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Functional Domains of LAG-2, a Putative Signaling Ligand for LIN-12 and GLP-1 Receptors in *Caenorhabditis elegans*

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The LAG-2 membrane protein is a putative signaling ligand for the LIN-12 and GLP-1 receptors of *Caenorhabditis elegans*. LAG-2, like its *Drosophila* homologues Delta and Serrate, acts in a conserved signal transduction pathway to regulate cell fates during development. In this article, we investigate the functional domains of LAG-2. For the most part, mutants were constructed in vitro and assayed for activity in transgenic animals. We find a functional role for all major regions except one. Within the extracellular domain, the N-terminal region, which bears no known motif, and the DSL domain are both required. By contrast, the region bearing epidermal growth factor-like repeats can be deleted with no apparent reduction in rescuing activity. The intracellular region is not required for activity but instead plays a role in down-regulating LAG-2 function. Finally, membrane association is critical for mutant rescue.

INTRODUCTION

Two membrane proteins, LAG-2 and APX-1, are putative signaling ligands for the LIN-12 and GLP-1 receptors of *Caenorhabditis elegans* (Lambie and Kimble, 1991; Henderson *et al.*, 1994; Mango *et al.*, 1994; Mello *et al.*, 1994; Tax *et al.*, 1994; Wilkinson *et al.*, 1994; Mickey *et al.*, 1996; Berry *et al.*, 1997). Both LAG-2 and APX-1 belong to the DSL family of proteins; other members include Delta and Serrate from *Drosophila*, as well as several vertebrate counterparts (for review, see Artavanis-Tsakonas *et al.*, 1995; Nye and Kopan, 1995). DSL proteins act as part of a signal transduction pathway with at least two other conserved components: LNG receptors (e.g., LIN-12 and GLP-1 in *C. elegans* and Notch in *Drosophila*) and CSL transcription factors [e.g., Su(H) in *Drosophila*, CBF1 in vertebrates, and LAG-1 in *C. elegans*] (Artavanis-Tsakonas *et al.*, 1995; for review, see Christensen *et al.*, 1996).

The identification of DSL proteins as signaling ligands relies on several lines of evidence. Genetic mosaic analyses demonstrate clearly that Delta functions in signaling cells (Heitzler and Simpson, 1991), and expression studies show that various DSL proteins are expressed specifically in signaling cells (Ghysen *et al.*, 1993; Henderson *et al.*, 1994; Wilkinson *et al.*, 1994; Mickey *et al.*, 1996; Moskowitz and Rothman, 1996). Furthermore, Delta-expressing tissue culture cells adhere to Notch-expressing cells, the two proteins colocalize to the intercellular junction, and Delta coimmunoprecipitates with Notch, consistent with a physical association between the two molecules (Fehon *et al.*, 1990; Rebay *et al.*, 1991; Lieber *et al.*, 1992). Also, cells expressing Jagged (a rat homologue of Serrate) inhibit muscle differentiation in adjacent myoblasts expressing rat Notch1 (Lindsell *et al.*, 1995). Therefore, DSL proteins may act as ligands for LNG receptors.

One key step in understanding the function and regulation of a protein is to delineate its critical domains. Although numerous mutations of DSL proteins have been reported (Lieber *et al.*, 1992; Henderson *et al.*, 1994; Muskavitch, 1994; Tax *et al.*, 1994; Chitnis *et al.*, 1995; Fitzgerald and Greenwald, 1995; Sun and Artavanis-Tsakonas, 1996), no systematic analysis has been done of any single DSL protein. From the col-

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lected mutants in all DSL proteins, certain regions have not been previously assayed at all, whereas others have been subjected to different manipulations (e.g., replacement vs deletion) that are not comparable. To address this lack of a consistent picture, we undertook a mutational analysis of the major functional regions of LAG-2.

Figure 1A summarizes the architecture of LAG-2, whereas Figure 1B shows that of APX-1 for comparison. Like all DSL proteins known to date, LAG-2 and APX-1 contain a signal sequence (SS), an N-terminal (NT) region with no known motif, a cysteine-rich DSL domain, a variable number of epidermal growth factor (EGF)-like repeats, a transmembrane (TM) domain, and an intracellular (IC) region (Henderson *et al.*, 1994; Mello *et al.*, 1994; Tax *et al.*, 1994). Because LAG-2 and APX-1 appear to be functionally interchangeable (Fitzgerald and Greenwald, 1995; Gao and Kimble, 1995), one might guess that regions conserved between them would be central to their activity. To date, the only domain that is known to be key for signaling by DSL proteins is the family-specific DSL domain (Henderson *et al.*, 1994; also see Fitzgerald and Greenwald, 1995). Other regions have either not been investigated in any depth or appear to play an ill-defined or nonessential role (see Discussion).

In this article, we explore each major region of LAG-2 for function in vivo. We find that all regions, except one, play a role in either function or regulation. Most critical for signaling are the NT and DSL domains. In addition, membrane association is crucial for mutant rescue, and the IC domain is involved in down-regulating LAG-2 activity. Only the EGF-like repeats can be deleted with no apparent phenotype in our assays. The minimal protein capable of mutant rescue contains the region encompassing the NT and DSL domains fused to the TM domain.

MATERIALS AND METHODS

Worm Strains and Generation of Transgenics

All transgenic lines were derived from wild-type *C. elegans* variant Bristol, strain N2 (Brenner, 1974) and grown at 20°C unless stated otherwise. Alleles used were *smg-1(r861)*, *lag-2(q411)*, and *lag-2(q477)*. The *lag-2* alleles (Lambie and Kimble, 1991; Henderson *et al.*, 1994) were balanced with a reciprocal translocation called *DnT1*. Unless specified differently, transgenic animals were generated by injection of a mixture of pRF4 (*rol-6* DNA, 100 μg/ml) and test plasmid at 10 μg/ml into N2 hermaphrodites, as described by Mello *et al.* (1991). For rescue experiments, *lag-2(q411)*, a nonsense mutant predicted to be a molecular null (Henderson *et al.*, 1994), was used as described below. For all transgenes assayed, at least two independently derived transgenic lines were assayed.

Plasmid Construction

Deletion variants were constructed using a modified polymerase chain reaction (PCR) mutagenesis protocol. To ensure fidelity of the sequences, all regions generated by PCR were sequenced to verify changes. Primer sequences for deletion constructs are shown in Table 1. PCR was done with primers possessing homologous ends as described by Jones and Howard (1991) with the following modifications: 1) to limit the chance of second site mutations, large amounts of template (2–4 μg) were used, and only eight cycles were performed; PCR conditions were 94°C for 30 s, 56°C for 1 min, and 72°C for 8 min with a 10-s extension per cycle on segment 3, using either *Taq* Extender (Stratagene, La Jolla, CA) or XL PCR (Perkin-Elmer, Norwalk CT); and 2) PCR products were purified using a QiaQuick spin PCR purification kit (Qiagen, Chatsworth, CA) and then digested with *Dpn*I to eliminate the original template. Digested PCR reactions were then transformed into *Escherichia coli* strain XL1-blue, in which homologous ends recombined to generate a circular plasmid. Mutagenized regions were cloned into *lag-2* expression vectors pJK559 and pJK374 (Henderson *et al.*, 1994) as described below.

