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GLP-1 is localized to the mitotic region of the *C. elegans* germ line

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SUMMARY

In *C. elegans*, germline mitosis depends on induction by the somatic distal tip cell (DTC) and on activity of the *glp-1* gene. Using antibodies to GLP-1 protein, we have examined GLP-1 on western blots and by immunocytochemistry. GLP-1 is tightly associated with membranes of mitotic germline cells, supporting its identification as an integral membrane protein. Furthermore, GLP-1 is localized within the germ line to the mitotic region, consistent with the model that GLP-1 acts as a membrane receptor for the distal tip cell signal. Unexpectedly, GLP-1 and the zone of mitosis extend further than the DTC processes. We present three models by which the DTC may influence GLP-1 activity and thereby determine the zone of mitosis. The spatial restriction of GLP-1 appears to be controlled at the translational level in hermaphrodites. We suggest that down-regulation of GLP-1 may be required to effect the transition from mitosis into meiosis.

Key words: GLP-1, membrane protein, receptor, cell-cell interactions, induction, *C. elegans*, mitosis, germ line

INTRODUCTION

How do cell-cell interactions control cell fate during the development of a multicellular organism? Examples of regulatory cell-cell interactions now abound, and components of the signal transduction pathways controlling them are being identified (Greenwald and Rubin, 1992). We have approached this question by focusing on a well defined cell-cell interaction in the nematode *Caenorhabditis elegans*. This cell interaction is remarkably simple: the signal derives from a single somatic cell and the decision regulated is mitosis or meiosis. The germ line is normally patterned with mitosis at one end and meiosis at the other (see Fig. 3 for germline anatomy). This simple pattern is controlled by localizing the distal tip cell (DTC) to one end of the germ line tube. If the DTC is killed, germ cells leave mitosis, enter meiosis and differentiate; if the DTC is relocated, the polarity of the pattern is similarly changed (Kimble and White, 1981). Thus, the DTC/germ line interaction controls both growth and polarity of the germ line.

The *glp-1* gene is essential for DTC control of germline proliferation (Austin and Kimble, 1987; Priess et al., 1987). A lack of *glp-1* has the same effect on germline proliferation as elimination of the DTC: germ cells that are normally mitotic enter meiosis and polarity is lost. Both genetic and molecular lines of evidence have suggested that GLP-1 protein may function as the receptor for a DTC signal. Firstly, analysis of genetic mosaics suggested that *glp-1* functions in the germ line and not in the DTC (Austin and Kimble, 1987). Secondly, sequence analysis suggested that GLP-1 is a transmembrane protein with conserved motifs in both the extracellular and intracellular domains (Yochem and Greenwald, 1989; Austin and Kimble, 1989; see Fig. 1). Furthermore, both extracellular and intracellular domains are important for GLP-1 function (Kodoyianni et al., 1992), and the intracellular domain is sufficient to direct cell fate in the vulva (Roehl and Kimble, 1993). Therefore, the extracellular domain of GLP-1 is predicted to receive a signal from the DTC and the intracellular domain is predicted to direct germline mitosis.

GLP-1 belongs to a family of transmembrane proteins that includes LIN-12 in *C. elegans*, Notch in *Drosophila*, and several vertebrate homologs (reviewed by Fortini and Artavanis-Tsakonas, 1993). We refer to these proteins as LNG proteins for the founding members, **L**IN-12, **N**otch and **G**LP-1. The LNG proteins are defined by their domain structure: all have an extracellular region containing cysteine-rich EGF-like (EGFL) and LNG repeats, a transmembrane domain, and an intracellular region with ankyrin (ANK) repeats. Like GLP-1, LIN-12 and Notch appear to act as receptors for cell-cell interactions that control cell fate during development (LIN-12: Seydoux and Greenwald, 1989; Notch: reviewed by Artavanis-Tsakonas et al., 1991). It is not understood how LNG proteins control cell fate. The intracellular domain has no catalytic domain, but does possess ANK repeats, which may foster protein-protein interactions.

Several cell-cell interactions controlled by GLP-1, LIN-12 and Notch are well characterized at single cell resolution. GLP-1 plays a central role in inductive interactions, which occur between cells of different developmental potential. In addition to its control of germline fate, maternally encoded GLP-1 controls at least two inductive interactions during early embryogenesis (Priess et al., 1987; Austin and Kimble, 1987; Evans et al., 1994; Mello et al., 1994; Hutter and Schnabel, 1994; Moskowitz et al., 1994; J. Rothman, personal communication).

By contrast, LIN-12 and Notch mediate lateral signalling, which occurs between cells of equivalent developmental potential. While induction and lateral signalling differ in some respects, both appear to rely on a common underlying molecular mechanism. Not only can GLP-1 substitute for LIN-12 in controlling lateral signalling (Lambie and Kimble, 1991; Mango et al., 1991; Roehl and Kimble, 1993; Fitzgerald et al., 1993), but also GLP-1 and LIN-12 appear to interact with a single ligand, called LAG-2 (Henderson et al., 1994; Tax et al., 1994).

In this paper, we focus on the role of GLP-1 in the DTC/germline interaction. We show that GLP-1 is associated with germline membranes and that GLP-1 is localized to the mitotic region. These findings are consistent with the proposed role for GLP-1 as a receptor in inductive interactions. In hermaphrodites, we find that *glp-1* mRNA, in contrast to GLP-1 protein, is not localized, suggesting that localization may depend on translational control. Finally, we discuss the implications of our results for the regulation and function of GLP-1 and the specification of cell fates in the germ line.

MATERIALS AND METHODS

C. elegans strains and maintenance

C. elegans were maintained as described by Brenner (1974). Mutations used in this study were: LG I: *gld-1(q365), gld-1(q268), gld-1(q485)*(T. Schedl, personal communication), *fog-1(q180)* (Barton and Kimble, 1991), *smg-1(r861)* (Hodgkin et al., 1990); LG III: *glp-1(q35)* (Austin and Kimble, 1987; Mango et al., 1991), *glp-1(q172), glp-1(q175)* (Austin and Kimble, 1987; Kodoyianni et al., 1992), *lin-12(n137e2032)* (Greenwald, 1985), *unc-32(e189)* (Brenner, 1974). hT2 is a translocation that balances both *gld-1* and *glp-1* (McKim et al., 1993). The strains *gld-1(q268); unc-32(e189)glp-1(q172)/hT2* and *fog-1(q180);gld-1(q485)unc-32(e189)glp-1(q175)/hT2* were kindly provided by T. Schedl.

Antibody production

Fragments of *glp-1* cDNA (Kodoyianni et al., 1992) were subcloned into pGEX (Pharmacia; Smith and Johnson, 1988) and pET (Studier et al., 1990) vectors, and fusion proteins overexpressed in *E. coli*. Regions encoded EGFL 7-10 (aa 315-471), LNG 1-3 (aa 477-627) and ANK 1-6 (aa 866-1171) (see Fig. 1; amino acids numbered according to Yochem and Greenwald, 1989). Rats were injected initially with 15-30 μg of protein suspended in Freund's complete adjuvant and boosted with 7.5-15 μg of protein suspended in incomplete Freund's adjuvant at 3 week intervals. Test bleeds were taken 7-10 days after each boost. Each antibody was affinity purified on a column of the corresponding pET-GLP-1 fusion protein coupled to affi-gel 10 (Bio-Rad).

Protein procedures

Wild-type animals were raised in 1 l S-basal liquid cultures at 15°C or 20°C (Wood, 1988). *smg-1(r861)*; *unc-32(e189)glp-1(q35)* homozygotes were grown on rich agarose plates (Wood, 1988) at 20°C. Homozygous *fog-1(q180);gld-1(q485)unc-32(e189)glp-1(q175)* animals were hand picked from rich agarose plates. After harvesting, all animals were stored at −80°C.

To extract proteins, five volumes of homogenization buffer (20 mM Tris, pH 7.4, 0.32 M sucrose, 1% Triton X-100) plus protease inhibitors (10 μg/ml aprotinin, 1 mM benzamidine, 40 μg/ml bestatin, 10 μg/ml chymostatin, 2.5 μg/ml E-64, 1 mM EDTA, 1 mM EGTA, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM PMSF) were added to frozen animals, the mixture allowed to thaw and then disrupted in a French pressure cell at 12,000 psi, 2× 30 seconds. The resulting homogenate was centrifuged at 10,000 ***g*** for 15 minutes at 4°C and the supernatant collected.

