (DAPI)-stained young adult hermaphrodites raised at 25°C and then calculated the number of germ cells produced as the number of sperm divided by four; for other mutants, we used Nomarski microscopy to count total germ nuclei in L3 animals raised at 25°C; the latter method was applied only to mutants in which all germ line nuclei were primary spermatocytes by the L3 stage.

The effect of each mutant on embryogenesis was assayed by scoring the percentage of embryos that hatched; hermaphrodites were transferred every day to fresh plates through their reproductive period of adulthood. For each plate, embryos were counted first when the hermaphrodite was transferred and then again after 24 h (25°C) or 48 h (15°C). Embryos that fail to hatch were defective for both pharyngeal induction and embryonic morphogenesis. Embryos that hatched might either die, due to a failure in pharyngeal induction, or they might survive. The percentage of embryos with normal pharynx development was determined by counting how many embryos hatched and grew to adulthood; embryos that hatched but then died as young larvae were scored as defective in pharyngeal induction.

When our phenotypic characterization differed from those previously reported by Priess et al. (1987), we repeated it with new strains sent by J. Priess, with the same allele after repeated backcrossing, or both. We have no explanation for the differences that remain.

Construction and Analysis of glp-1 Transheterozygotes

General. All transheterozygotes reported in Table 3 were generated by the matings as described below. For all constructions, hermaphrodites were purged of their own sperm by allowing them to lay eggs at 20°C for several days before being mated. All transheterozygotes (except for those containing q50; see below) were homozygous for unc-32, a marker closely linked to glp-1 on LGIII.

glp-1 (+, q415, or e2072)/glp-1(q175). Hermaphrodites of genotype unc-32 glp-1(+, q415 or e2072)/dpq-19 unc-69 were mated with unc-32 glp-1(q175)/dpq-19 unc-69; him-5 males at the indicated temperature (Table 3). Unc non-Dpy F1 progeny, presumably of genotype unc-32 glp-1(x)/unc-32 glp-1(q175) were picked onto separate plates as L4s and scored for fertility and embryonic viability 24 or 48 h later. For controls, homozygous unc-32 glp-1 hermaphrodites, derived as self progeny from heterozygous mothers, were scored concurrently.

unc-36 glp-1 (q50)/unc-32 glp-1(q175). Hermaphrodites of genotype unc-36 glp-1(q50)/unc-36 glp-1(q175) arose on the translocation chromosome eT1 (unc-36(e873)). Hermaphrodites of genotype unc-36 glp-1(q50)/dpq-19 unc-69 were mated with unc-32 glp-1 q175)/dpq-19 unc-69; him-5 males. All non-Dpy non-Unce F1 hermaphrodites were picked onto separate plates as L4s and scored 24 h later. unc-36 glp-1 (q50) hermaphrodites were picked as F1 progeny of heterozygous mothers and were scored concurrently.

unc-32 glp-1(e2142)/unc-32 glp-1 (q175). Hermaphrodites of genotype unc-32 glp-1 (e2142)/+ were raised at 15°C, purged of endogenous sperm, and mated either with unc-32 glp-1(q175)/dpq-19 unc-69; him-5 or unc-32 glp-1 (e2142)/+ males at either 15 or 25°C. Unc F1 hermaphrodite progeny from these crosses, presumed to be unc-32 glp-1(e2142)/unc-32 glp-1 (q175) or, for the control, unc-32 glp-1 (e2142), were transferred to individual plates as L4s and scored concurrently.

Examination of smg-1; glp-1(0) Double Mutants

Construction of smg-1(1r681); glp-1(q46). smg-1; him-5 males were mated with glp-1(q46)/et17 hermaphrodites. Male F1 progeny, presumably of genotype smg-1(+); q46/ et17/+; him-5/et17, were crossed to dpq-19 unc-69 hermaphrodites. Cross progeny of genotype smg-1(+); q46/ dpq-19 unc-69 were identified by progeny testing in individual L4 hermaphrodites. Among self progeny of this double heterozygote, smg-1; q46/ dpq-19 unc-69 were identified by the genitalia defect typical of homozygous smg-1 animals (Hodgkin et al., 1989) and by progeny testing.

Construction of smg-1(1r681); unc-32(e189) glp-1 (q175). smg-1; glp-1(q46)/dpq-19 unc-69 hermaphrodites were purged of endogenous sperm at 20°C and were mated to unc-32 glp-1(q175)/dpq-19 unc-69; him-5 males. Non-Dpy non-Unc hermaphrodites were transferred onto individual plates as L4 larvae and individuals of genotype smg-1(+); unc-32 glp-1(q175)/dpq-19 unc-69 identified by progeny testing. Among self progeny of this double heterozygote, smg-1; q175/ dpq-19 unc-69 were identified as described above.

Scoring smg-1(1r681) double mutants. To look for smg-1 effects on q46 and q175, complete broods of three smg-1; glp-1(q46)/dpq-19 unc-69 hermaphrodites and three smg-1; unc-32 glp-1(q175)/dpq-19 unc-69 hermaphrodites were scored for brood size and segregation of phenotypes at the expected ratios. In addition, heterozygous mothers were examined for vulval aberrations and fertility.
RESULTS

glp-1(lf) Mutations Fall into Three Phenotypic Classes

The 15 recessive glp-1(lf) alleles that we have examined can be grouped into three phenotypic classes.

1) Five glp-1 alleles (q46, q158, q172, q175, and oz25) are nonconditional, fully penetrant, and eliminate germline induction (Table 1). In homozygotes, the two germline precursor cells that are present at hatching undergo one or two mitotic divisions, enter meiosis prematurely, and differentiate. Therefore, hermaphrodites are sterile and produce no embryos. These five alleles have the strongest glp-1(lf) phenotype known and are likely to be null mutations.