Table 1. Oligonucleotides used for deletions

Name	Sequence
Δnt-1	5′-gtg gcg acc gag gtc aca tgt gct cgc aac tat ttc
Δnt-2	5′-tgt gac ctc ggt cgc cac tcg ggc ttg gag gac tgg
Δds1-1	5′-gtt gac ccc cgc aag tgc tca tgc gag aac gac gga
Δds1-2	5′-gca ctt gcg ggg gtc aac cgc cac gct caa cga aat tcc
Δegf-1	5′-gaa atc tca ctc acc acc acc acc cca act acc
Δegf-2	5′-ggt ggt gag tga gat ttc ctt gcg ggg gtc aac
Δtm-1	5′-cta gac ata gtg gca ggc gta ttc cga ggt aga gac
Δic-1	5′-gcc tgc cac tat gtc tag att ctt cct tta tcc
Δic-2	5′-cta gac ata gtg gca ggc ctt gaa ctt gta ctt gaa gca

pJK559. A *Stu*I/*Spe*I fragment containing the 3′ end of the *lag-2* gene, from a *lag-2* cDNA clone (pJK530), was ligated into *Stu*I/*Spe*I–cut *lag-2* genomic clone pJK254 (Henderson *et al.*, 1994) to create a smaller rescuing clone. pJK559 rescues *lag-2(q411)*.

pJK567. A *Sac*II fragment containing much of the *lag-2* gene, from pJK559, was cloned into the *Sac*II site of pBluescript II SK− (Stratagene).

LAG-2(ΔNT), LAG-2(ΔNT)::β-gal, LAG-2(ΔEGF), and LAG-2(ΔEGF)::β-gal. PCR was performed on pJK559 using primers Δnt-1/Δnt-2, or Δegf-1/Δegf-2 to generate LAG-2(ΔNT) and LAG-2(ΔEGF) derivatives of pJK559, respectively. To limit the region generated by PCR, a segment (a *Bst*EII/*Rsr*II fragment for LAG-2(ΔNT) and a *Bst*EII/*Stu*I fragment for LAG-2(ΔEGF)) from each derivative was cloned back into pJK559 to yield the final LAG-2(ΔNT) and LAG-2(ΔEGF) and into pJK374 to yield LAG-2(ΔNT)::β-gal and LAG-2(ΔEGF)::β-gal.

LAG-2(ΔDSL), LAG-2(ΔDSL)::β-gal, LAG-2(ΔIC), and LAG-2(extra 1–286). PCR was performed on a mutagenesis cassette, pJK567, using primer pairs Δdsl-1/Δdsl-2, Δic-1/Δic-2, and Δic-1/Δtm-1 to generate LAG-2(ΔDSL), LAG-2(ΔIC), and LAG-2(extra 1–286) derivatives, respectively. *Sac*II fragments of these clones were then ligated back into pJK559 to generate the final LAG-2(ΔDSL), LAG-2(ΔIC), and LAG-2(extra 1-286), respectively. A *Sac*II/*Stu*I fragment from the LAG-2(ΔDSL) derivative was cloned into *Sac*II/*Stu*I-digested pJK374 to generate LAG-2(ΔDSL)::β-gal.

LAG-2(ΔIC)::β-gal. LAG-2(ΔIC)::β-gal was previously called LAG-2::β-gal or pJK374 (Henderson *et al.*, 1994) and renamed for consistency with this work.

LAG-2(ΔEGF, ΔIC). The LAG-2(ΔIC) derivative of pJK567 was used as a template for primers Δegf-1 and Δegf-2 to create a LAG-2(ΔEGF, ΔIC) derivative of the mutagenesis cassette. A *Sac*II fragment from this derivative was cloned into pJK559 to yield the final LAG-2(ΔEGF, ΔIC).

LAG-2(extra 1–174)::GFP and LAG-2(extra 1–286)::GFP. Primers were designed from the *lag-2* genomic sequence to fuse LAG-2 to GFP at predicted LAG-2 amino acid positions 174 and 286. pJK559 was used as a template. *Bst*EII/*Kpn*I fragments from the PCR products were cloned into the vector pJK520 (Gao and Kimble, 1995) to

create LAG-2(extra 1–174)::GFP and LAG-2(extra 1–286)::GFP, respectively.

LAG-2(extra 1–286/ΔDSL)::GFP. A *Sac*II/*Rsr*II fragment from LAG-2(ΔNT) was cloned into a *Sac*II/*Rsr*II-digested LAG-2(extra 1–286)::GFP vector.

APX-1(extra 1–387)::GFP and APX-1(extra 1–387). Primers were designed from the *apx-1* cDNA sequence to fuse APX-1 to GFP at predicted amino acid position 387 of APX-1. The template was APX-1 cDNA. *Xma*I/*Kpn*I fragments of PCR products were cloned into pJK520 (Gao and Kimble, 1995). To remove the GFP tag, APX-1(extra 1–387)::GFP was digested with *Kpn*I/*Spe*I, and the remaining vector was ligated to a *Kpn*I/*Spe*I fragment from pJK559.

LAG-2(c-myc/NT) and LAG-2(GFP/NT). Primers were made to either the c-*myc* epitope or GFP sequence with flanking *Sac*II sites. PCR products were then cloned into the *Sac*II site of pJK254 (Henderson *et al.*, 1994).

Rescue of lag-2(q411)

Rolling males carrying the extrachromosomal array to be tested were mated to JK1223, [*lag-2(q411)/DnT1*] to generate Rol non-Unc males. These *q411/+;Ex* males were mated again to JK1223, and Rol Unc hermaphrodite progeny (*q411/DnT1;Ex* or *+/DnT1;Ex*) were picked to individual plates. Progeny from these Rol Uncs were then scored for Lag animals, indicating the presence of the *lag-2(q411)* allele. The balanced strain *q411/DnT1;Ex* was examined for the presence of fertile Rol non-Unc progeny. If the *q411/DnT1;Ex* strain failed to segregate fertile Rol non-Uncs after several generations, the array was considered to have failed to rescue. To distinguish between generation of Rol non-Unc animals by breakdown of the balancer or mutant rescue, the generation of siblings was scored. A strain was scored as *lag-2(q411);Ex* and hence rescued if it segregated a high percentage of Lags (>50%), a high percentage of rollers (>90%), and no Unc (*DnT1/+*) animals. In addition, certain constructs yielded fertile non-Unc animals, the progeny of which were either all Lag or "Glp-like" sterile, so that a homozygous *lag-2(q411)* line could not be maintained. Yet, because the mothers were viable and fertile, and because they generated 100% Lag or Glp progeny, the array was scored as having rescuing activity.

Assay for Generation of Lag Animals and Ectopic Signaling in a Wild-Type Background

Approximately 10 young adults of the genotype *N2;Ex* were picked to a seeded plate and allowed to lay eggs at 25°C. After several hours the mothers were removed, and the eggs were allowed to develop. The following day the numbers of unhatched embryos and Lag animals were counted. After 2 more days all rolling adults were counted and moved to a fresh plate. The total number of animals carrying the extrachromosomal array was considered to be the sum of rollers, dead eggs, and Lag animals; this number was used in calculating percent Lags. For scoring adult phenotypes of Pro (proximal proliferation) and Muv (ectopic vulval protrusions), 100 rollers from the above plates were removed and examined with a Leica (Nussloch, Germany) Kombi stereo microscope at 400×.

