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lag-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*

Samuel T. Henderson[1], Dali Gao[1], Eric J. Lambie[1,2] and Judith Kimble[1]

[1]Laboratory of Molecular Biology and Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA
[2]Gilman Laboratory, Dartmouth College, Hanover, NH 03755, USA

SUMMARY

The *C. elegans lag-2* gene is required for several cell-cell interactions that rely on the receptors GLP-1 and LIN-12. In this paper, we report that *lag-2* encodes a putative membrane protein with sequence similarity to *Drosophila* Delta, a proposed ligand for the Notch receptor. Furthermore, we show that the *lag-2* promoter drives expression of a reporter protein in the signaling distal tip cell (DTC) of the DTC/germline interaction. By in situ hybridization, we have found that endogenous *lag-2* mRNA is present in the DTC but not the germ line. One fusion protein, called LAG-2::β-gal(intra), rescues a *lag-2* null mutant and can be detected in both DTC and germ line. Taking these results together, we propose that *lag-2* may encode a signaling ligand for GLP-1/LIN-12 and that the entire LAG-2 protein may be taken up into the receiving cell during induction by GLP-1 and lateral signaling by LIN-12.

Key words: *lag-2*, *glp-1*, *lin-12*, induction, lateral signaling, *C. elegans*, *Delta*, signal transduction

INTRODUCTION

Intercellular signaling is essential for the establishment of pattern, specification of cell fates and control of tissue growth during development. Central to this paper are two classical types of cell-cell interaction. One type, called induction, occurs between cells with different ancestries and distinct developmental potential. In this case, one tissue induces a developmental change in the other. The second type, called lateral signaling, occurs between cells of equivalent developmental potential. These similar cells interact among themselves and thereby adopt different cell fates.

The signal transduction pathways that mediate induction and lateral signaling are beginning to be understood. Various molecular components or pathways have been implicated in inductive interactions, including the GLP-1 membrane protein for induction of the nematode germline and early embryonic fates (Austin and Kimble, 1987, 1989; Priess et al., 1987; Mello et al., 1994) and the EGF signaling pathway for induction of the nematode vulva (e.g. Sternberg and Horvitz, 1991) and the fly dorsal-ventral axis (Neumann-Silberberg and Schupbach, 1993). Lateral signaling, on the other hand, appears to be mediated by homologous pathways in nematodes and *Drosophila*. In nematodes, LIN-12 is required for lateral signaling between vulval precursor cells to allocate precursors to specific vulval fates (Greenwald et al., 1983). In *Drosophila*, a homolog of LIN-12, called Notch, is required for lateral signaling and the allocation of some cells as neuroblasts within an epidermal sheet of precursor cells (e.g. Artavanis-Tsakonas et al., 1991). *Notch* is one gene in the neurogenic pathway, which mediates numerous cell-cell interactions throughout fly development (Lehman et al., 1983; Greenwald and Rubin, 1992).

The *glp-1* and *lin-12* genes, central to induction and lateral signaling respectively, encode similar proteins (Yochem and Greenwald, 1989; Austin and Kimble, 1989; Kodoyianni et al., 1992). Furthermore, the GLP-1 and LIN-12 proteins appear to be functionally interchangeable (Lambie and Kimble, 1991; Mango et al., 1991; Fitzgerald et al., 1993; Roehl and Kimble, 1993). Therefore, GLP-1 and LIN-12 are likely to interact with similar or identical proteins.

Most components of the GLP-1 and LIN-12 signal transduction pathways have not yet been defined at the molecular level. GLP-1 and LIN-12 themselves appear to be membrane receptors (GLP-1: Austin and Kimble, 1987, 1989; Yochem and Greenwald, 1989; Roehl and Kimble, 1993; Crittenden et al., 1994; LIN-12: Yochem et al., 1988; Seydoux and Greenwald, 1989; Struhl et al., 1993). In addition, several genes have been identified genetically as possible candidates for other parts of the pathway (Lambie and Kimble, 1991; Maine and Kimble, 1993; Sundaram and Greenwald, 1993). Of particular importance to this paper are *lag-1* and *lag-2*, which are required for both LIN-12 and GLP-1(Lambie and Kimble, 1991). The strong loss-of-function phenotype of each *lag* gene is virtually the same as that of *lin-12 glp-1* double mutants. Thus, animals lacking *lag-1*, *lag-2*, or both *lin-12* and *glp-1* die as first stage larvae with a diagnostic set of cell fate transformations. In addition, weak loss-of-function mutations of *lag-1* and *lag-2* survive larval development, but exhibit defects in postembryonic development that are typical of *glp-1* or *lin-12*

single mutants. Therefore, the *lag-1* and *lag-2* genes have been predicted to encode proteins common to the GLP-1 and LIN-12 signal transduction pathways.

Analysis of the individual components of a signal transduction pathway is greatly facilitated by the separation of the signal and receptor into well-defined cells or tissues. The cells participating in an inductive interaction are clearly defined as signaling or receiving members of the interaction. By contrast, cells involved in lateral signaling are initially equivalent and must resolve into signaling or receiving members of the interaction. We have therefore focused on the role of LAG-2 in a single cell-cell interaction that is mediated by GLP-1: induction of the germline by the somatic distal tip cell (DTC). For a detailed description of DTC and germline anatomy, see Fig. 3 in the accompanying paper (Crittenden et al., 1994). Briefly, the DTC resides at the distal end of the germ line and induces mitotic divisions in the germline throughout postembryonic development and adulthood. Therefore, this GLP-1 mediated inductive interaction is both well-defined and accessible to manipulation.

In this paper, we demonstrate that *lag-2* encodes a membrane protein with structural similarity to Delta and Serrate (putative signals for Notch). Furthermore, we establish the *lag-2* null phenotype at the molecular level and propose that a family-specific cysteine-rich motif present in the predicted extracellular domain is crucial for LAG-2 function. In addition, we show that *lag-2* is expressed in the DTC, but not the germline. Finally, we provide evidence that the entire LAG-2 protein may be taken up by the germ line during induction. We propose that *lag-2* encodes a signal that interacts with both GLP-1 and LIN-12 in inductive interactions and lateral signaling during development.

MATERIALS AND METHODS

Worms strains and mutagenesis

All stocks were derived from wild-type *Caenorhabditis elegans* var. Bristol, strain N2 (Brenner,1974). For UV mutagenesis, hermaphrodites were exposed to 310 nm UV at 12 J/m^2/sec for 15 seconds. Trimethylpsoralen(TMP)/UV mutagenesis was done as described by Yandell et al. (1994). Table 2 lists the *lag-2* alleles discussed here.

Nucleic acid manipulations

Standard molecular protocols were used (Sambrook et al., 1989). Phage obtained are: JK#L70 and JK#L71, isolated from an EMBL3 genomic library (thanks to C. Cummins) by screening with pJK229 (see below); JK#L74 and JK#L77, isolated from embryonic cDNA libraries (thanks to P. Okkema and J. Ahringer) by screening with fragments from T27C3 and JK#L70. Subclones relying on pBluescript II KS- as vector (Stratagene) include: pJK229, 4 kb *Hin*dIII fragment from T27C3; pJK232: partial *Sal*I fragment from JK#L70 ; pJK254, *Eco*RV fragment from pJK232; pJK361, *Bam*HI fragment from JK#L74; pJK367, *Eco*RI fragment from JK#L77; pJK373, *Hin*dIII fragment from pJK361. Sequencing relied on Sequenase T7 polymerase (USB) following manufacturers protocol. Sequenced cDNAs were analyzed using the BLAST program (Altschul et al., 1990).

Transplicing was examined by RT-PCR. Total RNA was isolated from mixed stage N2 hermaphrodites and poly(A)$^+$ selected using Oligotex-dt (Qiagen). cDNA was made using a *lag-2*-specific primer (DG54). PCR was done with cDNA template using primers for SL1 or SL2 and a nested *lag-2*-specific primer (DG56). PCR products were cloned into pT7Blue (Novagen) and sequenced to confirm splice junction.