Glycoproteins were enriched using wheat germ lectin coupled to agarose following the procedure of Wilcox (1986). Briefly, 1 ml of 10× lectin binding buffer (0.2 M Tris-HCl pH 7.4, 2 M NaCl, 20 mM $CaCl_2$, 2% NP-40, 2% Triton X-100 plus the same protease inhibitors used for the homogenization buffer, see above) was added to 9 ml of the supernatant from *C. elegans* homogenates. 100 μl of wheat germ lectin agarose (Sigma) was added and the mixture was shaken for 2 hours at 4°C. After washing, the lectin beads were resuspended in SDS sample buffer, heated to 80°C and the supernatant was run on SDS gels and immunoblotted. For endoglycosidase F digestion, the protein bound to the lectin column was eluted during two 15-minute treatments with 150 μl of 300 mM *N*-acetyl glucosamine, 0.15% SDS in 20 mM Tris, pH 7.4.

N-linked sugars were removed from the lectin bead eluate using endoglycosidase F (Boehringer Mannheim). Briefly, the eluate was dialyzed into 100 mM KH_2PO_4 pH 7.0, 1% SDS and heated to 80°C for 2 minutes. 900 μl of 100 mM KH_2PO_4, 0.5% NP-40 was added and the solution heated again at 80°C for 2 minutes. After denaturation, samples with or without endoglycosidase F (1 unit) were incubated at 37°C for 18 hours. Samples were concentrated, run on SDS gels and immunoblotted.

Western blots were done by standard procedures (Towbin et al., 1979; Harlow and Lane, 1988). Briefly, proteins isolated by lectin binding from approximately 200 μl packed worms (about 3000 animals) were run in each lane of an 8% SDS polyacrylamide gel, transferred to nitrocellulose, blocked, and incubated with α-GLP-1 followed by alkaline phosphatase conjugated secondary antibodies. Molecular masses were determined by comparison with prestained molecular mass markers (Bio-Rad).

Immunofluorescence

5-10 adults were placed on a subbed slide in 5.0 μl of M9 containing 0.25 mM levamisole. Heads or tails were cut off using a 25 g syringe needle, extruding the gonad and intestine. The extruded gonad and intestine as well as body pieces remain attached to the slide and can be stained. For staining with α-GLP-1 and α-DNA antibodies, cut animals were fixed with 1.0% paraformaldehyde in PBS for 10 minutes and permeabilized with TBS containing 0.1% Triton X-100 and 0.5% BSA for 3 minutes. Finally, samples were blocked in TBS containing 0.5% BSA (TBSB) for at least 30 minutes. For double staining with α-LNG and α-tubulin, animals were dissected as described above and fixed with 100% methanol for 5 minutes followed by 1% paraformaldehyde in PBS for 20 minutes prior to blocking as described above. Under these conditions at least 90% of extruded gonads and intestines are stained with anti-DNA or anti-tubulin whereas only approximately 10% of the remaining body pieces are stained. Using methanol and acetone as fixatives (Evans et al., 1994 and references therein), approximately 10-50% of body pieces stain with anti-actin. We have not observed specific GLP-1 staining in either the extruded intestine or in body pieces, both of which stain with anti-DNA, anti-tubulin or anti-actin; however, we have observed weak staining in the adult hermaphrodite spermatheca and larval somatic gonads (S. L. C., unpublished observation).

Fixed worms were incubated with primary antibodies overnight at 4°C. Samples were washed 3 times with TBSB, incubated with FITC donkey anti-rat and LRSC donkey anti-mouse secondary antibodies (Jackson Immunoresearch) and 1 μg/ml diamidinophenolindole (DAPI) for 1-2 hours at room temperature, washed three times with TBSB and mounted in medium containing 1mg/ml DABCO and 1 mg/ml paraphenylenediamine. Other antibodies used were α-β tubulin (Amersham) and α-DNA (mAb 030, Chemicon). Fluorescence was observed on a BioRad 600 scanning laser confocal microscope with a 60× lens.

For competition, α-GLP-1 antibodies were preincubated for 30

minutes at room temperature with approximately 200 μg/ml of the fusion protein used for antigen. The antibody/antigen mix was then used to stain *C. elegans* as described above.

Identification of regions of the germ line

Fourth stage larvae (L4s) were placed at 15°C for 24 hours to reach the young adult stage. Using α-LNG, α-tubulin and DAPI, the distributions of membrane-associated GLP-1, mitotic spindles, and pachytene nuclei were determined. Identification of the proximal boundary of intense GLP-1 staining was subjective and was always done before scoring mitotic spindles.

In situ hybridization

Gonads were dissected as for immunofluorescence, fixed and hybridized as described (Henderson et al., 1994). Probes to *glp-1* were as described by Evans et al. (1994).

RESULTS

We have generated polyclonal antibodies to three distinct regions of GLP-1: EGF-like (EGFL) repeats 7-10 and LNG repeats 1-3 in the extracellular domain and ANK repeats 1-6 in the intracellular domain (Fig. 1). We refer to the antibodies individually as α-EGFL, α-LNG, α-ANK or generically as α-GLP-1. The α-GLP-1 antibodies are specific for GLP-1 on western blots and by immunocytochemistry (see below).

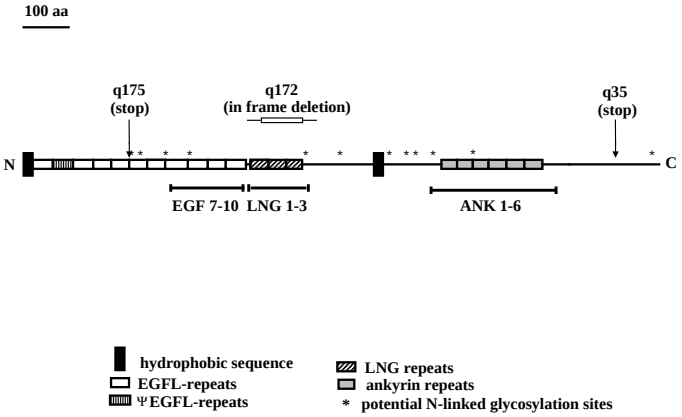

Fig. 1. Schematic of the GLP-1 protein. The regions of GLP-1 used for production of antibodies are indicated by bars below diagram. Sites of *glp-1* mutations (Mango et al., 1991; Kodoyianni et al., 1992) referred to in the text are indicated above diagram. Potential *N*-linked glycosylation sites are indicated by *.

GLP-1 is a glycoprotein

The GLP-1 sequence predicts a 142×10^3 M_r transmembrane protein with *N*-linked glycosylation sites in both the N and C termini (see Fig. 1). To examine GLP-1 on western blots, we enriched for glycoproteins using a lectin column. Antibodies to the extracellular region of GLP-1, α-EGFL and α-LNG, recognize a 160×10^3 M_r protein as well as a $110/100\times10^3$ M_r doublet (Fig. 2, lanes 1,3,7,9). Antibody to the intracellular region of GLP-1 (α-ANK) recognizes a 160×10^3 M_r protein as well as a 95×10^3 M_r doublet (Fig. 2, lanes 5, 11). To confirm that the detected proteins are products of the *glp-1* gene, we examined proteins from two *glp-1* mutants predicted to encode GLP-1 about 10×10^3 M_r smaller than wild type. A nonsense mutant, *glp-1(q35)*, is predicted to truncate GLP-1 by 122 amino acids (Mango et al., 1991; Fig. 1). In *glp-1(q35)* extracts, α-EGFL and α-LNG recognize a 150×10^3 M_r band and a doublet of $110/100\times10^3$ M_r, while α-ANK recognizes 150×10^3 M_r and 72×10^3 M_r bands (Fig. 2, lanes 2,4,6). A deletion mutant, *glp-1(q172)*, is predicted to remove 87 amino acids in the LNG region