Detection of β-Galactosidase and GLP-1 Protein

β-Galactosidase, c-myc epitope, and GLP-1 were detected by immunofluorescence using antibodies against β-galactosidase (5′-3′), c-myc epitope (Oncogene Science, Boulder, CO), and GLP-1 (Crittenden *et al.*, 1994). Secreted forms of LAG-2 and APX-1 fused to GFP were usually nonfluorescent and were detected with antibodies to GFP (Clontech, Palo Alto, CA).

RESULTS

Generation of Mutant Transgenes and Assays for Function

Mutants of LAG-2 or APX-1 were placed under control of the *lag-2* promoter (Henderson *et al.*, 1994) and introduced into wild-type nematodes as extrachromosomal arrays (*Ex*); at least two independently derived transgenic lines were assayed for each mutant (see Materials and Methods). In mutants with no signaling activity [e.g., LAG-2(ΔDSL)], we inferred the presence and distribution of protein from the β-gal derivative of the same mutant [e.g., LAG-2(ΔDSL)::β-gal]. These transgenic experiments are subject to the caveat that mutants are likely to be overexpressed because of multiple copies of transgenes on each extrachromosomal array.

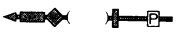
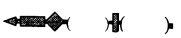
Mutants were tested for function in two ways. First, rescue of *lag-2(q411)* was attempted. The *lag-2(q411)* allele carries a nonsense mutation in the codon for amino acid 79 (see Figure 1A for relative position within LAG-2) and is predicted to be null (Henderson *et al.*, 1994). Whereas *lag-2(q411)* mutants normally die soon after hatching, rescued *lag-2(q411);Ex* animals survive to adulthood and are fertile. Second, transgenes were placed in a wild-type background, and phenotypes were sought that are typical of either loss-of-function or gain-of-function mutants of *lag-2*, *lin-12*, or *glp-1*. Specifically, we scored the following: 1) the Lag phenotype, an L1 lethality typical of *lag-2* null mutants (Lambie and Kimble, 1991); 2) the Pro phenotype, an overproliferation of germ line tissue seen in *glp-1* gain-of-function mutants (Berry *et al.*, 1997); and 3) the Muv phenotype, a generation of ectopic vulvae characteristic of both *glp-1* and *lin-12* gain-of-function mutants (Greenwald *et al.*, 1983; Mango *et al.*, 1991; Berry *et al.*, 1997). Our results are summarized in Tables 2 and 3.

One construct listed in Table 2 was previously analyzed (Henderson *et al.*, 1994) and is included here for comparison with other mutants. Also, some constructs similar to those in Table 3 have been tested by Fitzgerald and Greenwald (1995); however, conclusions drawn from the two studies are not identical (see Discussion).

Deletion/Insertion Mutations of the NT Domain

The NT region is a stretch of 105 amino acids extending between the SS and DSL domain (Figure 1A). A mutant *lag-2* protein deleted for the NT domain lacks *lag-2* activity: LAG-2(ΔNT) does not rescue *lag-2(q411)* (Table 2A). The same result is obtained when the deletion mutant is fused to *lacZ* (Table 2A). For the latter construct, the LAG-2(ΔNT)::β-gal fusion protein is present despite its lack of activity (see Figure 4A). Because an NT deletion might destroy LAG-2 activity

Table 2. Deletions and insertions of LAG-2

	Name	Schematic[a]	Rescue of *lag-2(q411)*	% Lag[b]	% Muv[b,c]	% Pro[b,c]
	LAG-2		+	0 (n = 141)	0	0
A	LAG-2(ΔNT)		−	7 (n = 102)	0	0
A	LAG-2(ΔNT)::β-gal		−	3 (n = 106)	0	0
A	LAG-2(c-myc/NT)		−	17 (n = 750)	0	0
A	LAG-2(GFP/NT)		−	35 (n = 864)	0	0
B	LAG-2(ΔDSL)		−	0 (n = 159)	0	0
B	LAG-2(ΔDSL)::β-gal		−	0 (n = 112)	0	0
C	LAG-2(ΔEGF)		+	0 (n = 186)	0	0
C	LAG-2(ΔEGF)::β-gal		+	0 (n = 121)	0	0
D	LAG-2(ΔIC)		+	0 (n = 218)	13	0
D	LAG-2(ΔIC)::β-gal[d]		+	0 (n = 125)	0	0
E	LAG-2(ΔEGF, ΔIC)		+	0 (n = 152)	0	0

[a] For description of domains, see Figure 1.

[b] Scored in +;*Ex* adults at 25°C.

[c] n = 100.

[d] Rescue by this construct was reported by Henderson *et al.* (1994); dominant effects were scored in this study.

by disrupting the DSL domain, we also examined the effect of in-frame insertions into the NT region between amino acids 55 and 56 so that amino acids flanking the DSL domain remain intact. LAG-2(c-myc/NT) inserted two copies of the c-*myc* epitope tag, whereas LAG-2(GFP/NT) inserted GFP at the same site. Like the NT deletion, neither insertion mutant rescued *lag-2(q411)*, even though mutant proteins were detectable by antibody staining to either the c-*myc* or GFP tag. Surprisingly, all NT mutants had a dominant negative effect; dead L1s with a typical Lag phenotype were found among the progeny of animals carrying LAG-2(ΔNT), LAG-2(ΔNT)::β-gal, LAG-2(c-myc/NT), or LAG-2(GFP/NT) in an otherwise wild-type background (Table 2A).

Deletion of the DSL Domain

Previous work showed that a missense mutation in the LAG-2 DSL domain eliminates activity and led to the suggestion that this domain is crucial for LAG-2 function (Henderson *et al.*, 1994). Consistent with this idea, mutant LAG-2 proteins deleted for the DSL domain lack *lag-2* activity: neither LAG-2(ΔDSL) nor LAG-2(ΔDSL)::β-gal rescued *lag-2(q411)* (Table 2B). Furthermore, neither mutant had a dominant effect (Table 2B), and LAG-2(ΔDSL)::β-gal protein was present (see Figure 4B).

Deletion of the EGF-like Region

The EGF-like region of LAG-2 carries one full EGF-like repeat and two potential partial EGF-like repeats (Figure 1-A). A mutant LAG-2 protein deleted for the EGF-like repeats retains full *lag-2* activity: LAG-2(ΔEGF) rescues *lag-2(q411)* (Table 2C). Similarly, LAG-2(ΔEGF)::β-gal was capable of *lag-2(q411)* rescue (Table 2C). However, LAG-2(ΔEGF) and LAG-2(ΔEGF)::β-gal differ in their strengths of rescue. Whereas LAG-2(ΔEGF) could rescue *lag-2(q411)* over many generations, LAG-2(ΔEGF)::β-gal rescued it for only a single generation. A similar effect was observed for LAG-2 deleted for both the EGF-like domain and the IC domain: LAG-2(ΔEGF, ΔIC) (Table 2E).

