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# Roles of the RAM and ANK domains in signaling by the *C.elegans* GLP-1 receptor

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**In *Caenorhabditis elegans*, the GLP-1 receptor acts with a downstream transcriptional regulator, LAG-1, to mediate intercellular signaling. GLP-1 and LAG-1 are homologs of *Drosophila* Notch and Su(H) respectively. Here, we investigate the functions of two regions of the GLP-1 intracellular domain: the ANK repeat domain, which includes six *cdc10*/ankyrin repeats plus flanking amino acids, and the RAM domain, which spans ~60 amino acids just inside the transmembrane domain. First, we demonstrate that both ANK and RAM domains interact with the LAG-1 transcription factor. The interaction between the ANK domain and LAG-1 is only observed in nematodes by a co-localization assay and, therefore, may be either direct or indirect. By contrast, the interaction between the RAM domain and LAG-1 is likely to be direct, since it is observed by co-precipitation of the proteins *in vitro* as well as by yeast two-hybrid experiments. Second, we demonstrate that the RAM domain, when expressed in nematodes without a functional ANK repeat domain, does not mimic the unregulated receptor in directing cell fates or interfere with signaling by endogenous components. Finally, we show in yeast that the ANK repeats are strong transcriptional activators. Furthermore, missense mutations that eliminate receptor activity also abolish transcriptional activation by the GLP-1 ANK repeats in yeast. We speculate that one possible function for the ANK repeat domain is to act as a transcriptional co-activator with LAG-1.**

*Keywords*: *C.elegans*/GLP-1/LAG-1/signal transduction

## Introduction

During metazoan development, many cell interactions are controlled by a group of related receptors, including GLP-1 and LIN-12 of *Caenorhabditis elegans* and *Drosophila* Notch (for review, see Artavanis-Tsakonas *et al.*, 1995). In *C.elegans*, two additional genes, *lag-1* and *lag-2*, are required for most intercellular signaling by GLP-1 and LIN-12 receptors (Lambie and Kimble, 1991). The LAG-1 protein shares a high degree of amino acid identity with *Drosophila* Su(H) and appears to act as a transcriptional regulator in the receiving cell (Christensen *et al.*, 1996). The LAG-2 protein, on the other hand, is similar to *Drosophila* Delta and acts upstream of GLP-1 and LIN-12, presumably as a signaling ligand (Henderson *et al.*, 1994; Tax *et al.*, 1994). A homologous signaling pathway has also been found in vertebrates (reviewed in Artavanis-Tsakonas *et al.*, 1995; Nye and Koplan, 1995). Vertebrate homologs of particular relevance to this study are murine Notch1 (Franco del Amo *et al.*, 1993) and CBF1 (also known as KBF2 or RBP-Jκ) (Matsunami *et al.*, 1989; Grossman *et al.*, 1994; Henkel *et al.*, 1994). Thus, the core elements of this pathway (signal, receptor, transcription factor) have been conserved throughout metazoan evolution.

The critical region of Notch-related receptors for directing cell fates resides in the intracellular domain (Fortini *et al.*, 1993; Lieber *et al.*, 1993; Rebay *et al.*, 1993; Roehl and Kimble, 1993; Struhl *et al.*, 1993; Kopan *et al.*, 1994; Nye *et al.*, 1994; this work). Within the intracellular domain, the *cdc10*/ankyrin, or ANK, repeats are critical for function. Two lines of evidence support this assertion. First, several loss-of-function mutations alter single conserved amino acids within the ANK repeats (Kodoyianni *et al.*, 1992; Diederich *et al.*, 1994). Second, a fragment composed primarily of the ANK repeats, when expressed from a transgene, directs cell fates in a manner similar to that of an unregulated receptor (Roehl and Kimble, 1993). Therefore, the ANK repeat domain appears to be both necessary and sufficient for GLP-1 signaling.

The molecular mechanism by which the ANK repeats regulate cell fate is not yet known. One clue is that the intracellular domains of *Drosophila* Notch and murine mNotch1 receptors physically interact with the transcription factors Su(H) and CBF1 respectively (Fortini and Artavanis-Tsakonas, 1994; Jarriault *et al.*, 1995; Tamura *et al.*, 1995; Hsieh *et al.*, 1996). However, reports vary with respect to the specific region of the intracellular domain important for interaction, and interpretations vary with respect to the function of the interaction. A second possible clue to the mechanism is that the constitutively acting intracellular domains of fly Notch and murine Notch1 (mNotch1) are found in the nucleus (Fortini *et al.*, 1993; Lieber *et al.*, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993; Kopan *et al.*, 1994; Nye *et al.*, 1994). However, the ANK repeat domain of GLP-1, when similarly expressed, is largely cytoplasmic (Roehl and Kimble, 1993), and the intracellular domains of endogenous receptors have not been detected in nuclei (Johansen *et al.*, 1989; Kooh *et al.*, 1993; Crittenden *et al.*, 1994). Therefore, although much attention has focused on the intracellular domains of the receptors, a clear picture has not yet emerged.

Here we investigate the regions of the intracellular domain of the GLP-1 receptor that are required for both interactions with the LAG-1 transcription factor and for signaling of cell fate. First, we find that the ANK repeat domain of GLP-1 interacts with LAG-1 *in vivo*, but do