2) Six glp-1 alleles (q224, q231, e2141, e2144, bn18, and sy56) are temperature sensitive (ts) in both germline and embryo (Table 1). When raised at permissive temperature (15°C), glp-1(ts) mutants appear wild-type, but when newly hatched larvae are shifted to restrictive temperature (25°C), germline induction fails and hermaphrodites are sterile. The germline effect of the glp-1(ts) mutations mimics that of putative glp-1 null alleles. When hermaphrodites are raised at 15°C and shifted to 25°C as young adults, they are fertile, but their embryos die at restrictive temperature.

3) Four glp-1 alleles (q415, q50, e2072, and e2142) have a more severe effect on embryogenesis than on germline induction (Table 1). These mutations range in strength. The weakest allele, e2142, is virtually wild-type for germline induction and temperature sensitive for embryonic lethality. The strongest allele, q415, is temperature sensitive for germline induction and nonconditional for embryonic lethality. Two alleles, q50 and e2072, are intermediate in strength; germline induction fails in some but not all animals, but embryos always die (Table 1). Among those q50 and e2072 homozygotes that produce embryos, 80% have two wild-type oocytes and 20% have one wild-type and one Glp-sterile oocyte.

Mapping the Molecular Defects of glp-1 Mutations

To determine their approximate locations, 11 of the 15 glp-1(lf) mutations were examined by one of two methods for mismatch detection: RNase A cleavage of RNA: DNA hybrids (Myers et al., 1985) or chemical modification of DNA:DNA hybrids followed by cleavage (Cotton et al., 1988). Probes that cover the glp-1 transcription unit plus 1.7 kb of 5' flanking sequence and 60 nucleotides of 3' flanking sequence were used to roughly localize each mutation (see MATERIALS AND METHODS). Once a region was identified as distinct from wild-type, the exact base change was determined by directly sequencing. In this way, we identified the sequence changes in 10 glp-1 mutants but not in glp-1(q50). We therefore sequenced the entire q50 coding region and found a single sequence change. In addition, the nucleotide changes of four other alleles were identified by sequencing a single region of glp-1 without the aid of mismatch detection (see MATERIALS AND METHODS). Finally, we identified the breakpoint of a lethal deletion mutant, qDF2, which breaks within the glp-1 gene (see MATERIALS AND METHODS).

The positions of the glp-1(lf) mutations within the glp-1 protein are diagrammed in Figure 2 and the molecular changes are summarized in Table 2. During the course of these experiments, we confirmed the glp-1 sequence reported previously (Yochem and Greenwald, 1989). In the discussion that follows, each amino acid motif is abbreviated (EGFL for EGF-like, LNG for lin-12/Notch/glpl, SW16 for cdc10/SW16) and each repeat is numbered, starting from the amino-terminus. Hence, the second EGF-like repeat from the amino-terminus is called EGFL-2 and the fourth cdc10/SW16 repeat from the amino-terminus is called SW16-4. In addition, glp-1 alleles are often referred to by allele number, e.g., q46 rather than glp-1(q46).

Molecular Basis of the Putative Null glp-1(1f) Mutations

The putative null alleles of glp-1 include nonsense, missense, and deletion mutations (Table 2). The reference allele, q46, is an ochre mutation in the middle of LNG-2 (Table 2, Figure 3), and q175 is an opal mutation at the end of EGFL-3 (Table 2; Figure 5). The q46 and q175 alleles are predicted to encode fragments of protein consisting of the amino-terminal 536 or 190 amino acids, respectively. These nonsense fragments should lack both the membrane-spanning and cytoplasmic domains of the glp-1 protein. Two other severe glp-1(1f) alleles, q158 and oz25, are missense mutations; q158 substitutes tyrosine for a conserved cysteine in LNG-2 (Figure 3), whereas oz25 makes the same amino acid substitution in EGFL-7 (Figure 5A). Both of these cysteines are conserved residues among all members of the LNG protein family. The remaining strong glp-1(1f) allele, q172, was previously identified as a small deletion (Austin and Kimble, 1989). We find that glp-1(q172) is an in-frame deletion of 261 nucleotides (Table 2, Figure 2). The q172 protein is predicted to lack the last 11 amino acids of LNG-1, which includes one of the conserved cysteines plus all of LNG-2 and LNG-3 (Figure 3).

The two nonsense mutations, glp-1(q46) and glp-1(q175), are likely to represent complete loss of glp-1 activity. The truncated proteins encoded by these two mutants should not be membrane associated and therefore should not function in signal transduction. Yet the q175 fragment contains three complete EGF-like repeats and the q46 fragment has all EGF-like repeats and one intact LNG motif. Either of these nonsense fragments might retain some function. One potential difficulty in detecting the activity of a nonsense fragment is that, in
Table 1. Phenotypes of glp-1 loss-of-function mutants

<table>
<thead>
<tr>
<th>Allele</th>
<th>Temperature (°C)</th>
<th>Germline induction (%)</th>
<th>Embryos hatch (%)</th>
<th>Pharynx induction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>15, 25</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>gtp-1(q46, q158, q172, q175, e225)</td>
<td>15, 25</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Temperature sensitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gtp-1(q224, q231, bn18, e2141, 2144, 956)</td>
<td>15</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Partial loss-of-function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gtp-1(q415)</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>gtp-1(q500)</td>
<td></td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>gtp-1(e2072)</td>
<td></td>
<td>30</td>
<td>0</td>
<td>0</td>
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<tr>
<td>gtp-1(e2144)</td>
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<td>80</td>
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<td>0</td>
</tr>
<tr>
<td>gtp-1(e2145)</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>gtp-1(e2146)</td>
<td></td>
<td>100</td>
<td>90</td>
<td>0</td>
</tr>
</tbody>
</table>

NA, not applicable.