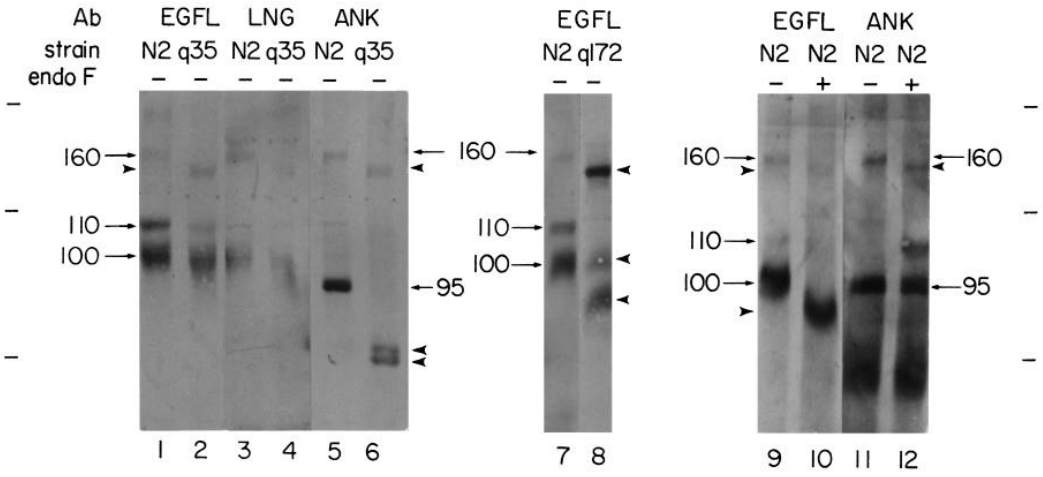

Fig. 2. Identification of GLP-1 on western blots. Positions of molecular mass markers (200, 116, 84, $\times10^3$ M_r) are indicated by dashes on both sides of figure. Lanes 1-6: Extracts from wild type (N2, lanes 1, 3 and 5) and *smg-1(r861);unc-32(e189)glp-1(q35)* homozygotes (q35, lanes 2, 4 and 6) were probed with α-EGFL (lanes 1 and 2), α-LNG (lanes 3 and 4) or α-ANK (lanes 5 and 6). *glp-1(q35)* homozygotes are fertile in the presence of *smg-1(r861)* (Mango et al., 1991). Arrows indicate full-length GLP-1, a $110/100\times10^3$ M_r doublet (lanes 1 and 3) and a 95×10^3 M_r doublet (lane 5); arrowheads indicate nearly full-length GLP-1 (lanes 2,4 and 6) and a 70×10^3 M_r (lane 6) doublet from *glp-1(q35)*. Faint bands present in both wild-type and mutant lanes are not seen consistently and do not appear to be products of the *glp-1* gene. Lanes 7 and 8: Extracts from wild-type (N2, lane 7) and *gld-1(q268); unc-32(e189)glp-1(q172)* homozygotes (q172, lane 8) were probed with α-EGFL. *glp-1(q172)* homozygous adults produce mitotic germ cells when doubly mutant with *gld-1(q268)* (Francis, Maine, and Schedl, unpublished data). Arrows indicate full-length GLP-1 and the $110/100\times10^3$ M_r doublet; arrowheads indicate nearly full-length GLP-1 and a $100/90\times10^3$ M_r doublet from *glp-1(q172)* mutants. Lanes 9-12: Lectin bound proteins incubated either with (+, lanes 10 and 12) or without (-, lanes 9 and 11) endoglycosidase F were probed with α-EGFL (lanes 9 and 10) or α-ANK (lanes 11 and 12). Arrows indicate glycosylated GLP-1 polypeptides; arrowheads indicate deglycosylated GLP-1 polypeptides. Both the 160×10^3 M_r protein and the $110/100\times10^3$ M_r doublet decrease in molecular mass when digested with endoglycosidase F (lane 10). The molecular mass of the 95×10^3 M_r doublet does not change (lane 12), indicating that it is not *N*-glycosylated.

(Kodoyianni et al., 1992; Fig. 1). In *glp-1(q172)* extracts, α-EGFL detects a 150×10^3 M_r band and a $100/90\times10^3$ M_r doublet (Fig. 2, lane 8). The reduced mobilities of the mutant proteins establish that the 160×10^3 M_r protein and the two doublets are products of the *glp-1* gene.

To learn which *glp-1* products are *N*-glycosylated and to examine their relative mobilities without their *N*-linked sugars, we treated extracts with endoglycosidase F (endoF). After endoF treatment, the 160×10^3 M_r and $110/100\times10^3$ M_r products all decrease approximately $5\text{-}10\times10^3$ M_r (Fig. 2, lanes 10,12), while the 95×10^3 M_r doublet does not change. Therefore, the 160×10^3 M_r and the $110/100\times10^3$ M_r products are *N*-glycosylated.

What are the various GLP-1 products observed by western blot? The 160×10^3 M_r band is likely to be full length GLP-1 protein, based on its detection by all three antibodies, the effects of *glp-1(q172)* and *glp-1(q35)* deletions, and its molecular mass which is consistent with the 142×10^3 M_r predicted by sequence. The $110/100\times10^3$ M_r doublet appears to contain extracellular GLP-1: this doublet is detected by both α-EGFL and α-LNG, but not α-ANK, antibodies (Fig. 2, lanes 1,3,5), it is smaller in *glp-1(q172)* (Fig. 2, lane 8), but not *glp-1(q35)* (Fig. 2, lanes 2,4), and it appears to be *N*-glycosylated (Fig. 2, lane 10). The 95×10^3 M_r doublet appears to contain intracellular GLP-1: this doublet is recognized by α-ANK but not α-EGFL or α-LNG (Fig. 2, lanes 5,1,3), it decreases in size in *glp-1(q35)* (Fig. 2, lane 6), and does not appear to be *N*-glycosylated (Fig. 2, lane 12).

We do not know if the GLP-1 fragments are generated in vivo or result from our extraction procedure. Protease inhibitors do not eliminate the doublets and incubation of extracts at room temperature does not increase the amount of fragments relative to full-length GLP-1. The possibility that GLP-1 may be cleaved into extracellular and intracellular fragments is intriguing in light of the finding that intracellular GLP-1 can function independently of the extracellular domain (Roehl and Kimble, 1993). However, using each of the three α-GLP-1 antibodies, we observe no difference in tissue or subcellular distribution (see staining results). Therefore, if cleavage does occur in vivo, we find no evidence for a difference in distribution of the resulting fragments.

GLP-1 is associated with the membranes of mitotic germ cells

Using immunohistochemistry, we find GLP-1 in the germ line of adult hermaphrodites and males (Fig. 4). Under the fixation conditions we have used, we have not detected strong GLP-1 staining in other adult tissues (see Materials and Methods; data not shown). This distribution is consistent with the germline-specific mutant phenotype of *glp-1* null alleles (Austin and Kimble, 1987). Therefore, we have focused on the distribution of GLP-1 in the germ line.