Two expression plasmids were constructed as follows: *lag-2 5′::lacZ* (pJK375): 3 kb *Sal*I fragment from pJK254 was ligated into the SalI site of pPD21.28 (Fire et al., 1990). This genomic fragment includes approximately 3 kb upstream of *lag-2*, ending 147 nucleotides upstream of the putative translational start site. *lag-2::lacZ(intra)* (pJK374): 4.4 kb *Kpn*I fragment from pJK254 containing much of the *lag-2* coding sequence as well as 3 kb of upstream sequence was ligated into the *Kpn*I site of pPD21.28 (Fire et al., 1990). The site of fusion is 16 amino acids downstream of the predicted transmembrane domain of LAG-2 protein.

Deficiency mapping by using PCR

The extents of deficiencies were established by using primer pairs and the polymerase chain reaction (PCR; Fig. 1). DNA was extracted from individuals homozygous for the given deficiency and amplified by PCR with tester and control primers to confirm the presence of DNA. Tester primer pairs were obtained from YACs and cosmids or directly from DNA sequences obtained from the Genome Sequencing Project (see Table1).

Generation of transgenic animals

Hermaphrodites were injected with a mixture of pRF4 (100 μg/ml) and a test DNA (10 μg/ml) as described by Mello et al. (1991). Transgenic nematodes roll due to the presence of pRF4 DNA; roller lines were established (Mello et al., 1991).

Rescue of *lag-2(ts)* by microinjection

For each test DNA, multiple roller lines were established at 15°C in a *lag-2(q420ts)* background. Rescue was assessed by counting progeny: at 25°C, wild-type hermaphrodites generate >100

Table 1. Primers used for deficiency mapping

Primer sequence: 5′ → 3′	Source*
EL4 CTT GTG ACT TTT CTT GTA CC EL5 GTT GCA GTT TCC AAA GAC AAC	ZK230
EL7 CTG CAT GAA TGG AAA TGC AGC EL8 CGG CTG CTC AAA CCT TAC CC	stP3
EL12 GTC TGG CAC CTC GCT GAT AAG EL13 GGG TGA ACT TCC GAG CTT TG	Y45G12
DG1 GAC CAC TGT CAG CAG CCT G DG2 GAG CTC AAA ACT ATA CAA TCT G	T18A6
DG3 CTG CCG GAG CCA TTT GCA C DG4 CTG CGA AGA ATC TCA ATG TC	cm11f10
DG5 GAC ATT GAG ATT CTT CGC AG DG6 GCT TGG AAC TTT GTG CGC G	cm11f10
DG7 CAA TTC ACA CAA CCG CTG GTC DG8 CCA TCA TCG ATG AAC AAT ACC	cm11e2
DG9 GGT ATT GTT CAT CGA TGA TGG DG10 GTA AGG GCA GTA GCA TAC TG	cm11e2
DG15 CAA ATC CTA CCC GTA TTT CCT DG16 AGA GAG TTA CTT CAC CGG ATG	F02C9
DG25 TGT CAT GTT GAC CTT GGG TC DG26 AAG AGA TAT ACT ACA CGA ATG C	ZK253
DG43 AGG ATG TGA GAA TGC ACT GAT DG44 AAC TTT CCT TCG GAC GAG AC	T27C3

*Sequence obtained from subcloned YAC (Y45G12), cosmids (ZK230, T18A6, FO2C9, ZK253, T27C3), known cDNAs (cm11f10 and cm11e2), or known polymorphism (stP3).

progeny/animal (*n*=20), whereas *lag-2(q420)* produces 2.6 progeny/animal (*n*=20). If at least one line yielded >60 progeny/hermaphrodite (*n*=20), the transgene was scored as positive; if none of at least five independent lines yielded an average of >60 progeny/hermaphrodite (*n*=20), it was scored as negative.

Identifying the molecular basis of *lag-2* mutations

Single L1s homozygous for a given *lag-2* mutation were examined for point mutations by PCR SSCP (PCR single strand conformational polymorphism; Orita et al., 1989). The segment with a polymorphism was amplified, subcloned into pT7 Blue (Novagen) and sequenced. If no change was detected by this method, fragments were amplified, cloned and sequenced until the lesion was found. For sequencing, at least four independently amplified clones were examined for each allele. In addition, since each point mutation alters a restriction site, PCR products obtained from mutant DNA were digested with the appropriate restriction enzymes to confirm the single base change.

In situ hybridization to *lag-2* RNA

In situ hybridization was performed as described (Evans et al., 1994) with the following modifications: hybridization time was shortened to 12 hours, and 100 μg/ml yeast tRNA was used in place of salmon sperm DNA and glycogen. Plasmid pJK373 was linearized with *Xho*I for T3 transcription to generate the sense probe and *Spe*I for T7 transcription to generate the antisense probe.

Detection of β-galactosidase fusion constructs and GLP-1

β-galactosidase and GLP-1 were examined either in intact transgenic animals or their dissected gonads, as described by Evans et al. (1994); Crittenden et al. (1994).

RESULTS

lag-2 encodes a small transmembrane protein that is structurally similar to *Drosophila* Delta

We cloned the *lag-2* gene by a combination of deletion mapping and mutant rescue (Fig. 1). In parallel, *lag-2* was cloned by another group (Tax et al., 1994); the results from the two labs are in agreement except for a few nucleotides in intron 1. Fig. 2 presents the *lag-2* coding region and its predicted product. The exon/intron boundaries were determined by comparing the nucleotide sequences of the *lag-2* cDNAs (pJK361 and pJK367, Fig. 2A) with that of the corresponding genomic region (Fig. 3, see legend). The two predicted introns both have acceptor and donor sequences typical of *C. elegans* introns (Emmons, 1988). Neither cDNA clone contained a translational start site; therefore the 5′ end of *lag-2* transcript is not known. A potential translational start, deduced from genomic sequence, begins an open reading frame of 1206 nucleotides, which is followed by a 269 nucleotide 3′ untranslated region (3′ UTR) (Fig. 3). A predicted polyadenylation signal AAAAAA, which is used in some *C. elegans* transcripts (T. Blumenthal, personal communication), was found. Because a perfect match to the consensus *C. elegans* splice acceptor occurs 7 nucleotides upstream of the predicted translational start, we asked whether *lag-2* might be transspliced. Using one primer to SL1 (Krause and Hirsh, 1984) or SL2 (Huang and Hirsh, 1989) and a second