* Temperature at which mutant was raised from early embryogenesis through adulthood. To examine the embryonic phenotype of certain alleles, fourth larval stage hermaphrodites were shifted from 15 to 25°C (15 → 25); in these cases, oogenesis, fertilization, and embryogenesis all took place at 25°C.

* Percent homozygotes able to produce embryos (see MATERIALS AND METHODS).

* All embryos are self-progeny of homozygous glp-1 hermaphrodites (see MATERIALS AND METHODS).

* For e2072 and e2142, the embryos that hatch are missing the anterior pharynx and die as L1 larvae (see MATERIALS AND METHODS).

* Each wild-type hermaphrodite makes ~2500 germ line descendants and produces ~300 embryos by self-fertilization.

* Putative null glp-1 homozygotes have few germline nuclei (4–8) and produce no embryos (Austin and Kimble, 1987; this article). See Austin and Kimble (1987) for phenotypes of glp-1(q46, q158, q172, and q175). For glp-1(e225), e225 homozygotes were Unc progeny of unc-32 glp-1(e225)/dpy-19 unc-69 mothers; all were sterile (n = 132, 25°C; n = 10, 15°C); germ cells totalled three to eight in homozygotes raised at 25°C (n = 10).

* Temperature sensitive glp-1 homozygotes have a reduced number of germline nuclei (6–56) when raised at 25°C from hatching but are virtually wild-type when raised at 15°C (Austin and Kimble, 1987; Preiss et al., 1987; this article). See Austin and Kimble (1987) for phenotypes of glp-1(q224 and q231) and see Preiss et al. (1987) for glp-1(e2141 and e2144). Our data for glp-1(bn18, e2141, e2144, 956) homozygotes raised at 25°C are as follows: for glp-1(bn18), germ cells totalled 20–56 (n = 12). For glp-1(e2141 and e2144), germ cells totalled 2–12 (n = 31) and 9–14 (n = 10), respectively. For glp-1(956), germ cells totalled 60–80 (n = 10).

* For glp-1(q415), germ cells in homozygotes raised at 25°C totalled 10–22 (n = 10); germ cells in homozygotes raised at 15°C totalled ~800. Older adults raised at 15°C were shown to continue germline mitoses by DAPI.

* Austin and Kimble (1987); among those q50 homozygous hermaphrodites that produce embryos, 73% have two wild-type oocytes and 27% have one wild-type and one Glp-sterile oocyte (n = 22, examined with Nomarski optics; this article).

* Preiss et al. (1987; this article: glp-1(e2072), homozygotes raised at 25°C fell into two distinct classes: 77% had few germline nuclei (the Gsp phenotype) and 23% had nearly a wild-type number (n = 172); among those animals producing embryos, 80% had two oocytes with a nearly wild-type number of germ cells and 20% had one nearly wild-type and one Gsp-sterile oocyte (n = 36, examined with Nomarski optics). The total number of germ cells in mutants with few germ cells was 6–11 (n = 16); the total number of germ cells in mutants with two nearly wild-type oocytes was ~600–1000 (n = 4).

* Preiss et al. (1987; this article: germ cells in glp-1(e2142) homozygotes raised at 25°C totalled 700–1200 (n = 10); older 25°C adults were examined after DAPI staining to confirm that mitosis continues as in wild-type (n = 20).

In C. elegans, mutations in any of six smg loci allow nonsense MRNAS to accumulate at wild-type levels (Hodgkin et al., 1989; Pulak and Anderson, personal communication). We previously showed that the loss-of-function phenotype of another glp-1 nonsense allele, q35, is suppressed by a mutation in smg-1 (Mango et al., 1991). Therefore, this suppression is likely to reflect an increase in the levels of both glp-1(q35) mRNA and protein.

To ask whether the nonsense fragments of q46 and q175 might have activity, we examined the phenotypes of glp-1(q46 or q175); smg-1 and glp-1(q46 or q175)/+; smg-1 animals. We found no effect of smg-1 on the phenotype of either mutant. Among the self progeny of glp-1(q46 or q175)/+; smg-1 hermaphrodites, one-quarter were sterile as expected. Furthermore, we saw no
Figure 2. Molecular basis of glp-1(lf) mutations. The positions of 15 recessive loss-of-function glp-1 mutations are shown on a schematic representation of the glp-1 protein (1295 amino acids; see Table 1 for the associated mutant phenotypes and Table 2 for a list of codon changes). The hydrophobic sequence at the N-terminus is likely to be a signal sequence and that found in the middle of the protein has been predicted to be a transmembrane domain. In this diagram, the extracellular domain lies to the left of the transmembrane domain and contains two sets of cysteine-rich amino acid motifs: 10 copies of an "EGF-like" motif followed by three copies of the LNG motif. The first and second EGF-like repeats are separated by a pseudo-EGF-like repeat (ψ-EGFL, previously called T + Y; Yochem and Greenwald, 1989), which contains seven cysteines. The putative cytoplasmic domain contains six copies of the cdc10/SWI6 motif. Arrows above the line indicate the positions of missense mutations; arrows below the line mark the sites of nonsense mutations and include glp-1(q35) (Mango et al., 1991). The deleted region in q172 is shown as an open box. Also shown is qDf2, a γ-ray-induced deficiency that breaks within EGF9 and removes all of glp-1 from that position extending 3'ward in the gene. The orientation of the glp-1 protein in this diagram is opposite that of the standard genetic map; therefore, qDf2 extends to the left from glp-1 on chromosome III.

The effect on viability or morphology among glp-1(q46 or q175); smg-1 or glp-1(q46 or q175)/+; smg-1 animals. One interpretation of these data is that the extracellular portion of the glp-1 protein, when produced as a nonsense fragment, is inactive and does not interfere with wild-type glp-1. Alternatively, the nonsense glp-1 fragment may be unstable. In either case, the mutant phenotype of glp-1(q46 and q175) is likely to reflect a complete lack of glp-1 activity.