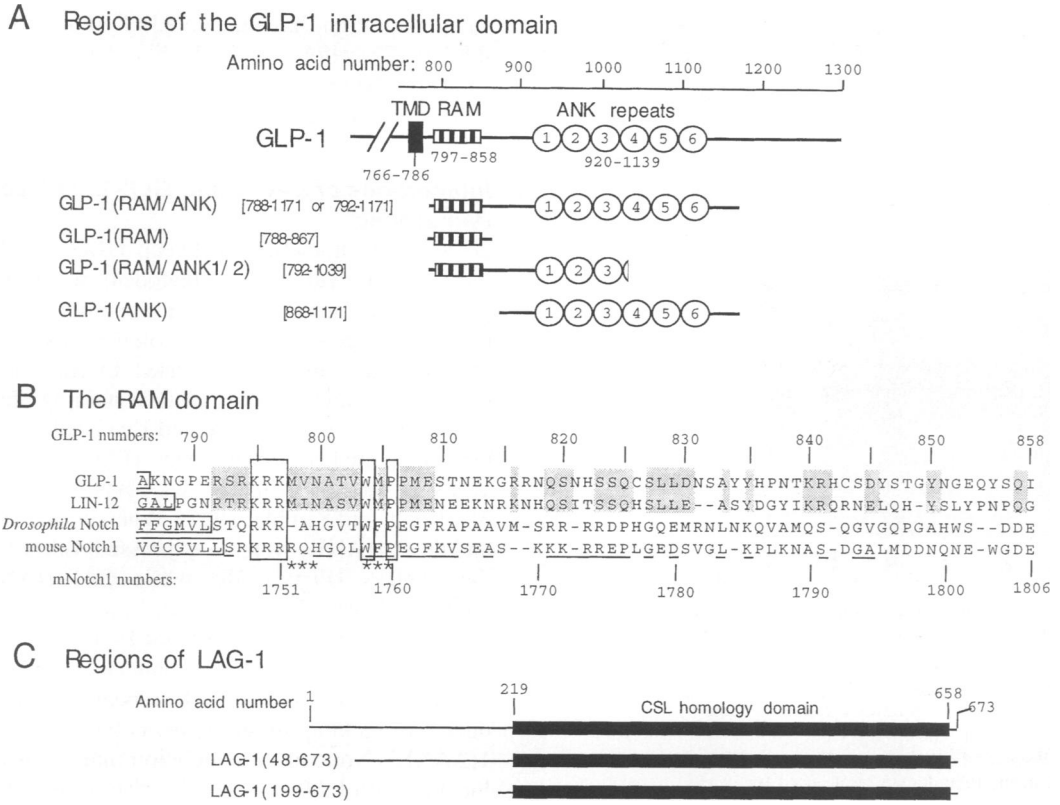


**Fig. 1.** The GLP-1 RAM and ANK domains and LAG-1. (**A**) Regions of the GLP-1 intracellular domain. Above, schematic of the intracellular domain with the scale of amino acid numbering taken from Yochem and Greenwald (1989). Three putative domains are shown: the transmembrane domain (TMD) spanning amino acids 766–786, the RAM domain spanning amino acids 797–858 by analogy with the RAM23 domain of mNotch1 (Tamura *et al.*, 1995) and the ANK repeats spanning amino acids 920–1139. Below, major GLP-1 fragments used in this study. RAM/ANK includes the RAM and ANK repeat domains; two separate constructs were used (788–1171 and 792–1171) for ease of cloning; RAM includes 788–867, RAM/ANK1/2 includes 792–1039 and ANK includes 868–1171, which includes both the ankyrin repeats plus flanking amino acids. (**B**) The GLP-1 RAM domain. The amino acid sequences of four receptors are aligned; amino acid numbering for GLP-1 is shown above and for mNotch1 below. The amino acid sequences are from Yochem and Greenwald (1989) for GLP-1, from Yochem *et al.* (1988) for LIN-12, from Wharton *et al.* (1985) for *Drosophila* Notch and from Franco del Amo *et al.* (1993) for mNotch1. The region shown includes the C-terminal portion of the TMD, and extends C-terminally to the amino acid defined by Tamura *et al.* (1995) as the end of the RAM23 domain. The TMD lies to the left and is partially enclosed; N-terminally a basic region and WXP are conserved among all four receptors (boxed). Comparison of GLP-1 and LIN-12 receptors from nematodes shows high conservation spanning this domain (shaded residues); comparison of fly and murine receptors also shows high conservation (underlined residues). However, comparison of nematode receptors with fly/murine receptors shows little conservation. Stars are placed under amino acids mutated in mNotch1 by Tamura *et al.* (1995) as described in the text. (**C**) Regions of LAG-1. Above, schematic of LAG-1 with the scale of amino acid numbering taken from Christensen *et al.* (1996). The conserved region in LAG-1, Su(H) and CBF1 is shown as a thick bar from amino acid 219 to 658. Below, major LAG-1 fragments used in this study.

not detect an equivalent interaction either by yeast two-hybrid assay or *in vitro*. Therefore, the interaction *in vivo* is likely to depend on post-translational modifications or additional proteins present in nematodes. Second, we observe a strong interaction between LAG-1 and a newly identified 'RAM' domain of GLP-1. This interaction is likely to be direct: it is observed in nematodes, *in vitro* and by yeast two-hybrid assay. Third, we demonstrate that a fragment composed of the RAM domain coupled to an active ANK repeat domain directs cell fates like an unregulated receptor, but that the RAM domain coupled to an inactive ANK repeat domain has no effect on cell fate. Fourth, we show that the GLP-1 ANK repeat domain activates transcription in yeast, but that mutant GLP-1 ANK repeat domains with single amino acid changes are not able to activate transcription in the same assay. Finally, we observe homotypic interactions between ANK repeat domains.

## Results

### *Regions of GLP-1 intracellular domain and LAG-1*

Figure 1A illustrates regions of the GLP-1 intracellular domain and the major constructs tested in this study. The GLP-1 ANK repeats span amino acids 920–1139; the GLP-1(ANK) construct encodes the ANK repeats plus flanking amino acids. We have not only examined wild-type GLP-1 ANK repeats, but also fragments bearing missense mutations residing in the *glp-1* ANK repeats that dramatically reduce receptor function (Austin and Kimble, 1987; Priess *et al.*, 1987; Kodoyianni *et al.*, 1992). Receptor activity in nematodes is virtually abolished in *glp-1(q224)* mutants and is severely diminished in *glp-1(q231)* and *glp-1(e2144)* mutants when raised at the restrictive temperature.

Between the transmembrane domain (TMD) and ANK repeats lies a region, which we dub 'RAM' by analogy

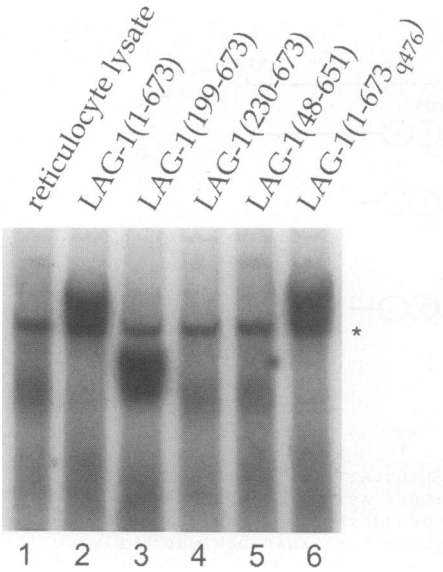


**Fig. 2.** Gel mobility shift analysis with *in vitro* translated LAG-1. Radiolabeled MB22 oligonucleotide containing the GTGGGAA consensus sequence was bound efficiently by LAG-1(1–673) (lane 2), LAG-1(199–673) (lane 3) and by mutant LAG-1(1–673$_{q476}$) (lane 6). No binding was observed with LAG-1(230–673) (lane 4) or LAG-1(48–651) (lane 5). The shifted band marked by an asterisk is present in the absence of LAG-1 (lane 1) and is due to DNA binding activity present in the reticulocyte lysate used for *in vitro* translation.

with the RAM23 domain of mNotch1 (Tamura *et al.*, 1995). The RAM23 domain binds directly to murine CBF1/KBF2/RBP-Jκ (henceforth referred to as CBF1), a homolog of LAG-1 (Tamura *et al.*, 1995; Hsieh *et al.*, 1996). The smallest region of RAM23 capable of CBF1 binding spans 56 amino acids (1751–1806); furthermore, amino acids 1752–1754 and 1758–1760 appear to be critical for binding (Tamura *et al.*, 1995). We define the RAM domain of GLP-1 as amino acids 797–858, which correspond by position to amino acids 1751–1806 of mNotch1. A comparison of amino acid sequences in the RAM and RAM23 domains of nematode GLP-1 and mNotch1 reveals little similarity except for amino acids 804 (W) and 806 (P), which correspond to mNotch1 1758 and 1760 respectively (Figure 1B).

Figure 1C shows a schematic of LAG-1 and the major LAG-1 constructs tested in this study. LAG-1 contains a total of 673 amino acids; in the C-terminal portion, a stretch of 439 amino acids is highly conserved among LAG-1, vertebrate CBF1 and *Drosophila* Su(H) (Christensen *et al.*, 1996). This conserved region extends from amino acid 219 through 658 of LAG-1 and is required for binding DNA (Figure 2). Whereas full-length LAG-1(1–673) and an N-terminally truncated fragment, LAG-1(199–673), bound the consensus DNA sequence (Figure 2, lanes 2 and 3), two other fragments, LAG-1(230–673) and LAG-1(48–651), failed to bind (Figure 2, lanes 4 and 5). Therefore, even small deletions of <15 amino acids from either end of the large conserved region abolish LAG-1 DNA binding. In addition, LAG-1(1–673$_{q476}$) bound DNA (Figure 2, lane 6); LAG-1(1–673$_{q476}$) contains the missense mutation present in the strong loss-of-function allele *lag-1(q476)* (Christensen *et al.*, 1996). Gel shift experiments performed with a mix of LAG-1(1–673) and LAG-1(199–673) failed to reveal intermediate bands that might have indicated oligomerization (data not shown). Therefore, LAG-1, like CBF1 (Chung *et al.*, 1994), probably binds DNA as a monomer rather than as a dimer or oligomer.