Deletion of the IC Domain

A mutant LAG-2 protein deleted for the IC region retains *lag-2*-rescuing activity (Table 2D, first line) but

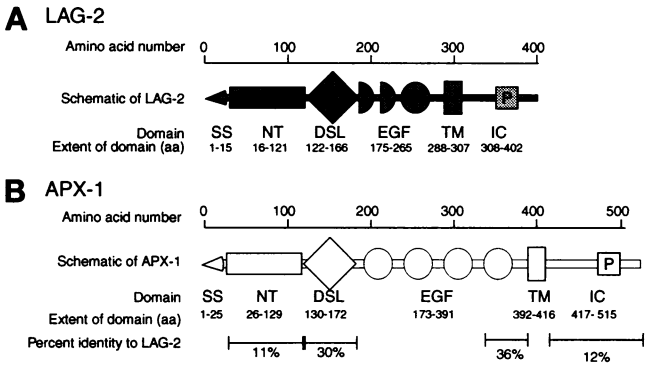


Figure 1. (A) Regions of LAG-2. All numbering based on predicted protein sequence of LAG-2 (Henderson *et al.*, 1994; Tax *et al.*, 1994); the N terminus is to the left. Regions include a hydrophobic SS (arrowhead), the N-terminal region (NT; shaded rectangle), DSL domain (shaded diamond), the EGF (shaded half or full circles), the TM (vertical bar), and the IC domain (IC). The EGF-like repeats include two incomplete repeats at aa 175–183 and 204–215 and one full repeat at aa 233–265. The potential PEST sequence within the IC is shown as P in a box. (B) Regions of APX-1. All numbering is based on predicted protein sequence of APX-1 (Mello *et al.*, 1994). The domains of APX-1 are unshaded and represented schematically as for LAG-2. Sequence identities between individual regions of LAG-2 and APX-1 are shown below. The full EGF-like repeat of LAG-2 is most similar to the fourth EGF-like repeat of APX-1, and percent identity is shown for this pair.

exhibits a dominant gain-of-function phenotype (Figure 2). In contrast to the single vulva typical of wild-type hermaphrodites (Figure 2A), LAG-2(ΔIC) confers a Muv phenotype in *+;Ex LAG-2(ΔIC)* animals (Figure 2B). The LAG-2(ΔIC) mutant protein does not, however, induce proximal proliferation of the germ line (Table 2D, first line; see Discussion). Unlike LAG-2(ΔIC), LAG-2(ΔIC)::β-gal does not generate a Muv phenotype (Table 2D, second line).

To test whether the Muv phenotype depends on endogenous wild-type LAG-2, we examined rescued lines for ectopic vulval protrusions. In *lag-2(q411);Ex LAG-2(ΔIC)*, 13% of the animals were Muv (Figure 2C), similar to the 14% seen in *+;Ex LAG-2(ΔIC)*. Therefore, the Muv effect of LAG-2(ΔIC) is intrinsic to the mutant protein and does not rely on an interaction of that protein with wild-type LAG-2.

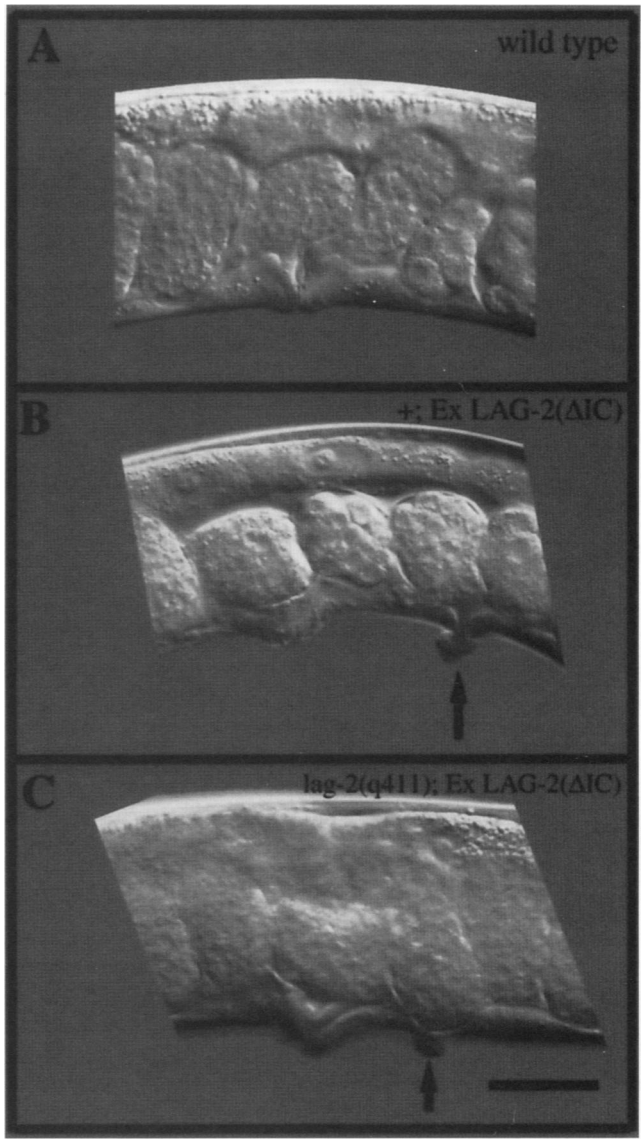


Figure 2. Hyperactivity of LAG-2(ΔIC). All panels are Nomarski images; anterior is left; dorsal is toward the top. (A) N2 hermaphrodite adult vulva. (B) *+;Ex LAG-2(ΔIC)* hermaphrodite; the arrow indicates ectopic vulval tissue. (C) *lag-2(q411);Ex LAG-2(ΔIC)* hermaphrodite; the arrow indicates ectopic vulval tissue. Scale bar, ~20 μm.

Removal of the Membrane Anchor: lag-2(q477)

A previous study suggested that membrane association is not required for LAG-2 function; APX-1(extra), a fragment composed solely of the APX-1 extracellular domain, was reported to be sufficient for *lag-2* rescue when expressed under control of a *lag-2* promoter (Fitzgerald and Greenwald, 1995). This result conflicts with the null phenotype of *lag-2(q477)*, a nonsense mutation predicted to generate a truncated fragment carrying the LAG-2 extracellular domain plus three amino acids from the TM domain (Henderson *et al.*, 1994).

One explanation of the difference between rescue by APX-1(extra) (Fitzgerald and Greenwald, 1995) and lack of activity of *lag-2(q477)* (Henderson *et al.*, 1994) might have been degradation of *lag-2(q477)* mRNA by nonsense-mediated mRNA decay (Theodorakis and Cleveland, 1996). In *C. elegans*, a mutation in *smg-1* abolishes nonsense-mediated mRNA decay and restores prematurely terminated messages to wild-type levels (Pulak and Anderson, 1993). To eliminate the

possibility that the *lag-2(q477)* null phenotype results from mRNA degradation, we examined a *lag-2(q477)/DnT1;smg-1* double mutant. This balanced strain segregates only Unc animals [*lag-2(q477)/DnT1;smg-1* heterozygotes], dead L1 larvae with a Lag phenotype [*lag-2(q477)* homozygotes], and dead eggs (*DnT1* homozygotes). No non-Unc *lag-2(q477)* homozygotes survived past L1. We conclude that the LAG-2(q477) extracellular fragment does not have detectable LAG-2 activity.