Six glp-1(ts) Alleles Are Missense Mutations in the cdc10/SWI6 Repeats

Six glp-1(ts) alleles are missense mutations in the cdc10/SWI6 repeats (Table 2, Figures 2 and 4). All six of these glp-1(ts) mutants are virtually wild-type at permissive temperature but are sterile at restrictive temperature (Austin and Kimble, 1987; Priess et al., 1987; this article). Four glp-1(ts) mutations map to SWI6-4 and change amino acids that are conserved between glp-1 and lin-

Table 2. Molecular lesions in glp-1(lf) mutants

<table>
<thead>
<tr>
<th>Allele</th>
<th>Nucleotide affected*</th>
<th>Base change</th>
<th>Codon change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>glp-1(0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>q46</td>
<td>6745</td>
<td>C → T</td>
<td>CAA → UAA</td>
<td>Gln 537 → ochre</td>
</tr>
<tr>
<td>q158</td>
<td>6615</td>
<td>G → A</td>
<td>UGU → UAU</td>
<td>Cys 560 → Tyr</td>
</tr>
<tr>
<td>q172</td>
<td>6709–6969</td>
<td>In-Frame Deletion</td>
<td>CGA → UGA</td>
<td>Arg 191 → opal</td>
</tr>
<tr>
<td>q175</td>
<td>5102</td>
<td>C → T</td>
<td>UGU → UAU</td>
<td>Cys 336 → Tyr</td>
</tr>
<tr>
<td>oo25</td>
<td>5538</td>
<td>G → A</td>
<td>UGU → UAU</td>
<td></td>
</tr>
<tr>
<td>glp-1(ts)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>q224</td>
<td>8721</td>
<td>G → A</td>
<td>GGA → GAA</td>
<td>Gly 1043 → Glu</td>
</tr>
<tr>
<td>q231</td>
<td>8763</td>
<td>G → A</td>
<td>GGA → GAA</td>
<td>Gly 1057 → Glu</td>
</tr>
<tr>
<td>bn18</td>
<td>8693</td>
<td>G → A</td>
<td>GCA → ACA</td>
<td>Ala 1034 → Thr</td>
</tr>
<tr>
<td>e2141</td>
<td>8378</td>
<td>C → T</td>
<td>CUU → UUU</td>
<td>Leu 929 → Phe</td>
</tr>
<tr>
<td>e2144</td>
<td>8378</td>
<td>C → T</td>
<td>CUU → UUU</td>
<td>Leu 929 → Phe</td>
</tr>
<tr>
<td>sy56</td>
<td>8678</td>
<td>C → T</td>
<td>CGG → UGG</td>
<td>Arg 1029 → Trp</td>
</tr>
<tr>
<td>glp-1(lf)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>q415</td>
<td>5208</td>
<td>G → A</td>
<td>GGA → GAA</td>
<td>Gly 226 → Glu</td>
</tr>
<tr>
<td>q50</td>
<td>4907</td>
<td>T → A</td>
<td>UGC → AGC</td>
<td>Cys 126 → Ser</td>
</tr>
<tr>
<td>e2072</td>
<td>4941</td>
<td>G → A</td>
<td>GGA → GAA</td>
<td>Gly 137 → Glu</td>
</tr>
<tr>
<td>e2142</td>
<td>2609</td>
<td>G → A</td>
<td>GGA → AGA</td>
<td>Gly 33 → Arg</td>
</tr>
</tbody>
</table>

* Nucleotide coordinates from Yochem and Greenwald (1989).
12 (Figure 4B). Two of these, sy56 and bn18, reside in a region with high similarity among all SWI6 repeats (see consensus in Figure 4A), whereas the other two, q224 and q231, are found in the less conserved half of SWI6-4 (Figure 4, A and B). Two other glp-1(ts) mutations, e2141 and e2144, though apparently isolated independently (Priess et al., 1987), carry the same nucleotide change, resulting in a leucine to phenylalanine substitution in SWI6-1 (Table 2, Figure 4A). This position is occupied by isoleucine in lin-12. The clustering of lesions that result in glp-1(ts) mutations points to the SWI6-4 repeat as playing a critical role in glp-1 function.

**glp-1 Alleles that Affect Embryos More Than the Germ Line are Missense Mutations in the Amino-Terminal EGF-Like Repeats**

Four glp-1 alleles (q50, q415, e2072, and e2142) are missense mutations in the amino-terminal EGF-like repeats (Figures 2 and 5); all four have a more severe effect on embryos than on the germ line (Table 1). Three of the four mutations affect residues that are highly conserved among all EGF-like repeats and one affects a glycine immediately adjacent to such a conserved residue (Figure 5, A and B). Assuming that such conservation reflects an underlying structural significance, these amino acid substitutions are likely to disrupt the structure of the EGF-like repeat in which they occur (see DISCUSSION).

The differential effect of these four alleles in embryos and the germline can be explained in two distinct ways. One possibility is that these mutations alter domains with embryo-specific functions. Alternatively, the amino acid substitutions may simply reduce glp-1 activity. If embryos are more sensitive than the germline to reduced levels of glp-1 activity, weak glp-1 alleles would affect embryos more severely than the germline. To distinguish between these explanations, we compared the phenotype of glp-1(x)/glp-1(x) homozygotes with that of glp-1(x)/glp-1(0) heterozygotes (where glp-1(x) represents each of the four glp-1 alleles that affect embryogenesis more than the germ line). We selected glp-1(q175) for glp-1(0), because this nonsense mutant truncates the glp-1 protein close to the amino-terminus (see above) and because the glp-1(q175) protein could not be detected using antibodies (Crittenden, Troemel, and Kimble, unpublished data). Although a glp-1 deficiency is preferable for this test, the only deficiency in this region, qDf2, leaves part of glp-1 intact (Figure 1). For the arguments presented below, we assume that q175 produces no functional glp-1 protein. If glp-1(x) disrupts an embryo-specific domain, a reduction in its copy number is predicted to enhance the embryo-specific defect preferentially without affecting other tissues (i.e., the germline). However, if the embryo were simply more sensitive to gene dose, a reduction in copy number is predicted to enhance the phenotype in all affected tissues.