### *Interactions of LAG-1 and GLP-1: co-localization in nematodes*

To analyze interactions between LAG-1 and the GLP-1 intracellular region in nematodes, we tested pairs of proteins that, when expressed alone under control of the heat shock promoter, were located in distinct subcellular regions. The intestine was used for this assay because there is no endogenous GLP-1 staining in this tissue. First, we examined *myc*-tagged LAG-1(48–673), which is primarily nuclear on its own (Figure 3A), and GLP-1(ANK), which is primarily cytoplasmic on its own (Figure 3D; Roehl and Kimble, 1993). When *myc*-LAG-1(48–673) and GLP-1(ANK) were co-expressed, LAG-1 remained in the nucleus (Figure 3E) while GLP-1(ANK) became nuclear (Figure 3F). Therefore, LAG-1(48–673) can alter the subcellular localization of GLP-1(ANK) in nematodes. Additionally, we examined a battery of ANK mutants that delete portions of the ANK repeat domain as well as only the flanking amino acids, collectively termed GLP-1(ΔANK). None of these deletion mutants was able to co-localize with LAG-1 (Table I). Therefore, the amino acids flanking the ANK repeats are required for co-localization with LAG-1.

Second, we examined *myc*-tagged LAG-1(199–673), which is cytoplasmic on its own (Figure 4A), and GLP-1(RAM/ANK-792–1171), which is nuclear on its own (Figure 4D). When *myc*-LAG-1(199–673) and GLP-1(RAM/ANK) were co-expressed, *myc*-LAG-1(199–673) became nuclear (Figure 4E) and GLP-1(RAM/ANK) remained in the nucleus (Figure 4F). To control for non-specific interactions, we examined GLP-1(RAM/ANK) with a C-terminal fragment of LAG-1(359–673) (Table I); this pair failed to co-localize to the nucleus. Therefore, GLP-1(RAM/ANK) interacts with LAG-1(199–673) specifically and can alter the subcellular localization of LAG-1(199–673) in nematodes.

We next asked if the missense mutations *glp-1(q224)* and *glp-1(e2144)*, which reside in the ANK repeats, interfere with GLP-1/LAG-1 co-localization. We found that GLP-1(ANK$_{q224}$) and GLP-1(ANK$_{e2144}$) fail to co-localize with LAG-1(48–673) (Figure 5). Therefore, the co-localization of GLP-1(ANK) and LAG-1 in nematodes appears to be specific to the active form of the ANK repeat domain.

To test whether the RAM domain can co-localize LAG-1 in the absence of an active ANK repeat domain, we used two constructs: GLP-1(RAM/ANK1/2-792–1039) and GLP-1(RAM/ANK$_{q224}$). Both alterations, when present in a GLP-1(ANK) fragment, abolish both the ability to co-localize with LAG-1 (see above, Table I) and to confer the Muv phenotype (see below, Table III). Yet, GLP-1(RAM/ANK1/2-792–1039) and GLP-1(RAM/ANK$_{q224}$) are still able to co-localize with LAG-1 (Table I). Therefore, the RAM domain, like the ANK repeat domain, appears to be sufficient to co-localize LAG-1 in nematodes.

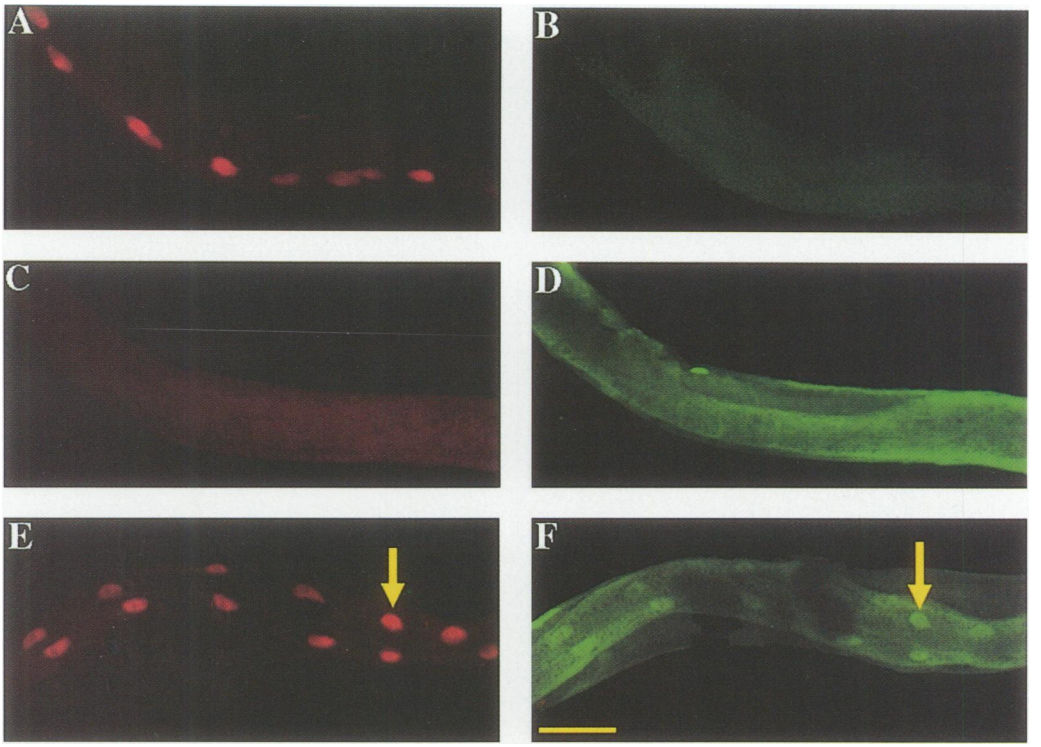


**Fig. 3.** Co-localization of LAG-1 and GLP-1(ANK) in transgenic *C.elegans*. Left column, immunostaining with antibody to c-*myc*; right column, immunostaining with antibodies to GLP-1 ANK repeats. (**A**) and (**B**) Transgenic animal carrying *myc*-LAG-1(48–673); (**C**) and (**D**) transgenic animal carrying GLP-1(ANK); (**E**) and (**F**) transgenic animal carrying both *myc*-LAG-1(48–673) and GLP-1(ANK). GLP-1(ANK) is predominantly cytoplasmic when expressed on its own (D), but is predominantly nuclear when co-expressed with *myc*-LAG-1 (F). Arrows point to the same intestinal nucleus in (E) and (F). Scale bar = 50 μm.

**Table I.** Co-localization of LAG-1 and GLP-1 in transgenic nematodes[a]

| Nuclear protein | Cytoplasmic protein | Co-localization |
|---|---|---|
| LAG-1(48–673) | GLP-1(ANK) | + |
| LAG-1(48–673) | GLP-1(ΔANK-868–1146) | – |
| LAG-1(48–673) | GLP-1(ΔANK-904–1171) | – |
| LAG-1(48–673) | GLP-1(ΔANK-904–1146) | – |
| LAG-1(48–673) | GLP-1(ΔANK-868–1039) | – |
| LAG-1(48–673) | GLP-1(ΔANK-1041–1171) | – |
| LAG-1(48–673) | GLP-1($ANK_{q224}$) | – |
| LAG-1(48–673) | GLP-1($ANK_{e2144}$) | – |
| GLP-1(RAM/ANK) | LAG-1(199–673) | + |
| GLP-1(RAM/ANK1/2) | LAG-1(199–673) | + |
| GLP-1(RAM/$ANK_{q224}$) | LAG-1(199–673) | + |
| GLP-1(RAM/ANK) | LAG-1(359–673) | – |

[a]GLP-1 amino acids are: ANK(868–1171), RAM/ANK (792–1171), RAM/ANK1/2 (792–1039) and various deletion mutants of GLP-1(ΔANK) with amino acids as shown. LAG-1 amino acids are shown.