Removal of the Membrane Anchor: Transgenic Experiments

A second explanation of the difference between rescue by APX-1(extra) (Fitzgerald and Greenwald, 1995) and lack of *lag-2(q477)* activity (Henderson *et al.*, 1994; this article) might have been a difference in the level of expression between the APX-1(extra) transgene and the *lag-2(q477)* endogenous gene. To address this idea, we tested a similar transgene, LAG-2(extra 1–286) for rescuing activity. LAG-2(extra 1–286) is predicted to consist of the LAG-2 extracellular domain (through amino acid 286) plus the C-terminal five amino acids of LAG-2; this construct uses the native *lag-2* stop codon and 3′ untranslated region. Consistent with the conclusion drawn from *lag-2(q477)*, LAG-2(extra 1–286) failed to rescue *lag-2(q411)* (Table 3, first line). We next tested APX-1(extra 1–387), which includes the APX-1 extracellular domain through amino acid 387, and found that it too was unable to rescue *lag-2(q411)* (Table 3, second line). In a final attempt to observe rescue with LAG-2(extra 1–286), we increased the concentration of injected plasmid by fivefold (from 10 to 50 μg/ml) but still were unable to rescue *lag-2(q411)*. We conclude that membrane association of LAG-2 and APX-1 is required for mutant rescue, and that the membrane association of LAG-2 and APX-1 proteins is essential for *lag-2* mutant rescue.

In the course of these experiments, we also tested LAG-2(extra 1–286)::GFP and APX-1(extra 1–387)::GFP. Surprisingly, we found that the GFP fusions were able to rescue *lag-2(q411)* (Table 3, third and fourth lines). To ask whether GFP might itself have signaling activity, we tested LAG-2(extra 1–286/ΔDSL)::GFP, a construct in which the DSL domain was deleted from the extracellular domain. However, this DSL deletion mutant failed to rescue *lag-2(q411)* (Table 3, fifth line). We conclude that GFP confers rescuing activity on either LAG-2(extra 1–286) or APX-1(extra 1–387) when fused to the C-terminus, and that the DSL domain is crucial for this rescue.

Finally, to test whether the EGF-like repeats are critical for rescue by LAG-2(extra 1–286)::GFP, we tested LAG-2(extra 1–174)::GFP. This construct deletes the entire EGF-like repeat region but leaves the NT and DSL domains intact. Consistent with our previous finding that the EGF-like repeats are not required for LAG-2-rescuing activity (Table 2C), LAG-2(extra 1–174)::GFP was able to rescue *lag-2(q411)* (Table 3, sixth line).

Ectopic Signaling by LAG-2(extra) and APX-1(extra)

LAG-2(extra) and APX-1(extra) were previously shown to have ectopic signaling activity (Fitzgerald and Greenwald, 1995). Because our experiments were different in detail from those of Fitzgerald and Greenwald (1995), we tested LAG-2(extra 1–286) and APX-1(extra 1–387), and variants thereof, for ectopic signaling. All such constructs induced proximal proliferation in the germ line and multivulvae in the ventral hypodermis (Table 3 and Figure 3B). We further found that the Pro phenotype induced by LAG-2(extra 1–286) is strongly dependent on temperature. At 15°C, only 3% of *+;Ex LAG-2(extra 1–286)* animals had germline tumors compared with 15% at 20°C and 65% at 25°C. In contrast to the relatively frequent Pro germ lines, Muv animals were significantly

Table 3. Secreted forms of LAG-2 and APX-1

Name	Schematic[a]	Rescue of *lag-2(q411)*	Muv[b]	Pro[b]
LAG-2(extra 1-286)		−	+	+
APX-1(extra, 1-387)		−	+	+
LAG-2(extra 1-286)::GFP	GFP	+	+	+
APX-1(extra, 1-387)::GFP	GFP	+	+	+
LAG-2(extra 1-286/ΔDSL)::GFP	GFP	−	−	−
LAG-2(extra 1-174)::GFP	GFP	+	+	+

[a] For domain architecture, see Figure 1.

[b] Muv and Pro phenotypes scored in *+;Ex* adults raised at 25°C.

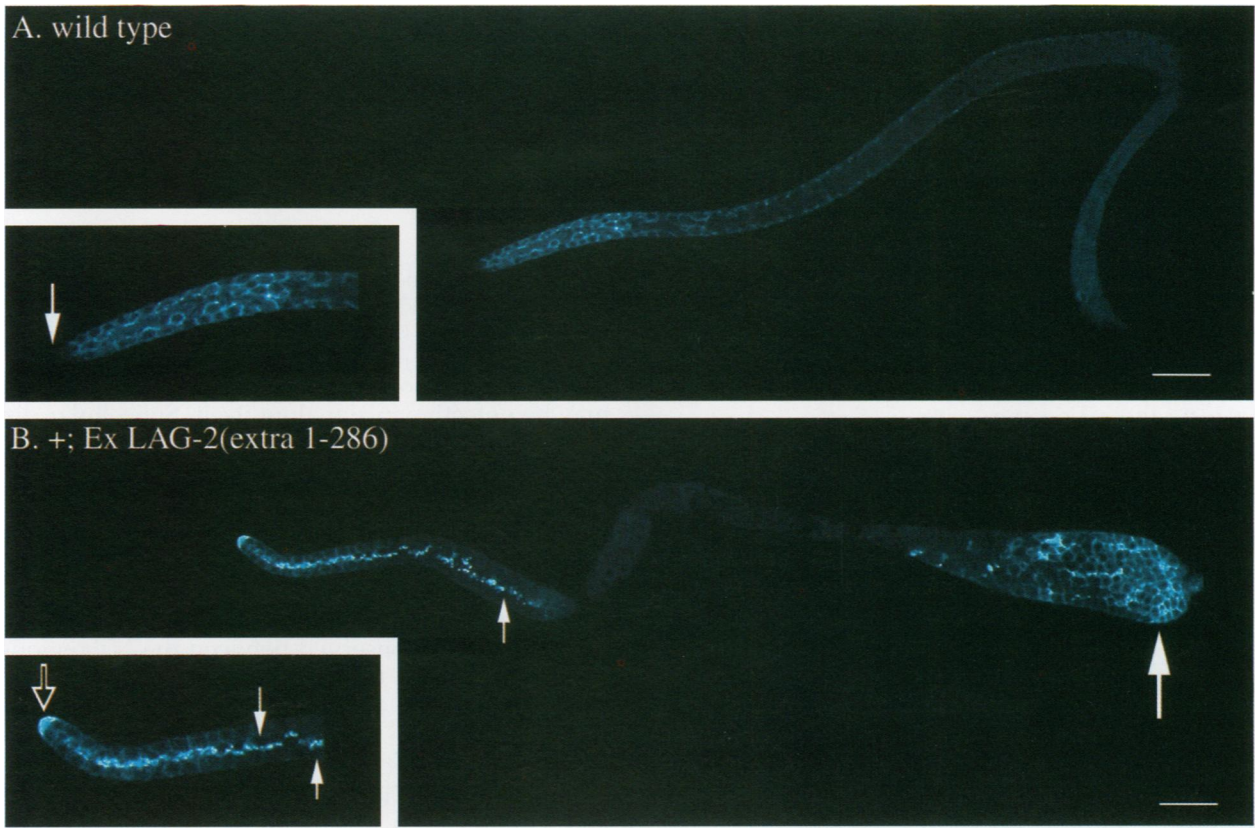


Figure 3. LAG-2(extra 1–286) induces germ line tumors and ectopic GLP-1. A composite of confocal images of dissected adult hermaphrodite germ lines is shown; distal is left, stained with antibodies to GLP-1 (Crittenden *et al.*, 1994). (A) Wild-type germ line. GLP-1 is associated with membranes at distal end of the germ line and is absent proximally. Inset, arrow indicates faint staining at the distal tip of the germ line. (B) *+;Ex LAG-2(extra 1–286)* germ line, raised at 25°C. GLP-1 is present both distally and proximally. Inset, open arrow indicates intense GLP-1 staining associated with the distal tip of the germ line; closed arrows indicate intense punctate staining within the germ line. Scale bar, 20 μm.

more rare (1–7%, 25°C) and were not examined for temperature sensitivity.