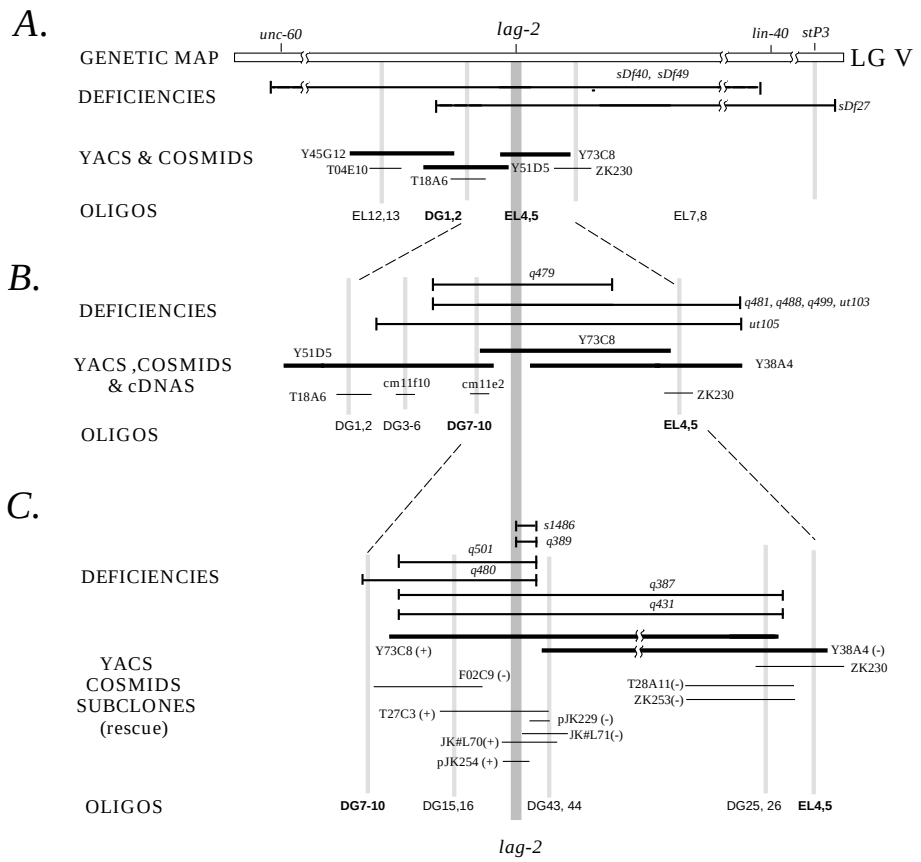


Fig. 1. Molecular cloning of *lag-2.* Cloning strategy shown as three successively expanded views with broken lines indicating changes in scale. Many genes, YACs and cosmids are omitted for clarity. YACs (Y prefix) and cosmids (F, T and ZK prefixes) were obtained from the *C. elegans* Genome Sequencing Project. Primers (e.g. EL7,8) are described in Table 1. In A and B, YAC Y73C8 (230 kb) provides a size reference; in C, pJK254 (7 kb) is the size reference. The vertical dark, broad line indicates the position of the *lag-2* locus; the vertical light, narrow lines indicate approximate locations of oligo primers. (A) Genetic map and deficiencies sDf27, sDf40, and sDf49 used to anchor *lag-2* onto the physical map. YACs and cosmids under region of deficiency overlap were identified. (B) Deficiencies obtained as *lag-2* mutations (*q479, q481, q488, q499, ut103* and *ut105)* were used to narrow the *lag-2* region. (C) Smaller deficiencies obtained as *lag-2* mutations (*q387, q389, q431, q480, q501, s1486*) were used to further narrow down the *lag-2* region. Mutant rescue experiments refined the *lag-2* location to a 7 kb plasmid (pJK254). Rescue of *lag-2(q420)* is indicated by (+) or (–) following clone names. We isolated several cDNAs, including pJK361 (Fig. 2), with restriction fragments derived from JK#L70 and T27C3. We selected for further study the cDNA pJK361 (Fig. 2) because its deduced amino acid sequence is similar to that of *Drosophila* Delta (Figs 3, 7), which we might have expected, and because part of the sequence was identical to that of *lag-2* as reported independently by Tax et al. (1994).

primer to *lag-2* coding sequence, we identified an SL1 transpliced product. Sequence of the PCR product revealed an SL1 transpliced leader at the predicted position.

The conceptual translation product of *lag-2* possesses 402 amino acids (see Fig. 3). At the N terminus is a putative signal sequence (von Heijne, 1985); more C-terminally is a second hydrophobic region with the hallmark features of a transmembrane domain complete with basic residues on its C-terminal side (Figs 2B, 3). Therefore, we suggest that LAG-2 is an integral membrane protein.

The LAG-2 amino acid sequence is most similar to that of the *Drosophila* proteins Delta and Serrate (22.5% similar to Delta and 23.4% to Serrate). More importantly, the LAG-2 architecture bears a striking resemblance to that of Delta and Serrate: all three proteins are predicted to be integral membrane proteins bearing a single transmembrane domain and all possess the same types of cysteine-rich motifs (see Fig. 7A and Discussion) The most N-terminal cysteine-rich motif of LAG-2, Delta, and Serrate is probably the most significant one. This motif exhibits a conserved number and spacing of cysteines, glycines, and phenylalanines/tyrosines and the spacing of these amino acids is unique. We suggest that this motif may be diagnostic of this family of proteins and refer to it as the DSL motif (for Delta, Serrate, and LAG-2) (Fig. 7B; see Discussion). Immediately C-terminal to the LAG-2 DSL motif is a half EGF-like repeat (Figs 2B, 3). The next cysteine-rich region conforms to a typical full EGF-like repeat (Figs 2, 3, 7C). The predicted intracellular domain consists of 94 amino acids, including a cluster of 14 amino acids, rich in proline (P), serine (S) and threonine (T) residues, similar to PEST sequences implicated in rapid degradation of proteins (Rogers et al., 1986).

Molecular basis of *lag-2* mutations

We have determined the molecular lesions associated with 16 *lag-2* mutations (Table 2, Figs 2, 3) (see Materials and Methods). Four mutations, *lag-2(q393)*, *lag-2(q411)*, *lag-2(q420)*, *lag-2(q477)*, carry single base changes within *lag-2*. The *lag-2(q411)* mutation creates a stop codon early in the gene and is likely to be a protein null; this allele results in the most severe Lag loss-of-function phenotype (Table 1, Figs 2, 3). The *lag-2(q393)* mutation changes one conserved cysteine in the DSL motif to a tyrosine. The strong loss-of-function phenotype associated with *lag-2(393)* suggests that the DSL motif may be essential for LAG-2 function. The temperature-sensitive *lag-2(q420)* mutation changes the invariant splice acceptor AG to AA and may result in inefficient splicing (Aorian et al., 1993). The temperature sensitivity of this mutant suggests that more functional transcripts may be made at lower temperature. The *lag-2(q477)* mutation generates a stop codon just within the predicted transmembrane domain and results in a strong loss-of-function phenotype. The presence of these molecular changes confirms the identity of this sequence as *lag-2*. The other 12 *lag-2* mutations are deletions (Table 2). The null phenotype is thus confirmed by the identification of numerous deficiencies and two nonsense mutants (Table 2). The *lag-2(q387)* mutation, which was first assigned as the *lag-2* canonical allele, is a large deficiency that removes not only *lag-2* but also neighboring DNA. By contrast, *lag-2(q411)* is a nonsense mutant mapping towards the 5' end of the *lag-2* coding region. In light of this molecular information, we reassign the canonical allele to be *lag-2(q411)*.

lag-2 is expressed in the distal tip cell

The similarity of LAG-2 with Delta and Serrate would suggest that LAG-2 may encode the signaling ligand for GLP-1 and LIN-12, much as Delta is thought to signal Notch (see Discussion). To test this prediction, we examined the expression of *lag-2* in cells participating in an inductive interaction that controls germline proliferation. In this cell-cell interaction, the somatic DTC is the signaling cell and the germ line is the receiving tissue (Kimble and White, 1981). We therefore asked

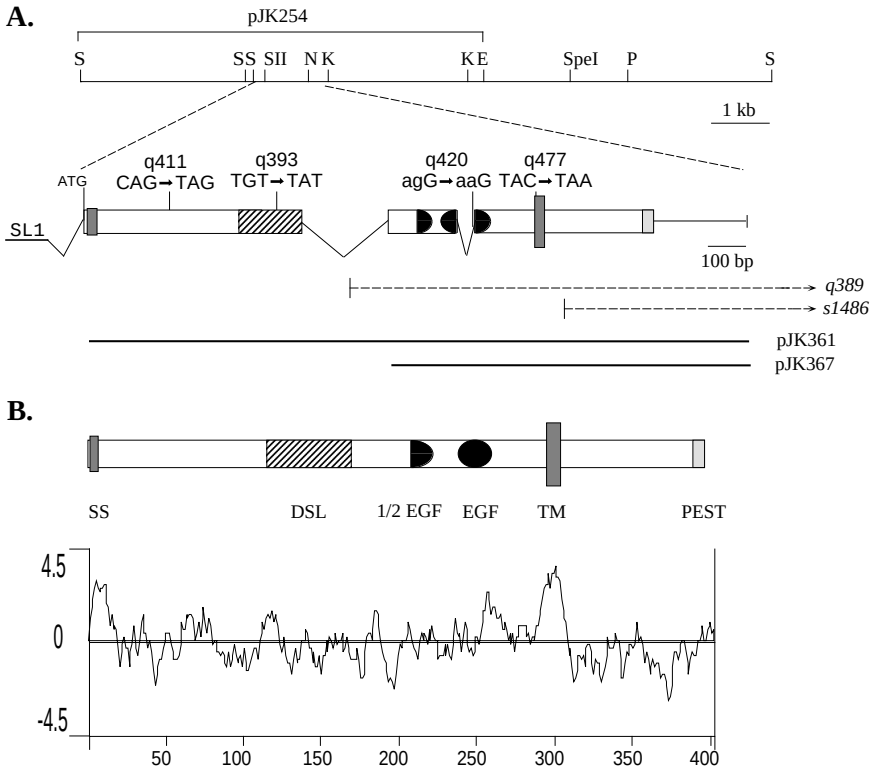


Fig. 2. Molecular map of *lag-2*. (A) Above, restriction map of *lag-2* genomic region with extent of the 7 kb rescuing plasmid, pJK254; S, *Sal*I; SII, *Sac*II; N, *Nar*I; K, *Kpn*I; E, *Eco*RV; and P, *Pst*I. Below, the deduced *lag-2* gene structure. Thin lines, non-coding regions, indicate postions of transpliced leader SL1 and introns; boxes, protein coding regions. The LAG-2 amino acid sequence was obtained from two cDNAs: pJK361 and pJK367, solid lines. Positions of point mutations and extents of deletions, *q389* and *s1486*, are shown. (B) Schematic of LAG-2 protein with the corresponding hydrophobicity plot (Kyte and Doolittle, 1982) shown below.