### *Interactions of LAG-1 and GLP-1: yeast two-hybrid assay*

The co-localization of LAG-1 and intracellular GLP-1 as detected by microscopy (Figures 3 and 4) might reflect either a direct or indirect interaction between the proteins. To investigate the interaction further, we used a yeast two-hybrid assay. Plasmids encoding hybrid proteins of either the GAL4 DNA binding domain (DB) or GAL4 activation domain (AD) fused with either LAG-1 or GLP-1 were constructed and transformed into yeast (see Materials and methods). In initial experiments, we found that DB–GLP-1 hybrids and DB–LAG-1(1–673) were able to activate reporter expression when transformed by themselves (data not shown). Therefore, all subsequent experiments relied on AD–GLP-1 hybrids and smaller DB–LAG-1 hybrids, which did not activate reporter transcription on their own.

Fragments of LAG-1 and intracellular GLP-1 clearly interact in yeast two-hybrid experiments (Table II). DB–LAG-1(199–673) and DB–LAG-1(230–673) interacted with both AD–GLP-1(RAM/ANK-788–1171) and AD–GLP-1(RAM). By contrast, both hybrids interacted poorly with either AD–GLP-1(ΔRAM/ANK-798–1171) or AD–GLP-1(ANK). AD–GLP-1(ΔRAM/ANK) lacks the 10 N-terminal amino acids of AD–GLP-1(RAM/ANK), emphasizing the importance of this N-terminal region of the RAM domain for interactions between LAG-1 and GLP-1. As negative controls, we showed that DB–LAG-1 hybrids failed to interact with the yeast SNF1 protein and that AD–GLP-1 hybrids failed to interact with the yeast SNF4 protein (Table II).

Because LAG-1 interacted so weakly with GLP-1(ANK), we tested the ANK region of a different *C.elegans* protein. FEM-1(ANK) consists of six ANK repeats from the sex-determining gene *fem-1* (Spence *et al.*, 1990). Because we detected a weak interaction between DB–LAG-1(230–673) and AD-FEM-1(ANK) (Table II), the interaction between LAG-1 and GLP-1(ANK) is likely to be non-specific.

Finally, we asked if a missense mutation in the GLP-1 ANK repeats might affect interactions with LAG-1. We found that DB–LAG-1(230–673) interacted equally well with AD–GLP-1(RAM/ANK) and AD–GLP-1(RAM/$ANK_{q224}$) (Table II). Furthermore, DB–LAG-1(230–673) interacted equally poorly with AD–GLP-1(ANK) and with

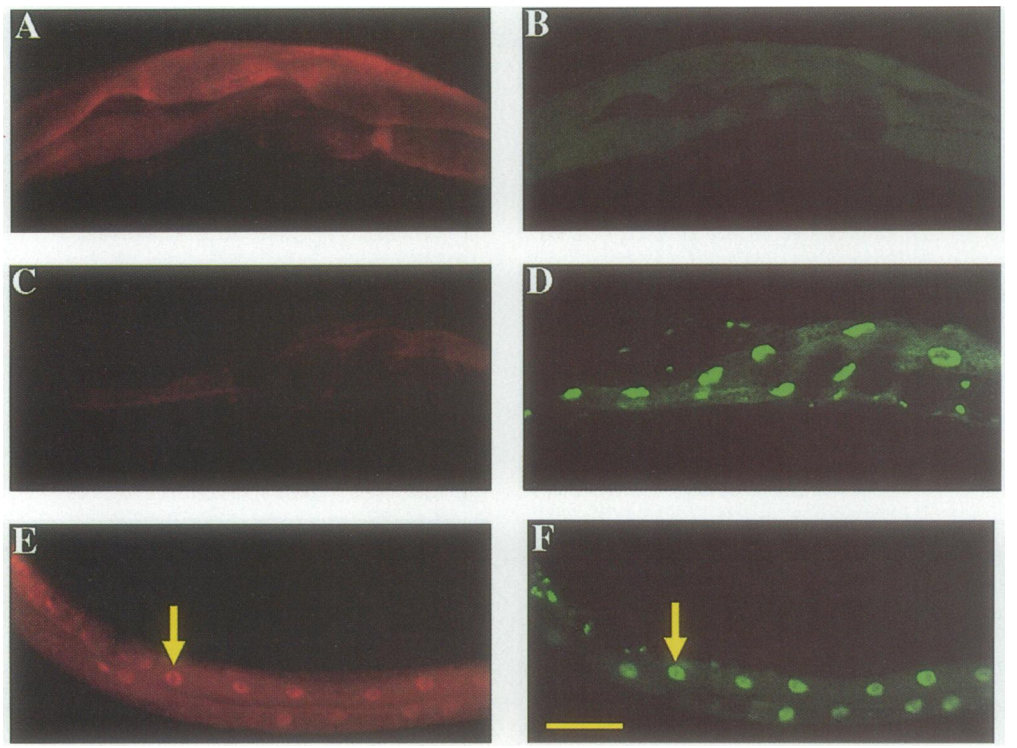


**Fig. 4.** Co-localization of LAG-1 and GLP-1(RAM/ANK) in transgenic nematodes. Left column, immunostaining with antibody to c-*myc*; right column, immunostaining with antibodies to GLP-1 ANK repeats. (**A**) and (**B**) Transgenic animal carrying *myc*-LAG-1(199–673); (**C**) and (**D**) transgenic animal carrying GLP-1(RAM/ANK-792–1171); (**E**) and (**F**) transgenic animal carrying both *myc*-LAG-1(199–673) and GLP-1(RAM/ANK). *myc*-LAG-1(199–673) is predominantly cytoplasmic when expressed on its own (A), but is predominantly nuclear when co-expressed with c-*myc*-LAG-1 (E). Arrows point to the same intestinal nucleus in (E) and (F). Scale bar = 50 μm.

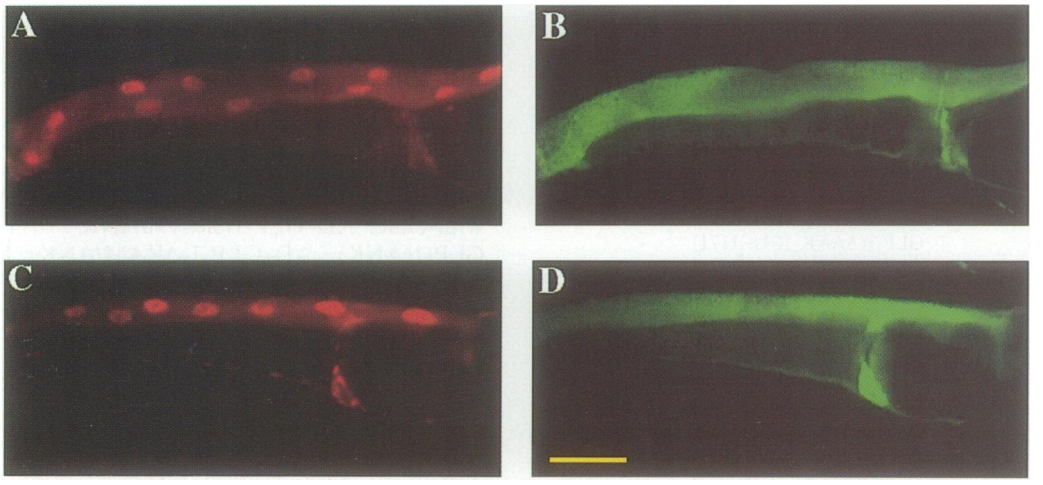


**Fig. 5.** Lack of co-localization of LAG-1 with inactive forms of GLP-1. Left column, immunostaining with antibody to c-*myc*; right column, immunostaining with antibodies to GLP-1 ANK repeats. (**A**) and (**B**) Transgenic animal expressing c-myc-LAG-1(48–673) and GLP-1($ANK_{q224}$). (**C**) and (**D**) Transgenic animal expressing c-myc-LAG-1(48–673) and GLP-1($ANK_{e2144}$). (A) and (C) c-myc-LAG-1(48–673) is nuclear; (B) GLP-1($ANK_{q224}$) is cytoplasmic; (D) GLP-1($ANK_{e2144}$) is cytoplasmic. These animals were raised at 20°C after heat shock, though the same result was obtained at either 12 or 15°C (data not shown). Scale bar = 50 μm.

three different missense mutants AD–GLP-1($ANK_{q224}$), AD–GLP-1($ANK_{q231}$) and AD–GLP-1($ANK_{e2144}$) (data not shown). Therefore, in the yeast two-hybrid assay, a strong interaction is observed with the RAM domain, and a weak and non-specific, but reproducible interaction is observed with the ANK domain. Furthermore, mutations in the ANK repeats do not affect either the strong or weak interaction.