To explore the mechanism by which LAG-2(extra 1–286) signals ectopically, we examined Pro germ lines for ectopic GLP-1. In wild-type worms, LAG-2 signals to GLP-1 in the distal mitotic region. GLP-1 is limited to this distal region and is primarily membrane associated (Crittenden *et al.*, 1994) (Figure 3A, inset). By contrast, worms expressing LAG-2(extra 1–286) possess GLP-1 in both distal and proximal regions of the germ line (Figure 3B). In the distal region, staining is intense at the very tip, where GLP-1 appears to be capped (Figure 3B, inset, open arrow), and is also observed internally along the length of the distal region (Figure 3B, inset, closed arrows). More proximally, GLP-1 is observed either in the proximal germ line (Figure 3B) or throughout the germ line.

To determine the cells required for ectopic induction of GLP-1 by LAG-2(extra 1–286), we ablated somatic gonadal cells that normally express *lag-2*, the distal tip cell (DTC) (Henderson *et al.*, 1994) and AC/VU precursors Z1ppp and Z4aaa (Wilkinson *et al.*, 1994). Neither ablation of the anterior DTC nor ablation of both DTCs (Z1aa and Z4pp) eliminated the Pro effect (Table 4). When the anterior DTC was ablated during the third larval stage (L3), 42% of the anterior arms were Pro (n = 19), compared with 52% for the unablated posterior arms (n = 19). Similarly, when both DTCs were killed during L2, the Pro phenotype was observed in 40% of the animals (n = 15). Ablation of Z1ppp and Z4aaa in L2 *+;Ex LAG-2(extra 1–286)* animals also failed to eliminate the Pro effect: 50% of the animals were Pro but vulvaless because of loss of the anchor cell (n = 10) (Table 4). Finally, we ablated during L2 both DTCs and AC/VU precursors. In this case, no animals developed the Pro phenotype (n = 10), suggesting that ectopic germ line proliferation can be induced by a signal from either the DTC or AC/VU precursors (Table 4).

Table 4. Effect of DTC and AC/VU cell ablations

Cells ablated	% Pro/Tum[a]	n
None	65	100
Z1aa	Ant. 42 Post. 52	19
Z1ppp, Z4aaa	50	10
Z1aa, Z4pp	40	15
Z1aa, Z4pp, Z1ppp (or Z1pp), Z4aaa (or Z4aa)	0	10

[a] Single number refers to entire germ line; Ant. refers to germ line in anterior gonadal arm and Post. to germ line in posterior gonadal arm.

Cellular Distribution of LAG-2 Mutant Proteins

The distribution of LAG-2 mutant proteins was examined with the use of β-gal-tagged versions and β-gal antibodies. Normally, LAG-2 is expressed in the DTC but not the germ line; however, LAG-2 is detected in both DTC and germ line, presumably because of uptake into the germ line during induction (Henderson *et al.*, 1994). All LAG-2 mutant proteins were present in the DTC (Figure 4). However, presence in the germ line correlates with activity. Thus, LAG-2(ΔNT)::β-gal and LAG-2(ΔDSL)::β-gal, which fail to rescue *lag-2(q411)*, were never seen within the germ line (n = 20) (Figure 4, A and B), whereas LAG-2(ΔEGF)::β-gal and LAG-2(ΔIC)::β-gal, which rescue *lag-2(q411)*, were found frequently within the germ line: 80% (n = 20) and 75% (n = 20), respectively (Figure 4, C and D). We suggest that uptake into the germ line may require active LAG-2 protein.

DISCUSSION

This article reports the first systematic deletion study of any DSL protein and provides four new conclusions. First, the NT region is essential for LAG-2-rescuing activity. Second, membrane association is also crucial. Third, the IC domain plays a role in down-regulation of LAG-2 activity. And fourth, EGF-like repeats are not required. As would be predicted from these findings, the minimal protein that can rescue a *lag-2(0)* mutant includes the NT and DSL regions plus a TM domain. Intriguingly, GFP can substitute for the TM domain and confer rescuing activity on an NT/DSL fragment. These conclusions are summarized in Table 5, together with conclusions from previous studies concerning the functional domains of DSL proteins. In the following sections, we discuss our findings in relation to these other studies.

The NT and DSL Domains Are Critical for LAG-2 Function

The NT region is essential for LAG-2 function. In addition, both a deletion of the NT region and insertions into it have a dominant negative effect. NT mutant proteins may be defective in a process within the signaling cell, such as processing or trafficking, or they may be defective in signaling itself. Subcellular distribution provides no hint: the NT mutants have the same distribution in the signaling cell as other LAG-2 mutants assayed. The dominant negative effect may result either from interference with endogenous LAG-2 or some protein required for LAG-2 function, such as the receptor.

Neither amino acid sequence nor predicted secondary structure is conserved between the NT regions of LAG-2 and APX-1 (Henderson *et al.*, 1994; Mello *et al.*, 1994; Tax *et al.*, 1994) (Gao, unpublished observations). Nonetheless, these two domains are likely to serve the same purpose, because the proteins are functionally interchangeable (Fitzgerald and Greenwald, 1995; Gao and Kimble, 1995). One feature they have in common is their length: the LAG-2 NT region possesses 105 amino acids, whereas that of APX-1 has 103. Perhaps a change in NT length, either by insertion or deletion, interferes with function. If so, perhaps the NT region acts as a measuring device during signaling.

Previous work suggested that the DSL domain is critical for LAG-2 function (Henderson *et al.*, 1994), an idea that was confirmed in this study. Furthermore, several studies agree that a region composed of the NT plus DSL domain is critical for LAG-2 function (Table 5). Thus, the NT plus DSL region is required for adhesion between Delta- and Notch-expressing tissue culture cells (Muskavitch, 1994); the same region can confer ectopic signaling in vivo (Fitzgerald and Greenwald, 1995; this study); and the same region fused either to a TM domain or GFP is sufficient for *lag-2(0)* rescue (this study). If LAG-2 is indeed the ligand for GLP-1 and LIN-12, we surmise that the NT and DSL domains may work together to provide binding activity.