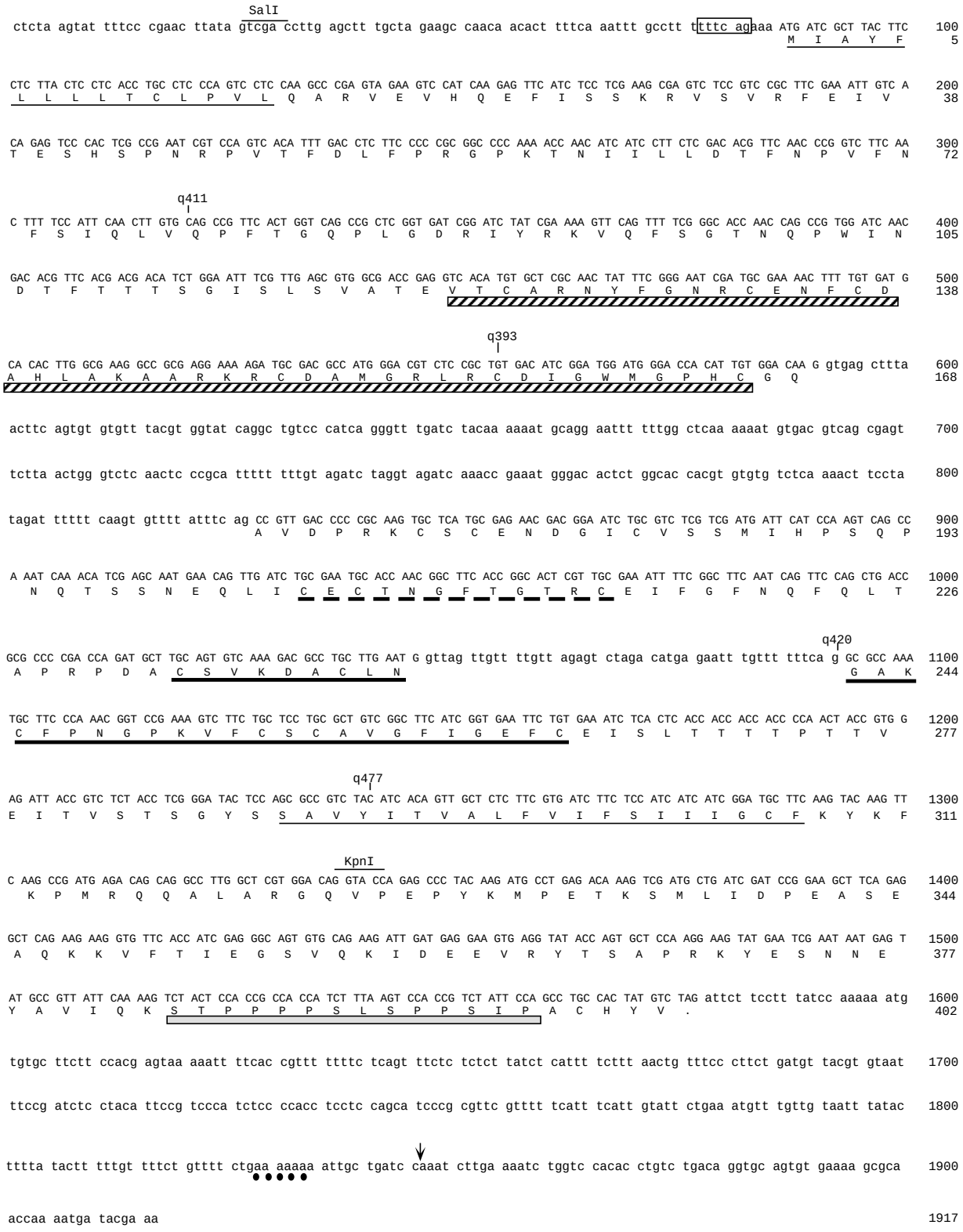


Fig. 3. The *lag-2* sequence. Genomic sequence of the region surrounding the *lag-2* gene; noncoding sequences in lowercase, predicted coding regions in uppercase. Transpliced leader SL1 splice acceptor site 5′ of predicted ATG is boxed. Hydrophobic regions underlined with thin line, predicted signal sequence found at amino terminus. DSL motif underlined by thick striped line. Half EGF-like region underlined with thick broken line. Full EGF-like region shown as thick solid line. Predicted transmembrane region shown as thin underlined sequences from amino acid positions 288-307. Predicted PEST sequence underlined with grey stippled box. Polyadenylation signal underlined with ●●●●●, site of polyadenylation indicated with arrow. Important restriction sites and known point mutations (Table 2) labeled above corresponding position.

whether LAG-2 was expressed in the DTC rather than in the germline.

To examine the site of *lag-2* expression, we constructed two reporter constructs (see Materials and Methods). First, *lag-2 5′::lacZ* fuses the 5′ flanking region of *lag-2* to a *lacZ* gene equipped with a nuclear localization signal (NLS) (see Fig. 4D, left). *lag-2 5′::lacZ* removes all predicted *lag-2* coding and downstream sequences. The reporter protein produced from *lag-2 5′::lacZ* should be localized in the nucleus of the cell that synthesizes it. Second, *lag-2::lacZ (intra)* fuses the *lacZ* coding region into *lag-2* at nucleotide +1341 (Fig. 3); this fusion removes the region that encodes most of the intracellular domain plus all downstream sequences. The reporter protein produced from this construct, called LAG-2::β-gal(intra), retains 16 amino acids of LAG-2 past the predicted transmembrane domain and replaces the remaining intracellular amino acids with β-galactosidase. (Fig. 4D, right). LAG-2::β-gal(intra) should be inserted into the membrane and might mark the subcellular location of native LAG-2.

In adult hermaphrodites carrying either *lag-2 5′::lacZ* or *lag-2::lacZ(intra)*, β-galactosidase was observed in the DTC (Fig. 4). The DTC is most clearly identified in gonads that have been extruded from the animal (Fig. 4A). However, most of our observations were made by staining whole animals. In adult hermaphrodites carrying *lag-2 5′::lacZ*, we observed β-galactosidase exclusively in the DTC nuclei (see Fig. 4B). β-galactosidase was found in 62% (30/48) of the DTCs examined in *lag-2 5′::lacZ* animals, but it was not observed in the germ line or in any non-gonadal cells of these hermaphrodites. Similarly, in adult hermaphrodites carrying *lag-2::lacZ(intra)*, we observed β-galactosidase in 100% (42/42) of the DTCs examined. In addition to the consistent DTC staining of *lag-2::lacZ(intra)* animals, we observed β-galactosidase within the germ line of 36% (15/42) of the gonads. This germline staining appeared to extend some distance from the distal tip cell (Fig. 4A,C; also see below). This germline staining was unexpected. LAG-2::β-gal(intra) is predicted to have β-galactosidase on the intracellular side of the fusion protein, which should reside within the cytoplasm of the distal tip cell. Some variable staining was also seen outside the gonad of *lag-2::lacZ(intra)* animals: a single uncharacterized cell stained positively with X-gal within the head of 2/21 animals, a few cells were positive in the pharynx of 1/21 animals, and a few uncharacterized cells were positive in the tail of 1/21 animals.