### *Interactions of LAG-1 and GLP-1: in vitro binding*

We examined protein binding *in vitro* using a co-precipitation assay. GLP-1 fragments were immobilized using

**Table II.** Interactions between LAG-1 and the GLP-1 intracellular domain

| Binding domain hybrid | Activation domain hybrid[a] | Colony lift assay[b] |
|---|---|---|
| LAG-1(199–673) | GLP-1(RAM/ANK) | ++++ |
| LAG-1(230–673) | GLP-1(RAM/ANK) | ++++ |
| LAG-1(199–673) | GLP-1(RAM) | ++ |
| LAG-1(230–673) | GLP-1(RAM) | ++++ |
| LAG-1(199–673) | GLP-1(ΔRAM /ANK) | +/– |
| LAG-1(230–673) | GLP-1(ΔRAM /ANK) | + |
| LAG-1(199–673) | GLP-1(ANK) | +/– |
| LAG-1(230–673) | GLP-1(ANK) | + |
| LAG-1(230–673) | FEM-1(ANK) | + |
| LAG-1(230–673) | GLP-1(RAM/ANK$_{q224}$) | ++++ |
| LAG-1(199–673) | SNF1 | – |
| LAG-1(230–673) | SNF1 | – |
| SNF4 | GLP-1(RAM/ANK) | – |
| SNF4 | GLP-1(RAM) | – |
| SNF4 | GLP-1(ΔRAM /ANK) | – |
| SNF4 | GLP-1(ANK) | – |
| SNF4 | SNF1 | ++++ |

[a]GLP-1 amino acids in these activation domain hybrids are: RAM/ANK(788–1171), RAM(788–867), ΔRAM /ANK(798–1171) AND ANK(868–1171).
[b]Filters with detectable blue color after 30 min were denoted (++++), after 1 h (+++), 2 h (++), 4 h (+), some blue colonies at 4 h (+/–) or no blue colonies at 4 h (–).

S-Tag-GLP-1 variants and agarose beads coupled to S-protein (see Materials and methods). After pre-incubation of S-Tag-GLP-1 agarose beads, $^{35}$S-labeled LAG-1(199–673) was added and the precipitate run on a gel. In the absence of S-Tag-GLP-1, LAG-1 was not retained on the beads (data not shown). With S-Tag-GLP-1(ANK), retention of LAG-1(199–673) was poor (Figure 6, lane 2), but with S-Tag-GLP-1(RAM/ANK) retention of the LAG-1(199–673) was efficient (Figure 6, lane 3). We conclude that the interaction between LAG-1 and the GLP-1 RAM domain is likely to be direct.

### *In vivo* receptor activity of ANK and RAM domains

When either LIN-12 or GLP-1 receptors are activated aberrantly, multiple vulvae are observed, i.e. the Muv phenotype (Greenwald *et al.*, 1983; Mango *et al.*, 1991). We previously showed that GLP-1(ANK), when placed under heat shock control and expressed at the stage of vulval determination, generates Muv animals, but that GLP-1(ANK$_{q224}$) does not (Roehl and Kimble, 1993). Here, we explore additional constructs by the same assay. DNAs encoding fragments of the GLP-1 intracellular domain were placed under heat shock control, introduced as transgenes into nematodes and assayed for their ability to induce Muv animals (see Materials and methods; Table III). The expression of all constructs was monitored *in vivo* with antibodies raised against the GLP-1 ANK repeat domain; all fragments produced stable products.

We first compared the effects of the ANK repeat domain with that of a RAM/ANK construct. Multiple transgenic lines were generated after injection of the same concentration of DNA (either 1 mg/ml or 5 mg/ml) for each construct. These two concentrations are intermediate between 0.5 mg/ml, which only sometimes gives lines that exhibit Muvs, and 10 mg/ml, which nearly always gives Muv animals (Roehl and Kimble, 1993). The strength

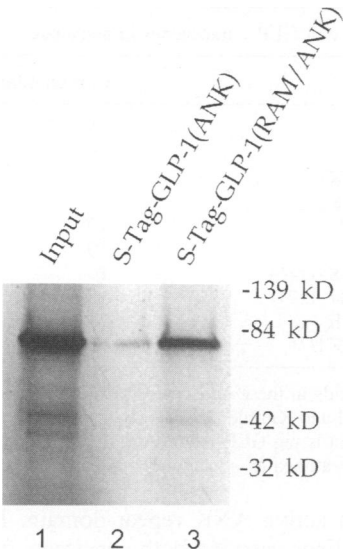


**Fig. 6.** Co-precipitation of LAG-1 with the intracellular domain of GLP-1. *In vitro* translated $^{35}$S-labeled LAG-1(199–673) was incubated with a 4-fold molar excess of S-Tag GLP-1(RAM/ANK) or S-Tag-GLP-1(ANK), precipitated on S-protein–agarose beads and washed four times. Samples were eluted by boiling in SDS sample buffer, separated by SDS–PAGE, and visualized by autoradiography. The input lane represents 20% of the $^{35}$S-labeled LAG-1(199–673) that was incubated with S-protein beads. LAG-1(199–673) was co-precipitated efficiently with S-Tag-GLP-1(RAM/ANK) (lane 3) and poorly with S-Tag-GLP-1(ANK) (lane 2).

of the Muv phenotype was scored by counting the number of pseudovulvae. We found that both ANK and RAM/ANK constructs generated Muv animals. Intriguingly, the RAM/ANK construct appeared to be more potent than the ANK construct [Table III, compare GLP-1(ANK) with GLP-1(RAM/ANK) effects at each of two concentrations]. Therefore, the RAM domain does not inhibit the activity of the ANK domain, but instead appears to enhance its activity.

Second, we examined the effect of mutant versions of GLP-1(ANK) to assess what parts of that receptor fragment are essential (Table III). The activity of GLP-1(ANK$_{e2144}$), which carries a single amino acid change in the first ANK repeat, was severely reduced: few Muv animals were found and those animals had few pseudovulvae. The effect of this mutation on the endogenous receptor is weaker that that of *glp-1(q224)*, which abolishes GLP-1 activity at the restrictive temperature (Kodoyianni *et al.*, 1992). Given the GLP-1(ANK$_{e2144}$) result, we repeated GLP-1(ANK$_{q224}$) at the same concentration, and confirmed our previous finding (Roehl and Kimble, 1993): no Muv animals were generated. We also examined whether the amino acids flanking the ANK repeats are required for GLP-1 ANK activity. These deletion constructs made detectable protein, but abolished induction of Muv animals (Table III). We conclude from these results that the activity of GLP-1(ANK) depends not only on the ANK repeats, but also on the flanking amino acids.