Gonadal anatomy

Fig. 3 shows the anatomy of an adult hermaphrodite ovotestis. The U-shaped germline tissue consists of distal and proximal arms and contains about 1000 nuclei. The germ line is syncytial (Fig. 3B,C; Hirsh et al., 1976; Wolf et al., 1978); for simplicity, we refer to each germline nucleus with its surrounding cytoplasm and partially enclosing membranes as a 'cell' (Fig. 3C). Distally, germ cells are in the mitotic cell cycle; more proximally along the distal arm, germ cells enter meiosis; in the proximal arm, germ cells differentiate as gametes (Fig. 3A; Hirsh et al., 1976). The same arrangement of mitosis and meiosis along the distal-proximal axis occurs in the male germ line (Klass et al., 1976). Within the syncytium, germ cells are arranged in a ring around a central core of cytoplasm (Fig. 3C); the core is less distinct in the

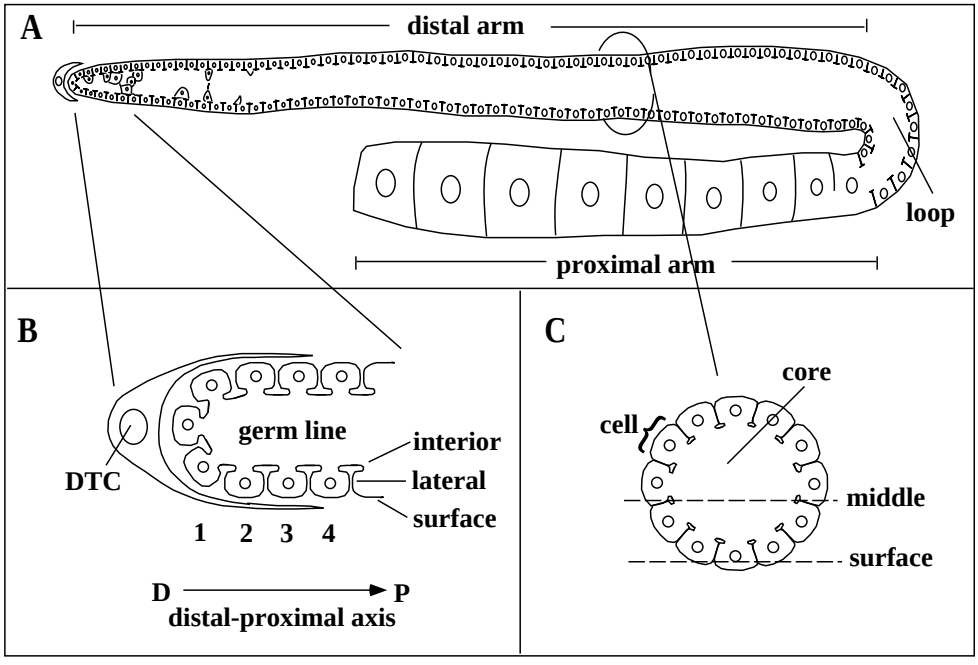

Fig. 3. Anatomy of the hermaphrodite gonad. (A) A hermaphrodite ovotestis. The germ line and somatic DTC are shown. Each hermaphrodite ovotestis contains approximately 1000 germ nuclei arranged in a U shaped tube. In the *distal arm*, germ cells closest to the DTC are mitotic, those further proximal are arrested in meiotic pachytene. In the *loop*, gametogenesis begins and in the *proximal arm*, gametes mature. For simplicity, we have only shown the DTC since it is the only somatic gonad cell found near the mitotic region of the germ line. (B) Close-up of the distal gonad. The somatic DTC, which is required for germline mitosis (Kimble and White, 1981), resides at the distal tip of the gonad and extends processes around the germline (D. Hall, personal communication). Germ cells closest to the DTC are numbered 1, their proximal neighbors 2, etc., according to their position relative to the distal end of the germ line. Since germ cells form a ring around the central core (Fig. 3C) and occasionally cross it, multiple germ cells occupy each position along the distal-proximal axis. *Surface*, *lateral* and *interior* parts of the plasma membrane are labeled. (C) Cross section of the germ line. We refer to each germline nucleus, surrounding cytoplasm and membranes as a germ *cell*. Germ 'cells' are organized around a central *core* of cytoplasm (Hirsh et al., 1976). Confocal sections shown in Fig. 5 were taken in *surface* or *middle* focal planes.

mitotic region because germ cells cross it occasionally. In our description of the GLP-1 distribution, we refer to specific regions of the plasma membrane associated with each cell (surface, lateral, internal; Fig. 3B). Positions along the distal-proximal axis are assigned by the number of cell diameters from the distal tip (Fig. 3B). For example, the somatic DTC in the hermaphrodite sends out cytoplasmic processes that extend about three cell diameters along the distal-proximal axis (Fig. 3B; D. Hall, personal communication). Finally, in Fig. 3C, we indicate the surface and middle focal planes of the confocal sections shown in Fig. 5.

GLP-1 is membrane-associated

To learn the location of GLP-1 within the germ line, we dissected gonads from wild-type adult hermaphrodites (Figs 4, 5) and males (data not shown) and stained them with all three α- GLP-1 antibodies. GLP-1 is tightly associated with the plasma membranes of the distal germ cells using both α-LNG (Fig. 4A,C), which recognizes the extracellular domain, as well as α-ANK (Fig. 4B), which recognizes the intracellular domain. No GLP-1 is detected in the somatic DTC (Figs 4A,C; 5A,C). The tight membrane association is consistent with the prediction that GLP-1 is a transmembrane protein (Yochem and Greenwald, 1989; Austin and Kimble, 1989). Although the association of GLP-1 with germ cell membranes is consistent with the idea that GLP-1 is a receptor for the DTC signal, we find little GLP-1 in surface membranes of the germ cells (Fig. 4C), even immediately adjacent to the DTC. Instead, GLP-1 is dramatically enriched in the lateral and interior plasma membranes (Fig. 4C; see Fig. 3B for anatomy). The significance of this subcellular distribution is not understood. However, Notch (Johansen et al., 1989; Kidd et al., 1989; Fehon et al., 1991) and other membrane receptors such as Toll (Hashimoto et al., 1991) are also enriched in membranes between cells.

In addition to the membrane-associated GLP-1 described above, GLP-1 is found in a punctate pattern through most of the distal germline arm (Fig. 4D). This punctate staining is closely associated with internal membranes; it may represent