Remarkably, *lag-2::lacZ(intra)* rescues *lag-2* mutants. Not only can *lag-2::lacZ(intra)* rescue the temperature sensitive allele *lag-2(q420)*, but it also rescues the null mutant *lag-2(q411)*. Thus, *lag-2(q420)* and *lag-2(q411)* can live, reproduce, and be maintained as homozygous stocks when the *lag-2::lacZ(intra)* transgene is present. Only Roller animals survive in these strains, showing that the presence of the transgene is essential. Based on this result, we suggest that the specific amino acid sequence of the LAG-2 intracellular domain is not required for LAG-2 function.

Table 2. Molecular basis of the *lag-2* loss-of-function mutations

Allele	Mutagen*	Molecular defect	Position	Phenotype
q411†	EMS	CAG (Q) → TAG amber	320‡	null
q393†	EMS	TGT (C) → TAT (Y)	555‡	strong lf§
q420†	EMS	agG → aaG splice acceptor	1092‡	ts
q477	UV	TAC (Y) → TAA ochre	1241‡	null
q389† *s1486*†	EMS EMS	3′ deletion (<5kb)	see Fig. 2	strong lf§
q501 *q480*	TMP/UV UV	Complete deletion (~40kb)	see Fig. 1	null
q387 *q431* *q479*	EMS EMS TMP/UV	Complete deletion (~200kb)	see Fig. 1	null
q481 *q488* *q499* *ut103* *ut105*	TMP/UV TMP/UV TMP/UV *mut-6* *mut-6*	Complete deletion (>400kb)	see Fig. 1	null

*EMS=ethyl methanesulfonate, TMP/UV=trimethyl psoralen/ultraviolet, see Materials and Methods; for *mut-6* see Mori et al. (1988).

†For complete description of these alleles see Lambie and Kimble (1991).

‡Positions relative to Fig. 3.

§When homozygous, viability at 15°C is <5% , and at 25°C is 0%.

Endogenous *lag-2* mRNA is present in the DTC but not the germline

The production of β-galactosidase in the DTC by *lag-2 5′::lacZ* or *lag-2::lacZ(intra)* suggests strongly that endogenous *lag-2* is expressed in the DTC. However, *lag-2* germline expression may not have been observed in these experiments because expression of β-galactosidase from a transgene has never been detected in the *C. elegans* germ line (Fire et al. 1990; our unpublished observations). To investigate the possibility of *lag-2* germline expression we examined, by in situ hybridization, the location of endogenous *lag-2* RNA in dissected gonads from wild-type adult hermaphrodites (see Materials and Methods). With the *lag-2* anti-sense probe, we found that *lag-2* RNA is limited to the DTC and is absent from the germline (Fig. 5A). With the corresponding *lag-2* sense probe, we observed no staining in either DTC or germline (Fig. 5B). Other transcripts (e.g. *glp-1*, *fem-3)* are readily detected in the germ line using this technique (Crittenden et al., 1994; T. Evans, personal communication). We conclude that endogenous *lag-2* expression in the adult gonad is limited to the DTC and excluded from the germ line.

LAG-2::β-gal co-localizes with GLP-1 in the germline

Since the LAG-2::β-gal(intra) protein is functional and can rescue *lag-2* mutants, the subcellular location of this fusion protein may well mimic that of native LAG-2. To examine the distribution of LAG-2 and GLP-1 protein in the same gonad, we used antibodies to β-galactosidase and GLP-1 to stain dissected gonads from adult hermaphrodites that carried *lag-2::lacZ(intra)* (see Materials and Methods). The pattern of GLP-1 is identical to that reported by Crittenden et al. (1994): most staining is associated with the germline plasma membranes in the distal mitotic region of the germ line (Fig. 6B). The pattern of β-galactosidase is similar to that seen with X-gal: intense staining is found in the DTC and punctate staining is observed in the germline (Fig. 6C). The image of

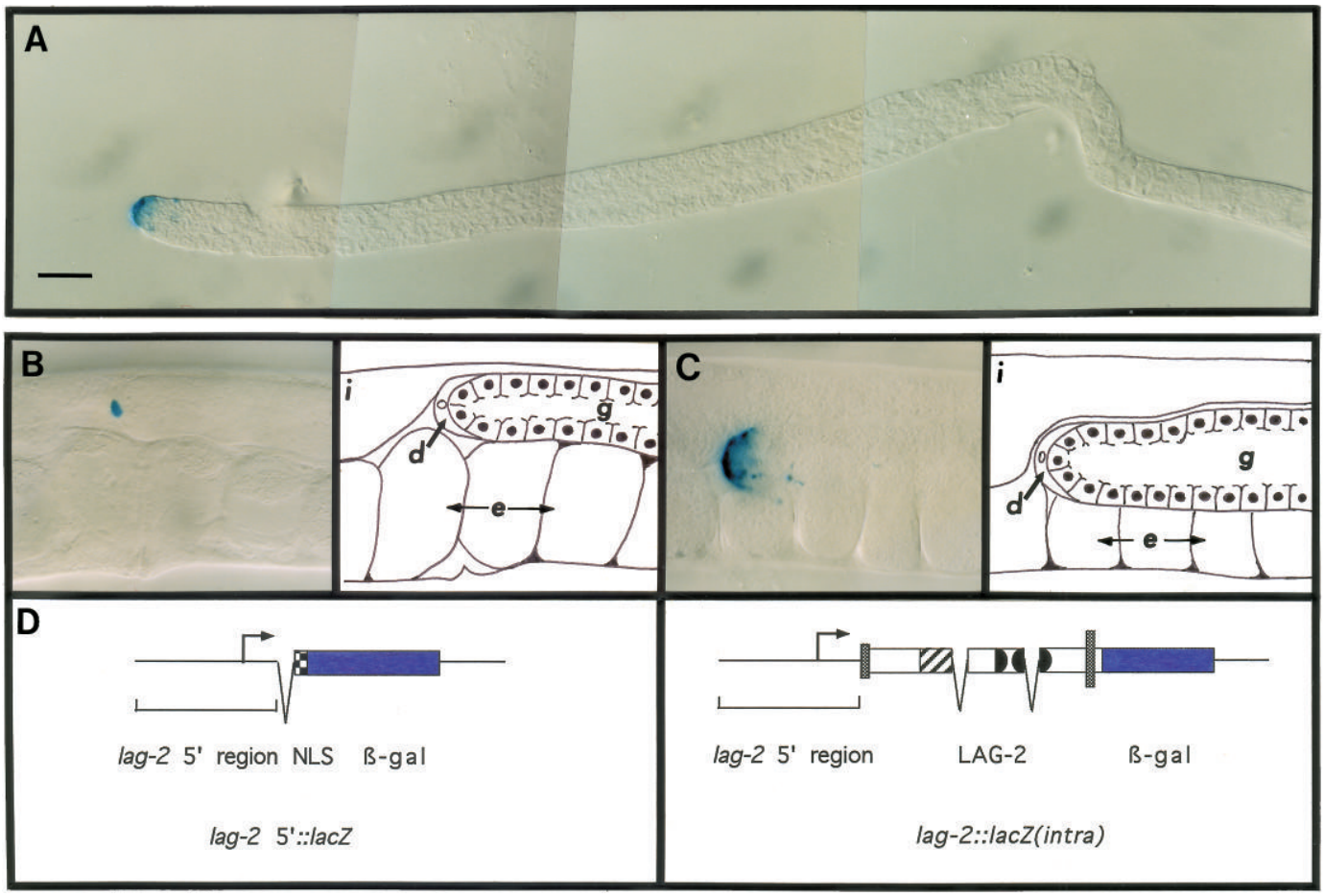


Fig. 4. The distal tip cell expresses β-galactosidase in gonads carrying *lag-2 5′::lacZ or lag-2::lacZ(intra).* Gonads from transgenic adult hermaphrodites carrying either *lag-2 5′::lacZ* or *lag-2::lacZ(intra).* X-gal is used to detect β-galactosidase. In schematic diagrams, d, distal tip cell; i, intestine; e, embryos; g, germline; black circles, mitotic germ cells; open circle, DTC nucleus. Scale bar, 20 μm. (A) Gonad extruded from an adult hermaphrodite carrying *lag-2::lacZ(intra)*. Distal is left; β-galactosidase is found in a cell residing at the distal end of the gonad. This cell is the DTC. (B) β-galactosidase is limited to the DTC nucleus. Left, intact adult hermaphrodite carrying *lag-2 5′::lacZ*; right, schematic diagram. (C) Some β-galactosidase is evident in the germ line at some distance from the DTC. Left, intact adult hermaphrodite carrying *lag-2::lacZ(intra)*; right, schematic diagram. (D) Constructs are shown beneath the corresponding X-gal staining pattern. In both, β-galactosidase shown in blue. NLS, nuclear localization signal.