Two distinct constructs were used next to examine the effect of the RAM domain in the absence of an active ANK repeat region. We found that neither GLP-1(RAM/ANK$_{q224}$) nor GLP-1(RAM/ANK1/2-792–1039) is active in generating a Muv phenotype (Table III). Therefore, the RAM domain does not mimic unregulated receptor in the

**Table III.** Activity of GLP-1 transgenes in nematodes

| Transgene[a] | Concentration of injected DNA[c] | Average No. of Ψvulvae[d] | Total No. of animals |
|---|---|---|---|
| GLP-1(ANK) | 1 | 1.74 | 124 |
| GLP-1(ANK) | 5 | 2.70 | 141 |
| GLP-1(RAM/ANK) | 1 | 2.42 | 141 |
| GLP-1(RAM/ANK) | 5 | 4.17 | 164 |
| GLP-1($ANK_{q224}$)[b] | 10 | 0 | 25 |
| GLP-1($ANK_{e2144}$) | 10 | 0.26 | 49 |
| GLP-1(ΔANK-868–1146) | 10 | 0 | 35 |
| GLP-1(ΔANK-904–1171) | 10 | 0 | 21 |
| GLP-1(RAM/$ANK_{q224}$) | 10 | 0 | 49 |
| GLP-1(RAM/ANK1/2) | 20 | 0 | 33 |

[a]GLP-1 amino acids in these transgenes are: ANK(868–1171), RAM/ANK (792–1171), RAM/ANK1/2 (792–1039).
[b]Data from Roehl and Kimble (1993).
[c]The concentration is mg GLP-1 DNA/ml; the concentration of *rol-6(su1006)* DNA is constant for all injections at 100 mg/ml.
[d]Animals were examined as young adults after a heat shock during L2 lethargus. The number of pseudovulvae (Ψvulvae) ranges from 0 to 5.

absence of an active ANK repeat domain. Furthermore, we examined lines carrying both constructs for dominant-negative effects. Specifically, we sought dead embryos, dead larvae, sterile animals or animals with protruding vulvae, any of which might have indicated a dominant-negative effect. No dominant-negative effect was observed for either GLP-1(RAM/ $ANK_{q224}$) or GLP-1(RAM/ANK1/2-792–1039) (data not shown).

### *Mutations in the ANK repeats eliminate GLP-1-mediated transcriptional activation in yeast*

In designing hybrid proteins for yeast two-hybrid experiments, we found that DB–GLP-1(ANK) induces reporter expression on its own (Table IV, line 1). We next asked if the missense mutations that reduce or abolish endogenous receptor activity and that eliminate co-localization *in vivo* (Figure 5; Table I) might also influence transcriptional activation by GLP-1(ANK). Intriguingly, the three ANK repeat mutations tested abolished activation of reporter expression by GLP-1(ANK) (Table IV, lines 2–4). Whereas wild-type GLP-1(ANK) induces reporter expression that is easily visible within 30 min of incubation with substrate, mutant GLP-1(ANK) failed to show reporter activity even after 24 h. These results in yeast raise the possibility that the GLP-1 ANK repeats may normally play a role in transcriptional activation and that this function may be compromised by the mutations.

### *Homotypic interactions betwen the GLP-1 ANK repeats*

Because DB–GLP-1($ANK_{q224}$) does not activate transcription on its own, we used this hybrid protein to test for homotypic interactions between GLP-1 ANK repeat domains. We found that DB–GLP-1($ANK_{q224}$) interacted strongly with AD–GLP-1(ANK) and with AD–GLP-1($ANK_{q224}$), that it interacted weakly with AD–LIN-12(ANK) and failed to interact with AD-FEM-1(ANK) (Table V). Therefore, the GLP-1 ANK repeat domains appear to interact specifically, and this interaction is not disrupted by the *glp-1(q224)* mutation.

**Table IV.** ANK mutations block GLP-1-mediated transcriptional activation in yeast

| Binding domain hybrid[a] | Colony lift assay[b] |
|---|---|
| GLP-1(ANK) | ++++ |
| GLP-1($ANK_{q224}$) | – |
| GLP-1($ANK_{q231}$) | – |
| GLP-1($ANK_{e2144}$) | – |

[a]All ANK constructs carry amino acids 868–1171 of GLP-1; ANK carries the wild-type sequence; $ANK_{q224}$ carries the *glp-1(q224)* missense mutation, which substitutes G for E at position 1043; $ANK_{q231}$ carries the *glp-1(q231)* missense mutation, which substitutes G for E at position 1057; $ANK_{e2144}$ carries the *glp-1(e2144)* missense mutation, which substitutes L for F at position 929.
[b]Calibration for colony lift assay as in footnote b of Table II.

**Table V.** ANK–ANK interactions

| Binding domain hybrid[a] | Activation domain hybrid[a] | Colony lift assay[b] |
|---|---|---|
| GLP-1($ANK_{q224}$) | – | – |
| GLP-1($ANK_{q224}$) | GLP-1(ANK) | +++ |
| GLP-1($ANK_{q224}$) | GLP-1($ANK_{q224}$) | +++ |
| GLP-1($ANK_{q224}$) | LIN-12(ANK) | + |
| GLP-1($ANK_{q224}$) | FEM-1(ANK) | – |
| GLP-1($ANK_{q224}$) | SNF1 | – |

[a]GLP-1(ANK) is as described in footnote a of Table IV. Other constructs are described in Materials and methods.
[b]Calibration for colony lift assay as in footnote b of Table II.

## Discussion

### *Interaction of the GLP-1(ANK) domain with LAG-1*

Interactions between fly Su(H) and Notch ANK repeats have been reported (Fortini and Artavanis-Tsakonas, 1994) and refuted (Tamura *et al.*, 1995). These reports were based on tissue culture co-localization, two-hybrid and *in vitro* binding assays. Here, we extend the investigation of this putative interaction to the homologous nematode proteins, LAG-1 and GLP-1. When examined in transgenic nematodes by a co-localization assay, we find a clear interaction between LAG-1 and the GLP-1(ANK) repeat domain (Figures 3 and 4). Furthermore, single amino acid changes in the GLP-1(ANK) repeat domain abolish co-localization. Since the GLP(ANK) repeat domain itself contains 303 amino acids, single amino acid changes would not *a priori* be expected to eliminate co-localization totally. However, these same missense mutations destroy or severely reduce receptor activity—whether assayed as an endogenous receptor (Kodoyianni *et al.*, 1992) or as a transgenic fragment (Roehl and Kimble, 1993; Table III).

Therefore, we conclude that co-localization of LAG-1 with the GLP-1(ANK) repeat domain depends on an active form of that domain.

Co-localization at microscopic resolution does not address how direct an interaction might be. To examine further the LAG-1–GLP-1(ANK) interaction, we used the yeast two-hybrid assay and *in vitro* binding studies. By both assays, we reproducibly observed a weak interaction; however, a similar interaction was observed with FEM-1(ANK) repeats. Therefore, this interaction appears to be both weak and non-specific in yeast and *in vitro*.

The strong and specific interaction between LAG-1 and an active GLP-1(ANK) domain in transgenic nematodes contrasts with the weak and non-specific interaction observed by yeast two-hybrid and *in vitro* binding studies. Two simple explanations can solve this paradox: one or both proteins may be modified *in vivo* to strengthen their interaction, or they may interact with other proteins to achieve co-localization in nematodes.

### *Interaction of the GLP-1(RAM) domain with LAG-1*

The RAM domain of Notch-related receptors (Tamura *et al.*, 1995) interacts with CSL [for CBF1, Su(H), LAG-1] proteins of nematodes (this work), flies (Tamura *et al.*, 1995) and vertebrates (Tamura *et al.*, 1995; Hsieh *et al.*, 1996). The extent of the RAM domain was narrowed down to 56 amino acids in mNotch1 (Tamura *et al.*, 1995). Within this minimal RAM domain, fly and vertebrate proteins are similar to each other (underlined amino acids in Figure 1B), and the two nematode receptors, GLP-1 and LIN-12, are similar to each other (shaded amino acids in Figure 1C), but few amino acids are conserved throughout phylogeny (boxed amino acids in Figure 1B). Two mutants tested with vertebrate proteins destroyed the interaction of the RAM domain with CBF1 (Tamura *et al.*, 1995); the amino acids changed in these mutants are marked by stars in Figure 1B and include one cluster (1752–1754) that is not conserved with GLP-1/LIN-12 and one cluster (1758–1760) that is conserved.