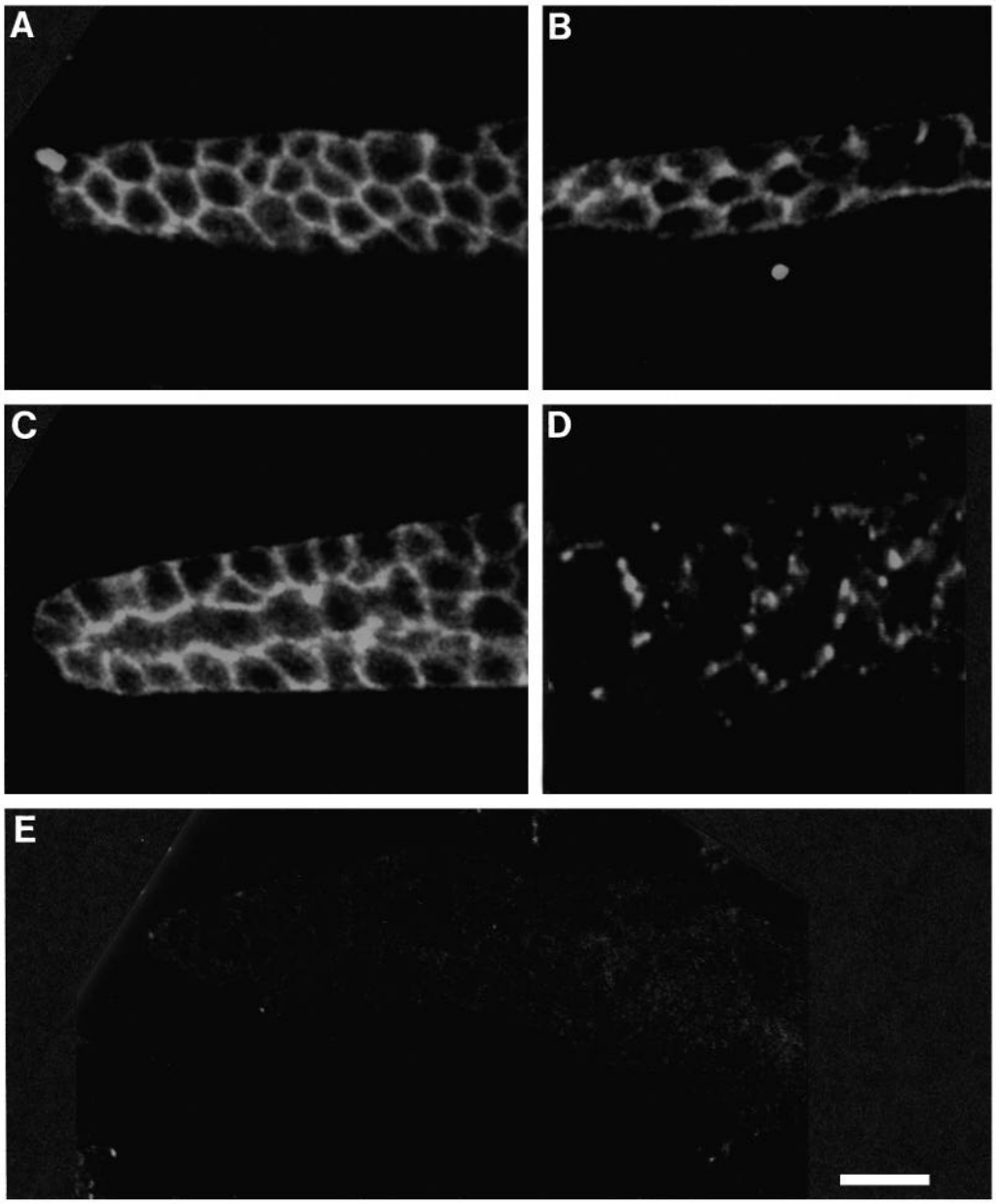

Fig. 4. Subcellular distribution of GLP-1. All panels show hermaphrodite gonads. (A) Surface focal plane of distal region (‘a’, Fig. 5A) stained with α-LNG. GLP-1 outlines each germ cell. (B) Surface focal plane of distal region stained with α-ANK. Distribution is the same as that observed with α-LNG; note lack of nuclear GLP-1. (C) Middle focal plane of distal region (‘b’, Fig. 5C) stained with α-LNG. GLP-1 is enriched in lateral and internal membranes. Note punctate GLP-1 adjacent to core, which appears similar to the internal, punctate pattern observed more proximally (see 4D). (D) Middle focal plane of more proximal region (‘c’, Fig. 5C) stained with α-LNG. GLP-1 is found in an internal, punctate pattern. (E) Surface focal plane of distal gonad homozygous for the nonsense mutant *glp-1(q175)* (Kodoyianni et al., 1992; Fig. 1). The *glp-1(q175)* single mutant has a Glp-1 null phenotype with no germline mitosis; however, as a triple mutant with *gld-1(q268)* and *fog-1(q180)*, some germline mitosis occurs (T. Schedl, personal communication). The gonad shown here is a *fog-1(q180)gld-1(q268); unc-32 (e189)glp-1(q175)* homozygote stained with α-LNG. GLP-1 is not detected, confirming the specificity of the antibody. Scale bars, 20 μm.

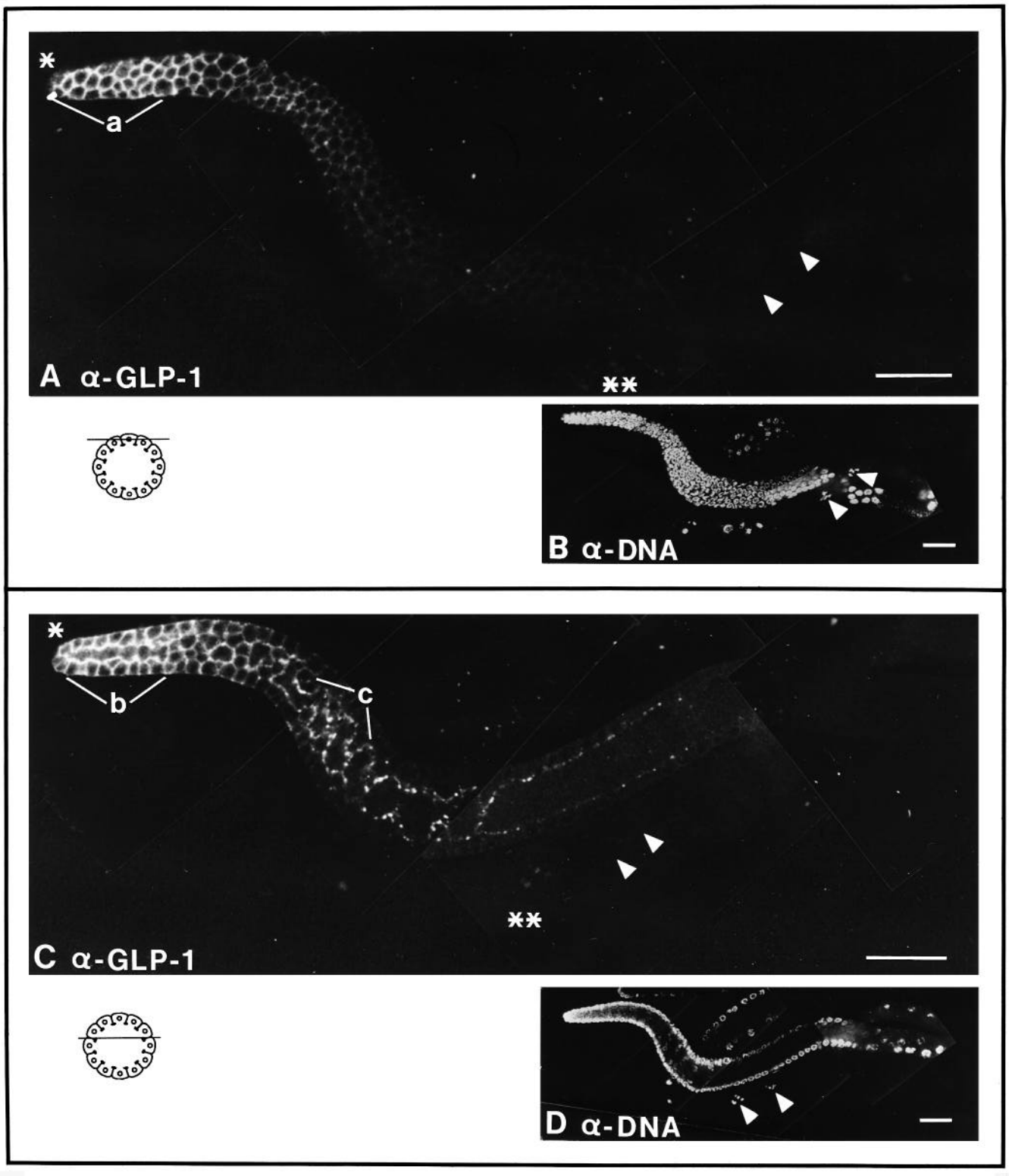

Fig. 5. Localization of GLP-1 to the distal end of the germ line. Confocal sections of a wild-type hermaphrodite gonad co-stained with α-LNG (A,C) and α-DNA (B,D). The distal end is at the upper left (*); the proximal end is at the lower left (**). Scale bars, 20 μm. Oocytes are marked by arrowheads. (A) Surface focal plane. Intense membrane-associated GLP-1 is found in the most distal germ cells (‘a’). More proximally, the intensity of membrane-associated GLP-1 staining decreases rapidly. GLP-1 is undetectable in maturing germ cells and oocytes. (B) Surface focal plane. DNA in nuclei is visible throughout the germ line. (C) Middle focal plane. Membrane-associated GLP-1 is intense distally and decreases proximally. Internal, punctate GLP-1 extends further proximally before it decreases and becomes undetectable. (D) Middle focal plane. DNA is visible in nuclei throughout germ line.

GLP-1 in internal membranes, alternatively it may represent GLP-1 in distinct organelles. We did not observe GLP-1 in nuclei (Fig. 4A-D), even with antibodies specific to the intracellular ANK repeats (Fig. 4B).

The antibodies appear to be specific for GLP-1: all three antibodies show the same staining pattern, no GLP-1 staining is seen in animals homozygous for *glp-1(q175)*, which carries a stop codon after the fourth EGF-like repeat of GLP-1 (Kodoyianni et al., 1992; see Fig. 1) (Fig. 4E), and for each antibody, staining is eliminated by preincubation with the corresponding fusion protein (data not shown).