We find that, for all four alleles, the germ line is more defective in glp-1(x)/glp-1(0) than in glp-1(x)/glp-1(x) animals (Table 3). For three alleles, (q50, q415, and e2072), the effect is dramatic, and for one allele, e2142, the effect is slight. The most probable explanation is that at least three of the four alleles are weak or partial loss-of-function glp-1 mutants; e2142 could either be a partial loss-of-function mutation or interfere with an embryo-specific function. We also examined the embryonic phenotypes of these animals. As might be expected, viability was reduced in glp-1(e2142)/glp-1(0) compared with glp-1(e2142)/glp-1(e2142) embryos (Table 3). Unexpectedly, e2072/q175 embryos were more viable than e2072/e2072 embryos. This might be explained by interference of the e2072 mutant protein, either with itself or another protein, which is alleviated when the copy number is reduced. In summary, we suggest that three of the four missense mutations in the N-terminal EGF-like repeats are partial loss-of-function mutations and that the embryo is more sensitive to the
A  
cdc10/SWI6 repeats in glp-1

<table>
<thead>
<tr>
<th>Repeat</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWI6-1</td>
<td>(920) K Y R R V R L H W L A N V R G K P E X o L K A G A D V N A X - e2141</td>
</tr>
<tr>
<td>SWI6-3</td>
<td>(993) N S E R S A L H E A V N K D L R I L R H L L L D K R L L X -</td>
</tr>
<tr>
<td>SWI6-4</td>
<td>(1029) R N G M T A L M L V A R E L G K H Q V X - 1 L L S K A G K L D Y X o</td>
</tr>
<tr>
<td>SWI6-5</td>
<td>(1073) Y K O R T A L H Y A M H D N E E M V I M V R R S S N K D K X -</td>
</tr>
<tr>
<td>SWI6-6</td>
<td>(1106) E D C G T P I M L A K E G C E K T V Q Y A L N D A S L G I X -</td>
</tr>
</tbody>
</table>

**Figure 4.** glp-1 mutations mapping to the cdc10/SWI6 repeats. (A) The six cdc10/SWI6 repeats of glp-1 are aligned to maximize homology. The number of the first amino acid of each repeat, shown in parentheses, is based on the numbering of Yochem and Greenwald (1989) (X, amino acid). Asterisks mark sites of amino acid substitutions in glp-1 mutants and a circle indicates the amino acid substitution of lin-12(n653) (Greenwald and Seydoux, 1990). Residues identical in at least three of the six repeats are boxed and appear in the glp-1 consensus. The consensus sequences derived from the cdc10/SWI6 repeats of fem-1 (Spence et al., 1990) and ankyrin (Lux et al., 1990) are shown for comparison. (B) Alignment of the SWI6-4 repeats of glp-1 and lin-12, with emphasis on the region with mutations. Identical amino acids are connected with vertical lines and similar amino acids with broken lines. The region of highest homology is boxed. The changes in glp-1 mutations are shown below the repeat and that associated with lin-12(n653) (Greenwald and Seydoux, 1990) is above the repeat.

dose of glp-1 than the germ line. Furthermore, we suggest that glp-1(e2142) may have an embryo-specific defect.

**DISCUSSION**

All glp-1(1f) Mutations Map Within the EGF-like, LNG, and cdc10/SWI6 Repeats

glp-1 is required for inductive cell interactions, during both embryonic and postembryonic development of C. elegans (Austin and Kimble, 1987; Priess et al., 1987). The glp-1 protein belongs to a small family of proteins, the LNG family (see INTRODUCTION), and is likely to function as a receptor (Austin and Kimble, 1987, 1989; Yochem and Greenwald, 1989). Repetitive motifs (EGF-like, LNG, and cdc10/SWI6) make up ∼60% of the glp-1 protein. In addition to the evolutionary conservation of these motifs among all LNG proteins, molecular analyses of mutations in Notch and lin-12 indicate that certain repeats are important for the function and regulation of individual LNG proteins (Hartley et al., 1987; Kelley et al., 1987; Kidd et al., 1989; Greenwald and Seydoux, 1990).

In this article, we report the characterization of 15 glp-1(1f) alleles. Two are nonsense mutations, 12 are missense mutations, and 1 is an in-frame deletion. All of these mutations map within the repetitive motifs. Therefore, the integrity of the EGF-like, LNG, and cdc10/SWI6 repeats must be crucial to glp-1 function. By analogy, these repeats will surely be essential to the function of all LNG proteins. In the following discussion, we present the major conclusions that can be drawn from our analysis and provide ideas for how the various repeated motifs of the glp-1 protein function to mediate cell interactions.