both GLP-1 and β-galactosidase antibodies reveals a striking co-localization of β-galactosidase and GLP-1 within the germline at some distance from the DTC (Fig. 6D). This co-localization demonstrates that the intracellular region of LAG-2::β-gal is internalized by the germline and suggests that LAG-2 and GLP-1 may be internalized together into the same subcellular compartment (see Discussion).

DISCUSSION

Similarity of LAG-2 to Delta and Serrate suggests a role of LAG-2 in signaling LIN-12 and GLP-1

The predicted LAG-2 protein of *C. elegans* is similar in architecture to the predicted Delta and Serrate proteins of *Drosophila* (Fig. 7A) (Vassin et al., 1987; Kopczynski et al., 1988; Thomas et al., 1991; Fleming et al., 1990; Tax et al., 1994; this paper). All three proteins are putative integral membrane proteins, with both an N-terminal signal sequence and a single transmembrane domain. In their extracellular domains, all three have EGF-like motifs: Delta and Serrate possess multiple EGF-like repeats, whereas LAG-2 has only a single full EGF-like repeat and half EGF-like repeat. Most importantly, all have a cysteine-rich region that is found in no other reported protein and that we suggest may define a family-specific motif (see next section). These structural similarities strongly suggest that LAG-2, Delta and Serrate are evolutionary homologs.

The similarity of LAG-2 with Delta and Serrate provides an important clue to LAG-2 function. The following description focuses on Delta, but for the most part, similar arguments can also be made for Serrate. *Delta* is one of the genes of the neurogenic pathway in *Drosophila* (Lehmann et al., 1983; see Introduction). Two results have suggested that Delta may be a signaling ligand for the receptor Notch. First, Notch acts autonomously whereas Delta acts non-autonomously (Heitzler and Simpson, 1991). Second, in tissue culture, cells expressing Notch adhere specifically with cells expressing Delta

(Fehon et al., 1990; Rebay et al., 1991). Since LIN-12 and GLP-1 are structurally similar to Notch (Wharton et al., 1985; Kidd et al., 1986) and LAG-2 is structurally similar to Delta, we propose that LAG-2 may be a signaling ligand for LIN-12 and GLP-1.

A family-specific cysteine-rich motif in LAG-2, Delta, and Serrate

One unusual feature of LAG-2, Delta, and Serrate proteins is the most N-terminal cysteine-rich region. From the amino acid sequences of Delta and Serrate, the C-terminal half of this motif was recognized by its similarity to EGF-like repeats. This region was therefore classified as an 'incomplete' (Vassin et al., 1987; Thomas et al., 1991) or 'degenerate' EGF-like repeat (Fleming et al., 1990). However, comparison of the LAG-2, Delta, and Serrate sequences indicates that this incomplete EGF-like repeat is in fact a small part of a new cysteine-rich motif. We suggest that this motif may be diagnostic of the Delta/Serrate/LAG-2 family; a similar conclusion has been drawn by Tax et al. (1994). The number and spacing of cysteines, several hydrophobic amino acids, and glycines are strictly conserved in this DSL motif (for Delta, Serrate, and LAG-2) of all three proteins (Fig. 7B). A DSL motif is also found in a second Delta homolog in *C. elegans*, the conceptual APX-1 protein (Mello et al., 1994). The similarity of the most C-terminal part of the DSL motif to that of EGF-like repeats is striking and may indeed reflect an evolutionary relation of the DSL motif with the EGF-like motif. Nonetheless, the bulk of the DSL motif differs significantly from an EGF-like repeat.

The DSL motif appears to be crucial for LAG-2 function: alteration of one of the conserved cysteine residues in the motif results in a strong loss-of-function Lag mutant phenotype [*lag-2(q393)*, Table 2]. In GLP-1, similar missense mutations in EGF-like repeats do not significantly alter protein stability (Kodoyianni et al., 1992; Crittenden et al., 1994). By analogy, the *lag-2(q393)* missense mutation may also affect function rather than stability. If true, this mutation indicates a central role for the DSL motif in LAG-2 function, and furthermore, suggests that the DSL motif is crucial for the function of Serrate, Delta and other members of the family as well.

Function of the LAG-2 intracellular domain

The intracellular domains of LAG-2, Delta, and Serrate have little similarity to each other or to other proteins in the data base (Vassin et al., 1987; Kopczynski et al., 1988; Thomas et al., 1991; Fleming et al., 1990; this paper). The lack of similarity among the intracellular domains plus the lack of mutations mapping to this region provides no information about a possible function of the domain.

We find that the LAG-2 intracellular domain per se is not important for function. Specifically, most of the LAG-2 intracellular domain can be replaced with β-galactosidase without eliminating function. The LAG-2::β-gal(intra) fusion protein possesses only 16 amino acids of the intracellular domain, but nonetheless rescues both *lag-2(ts)* and *lag-2(0)* mutants. Why then is the intracellular domain so large, with at least 90 amino acids (LAG-2 94, Serrate 160, and Delta 214) in all three proteins of the family? The PEST sequence of LAG-2 suggests that the intracellular region of LAG-2 may regulate protein turnover, but this function is apparently not essential. We speculate that the intracellular domain may serve to anchor the protein in the membranes of specific cells and limit the range of contact with other cells.

lag-2 is expressed in the signaling DTC but not in the receiving germline

The homology of LAG-2 with Delta and Serrate suggested that *lag-2* may encode the signaling ligand for the LIN-12 and GLP-1 receptors. To test this idea, we examined the cellular location of *lag-2* expression, focusing on the DTC/germline interaction (see Introduction and Crittenden et al., 1994). We find that *lag-2* expression, as assayed by either expression of reporter constructs or by in situ hybridization, is limited to the DTC in adult hermaphrodites; expression during larval development is more complex and will be described elsewhere (S. Henderson and D. Gao, unpublished). The localization of *lag-2* to the DTC and not the germline, coupled with the Glp germline phenotype of weak *lag-2* alleles, strongly supports the idea that LAG-2 is indeed part of the DTC signal required for germline proliferation.

By analogy with its proposed function as a signal for GLP-1 in the DTC/germline interaction, we propose that LAG-2 also functions as a signal for LIN-12 during late embryogenesis and larval development. The mutant phenotype of strong loss-of-function *lag-2* alleles implicates LAG-2 in cell inter-