Membrane-associated GLP-1 is restricted to mitotic germ cells

GLP-1 is limited to the distal germline arm in both hermaphrodites (Fig. 5) and males (data not shown). Since the intensity and subcellular distribution of GLP-1 changes along the distal-proximal axis, we asked whether the changes in GLP-1 staining correspond to changes in cell fates. To this end, we first defined the regions of GLP-1 staining and the regions of mitosis and meiosis. Intense, membrane-associated GLP-1 staining is found in germ cells up to 20 cell diameters away from the distal end (Fig. 5A,C). More proximally, the membrane-associated GLP-1 decreases rapidly in a steep gradient extending approximately 10-15 germ cell diameters along the distal-proximal axis (Fig. 5A,C) until it is undetectable. In contrast to the membrane-associated GLP-1, internal, punctate GLP-1 is easily detectable up to 35 cell diameters away from the distal end, and then decreases over the next 10-15 cell diameters (Fig. 5C). No GLP-1 is seen in the loop region (Fig. 5A,C) or in the proximal arm, either in oocytes (Fig. 5A,C) or sperm (not shown). The same pattern is observed using α-EGFL, α-LNG, and α-ANK. Control antibodies, on the other hand, detect protein throughout the germ line (e.g. α-DNA, Fig. 5B,D; α-actin and α-tubulin, data not shown), indicating that the entire germ line is accessible to antibodies.

We next defined three regions of cell fate in the distal germ line of young adult hermaphrodites: the *mitotic*, *transitional*, and *pachytene* regions. We stained germ lines with α-tubulin to observe mitotic spindles, and with DAPI to identify pachytene nuclei. The mitotic region is defined by the presence of mitotic spindles; this region always begins with the distal-most germ cells and extends approximately 24 cell diameters proximally along the distal-proximal axis (Fig. 6A). The mitotic region is typically smaller in older animals. In the transitional region, germ cells leave mitosis and enter meiosis. The distal boundary of the transitional region is defined by a lack of mitotic spindles and the proximal boundary by the first complete ring of nuclei in meiotic pachytene (Fig. 6A). The pachytene region extends from the first complete ring of pachytene nuclei proximally to the loop region; in this region, all nuclei have entered meiotic pachytene (Fig. 6A).

Changes in the intensity and subcellular distribution of GLP-1 coincide remarkably well with the regions of mitosis and meiosis defined above (Fig. 6A,B). The extent of intense membrane-associated GLP-1 corresponds well with the mitotic region in both hermaphrodites (Fig. 6A) and males

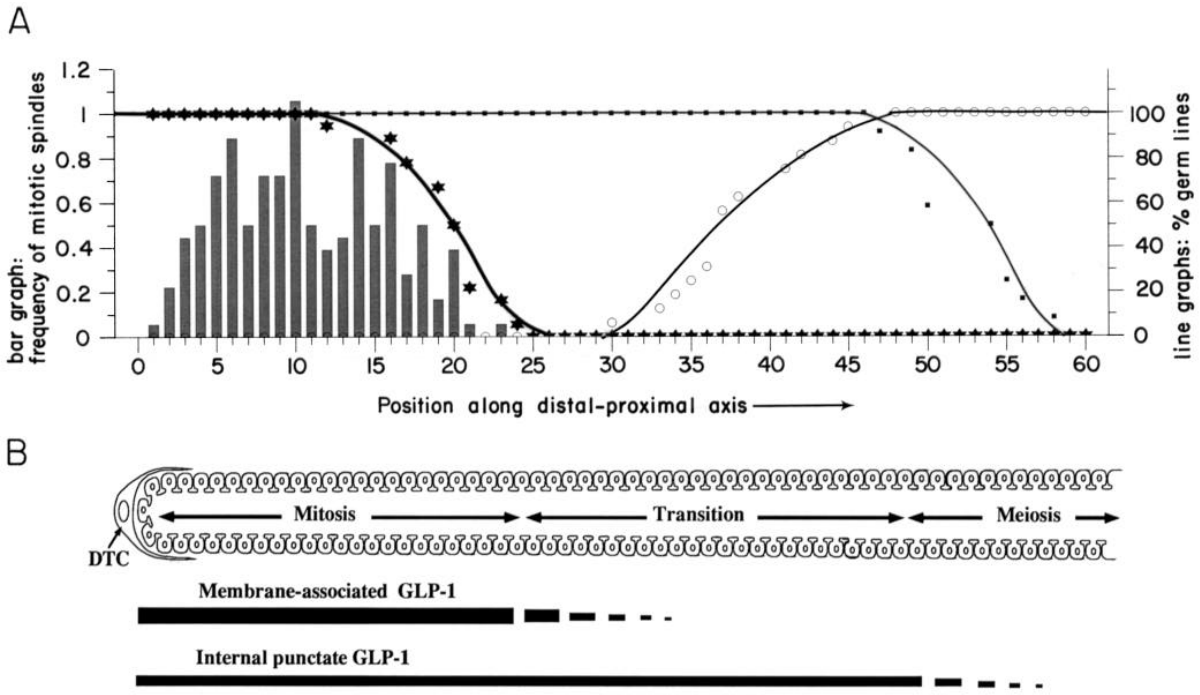

Fig. 6. Membrane associated GLP-1 is restricted to the mitotic germ line. (A) Extents of mitosis and meiosis within the germline were determined by examining the positions of mitotic spindles and pachytene nuclei along the distal-proximal axis (see Materials and Methods). In this way we define a mitotic region, a transitional region, and a pachytene region from distal to proximal. We next compared these regions to the distribution of GLP-1 in 18 young adult hermaphrodites. Bars show frequency of mitotic spindles (total number of spindles at each position in 18 germ lines divided by 18); stars represent percentage of germ lines with intense membrane-associated GLP-1; closed squares represent percentage of germ lines with internal, punctate GLP-1; open circles represent percentage of germ lines with complete rings of pachytene nuclei. (B) Pattern of cell fates and GLP-1 in the hermaphrodite germ line: a summary of data provided in A.

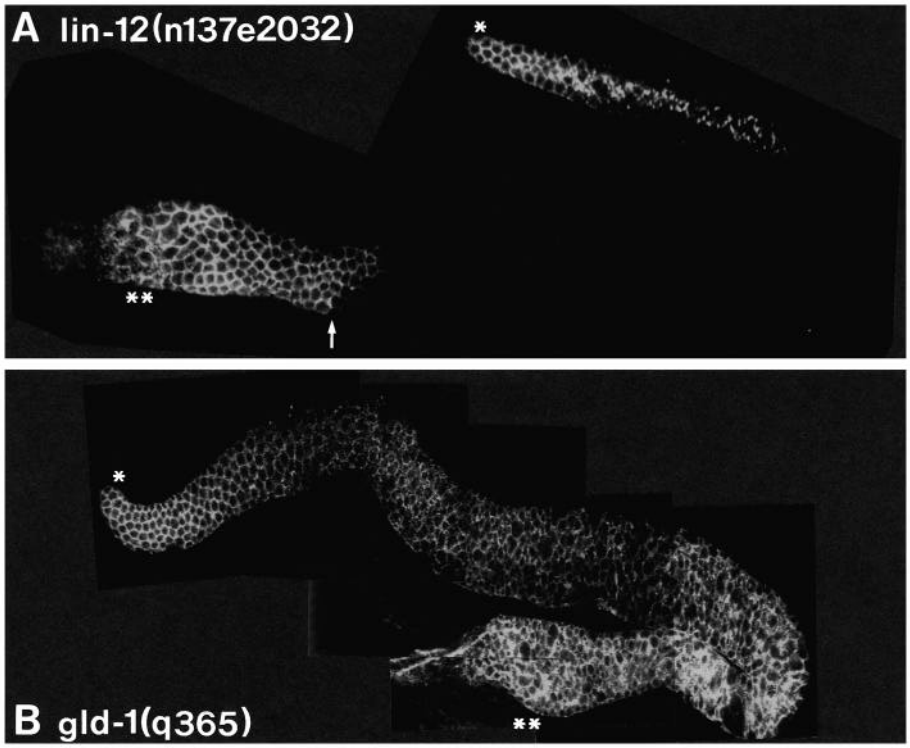

Fig. 7. Ectopic mitotic germ cells possess ectopic GLP-1. (A) Gonad from a *lin-12(n137e2032)* homozygous hermaphrodite with ectopic mitosis in the proximal germ line, stained with α-LNG. GLP-1 is present in the distal mitotic region (*); it decreases and disappears in the transitional and pachytene regions, as in wild type. Also, GLP-1 is present ectopically in the proximal mitotic region (**). No transitional zone exists between the proximal GLP-1-bearing cells and more distal meiotic cells; instead the ectopic region of mitosis and GLP-1 has a clear boundary (arrow). Scale bar, 20 μm. (B) Gonad from a *gld-1(q365)* homozygous hermaphrodite with ectopic mitoses scattered throughout the germ line, stained with α-LNG. GLP-1 is associated with membranes throughout the germ line. Distal (*) and proximal (**) ends of the germ lines are marked. Scale bar, 20 μm.