**Nonsense Mutants Define the glp-1 Null Phenotype**

The most severe phenotype of glp-1(1f) mutations is the failure of germline induction. Two mutations with this severe glp-1(1f) phenotype, glp-1(ql75 and q46), are nonsense mutants: one truncates the glp-1 protein just after EGF-3 and the other within LNG-2 (Figure 1).
Previously, we identified a different nonsense mutant, glp-1(q35), that truncates the glp-1 protein by 122 amino acids from the carboxy-terminus (Mango et al., 1991). The glp-1(q35) nonsense mutant has a weak loss-of-function phenotype that is suppressed by a mutation in smg-1 and a weak gain-of-function phenotype that is enhanced by the same mutation (Mango et al., 1991). The smg mutations allow nonsense mRNAs to accumulate at a wild-type level (Pulak and Anderson, personal communication). The influence of the smg-1 mutation on the glp-1(q35) phenotype implies that the truncated glp-1(q35) protein is functional when produced in sufficient quantity. By contrast, we show in this article that the amino-terminal nonsense mutants, glp-1(q46 and q175), are not affected by smg-1. A more standard genetic test for a null allele depends on the use of a deficiency; however, the only deficiency in this region, qDf2, only deletes a portion of glp-1 and its products have not yet been analyzed. Therefore, the standard tests cannot be done. Recent evidence with antibodies to the glp-1 protein supports the identification of glp-1(q175) as a protein null: no glp-1 protein is detected in glp-1(q175) homozygotes that nonetheless have a proliferative germ line due to the presence of other mutations that are not in glp-1 (Crittenden, Troemel, and Kimble, unpublished data). Therefore, glp-1(q46 and q175) lack glp-1 activity by both genetic and molecular criteria and are likely to be null mutants.

**Missense Mutations and an In-Frame Deletion may Identify Functional Domains in the glp-1 Protein**

Thirteen mutants are predicted to produce glp-1 proteins that are either full-length or nearly full-length. These 13 mutations fall into three phenotypic classes. The first class includes three alleles with a strong nonconditional phenotype, which is identical to that of the two nonsense mutants. Among these, oz25 is a missense mutation in EGFL-7; q158 is a missense mutation in LNG-2, and q172 is an in-frame deletion that removes most of the LNG repeats. In the second class are six glp-1(ts) alleles with a strong loss-of-function phenotype at re-
Table 3. Genetic analysis of glp-1 mutations with a more severe effect on embryogenesis than germline development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature (°C)</th>
<th>% fertile (n)</th>
<th>% hatching (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>15</td>
<td>100 (24)</td>
<td>100 (564)</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>100 (20)</td>
<td>100 (574)</td>
</tr>
<tr>
<td>+/q175</td>
<td>15</td>
<td>100 (17)</td>
<td>98 (605)</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>100 (68)</td>
<td>99 (914)</td>
</tr>
<tr>
<td>q415/q415</td>
<td>15</td>
<td>97 (60)</td>
<td>0 (738)</td>
</tr>
<tr>
<td>q415/q175</td>
<td>15</td>
<td>3 (31)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>q50/q50</td>
<td>25</td>
<td>33 (48)</td>
<td>0 (520)</td>
</tr>
<tr>
<td>q50/q175</td>
<td>25</td>
<td>0 (40)</td>
<td>NA</td>
</tr>
<tr>
<td>e2072/e2072</td>
<td>15</td>
<td>65 (194)</td>
<td>41 (3438)</td>
</tr>
<tr>
<td>e2072/q175</td>
<td>15</td>
<td>42 (161)</td>
<td>70 (3517)</td>
</tr>
<tr>
<td>e2072/e2072</td>
<td>25</td>
<td>22 (82)</td>
<td>74 (1176)</td>
</tr>
<tr>
<td>e2072/q175</td>
<td>25</td>
<td>7 (162)</td>
<td>80 (436)</td>
</tr>
<tr>
<td>e2142/e2142</td>
<td>15</td>
<td>100 (45)</td>
<td>97 (1666)</td>
</tr>
<tr>
<td>e2142/q175</td>
<td>15</td>
<td>95 (66)</td>
<td>0 (737)</td>
</tr>
<tr>
<td>e2142/e2142</td>
<td>25</td>
<td>100 (87)</td>
<td>21 (2219)</td>
</tr>
<tr>
<td>e2142/q175</td>
<td>25</td>
<td>100 (72)</td>
<td>0 (1285)</td>
</tr>
</tbody>
</table>

NA, not applicable.
* Transheterozygotes were generated from matings (see MATERIALS AND METHODS). All animals (except those containing e50) were homozygous for unc-32, a closely linked marker on chromosome III (see MATERIALS AND METHODS).
* All cross-progeny of at least three matings were scored.
* n, number of animals scored.
* N, number of embryos scored.
* Only one hermaphrodite produced four eggs, none of which hatched.

strictive temperature and a virtually wild-type phenotype at permissive temperature. All six are missense mutations in the cdc10/SWI6 repeats. Finally, the third class includes four alleles with a stronger effect on embryos than the germline. All four are missense mutations in the amino-terminal EGF-like repeats. Therefore, mutations in the latter two phenotypic classes correspond to clustered changes within the glp-1 protein.

Our finding that 12 missense mutations and 1 in-frame deletion of glp-1 alter the repeated motifs of the glp-1 protein suggests that the repeated motifs are critical to glp-1 function. Although we cannot rule out an effect on protein stability, it seems unlikely that all of these mutations yield unstable products. Therefore, in the discussion that follows, we discuss specific amino acid substitutions with the idea that they perturb protein function rather than stability.

**Individual EGF-Like Repeats May Have Distinct Functions**

Five missense mutations in glp-1 alter the EGF-like repeats of the glp-1 protein (Figure 5). Many proteins other than LNG proteins possess arrays of EGF-like repeats, but the function of these tandem arrays of EGF-like repeats is not understood. Some participate in protein–protein interactions (EGF, Cohen et al., 1980; thrombomodulin, Kuroswa et al., 1988; urokinase, Appella et al., 1987; Notch, Rebay et al., 1991), whereas those in the LDL receptor have been implicated in endocytosis (Davis et al., 1987). Characterization of mutations in the glp-1 EGF-like repeats may therefore provide insight into the function of this array in the glp-1 protein.