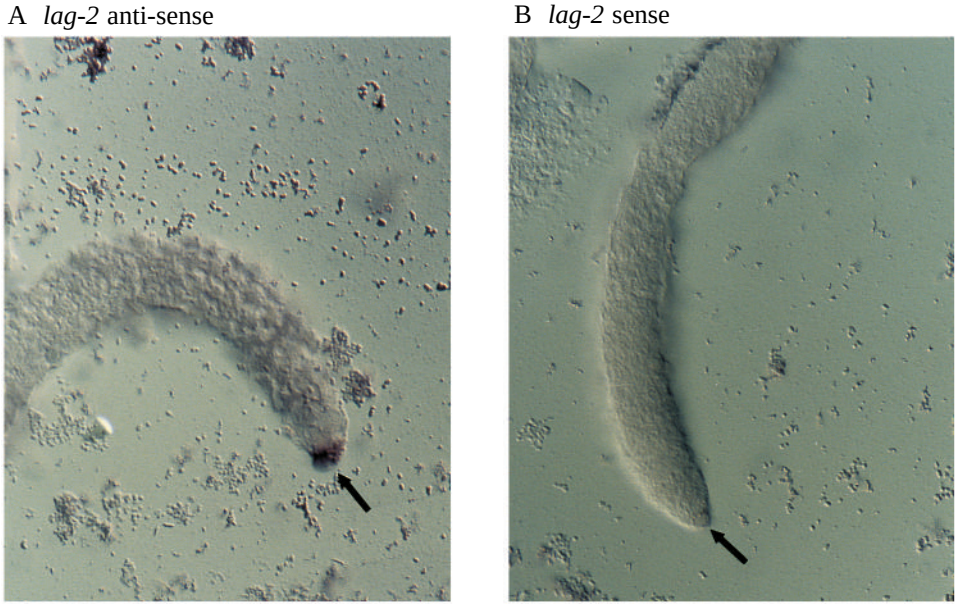
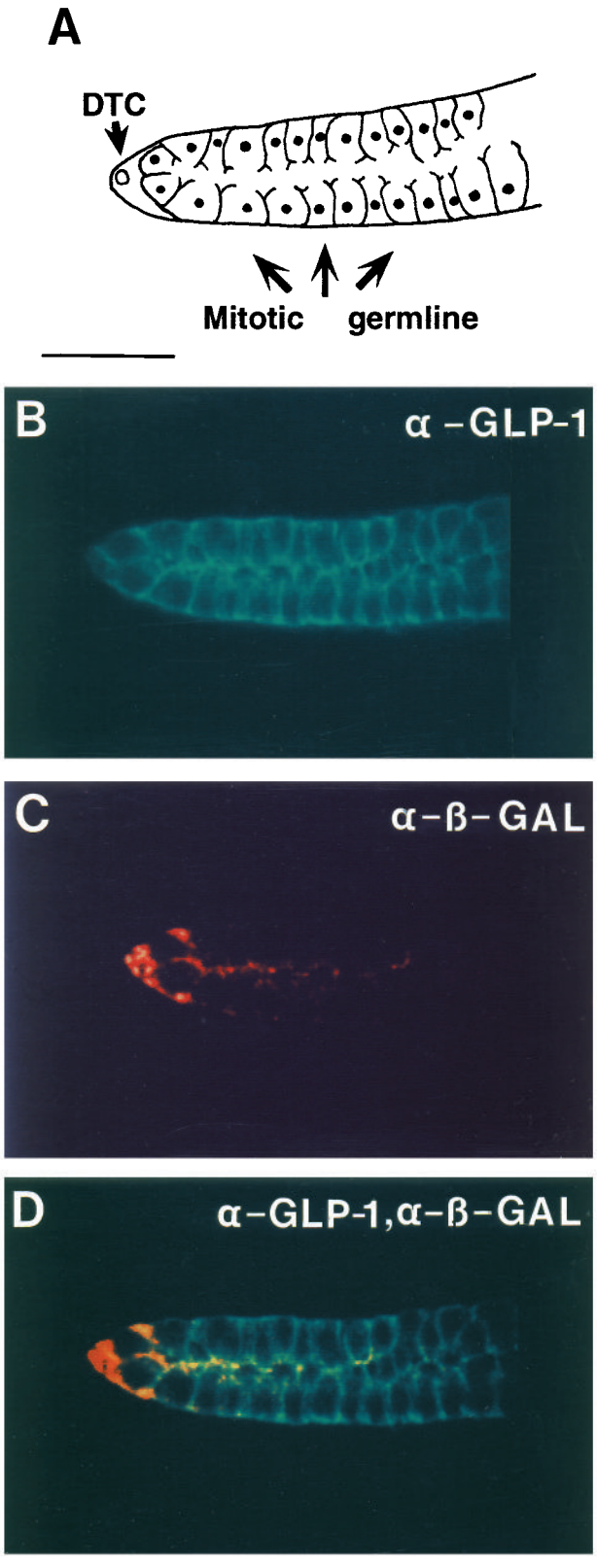


Fig. 5. RNA in situ hybridization of adult germlines. Dissected adult hermaphrodite germlines were probed with *lag-2* anti-sense probe (A) or sense probe (B). Arrow indicates position of DTC. No germline *lag-2* RNA was observed.

actions occurring during late embryogenesis, whereas the phenotype of weak *lag-2* alleles implicates it in the AC/VU decision in the somatic gonad among others (Lambie and Kimble, 1991). Further analysis will be required to resolve the full extent of the LAG-2/LIN-12 interactions during larval development.

The *lag-2* gene does not appear to be required for all *glp-1*-mediated inductive events. Specifically, unlike *glp-1*, *lag-2* does not appear to function in the early embryo. Maternal *glp-1* is translated to mediate at least two inductive cell interactions at the 4-28 cell stage of embryogenesis (Evans et al., 1994; Priess et al., 1987; Austin and Kimble, 1987; Mello et al., 1994; Mango et al., 1994; Hutter and Schnabel, 1994; J. Rothman, personal communication). By contrast, maternal *lag-2* is not required in the early embryo, *lag-2* RNA is not present in oocytes, and *lag-2* is apparently not expressed in early embryos (E. Lambie and S. Henderson, unpublished; this paper). Indeed, the *apx-1* gene has recently been identified as a Delta homolog that is a good candidate for the GLP-1 signaling ligand in the embyonic induction of ABp by P2 (Mello et al., 1994). Therefore, we propose that *lag-2* is not the ligand for GLP-1 in the early embryo, but instead that GLP-1 has one or more embryo-specific ligands.



LAG-2 becomes internalized by the germline during induction

The LAG-2::β-gal(intra) fusion protein, which is produced by the *lag-2::lacZ(intra)* transgene, is detected in both DTC and germline (Fig. 6D). It seems likely that the presence of LAG-2::β-gal(intra) in both DTC and germline reflects the normal location of endogenous LAG-2, largely because LAG-2::β-gal can rescue a *lag-2(0)* mutant. We suggest that LAG-2::β-gal fusion protein is initially synthesized by the DTC and then is taken up by the germline tissue during induction. By analogy we suggest that endogenous LAG-2 is transported from DTC to germline during induction. The co-localization of GLP-1 and LAG-2::β-gal(intra) suggests that the two proteins are taken up together into the same intracellular site.

The uptake of LAG-2::β-gal by the germline is similar to the uptake of Delta by Notch-expressing cells in tissue culture (Fehon et al., 1990) and the uptake of the boss signaling ligand by the R7 cell in the *Drosophila* eye (Kramer et al., 1991; Cagan et al., 1992). In all three cases, a predicted membrane protein that acts as a signaling ligand (LAG-2, Delta, or boss) is observed in cells that do not express that protein and that are receiving cells in a cell-cell interaction. Furthermore, in all three cases, the putative receptor protein (GLP-1, Notch, sevenless) co-localizes with putative ligand. Therefore, these cell-cell interactions may rely on a common underlying molecular mechanism.

Fig. 6. Distribution of LAG-2::β-gal and GLP-1 in adult germline. (A) Schematic representation of distal region of adult germline, showing DTC on left. DTC nucleus shown as open circle, mitotic germ nuclei shown as black circles. Thin lines represent cell membranes, mitotic germ line is a syncytium. Scale bar, 20 μm. (B-D) Confocal images of dissected germlines that have been double stained with both anti-β-galactosidase (red) and anti-GLP-1 (green) antibodies. (B) GLP-1 is present in germline membranes. See Crittenden et al. (accompanying paper) to see definitively that the DTC does not contain GLP-1. (C) The LAG-2::β-gal fusion protein is present in the DTC and also near the center of the germline. (D) Simultaneous detection of anti-GLP-1 and anti-β-gal using merged fluorescein and rhodamine confocal image. Note co-localization of GLP-1 staining and β-gal staining within membranes of the germline.