Possible Roles for the EGF-like Repeats

All known DSL family members possess at least one EGF-like repeat, ranging from the one full repeat in LAG-2 (Henderson *et al.*, 1994; Tax *et al.*, 1994) to 16 repeats in Jagged (Lindsell *et al.*, 1995). Previously described mutations mapping to the EGF-like repeats suggest that they may interact with the receptor; mu-

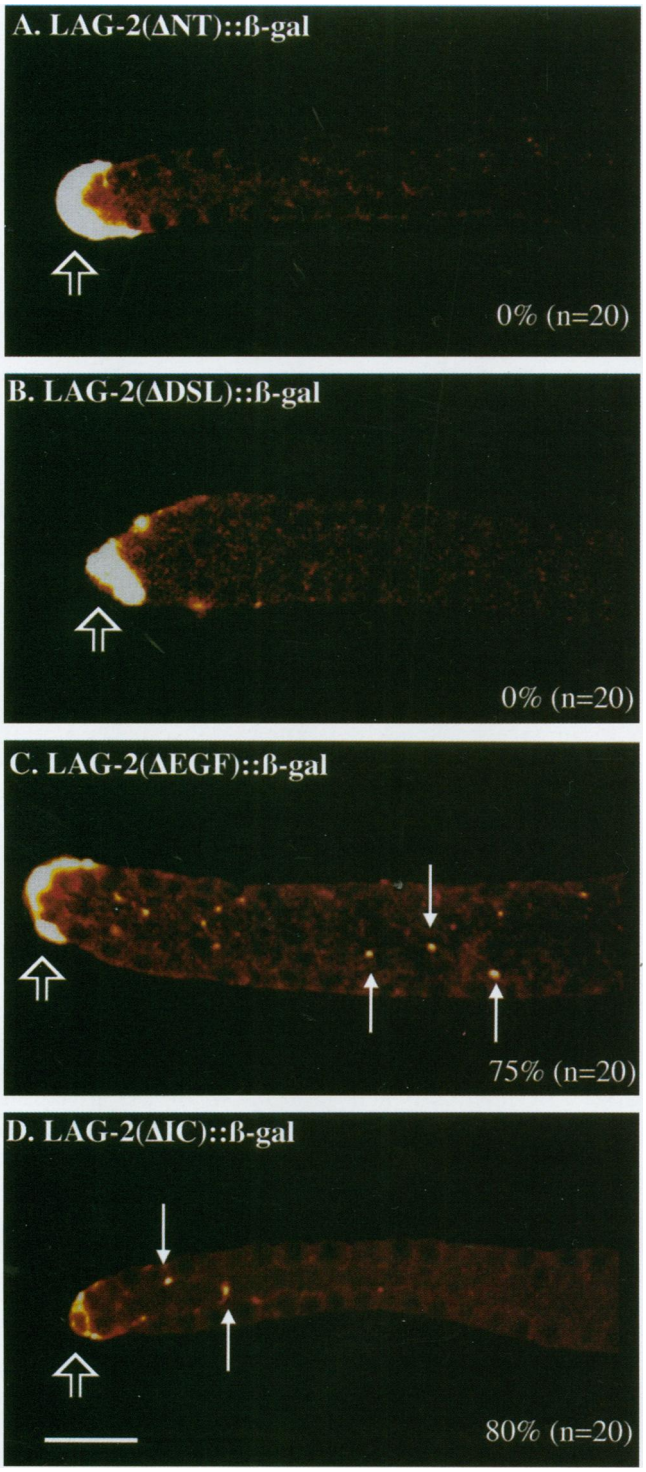


Figure 4. Distribution of LAG-2::β-galactosidase fusion constructs detected by antibodies to β-gal. All panels are confocal images at level of the core of dissected adult hermaphrodite germ lines. DTC (open arrow) is at the left; the germ line syncytium extends to right; percent germ lines with internal staining are shown at the lower right. (A) LAG-2(ΔNT)::β-gal accumulates in DTC and is not seen within the germ line syncytium. (B) LAG-2(ΔDSL)::β-gal accumulates in DTC and is not found in the germ line. (C) LAG-2(ΔEGF)::β-gal is present in both DTC and the germ line (arrows). (D) LAG-2(ΔIC)::β-gal in both DTC and the germ line. Solid arrows indicate punctate staining that colocalizes with anti-GLP-1 staining (Crittenden *et al.*, 1994; Henderson *et al.*, 1994). Scale bar, 20 μm.

tations in the fourth and ninth EGF-like domains of Delta, Dl^{sup4} and Dl^{sup5}, respectively, were isolated as suppressors of the Notch allele *split* (Lieber *et al.*, 1992). Each of these alleles is homozygous viable yet lethal or weakly viable over a deficiency (Brand *et al.*, 1990), suggesting a requirement for the EGF-like repeats under these conditions. In *C. elegans*, a mutation in the LAG-2 full EGF-like repeat, which changes a conserved glycine to aspartic acid, was isolated as a suppressor of a dominant *lin-12* allele. This suppressor mutation is homozygous viable and has no apparent phenotype (Tax *et al.*, 1994).

Three constructs reported here lack the EGF-like repeats of LAG-2 but nonetheless have rescuing activity: LAG-2(ΔEGF), LAG-2(ΔEGF, ΔIC), and LAG-2(extra 1–174)::GFP. Therefore, the EGF-like repeats are not required for signaling. Furthermore, the number of EGF-like repeats in APX-1(extra) does not have any apparent phenotypic consequence (Henderson, unpublished observations). We suggest that the EGF-like repeats may act normally either in oligomerization or stabilization of the receptor-ligand complex. Such a function might not be essential, or it might become unnecessary when the DSL protein is overexpressed. Alternatively the function of the EGF-like repeats may be redundant within the protein. Regarding this latter idea, we note that the LAG-2(ΔEGF, ΔIC), which is deleted for both EGF-like repeats, and the IC domain exhibited a significantly less robust rescue than either LAG-2(ΔEGF) or LAG-2(ΔIC). Perhaps the EGF-like repeats and the IC domain have some function in common, despite their distinct sequences and positions within the protein.

A Critical Role for the TM Domain

All known DSL proteins are predicted to be membrane proteins with a single TM-spanning sequence. In this article, we report two complementary experiments that suggest membrane association to be critical. First, a *lag-2* nonsense mutant predicted to leave a fragment equivalent to LAG-2(extra) has no detectable rescuing or signaling activity. Second, neither transgenic LAG-2(extra 1–286) nor APX-1(extra 1–387) rescues *lag-2* null; these protein fragments carry the entire extracellular region but lack the TM domain. By contrast, when that TM domain is also included, as in LAG-2(ΔIC), rescue is obtained.

Fitzgerald and Greenwald (1995) reported that APX-1(extra 1–402) can rescue a loss-of-function *lag-2* allele

Table 5. Summary of results concerning DSL functional domains.

Region(s)	Protein	Function of region	Reference[a]
NT	LAG-2	Essential for mutant rescue; disruption is dominant negative	This work
DSL	LAG-2	Essential for mutant rescue	1, This work
NT and DSL	LAG-2	Sufficient for ectopic signaling	2, This work
	Delta	Requried for adhesion in tissue culture assay	3
EGF	LAG-2/Delta	Site of suppressor mutations, role unknown	4,5
	LAG-2	Not essential	This work
IC	LAG-2/APX-1	Specific sequence not essential	1,2
	LAG-2	Not essential, required for negative regulation of LAG-2	This work
X-Delta-1, Serrate	Delta	Dominant negative effect, unknown function	6,7
Membrane association	LAG-2/APX-1	Essential, prevents ectopic signaling	This work
	APX-1	Not essential, prevents ectopic signaling	2
NT and DSL and TM	LAG-2	Sufficient for mutant rescue	This work

[a] 1, Henderson *et al.*, 1994; 2, Fitzgerald and Greenwald, 1995; 3, Muskavitch, 1994; 4, Lieber *et al.*, 1992; 5, Tax *et al.* 1994; 6, Chitnis *et al.*, 1995; 7, Sun and Artavani-Tsakonas, 1996.

and concluded that membrane attachment is not required for rescue. By contrast, we report that APX-1(extra 1–387) cannot rescue a *lag-2* null allele and conclude that the TM domain is important. We do not understand the differences between the results from the two laboratories but suspect that differences in the details of our experiments may account for the discrepancy. For example, Fitzgerald and Greenwald (1995) used *lag-2(s1486)* for mutant rescue. This *lag-2* allele carries a 3′ deletion (Henderson *et al.*, 1994), which theoretically could be partially suppressed in the *smg-1* mutant background used in their assays (Pulak and Anderson, 1993). Alternatively, the APX-1(extra 1–402) construct used by Fitzgerald and Greenwald (1995) includes 10 amino acids of the predicted TM domain, which may allow partial membrane anchoring or promote aggregation.