An EGF-like repeat is defined by its similarity to EGF. All EGF-like repeats contain at least six cysteines, three glycines, and three tyrosines/phenylalaines in a conserved pattern (Figure 5). In EGF, the six cysteines establish disulfide bridges that are essential to its secondary structure (Cooke et al., 1987; Montelione et al., 1987) (Figure 5B). The conserved amino acids in the EGF-like repeats may therefore be required for the repeat to assume a structure similar to that of EGF. Among the glp-1 missense mutations, two alter a conserved cysteine, two alter a conserved glycine, and one alters a glycine that lies adjacent to a conserved phenylalanine. One mutation, e2142, makes a change analogous to that found in Factor IX in two independent cases of Hemophilia B (Denton et al., 1988). The conserved nature of the amino acids altered in these mutants suggests that each mutation may change the secondary structure of its EGF-like domain.

Four missense mutations in the EGF-like repeats affect embryos more severely than the germline. The differential effect of these alleles on embryos could be attributed either to an embryo-specific function of the mutated EGF-like repeats or to a more stringent requirement for glp-1 activity in embryos than in the germline. We found that the germline defect was more pronounced in animals with only one dose, although the effect of one mutation, e2142, was only slightly enhanced. These results suggest that mutations in EGFL-1, EGFL-2, and EGFL-4 reduce glp-1 activity partially and that the embryo therefore has a lower tolerance for reduced glp-1 activity than the germline. Furthermore, the mutation in EGFL-1, e2142, may interfere with an embryo-specific function.

One missense mutation in the EGF-like repeats, glp-1(oz25), results in a null phenotype. This mutation substitutes tyrosine for a conserved cysteine in EGFL-7 (Figure 5). The sequence of EGFL-7 has two features that are unique among the glp-1 EGF-like repeats. First, EGFL-7 possesses eight rather than six cysteines. Second, EGFL-7 contains the sequence CDPGGYIGSR, which is also found in laminin (Figure 6). Domain III of the B1 subunit of laminin has eight EGF-like repeats, each with eight rather than six cysteines; its sequence is conserved in Drosophila (Montell and Goodman, 1988), mouse (Sasaki et al., 1987), and human (Pikkarainen et al., 1987). The fourth EGF-like repeat of domain III of laminin from all three species includes the recognition site for the laminin receptor, CDPGYIGSR; the binding of laminin to laminin receptor promotes cell attachment and migration (Graf et al., 1987). There-
fore, EGFL-7 of the glp-1 protein shares two characteristics of the laminin-subclass of EGF-like repeats. It is intriguing that the only missense mutation in the EGF-like repeats that appears to eliminate glp-1 function is located in EGFL-7. One interpretation is that EGFL-7 is a functionally unique domain and that its function is essential for glp-1 activity. Indeed, this domain might mediate interactions between the glp-1 protein and the extracellular matrix (Maine and Kimble, 1989).

Positive Regulation of glp-1 may be Mediated by the EGF-Like Repeats

The two mutations located in EGFL-2 have a unique "all or none" effect on germline proliferation. In glp-1(q50 or e2072) homozygotes, germline proliferation is either severely defective or nearly wild-type. Even within an individual animal, one ovotestis can be severely defective and the other virtually wild-type. This phenotype is rare or undetectable (e.g., <1%) among other glp-1 mutants (Austin and Kimble, 1987; Kodoyianni, unpublished data). The finding that a gonadal arm can be phenotypically Glp, whereas the other is phenotypically wild-type suggests that glp-1 is regulated independently in the two arms.

An all or none effect on germline proliferation can be explained by invoking a positive feedback control that maintains glp-1 in an active state. The altered glp-1 protein in glp-1(q50 or e2072) may provide sufficient activity in some ovotestes to initiate the positive feedback, whereas in other ovotestes, the defective protein may have too little activity to trigger this control. The positive control might be autoregulatory or it might act through a positive feedback loop.

Why is the all or none effect on germline proliferation observed in the two EGFL-2 mutants but not in other glp-1 mutants? One model is that EGFL-2 itself might be required for initiating the positive regulation. This interpretation is consistent with the finding that individual EGF-like repeats of Notch have distinct functions (Kelley et al., 1987; Rebay et al., 1991). An alternative model is that the EGF-like repeats act together to mediate the positive regulation of glp-1 activity in the germline. Consistent with this idea is the finding that the five missense mutations in the EGFL-repeats can be placed loosely into an allelic series, with the relative strength of the mutations corresponding to their distance from the amino-terminus. Thus, the weakest allele is glp-1(e2142) in EGFL-1 and the strongest is glp-1(oz225) in EGFL-7 (Table 1, Figure 5A). Mutations in EGFL-2 (q50 and e2072) are intermediate in strength and exhibit the all or none character described. Mutations in EGFL-4 (q415) are temperature sensitive for germline activity. A mutation that inactivates EGFL-1 would leave the majority of repeats intact, whereas mutations located more internally, e.g., EGFL-4 or EGFL-7; might severely disrupt the array and inactivate it. The position of EGFL-2 in the array would be poised so that some proteins with EGFL-2 defects remain functional, whereas others do not. Therefore, in some ovotestes, the level of glp-1 activity may reach a threshold level required to promote wild-type germline proliferation, and in other ovotestes, that threshold level may not be obtained. It is possible that elements of each model are correct. For example, the EGF-like repeats may act together to achieve positive regulation, but EGFL-2 may have evolved to play a more critical role in this function than the other EGF-like repeats.