Given the lack of amino acid conservation between the RAM domains of flies and vertebrates on the one hand and those of *C.elegans* on the other, the two domains may or may not have been equivalent in function. We therefore tested the putative GLP-1 RAM domain for binding to LAG-1 and found strong binding by the yeast two-hybrid assay, by *in vitro* co-precipitation studies and by co-localization in nematodes. The GLP-1 RAM domain is defined as amino acids 788–867, by analogy with the minimal RAM23 fragment found for vertebrate proteins (Tamura *et al.*, 1995).

The interaction between LAG-1 and the GLP-1 RAM domain, as assayed in yeast two-hybrid experiments, was virtually unaffected by the presence of the ANK repeat domain. Thus, GLP-1(RAM/ANK) and GLP-1(RAM) constructs bound similarly to LAG-1 proteins. Binding by GLP-1(RAM/ANK) was somewhat stronger than GLP-1(RAM) for one LAG-1 protein (Table I), suggesting that the weak interaction with the ANK domain might reinforce the RAM–LAG-1 binding. Furthermore, mutations in the ANK repeats did not interfere with binding between GLP-1(RAM) and LAG-1 in either yeast two-hybrid or co-localization assays. Therefore, it seems likely that the RAM–LAG-1 interaction does not rely on the ANK repeats.

An N-terminal deletion of only 10 amino acids from the RAM domain significantly reduced its interaction with LAG-1 (Table II). This N-terminal deletion removes the conserved basic region from the predicted RAM/ANK fragment, and suggests that that basic region may be crucial for interactions. Alternatively, this truncation may interfere with a neighboring region such as that identified by mutations of mNotch in amino acids 1752–1754 or 1758–1760 (Tamura *et al.*, 1995).

### *Functional significance of RAM and ANK interactions with LAG-1*

The interactions reported between CSL proteins and the intracellular domains of Notch-related receptors (Fortini and Artavanis-Tsakonas, 1994; Tamura *et al.*, 1995; Hsieh *et al.*, 1996; this work) are puzzling when compared with the activities of these proteins in transgenic animals. On one hand, the RAM domain interacts well with CSL proteins, and the ANK repeat domain interacts poorly and non-specifically. However, on the other hand, the GLP-1(ANK) repeat region has constitutive activity on its own in transgenic nematodes (Roehl and Kimble, 1993), as do transgenes of *Drosophila* Notch or mNotch1 that lack an intact RAM domain (Fortini *et al.*, 1993; Lieber *et al.*, 1993; Jarriault *et al.*, 1995).

One possibility might have been that the RAM and ANK domains were functionally redundant in their ability to direct cell fates. However, constructs carrying an intact RAM domain but an inactive ANK domain are not able to direct cell fates *in vivo*. A second possiblity might have been that the RAM domain competes with the ANK domain for binding LAG-1. However, the transgenes carrying an intact RAM domain but an inactive ANK domain do not interfere with endogenous signaling. Furthermore, a construct carrying both an intact RAM domain and an intact ANK domain does not have reduced activity in directing cell fates, but instead appears to be somewhat more potent.

The function of the RAM domain in signaling cell fates, therefore, remains unknown. We suggest that the RAM and ANK domains are partially redundant in GLP-1 for at least one activity, that of interacting with LAG-1. However, we also suggest that the ANK repeat domain has at least one additional essential function that is not shared with the RAM domain. One possibility for that additional function is transcriptional activation (see below).

### *Speculations on the molecular mechanism of GLP-1 signal transduction*

What role might interactions between a DNA binding protein and the intracellular domain of a transmembrane receptor play in signal transduction? Mutants that lack either GLP-1 or LAG-1 activity are incapable of intercellular signaling. Therefore, the GLP-1 receptor must function positively to activate LAG-1 rather than acting primarily negatively to inhibit LAG-1.

We envisage two general models for how an interaction between these two proteins might achieve activation of downstream genes (Figure 7). In both models, LAG-1 is proposed to activate transcription at GLP-1-responsive

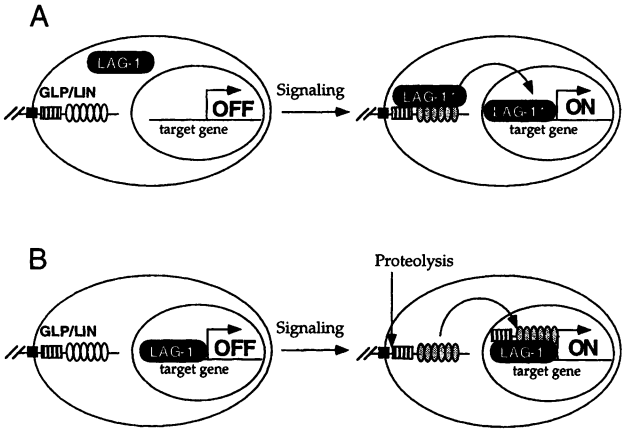


**Fig. 7.** Two models by which interactions between LAG-1 and the intracellular domain of GLP-1 may lead to activation of downstream genes. See text for explanation. Note that the extracellular domains of GLP-1/LIN-12 are not shown for clarity and that LAG-1* represents a potential activated form of LAG-1.

promoters, an idea based on work in flies and vertebrates showing that Su(H) and CBF1 activate Notch-responsive and mNotch1-responsive promoters respectively (Bailey and Posakony, 1995; Jarriault *et al.*, 1995; Lecourtois and Schweisguth., 1995). In the first model, GLP-1 binds and activates LAG-1 in a signal-dependent process; activated LAG-1 enters the nucleus and regulates transcription of target genes (Figure 7A). According to this model, activation might involve a conformational change of LAG-1, a modification to LAG-1 or a change in other proteins binding LAG-1. In the second model, the intracellular domain of GLP-1 is cleaved proteolytically in a signal-dependent process; this GLP-1 fragment enters the nucleus, joins LAG-1 at the target promoter, and a joint LAG-1–GLP-1 complex activates transcription (Figure 7B). Although these two models represent two extremes, mechanisms combining aspects of each remain possible.

Support for the first model comes from the cytoplasmic co-localization of Notch and Su(H) in tissue culture cells, and the entry of Su(H) into the nucleus following signaling (Fortini and Artavanis-Tsakonas, 1994). However, when examined during fly development, Su(H) is nuclear both in cells with unstimulated Notch and in those undergoing Notch signaling. Support for the second model comes from several lines of evidence, though none is definitive. First, Su(H) is predominantly a nuclear protein by immunostaining, and fails to co-localize with the Notch receptor at the membrane (Gho *et al.*, 1996). Second, an activated form of mNotch1 is processed in tissue culture cells, releasing an intracellular fragment that enters the nucleus (Kopan *et al.*, 1996). However, this processing in tissue culture is not ligand-dependent, and no equivalent proteolytic fragment from endogenous receptor has been detected in the nucleus (Johansen *et al.*, 1989; Kooh *et al.*, 1993; Crittenden *et al.*, 1994). Third, the intracellular domains of GLP-1 and a human Notch homolog called TAN-1 activate transcription in yeast (Aster *et al.*, 1994; this work) and the intracellular domain of mNotch1 activates transcription in tissue culture cells (Hsieh *et al.*, 1996). Intriguingly, missense mutations in the ANK repeats that abolish signaling in nematodes also abolish transcriptional activation in yeast. A provocative interpretation, though speculative, is that the ANK repeat domain may provide an activation domain that functions in conjunction with the LAG-1 DNA binding protein to activate transcription.