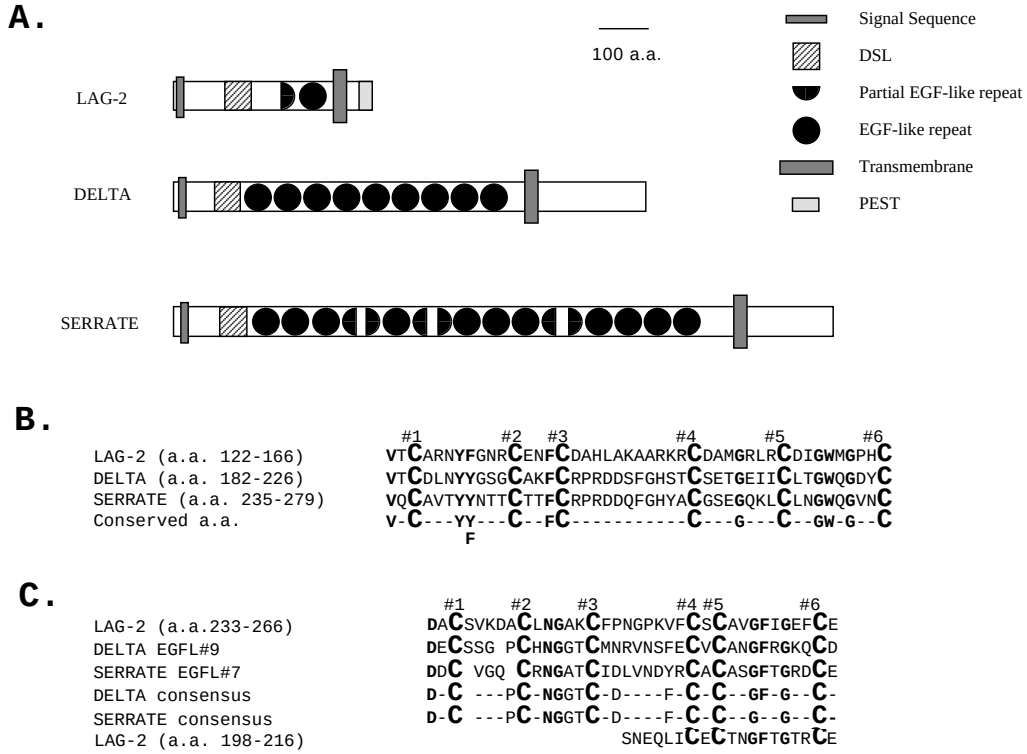


Fig. 7. Comparison of LAG-2, Serrate and Delta. (A) Predicted LAG-2, Serrate and Delta proteins; motifs identified by key. (B) Sequence comparison of DSL motifs from LAG-2, Delta and Serrate. Note conserved amino acids and spacing. (C) Sequence comparison of EGF-like motifs of LAG-2, Serrate and Delta. EGF-like motifs from LAG-2, Serrate and Delta that are most similar to LAG-2 EGF-like motif, consensus derived from all EGF-like repeats of Delta and Serrate, and the half EGF-like repeat from LAG-2. The half EGF-like motif matches well with the consensus for EGF-like repeats of Delta and Serrate. The LAG-2 full EGF-like repeat contains neither a tyrosine residue immediately preceding Cys#2 nor an arginine immediately preceding Cys#6 which are found in growth factors. The LAG-2 EGF-like repeat does not match the consensus --C-D/N----F/Y-C-C-- believed to be involved in hydroxylation and calcium binding (Selander-Sunnerhagen et al., 1992).

Delta is believed to be internalized with Notch protein in vivo (Kooh et al., 1993). The uptake of LAG-2::β-gal by the germline extends previous observations with Delta in two ways. First, β-gal provides a tag for the intracellular domain, whereas antibodies used to detect Delta were raised against its extracellular domain. Therefore, the entire LAG-2 protein, and perhaps other Delta-like proteins, may be endocytosed into the receiving cell or tissue. Second, within *Drosophila*, Delta and Notch are expressed in the same cells and therefore signaling and receiving cells cannot be distinguished (Kooh et al., 1993). By contrast, in our work, the signaling and receiving cells are distinct and LAG-2::β-gal is clearly present in the receiving tissue.

How does LAG-2 control cell fate?

The homology of LAG-2 with Delta and Serrate coupled to the localization of LAG-2 in the signaling DTC provides strong evidence that LAG-2 is the signal for GLP-1 and LIN-12. How then might LAG-2 influence GLP-1/LIN-12 activity? Since loss-of-function mutations of the *lag-2*, *glp-1*, and *lin-12* genes effect similar cell fate transformations (Greenwald et al., 1983; Austin and Kimble, 1987; Lambie and Kimble, 1991), it is likely that LAG-2 acts positively on GLP-1 to control cell fate. However, there is little rigorous evidence that bears on the molecular mechanism involved. LAG-2 might act as a membrane protein or it might be processed to release a diffusible factor. Given the uptake of the intracellular domain of LAG-2 into the germline, we favor the idea that LAG-2 acts without being cleaved.

One model for LAG-2/GLP-1 function that now appears to be unlikely is that the extracellular domains of LAG-2 and GLP-1/LIN-12 bind together to bring the signaling and receiving cells in close proximity, and some other signal transduction mechanism is operating. The primary evidence against this model is that the extracellular domain of GLP-1/LIN-12 is not essential for function (Roehl and Kimble, 1993; Struhl et al., 1993). Instead, the intracellular ankyrin repeats of GLP-1 are capable of activity on their own (Roehl and Kimble, 1993). Therefore, LAG-2 must act by influencing the activity of the intracellular domain of GLP-1.

Control of GLP-1/LIN-12 activity by LAG-2 could occur by any of several mechanisms. One simple idea is that LAG-2 sta-

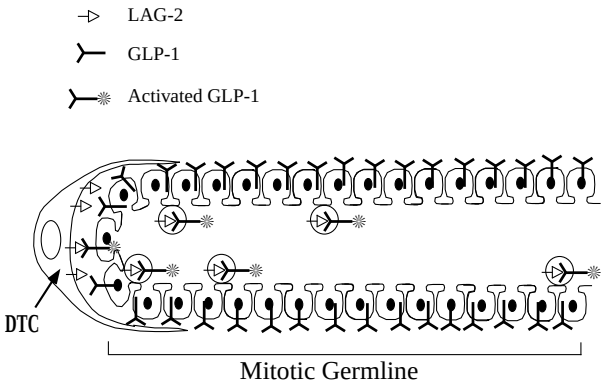


Fig. 8. Model of LAG-2 and GLP-1 action in germline. LAG-2 is synthesized in DTC and inserted into its membrane. GLP-1 is synthesized in the germline and inserted into germline membranes. We propose that LAG-2 binds and activates GLP-1, which in turn induces nearby germ cells to remain in mitotic cell cycle. Both LAG-2 and GLP-1 are endocytosed into the germline syncytium. In the diagram, we show the endocytosed form as an active complex, which is down regulated after moving in the germline syncytium. Alternatively, the endocytosed complex may represent an inactive form.

bilizes GLP-1/LIN-12 and prevents its degradation; by this scenario, LAG-2 would control the presence of GLP-1/LIN-12 rather than its functional state. According to this model, absence of LAG-2 would lead to GLP-1/LIN-12 degradation, a prediction that has not yet been tested. A second model is that LAG-2 binding might induce a change in GLP-1/LIN-12 and thereby lead to activation (Fig. 8). Such a change might involve, for example, dimerization or endocytosis. The subcellular co-localization of LAG-2 and GLP-1 and its distribution in the germ line suggests that the uptake of both signal and receptor may be important for subsequent events that control cell fate.

Conclusions and prospects

In summary, LAG-2 appears to be a signaling ligand for the GLP-1 and LIN-12 membrane receptors. Therefore, two central components, signal and receptor, of a signal transduction pathway that controls cell fate during development, have been conserved between nematodes and *Drosophila*. Homologs of Notch and Delta have also been identified in vertebrates (e.g. Fortini and Artavanis-Tsakonas, 1993, for review; D. Ish-Horowicz, personal communication), suggesting that this pathway has been conserved in all multicellular organisms. An indepth understanding of this signal transduction pathway will therefore have broad significance. Now that some of the principle components of the pathway have been identified in a simple inductive cell interaction, we can take the next step to ask how LAG-2 activates GLP-1 and how that activation regulates cell fate.

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