LNG Repeats are Required for glp-1 Function

Two mutations that affect the LNG repeats of the glp-1 protein lead to a severe loss-of-function phenotype that is indistinguishable from that of null mutants. In glp-1(q172), 87 amino acids within the LNG repeats are deleted; in glp-1(q158), a conserved cysteine in LNG-2 is replaced by a tyrosine (Figure 3). The only other known mutations in LNG repeats are single amino acid substitutions in LNG-2 and LNG-3 of lin-12, which elevate rather than reduce lin-12 activity (Greenwald et al., 1983; Greenwald and Seydoux, 1990). One of the lin-12(gf) mutants, lin-12(n302), occurs within a stretch of eight amino acids in LNG-3 that is identical in glp-1 and lin-12 and may be a common site of regulation. The finding that mutations in the LNG repeats can lead to both loss- and gain-of-function suggests that the LNG region has a pivotal role in the function of LNG proteins.

cdc10/SWI6 Repeats of glp-1 may bind Proteins that Control Cell Fate

Six glp-1 mutations alter the cdc10/SWI6 or ankyrin repeats in the intracellular domain (Figures 2 and 4). The germline phenotype of these glp-1(ts) mutants, when raised at restrictive temperature, is similar to that of glp-1(0) mutants, as defined above. Therefore, it is likely that the glp-1(ts) proteins are not active at restrictive temperature. This inactivity may reflect either degradation of the glp-1 protein or inability of that protein to function. In either case, the embryonic defect of the glp-1(ts) mutations, which cannot be observed in glp-1(0) mutants, is likely to represent the effect of a lack of glp-1 activity in the early embryo.

\[\text{glp-1 EGFL-7 (aa 347-359)}\]

\[\text{V C E P D Y I G D R C} \]

\[\text{human Laminin B1 (aa 944-956)}\]

\[\text{C V C D P O V I G S R C D} \]

\[\text{mouse Laminin B1 (aa 944-956)}\]

\[\text{C V C D P O V I G S R C D} \]

\[\text{drosophila Laminin B1 (aa 937-949)}\]

\[\text{C H C Q E G Y S G S R C E} \]

Figure 6. Similarity in sequences of EGFL-7 of glp-1 and the EGF-like repeats of domain III of the B1 subunit of laminin from human (Pikkarainen et al., 1987), mouse (Sasaki et al., 1987), and Drosophila (Montell and Goodman, 1988). Identical or similar (Asp [E] and Glu [D]) residues are boxed. EGF-like repeat 10 of lin-12, which also has eight cysteines, does not contain the sequence found in laminin.
Remarkably, four of the glp-1(ts) mutations cluster in SWI6-4 (Figure 2). Furthermore, a lin-12(ts) mutation maps to the same repeat (Greenwald and Seydoux, 1990). These findings suggest that SWI6-4 is essential either to the structure of the intracellular domain or its function. Comparison of the sequence of SWI6-4 to the sequences of the other cdc10/SWI6 repeats reveals no obvious unique domains within SWI6-4. Therefore, the significance of SWI6-4 may lie in its position within the group of repeats or on some unique feature of SWI6-4 that we cannot recognize.

The cdc10/SWI6 repeats occur in diverse proteins and have been implicated in mediating protein–protein interactions. Three mammalian proteins exemplify this function. First, four cdc10/SWI6 repeats in the β1 subunit of the GA binding protein (GABP) mediate a stable interaction between the β1 and α subunits of GABP (LaMarco et al., 1991; Thompson et al., 1991). Second, five cdc10/SWI6 repeats are present in IkB, a negative regulator of the transcription factor NF-κB (Haskill et al., 1991). NF-κB is maintained in an inactive cytoplasmic form by binding to IkB (Nolan et al., 1991). Third, the 22 cdc10/SWI6 repeats in ankyrin appear to tether membrane proteins (Lux et al., 1990). By analogy, it is likely that the six cdc10/SWI6 repeats of LNG proteins are important for protein–protein interactions. The temperature sensitivity of the mutations mapping to the cdc10/SWI6 repeats of glp-1 is consistent with this hypothesis. Many cdc10/SWI6 repeat-containing proteins bind and/or regulate a putative transcription factor: SWI4 and SWI6 of S. cerevisiae (Andrews and Herskowitz, 1989a,b), fem-1 of C. elegans (Spence et al., 1990), cactus of Drosophila (Nusslein-Volhard, personal communication), IkB (Haskill et al., 1991) and the β1 subunit of GABP from mammals (Thompson et al., 1991). The exceptions are ankyrin and α-latrotoxin, which are also exceptional in their high number of repeats (Kiyatkin et al., 1990; Lux et al., 1990). An intriguing possibility is that the cdc10/SWI6 repeats in the LNG proteins may bind a transcriptional regulator. If true, they may regulate entry of a protein into the nucleus by sequestering it to the cytoplasm. In the germ line of C. elegans, the bound factor might be predicted to instruct entry into meiosis or to negatively regulate mitosis.

CONCLUSION

A knowledge of the amino acid changes in mutant glp-1(If) proteins, when coupled with a description of their phenotypic effects, provides insight into how the glp-1 protein functions. In this article, we provide molecular evidence for the glp-1 null phenotype and identify two mutants that are likely to be protein nulls. Furthermore, we find clusters of amino acid changes that may identify functional domains in the glp-1 protein. In addition, we present ideas about molecular mechanisms that may underlie certain aspects of glp-1 regulation and function. First, we speculate that the unusual “all or none” effect of EGFL-2 mutants on germline proliferation may reflect a role of the EGF-like repeats in a positive feedback control that regulates glp-1 activity. Positive regulation of glp-1 could serve to maintain glp-1 in an active state and commit cells carrying glp-1 to a certain fate. Second, we speculate that the EGFL-7 repeat may play a modular role in glp-1 function. This repeat is unique among the EGF-like repeats of glp-1, but it shares features with the laminin-subclass of EGF-like repeats. EGFL-7 may therefore mediate interactions of the glp-1 protein with the extracellular matrix or receptors of the extracellular matrix. Third, we speculate that SWI6-4 may be central to the function of the glp-1 intracellular domain. We further postulate that the cdc10/SWI6 repeats of the LNG proteins may function by sequestering a regulatory protein, perhaps a transcription factor, to the membrane and by preventing its transport into the nucleus.

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Molecular Basis of glp-1(II) Mutations