## Materials and methods

### *Plasmids*

The plasmids used for the two-hybrid experiments, pAS1-CYH and pACTII, and the Y190 reporter strain were kindly provided by Steve Elledge (Durfee *et al.*, 1993). The pCITE-4a+ vector (Novagen) was used for *in vitro* transcription/translation. pCITE-LAG-1(1–673) was constructed by subcloning LAG-1(48–673) into pCITE-4a+ (Christensen *et al.*, 1996) and subsequently replacing the sequence encoding amino acids 48–317 of LAG-1 with a PCR-generated fragment encoding amino acids 1–317 of LAG-1. All PCR-derived sequences were confirmed by sequencing. pCITE-LAG-1(199–673) was constructed by ligating the *Hpa*I–*Bgl*II framgent of pCITE-LAG-1(1–673) into *Nde*I- and mung bean nuclease-treated pCITE-4a+ that subsequently was digested with *Bgl*II. pCITE-LAG-1(230–673) was prepared by digesting pCITE-LAG-1(1–673) with *Nde*I and mung bean nuclease and religating. pCITE-LAG-1(48–651) was prepared by digesting pCITE-LAG-1(48–673) with *Acc*I, *Bgl*II and mung bean nuclease and religating. pCITE-LAG-1(1–$673_{q476}$) was constructed by replacing the wild-type LAG-1(1–317) sequence with a PCR-derived fragment containing the missense mutation in *lag-1(q476)*. pAS-LAG-1(1–673) was prepared by subcloning the *Nco*I–*Bgl*II fragment of pCITE-LAG-1(1–673) into *Nco*I- and *Bam*HI-digested pAS1-CYH. The *Hpa*I–*Bgl*II fragment of pCITE-LAG-1(1–673) was ligated into *Sma*I- and *Bam*HI-digested pAS1-CYH to generate pAS-LAG-1(199–673). LAG-1(230–673) was derived from pAS1-LAG-1(1–673) by *Nde*I and mung bean exonuclease digestion and religation. LAG-1(359–673) was constructed by treating pAS1-LAG-1(1–673) with *Nco*I, *Bsa*BI, Klenow fragment and religating. LAG-1(230–656) was constructed by subcloning the *Cla*I–*Bsa*I fragment of LAG-1 into *Cla*I- and *Sma*I-digested LAG-1(230–673). LAG-1(230–546) was derived from *Cla*I-, *Sma*I- and Klenow-treated LAG-1(230–673). The *Nco*I–*Bam*HI fragment of pCITE-LAG-1 was ligated into *Nco*I- and *Bam*HI-digested pAS1-CYH to prepare LAG-1(1–317). pAS-GLP-1(ANK) contains the *Eco*RV fragment of GLP-1 (amino acids 868–1171). The ANK repeats of the *glp-1(q224)*, *glp-1(q231)* and *glp-1(e2144)* alleles were prepared by subcloning the *Pst*I–*Eco*RV fragments from genomic subclones of the respective mutant DNAs into a pCITE-GLP-1(ANK) shuttle vector, and susequently subcloning the *Nco*I–*Bgl*II fragments into the pAS1-CYH and pACTII vectors. GLP-1(RAM/ANK), GLP-1(ΔRAM/ANK) and GLP-1(RAM) inserts were prepared by PCR, sequenced and contain amino acids 788–1171, 798–1171 and 788–867 of GLP-1 respectively (numbering as per Yochem and Greenwald, 1989). GLP-1(RAM/$ANK_{q224}$) contains the same N- and C-termini as GLP-1(RAM/ANK) but also contains the *glp-1(q224)* mutation in the fourth ANK repeat. LIN-12(ANK) and LIN-12(RAM/ANK5) were prepared by PCR and contain amino acids 1004–1302 and 930–1232 respectively (numbering as per Yochem *et al.*, 1988). FEM-1(ANK) (Spence *et al.*, 1990) was derived from a PCR product and contains six ANK repeats and similar lengths of N- and C-terminal flanking sequence relative to GLP-1(ANK). The oligonucleotides used for the FEM-1 PCR were 5′-CGGGCCATGGGCCGTATGACACCAAATGGACAT-3′ and 5′-CGG-CGGATCCTATGCATGAAGTGGAACTTCCAT-3′.

The co-localization constructs HS-ANK, HS-RAM/ANK and HS-myc-LAG-1 were constructed from a modified version of pPD49.83 (kindly provided by A.Fire), which contains the heat shock promoter, HSP16-14 and a translation start site. HS-ANK encodes amino acids 868–1171 (Roehl and Kimble, 1993) and HS-RAM/ANK encodes amino acids 792–1171 of GLP-1. HS-myc-LAG-1 encodes amino acids 48–673 of LAG-1 and an N-terminal peptide epitope from human c-*myc* (ARAEEQKLISEEDLL).

### *Generation of transgenic nematodes and immunostaining*

Transgenic animals were generated in wild-type hermaphrodite animals (variety Bristol) using *rol-6(su1006)* as a co-transformation marker (Mello *et al.*, 1991). Transgenic animals were heat shocked for 2 h at 33°C and then were allowed to recover for 2 h at 20°C. Animals were then fixed and incubated with primary and secondary antibodies as described (Finney and Ruvkun, 1990). Animals were stained for GLP-1 using rat polyclonal antibodies raised to the GLP-1 ANK repeat region (Crittenden *et al.*, 1994). Animals were stained for myc-tagged LAG-1 using the 9E10 monoclonal antibody (Oncogene Science) raised against

human c-myc. Images were recorded using a Bio-Rad MRC 1024 confocal microscope.

### Electrophoretic mobility shift assay

LAG-1 protein was synthesized using the TnT *in vitro* transcription/translation kit (Promega) as per the manufacturer's instructions. Electrophoretic mobility shift assays were performed as previously described (Christensen *et al.*, 1996). The sequences of the complementary MB22 oligonucleotides used as a probe are 5′-GATCGACGTGGGAAAG-3′ and 5′-GATCTTTCCCACGTC-3′.

### Yeast transformations and colony lift assays

The Y190 strain was used for all of the yeast two-hybrid experiments (Fields and Song, 1989). Yeast transformations were performed as described (Gietz *et al.*, 1992) and transformants were selected on synthetic complete media lacking the appropriate amino acids. Colonies that had grown between 3 and 5 days were lifted onto Hybond-N nylon filters and permeabilized by immersion into liquid nitrogen for 10 s. The filters were placed on Whatman filter paper pre-soaked in Z-buffer (Ausubel *et al.*, 1987) containing 1 mg/ml X-gal. Filters were incubated at 30°C and monitored frequently for the appearance of blue color. The scale used to rate reporter induction is described in Figure 3. All transformations and colony lift assays were performed at least three times. Levels of fusion protein in yeast were monitored by Western immunoblotting, using anti-GAL4(bd) (Upstate Biotechnology Incorporated), anti-GAL(ad) antibodies (Upstate Biotechnology Incorporated) or anti-GLP-1 antibodies (Crittenden *et al.*, 1994), especially in cases where a protein–protein interaction was not detected.

### Co-precipitation experiments

[$^{35}$S]LAG-1(199–673) was synthesized *in vitro* using pCITE-LAG-1(199–673) as template, [$^{35}$S]methionine and the TnT *in vitro* transcription/translation kit (Promega) as per the manufacturer's instructions. S-Tag-GLP-1(RAM/ANK) and S-Tag-GLP-1(ANK) were also synthesized *in vitro* using the TnT kit using the pCITE-GLP-1(RAM/ANK) and pCITE-GLP-1(ANK) templates. A 4-fold molar excess of pCITE-GLP-1(RAM/ANK) or pCITE-GLP-1(ANK) was incubated with [$^{35}$S]LAG-1(199–673) for 3 h at 4°C in 1 ml of buffer A (50 mM HEPES pH 7.5, 250 mM KCl, 0.2% NP-40). Then 50 μl of S-protein–agarose (Novagen) was added and samples were incubated for an additional 30 min at 4°C. The S-protein and S-Tag are fragments of RNase S that have high affinity for one another and therefore can be used in co-precipitation assays (Kim and Raines, 1993). The beads were collected by centrifugation and washed four times in buffer A. The samples were eluted at 95°C in SDS sample buffer and separated on 8% SDS–PAGE. The input lane represents 20% of the [$^{35}$S]LAG-1(199–673) that was incubated with beads. The results shown are representative of a total of four independent co-precipitation experiments.

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