(not shown). When the germ line was co-stained with both α-GLP-1 and α-tubulin, mitotic spindles were only rarely seen proximal to the strongest membrane staining of GLP-1 (data not shown). Membrane-associated GLP-1 rapidly decreases in the transitional region, and no membrane-associated GLP-1 is detected in the pachytene region. By contrast, internal, punctate GLP-1 is present with about equal intensity in both the mitotic and transitional regions, but decreases within the first 10 rings of germline cells in the pachytene region (Fig. 6A). The pattern of GLP-1 in the male germ line is similar to that in the hermaphrodite: both membrane-associated and internal, punctate GLP-1 are found in the mitotic region, only internal, punctate GLP-1 is detectable more proximally, and no GLP-1 is observed in the proximal germ line (data not shown).

Thus, membrane-associated GLP-1 is found in mitotic germ cells, which require *glp-1* activity to remain mitotic, and it disappears as germ cells enter meiosis. This observation is consistent with the idea that GLP-1 is a receptor for a mitotic signal and suggests that GLP-1 is active in mitotic germ cells. By contrast, internal, punctate GLP-1 is found equally in both mitotic and transitional regions and disappears after germ cells have arrested in meiotic pachytene. The internal, punctate GLP-1 observed in pachytene cells, may be inactive.

Ectopic membrane-associated GLP-1 correlates with ectopic germline mitosis

We next asked whether membrane-associated GLP-1 is found in mitotic germ cells that are not found in the distal germ line. Two mutants exhibit such ectopic germline mitosis. First, the germ line of *lin-12* loss-of-function *(lf)* mutants is mitotic proximally where gametogenesis normally occurs (Seydoux et al., 1990), and we find membrane-associated GLP-1 in the *lin-12(lf)* proximal germ line (Fig. 7A). Second, the germ line of *gld-1(lf)* mutants is tumorous, with mitoses distributed throughout (T. Schedl, personal communication); similarly, membrane-associated GLP-1 is present throughout the *gld-1(lf)* germ line (Fig. 7B). Thus, as in wild type, the presence of membrane-associated GLP-1 correlates with mitosis.

glp-1 mRNA is present in the mitotic regions of hermaphrodite and male germ lines

Because GLP-1 protein is localized in the distal germ line, we next asked if *glp-1* mRNA is similarly localized. To this end, we used digoxigenin-labeled RNA probes to detect *glp-1* mRNA by in situ hybridization. We found *glp-1* mRNA evenly distributed throughout the adult hermaphrodite germ line, except in sperm (Fig. 8A). In adult males, *glp-1* mRNA is most abundant distally, decreases proximally, and is undetectable in mature sperm (Fig. 8C). By contrast, no signal was detected in either hermaphrodite or male gonads with either a sense *glp-1* RNA probe (Fig. 8B,D) or an anti-sense pharyngeal myosin RNA probe (data not shown). Therefore, at least in hermaphrodites, the localization of GLP-1 protein is likely to be achieved by a post-transcriptional control (see discussion and Evans et al., 1994). The presence of *glp-1* mRNA, but lack of GLP-1 protein, in oocytes indicates that maternal *glp-1* mRNA but not protein is contributed to embryos.

DISCUSSION

GLP-1 may be a receptor for the distal tip cell signal

The idea that GLP-1 is a receptor for a distal tip cell (DTC) signal is based on genetic and sequence data (see Introduction). In this paper, we present two additional lines of evidence to support this model. First, GLP-1 is tightly associated with

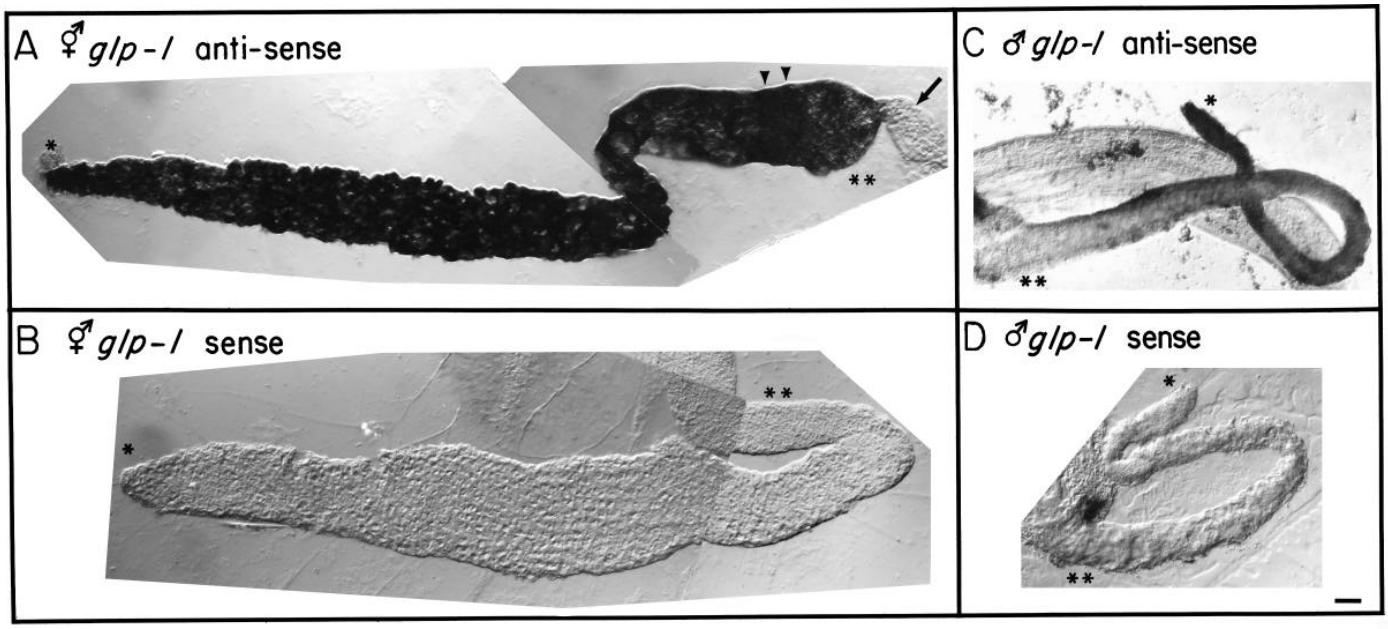

Fig. 8. In situ hybridization of *glp-1*. Distal (*) and proximal (**) ends are marked. (A) Dissected adult hermaphrodite gonad hybridized with *glp-1* anti-sense RNA probe. *glp-1* mRNA is uniformly distributed throughout the germ line, including oocytes (arrowheads). No *glp-1* mRNA is detected in sperm (arrow). (B) Dissected adult hermaphrodite gonad hybridized with *glp-1* sense RNA probe. No signal is detected. (C) Dissected adult male gonad hybridized with *glp-1* anti-sense RNA probe. *glp-1* mRNA is most abundant distally, but decreases more proximally. No *glp-1* mRNA is detected in mature sperm. D. Dissected adult male gonad hybridized with *glp-1* sense RNA probe. No signal is detected. Scale bar, 10 μm.

membranes and is *N*-glycosylated, suggesting that it is an integral membrane protein. While we have not definitively shown that GLP-1 spans the membrane, Notch, a homologous LNG protein, does indeed span the membrane (Johansen et al., 1989; Kidd et al., 1989). Second, GLP-1 is observed in the receiving cells of the DTC/germ line interaction: mitotic germ cells, but not the DTC, possess GLP-1. Similarly, GLP-1 is present in blastomeres that receive inductive signals of the early embryo (Evans et al., 1994). Thus, the distribution of GLP-1 in both the germ line and the embryo supports the idea that it is a receptor for inductive interactions.