GFP Can Confer Rescuing Activity on LAG-2(extra 1–286) and APX-1(extra 1–387)

How might GFP confer rescuing activity on LAG-2(extra 1–286) and APX-1(extra 1–387)? Several possibilities exist. One idea is that GFP contains a stretch of hydrophobic amino acids that may serve as a weak synthetic TM domain (Henderson, unpublished observation). Another possibility is that the extracellular domain, when released from the membrane, is unstable; if true, GFP might stabilize the protein. Finally, the addition of GFP may promote aggregation of the secreted protein, either by decreasing its solubility or by enhancing multimerization. GFP has been reported to be insoluble under some circumstances (Crameri *et al.*, 1996). Because GFP can substitute for the membrane association of LAG-2, the TM domain per se is clearly not essential for signaling. However, that domain is likely to provide some key function that can be substituted by GFP. We surmise that membrane association may function to limit the diffusion of LAG-2 and also to increase its local concentration.

Ectopic Activation of GLP-1 by LAG-2(extra 1–286)

LAG-2(extra 1–286) and APX-1(extra 1–387) can activate the receptor ectopically, even though they cannot rescue a *lag-2* null mutant (this article; also see Fitzgerald and Greenwald, 1995). Normally, LAG-2 and GLP-1 direct germ line cells to continue mitosis distally (Austin and Kimble 1987; Lambie and Kimble, 1991; Crittenden *et al.* 1994; Henderson *et al.*, 1994). Although LAG-2 is expressed transiently in the AC/VU precursors, which reside at the proximal-most point in the gonad (Wilkinson *et al.*, 1994), LAG-2 does not normally activate GLP-1 in the proximal germ line. We find that LAG-2(extra 1–286) induces ectopic GLP-1 in the proximal germ line, and that the signal can derive from either the DTC or the AC/VU precursors.

How does LAG-2(extra 1–286) induce ectopic GLP-1 and ectopic germ line proliferation? Because LAG-2(extra 1–286) produced from the DTC can induce proximal proliferation in the absence of the AC/VU precursors, LAG-2(extra 1–286) appears to act at a distance. One simple explanation is that secreted LAG-2(extra 1–286) diffuses to ectopic sites. Alternatively, as the germ line grows and the DTC migrates away from its initially central position, LAG-2(extra 1–286) may be deposited into the extracellular matrix along the route of the DTC. If stable, LAG-2(extra 1–286) may signal to germ cells even after the DTC has moved away. In either case, membrane association of LAG-2 must normally limit the extent of LAG-2 and GLP-1 interactions to the distal end.

LAG-2(extra 1–286) clearly retains the ability to signal postembryonically but does not have rescuing activity. Why not? One possibility might have been that a *lag-2(0)* mutant expressing LAG-2(extra 1–286) dies because of too much signal rather than too little. However, expression of LAG-2(extra 1–286) does not appear to alter the Lag phenotype of *lag-2(0)* mutant larvae and does not generate dead embryos or larvae in otherwise wild-type animals. A second possibility is that signaling might be enhanced postembryonically by the presence of an extracellular matrix, which might stabilize or restrict diffusion of secreted LAG-2. In the embryo, little extracellular matrix is observed at the time of signaling (Kramer, personal communication). A third possibility is that signaling in the embryo may be more sensitive to a requirement for a localized signal than in larvae. The embryonic expression of *lag-2* and *lin-12* may be regulated by a signaling cascade; early signaling sets up later expression patterns (Moskowitz and Rothman, 1996). Loss of precise cell-cell signaling in early interactions would lead to extensive later misexpression and failure to rescue.

The IC Domain of LAG-2 Negatively Regulates Its Activity

Deletion of the LAG-2 IC domain increases activity of the mutant LAG-2(ΔIC) protein. LAG-2(ΔIC) can rescue a null *lag-2* mutant, and it can also generate ectopic vulval tissue. The Muv effect does not depend on endogenous wild-type LAG-2, because it is also observed in *lag-2* null animals carrying LAG-2(ΔIC). One possible explanation of the LAG-2(ΔIC) dominant phenotype might have been the production of a molecule similar to LAG-2(extra 1–286) because of a poor anchor in the membrane. However, this is unlikely. The IC domain of LAG-2(ΔIC) is predicted to bear 10 amino acids, including a group of positively charged residues immediately inside the predicted TM domain. This IC domain together with the TM-spanning domain should be sufficient to anchor the protein in the membrane (Yost *et al.*, 1983; von Heijne and Gavel, 1988). Furthermore, activation by LAG-2(ΔIC) and LAG-2(extra 1–286) are distinct: LAG-2(extra 1–286) induces germ line tumors, whereas LAG-2(ΔIC) does not.

A more plausible explanation is that the dominant Muv phenotype of LAG-2(ΔIC) results from protein stabilization. Because LAG-2 is normally expressed in cells that are precursors to the vulval precursor cells (Henderson, unpublished observation), the Muv effect of LAG-2(ΔIC) mutants may result from residual signal that persists from earlier expression. LAG-2 harbors a potential PEST sequence in its IC domain (Henderson *et al.*, 1994; Tax *et al.*, 1994). We suggest that the dominant effect of LAG-2(ΔIC) may result from removal of that PEST sequence. Consistent with this idea, the Muv phenotype was not observed in LAG-2(ΔIC)::β-gal animals; β-gal is known to turn over rapidly in *C. elegans* (Fire *et al.*, 1990). Because PEST sequences are thought to signal protein degradation (Rechsteiner and Rogers, 1996), removal of such a sequence from LAG-2 may stabilize and hyperactivate the protein.

Functions of the IC domains appear to differ among DSL proteins. Potential PEST regions are found in LAG-2 and APX-1 but not in fly or vertebrate homologues. In addition, deletion of the IC domain from X-Delta-1 of *Xenopus* or from Delta or Serrate of *Drosophila* generates a dominant negative protein (Chitnis *et al.*, 1995; Sun and Artavanis-Tsakonas, 1996). By contrast, LAG-2(ΔIC) has no dominant negative effect but instead is more active than normal. The hyperactivity of LAG-2(ΔIC) may be analogous to that of Ser^{D}. Ser^{D} removes potential RNA degradation signals from the Serrate transcript and results in an increased level of both Serrate RNA and protein (Thomas *et al.*, 1995). Perhaps LAG-2 and APX-1 levels are regulated by protein degradation in *C. elegans*, whereas Serrate levels are regulated by RNA degradation in *Drosophila*.

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