What is the active form of GLP-1?

Little is known about how LNG proteins function to influence cell fates. The simplest model is that they are activated, like most receptors, in their intact, membrane-bound form; however, the idea has emerged that the LNG receptors may be novel, with the intracellular region being processed from the intact protein for activity in the nucleus (Lieber et al., 1993; Struhl et al., 1993; Fortini et al., 1993; Kopan et al., 1994). Although we cannot exclude this second model, we find no evidence to support it. Using antibodies to both extracellular and intracellular GLP-1, we find GLP-1 in the plasma membrane and possibly in an intracellular organelle, but not in the nucleus. In addition, the presence of membrane-associated GLP-1 coincides precisely with the occurence of germline mitoses. One finding that might support the intracellular processing model is the presence of cleavage products on western blots. However, the intracellular GLP-1 fragment was isolated on a lectin column even though only the extracellular fragment appears to be *N*-glycosylated; this suggests that the N- and C-terminal fragments remain associated with each other, even if GLP-1 is clipped in vivo. The simplest interpretation of these results is that membrane-associated GLP-1 is indeed the active form.

How does the distal tip cell control mitoses at a distance?

The extent of the physical association between the DTC and the germ line does not correlate with the extent of germline mitoses. Thus, the DTC processes embrace the germline tip and extend about three germ cell diameters along the distal-proximal axis (D. Hall, personal communication), whereas mitotic spindles and membrane-associated GLP-1 extend over 20 germ cell diameters along that axis (this paper). How does the DTC control mitosis over a distance? Fig. 9 shows three simple models. All three models rely on the idea that the DTC controls mitosis by producing a GLP-1-activating ligand; this ligand is likely to be the product of the *lag-2* gene (Henderson et al., 1994; Tax et al., 1994). The molecular mechanism for GLP-1 activation is unknown, but may involve stabilization, proteolysis, oligomerization or a conformational change.

In model 1, a membrane-bound DTC signal binds and activates GLP-1. Active GLP-1 moves proximally, either by moving with the germ cells or by diffusion, and is finally degraded. By this model, the zone of mitosis is defined by the rates of turnover and movement of active GLP-1; germ cells leave mitosis when the amount of active GLP-1 falls below a certain level. In model 2, the membrane-bound DTC signal again binds and activates GLP-1, but here active GLP-1 generates a diffusible second messenger. By this model, the zone of mitosis is determined by the extent of second messenger diffusion in the germline syncytium; germ cells leave mitosis when the amount

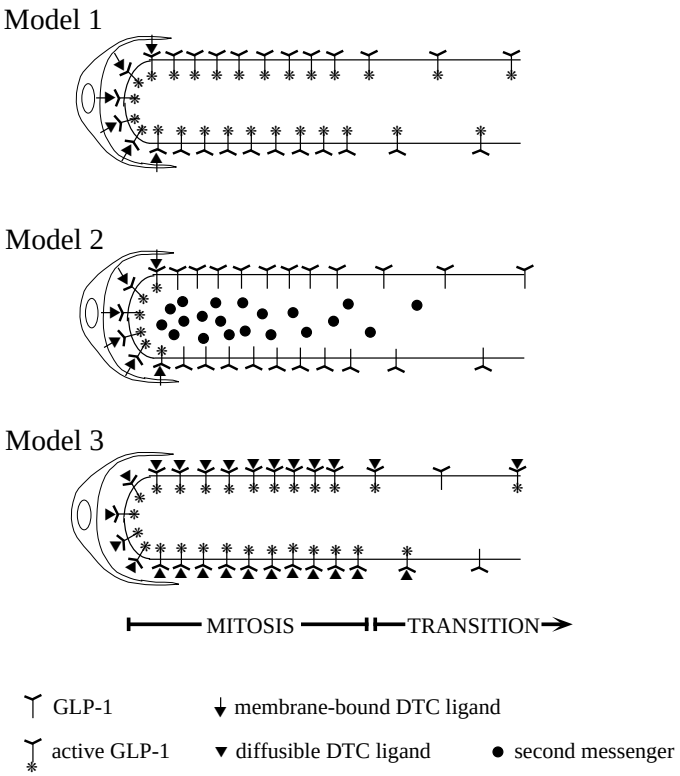

Fig. 9. Models for the control of germline mitosis by GLP-1 and the somatic distal tip cell. The processes of the DTC are in contact with the distal-most germ cells, whereas mitosis occurs both in those cells and also more proximally (see Fig. 6B). Model 1 suggests that the extent of the mitotic region is determined by GLP-1 turnover; model 2 suggests that the mitotic zone is determined by diffusion of an intracellular second messenger; model 3 suggests that the mitotic zone is determined by diffusion of an extracellular ligand. See text for explanation.

of second messenger falls below a certain level. We do not favor this model, because the level of membrane-associated GLP-1 is apparently constant throughout the mitotic region. However, we cannot exclude it at the present time. In model 3, the DTC signalling ligand is diffusible and acts at a distance to activate GLP-1. By this model, the zone of mitosis is defined by the extent of signal diffusion; germ cells leave mitosis when the amount of signal falls below a certain level.

A gene that encodes a candidate signalling molecule, LAG-2, has been identified based on its genetic interactions with *glp-1* and *lin-12* (Lambie and Kimble, 1991). *lag-2* encodes a transmembrane protein with similarity to *Drosophila* Delta, a putative ligand for Notch (Henderson et al., 1994; Tax et al, 1994). At this point it is not clear whether LAG-2 remains membrane bound or is diffusible. In support of models 1 and 2, which suggest that the ligand is membrane-bound, it appears that GLP-1-mediated induction in the embryo may depend on short range interactions and possibly direct cell-cell contacts (Mango et al., 1994). The intracellular proteins that GLP-1 interacts with in the germ line have not yet been identified. Knowledge of the location within the germ line and the subcellular location of such intracellular proteins may help distinguish among these models.

How is the localization of GLP-1 in the germ line achieved?

GLP-1 protein is spatially restricted in the germline syncytium of both hermaphrodites and males: GLP-1 is abundant in the distal mitotic region, levels taper off through the transitional and pachytene regions and no GLP-1 is detected in gametes. The mechanism for localizing GLP-1 to the distal germline appears to differ in hermaphrodites and males. In males, *glp-1* mRNA decreases through the transitional and meiotic regions, suggesting a control at the level of transcription or mRNA stability. By contrast, *glp-1* mRNA is present in similar amounts throughout the adult hermaphrodite germ line, indicating that localized GLP-1 protein must result from a post-transcriptional repression in meiotic germ cells. Consistent with this idea, reporter RNA carrying the *glp-1* 3′ UTR is not translated in germ cells in the proximal arm of the hermaphrodite germ line (Evans et al., 1994). Thus, in germ lines producing oocytes, translational repression of *glp-1* mRNA appears to be responsible for the absence of GLP-1 protein in meiotic germ cells. The different mechanisms of GLP-1 protein localization used by hermaphrodites and males may reflect the embryonic requirement for maternal *glp-1* mRNA.

Control over the presence or absence of LNG proteins may be essential for cell fate determination

The presence of GLP-1 in cells requiring its activity and its absence from cells not requiring its activity is striking. In the germline, mitotic cells possess GLP-1 and meiotic cells lack it (this paper); in the embryo, AB descendants possess GLP-1 and P_1 descendants lack it (Evans et al., 1994). Furthermore, GLP-1 functions during the 4-28 cell stage of embryogenesis (Priess et al., 1987; Austin and Kimble, 1987), and GLP-1 is not observed past the 28-cell stage (Evans et al., 1994). These findings suggest that GLP-1 may be controlled at the level of its presence or absence, which stands in stark contrast to the uniform distribution of Toll (Hashimoto et al., 1991) and torso (Casanova and Struhl, 1991), two receptors that control pattern in the *Drosophila* embryo. Thus, for GLP-1, and perhaps for other LNG proteins, pattern control may be imposed by spatially restricting the receptor.

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