aph-2 encodes a novel extracellular protein required for GLP-1-mediated signaling

Caroline Goutte¹, *, ‡, William Hepler¹, ², Katherine M. Mickey¹ and James R. Priess¹, ²

¹Division of Basic Sciences and Molecular and Cellular Biology Program, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
²Howard Hughes Medical Institute
*Current address: Department of Biology, Amherst College, Amherst, MA 01002, USA
‡Author for correspondence (e-mail: cegoutte@amherst.edu)

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SUMMARY

In animal development, numerous cell-cell interactions are mediated by the GLP-1/LIN-12/NOTCH family of transmembrane receptors. These proteins function in a signaling pathway that appears to be conserved from nematodes to humans. We show here that the aph-2 gene is a new component of the GLP-1 signaling pathway in the early Caenorhabditis elegans embryo, and that proteins with sequence similarity to the APH-2 protein are found in Drosophila and vertebrates. During the GLP-1-mediated cell interactions in the C. elegans embryo, APH-2 is associated with the cell surfaces of both the signaling, and the responding, blastomeres. Analysis of chimeric embryos that are composed of aph-2(+) and aph-2(−) blastomeres suggests that aph-2(+) function may be provided by either the signaling or responding blastomere.

Key words: Caenorhabditis elegans, Cell-cell interaction, NOTCH, GLP-1, aph-2

INTRODUCTION

The GLP-1 and LIN-12 proteins of C. elegans, and the related NOTCH proteins in Drosophila and vertebrates, function in numerous cell-cell interactions in development. These proteins are transmembrane receptors that allow cells to respond to ligands expressed in neighboring cells (see Artavanis-Tsakonas et al., 1999; Greenwald, 1998; Kimble and Simpson, 1997 for reviews). The ligands are a conserved family of transmembrane proteins such as APX-1 and LAG-2 in C. elegans, and DELTA and SERRATE in Drosophila. Signal transduction appears to result in a cleavage event that releases the intracellular part of the receptor into the cytoplasm (see Greenwald, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). The intracellular domain is thought to associate with a conserved transcription factor encoded by lag-1 in C. elegans, Suppressor of hairless [Su(H)] in Drosophila and CBF1 in mammals (Jarriault et al., 1995). The intracellular NOTCH fragment and LAG-1/Su(H)/CBF1 complex appears to enter the nucleus where they regulate the expression of target genes (Bailey and Posakony, 1995; LeCourtiois and Schweisguth, 1995; Christensen et al., 1996, Kopan et al., 1996).

In addition to these “core” components of the GLP-1/LIN-12/NOTCH signaling pathway, genetic studies have identified several proteins that alter the responsiveness of cells to NOTCH signaling. For example, the intracellular portion of the Notch receptor may interact with cellular proteins, such as Drosophila DELTEX (Matsuno et al., 1995) and NUMB (Guo et al., 1996) and nuclear components such as the EMB-5 protein of C. elegans (Hubbard et al., 1996).

Genetic studies have also identified proteins that appear to function in processing or modifying one of the core components. For example, several genes that are required for full receptor activity encode proteins with possible proteolytic activity. The C. elegans SUP-17 protein and the Drosophila KUZBANIAN protein are related to members of the ADAM family of metalloproteases (Rooke et al., 1996; Pan and Rubin, 1997; Sotillos et al., 1997; Wen et al., 1997) and the C. elegans SEL-12 and HOP-1 proteins are presenilin-related multipass transmembrane proteins (Levitan and Greenwald, 1995; Li and Greenwald, 1997). Although the specific functions of these proteins are not known, several distinct proteolytic cleavage events have been implicated in the processing of receptors in the NOTCH family. First, NOTCH appears to be cleaved before reaching the cell surface where it is then presented as a heterodimer (Blaumueller et al., 1997; Logeat et al., 1998). Additional proteolytic processing steps may occur at the cell surface and, in some cases, may even release a soluble form of the NOTCH ligand (Qi et al., 1999). The importance of receptor turnover has also been highlighted by the characterization of two genes, sel-1 and sel-10, that act to negatively regulate GLP-1/LIN-12 activity in C. elegans. The sel-1 gene encodes a secreted protein that localizes to intracellular vesicles that are thought to be involved in protein turnover, while the sel-10 gene encodes a protein that is believed to promote ubiquitin-mediated turnover of NOTCH proteins (Grant and Greenwald, 1996; Hubbard et al., 1997).
The ability of receptor and ligand to associate and the consequences of this interaction are likely to be influenced by additional extracellular factors. For example, differences in extracellular components may explain why soluble forms of NOTCH ligands can be active in some studies, and yet block NOTCH activation in other studies (Fitzgerald and Greenwald, 1995; Sun and Artavanis-Tsakonas, 1997; Varum-Finney et al., 1998). Furthermore, the mechanism by which ligand-receptor association stimulates receptor cleavage is unclear and may require accessory proteins that have not yet been identified.

In this paper, we present the identification of a new component of GLP-1-mediated cell interactions in the C. elegans embryo. GLP-1 is expressed on the surfaces of a group of early embryonic blastomeres. One of these blastomeres is exposed to ligand at the 4-cell stage of embryogenesis and, in response, adopts a new pattern of differentiation. Two additional GLP-1-expressing blastomeres become exposed to a second signal at the 12-cell stage of embryogenesis, and consequently adopt unique cell fates. We show that a novel cell-surface protein, APH-2, is required for both the 4-cell and 12-cell interactions, and that mutations in the aph-2 gene result in phenotypes that are indistinguishable from those of glp-1 mutant embryos. Using the 12-cell-stage interaction to assay aph-2 function, we find that aph-2(+) can be supplied by either the signaling or the responding cell. Predicted proteins with sequence similarity to APH-2 are encoded in both the Drosophila and Human genomes, suggesting the possibility that these proteins may also function in NOTCH cell signaling pathways.

MATERIALS AND METHODS

Strains and alleles

Bristol strain N2 was used as the standard wild-type strain (Brenner, 1974). Genetic markers, deficiencies and balancer chromosomes used are listed by linkage group: LGI, dpy-5(e61), unc-13(e1091), unc-29(e1072), lin-11(n566), aph-2(zu172), aph-2(zu179), aph-2(zu181), aph-2(zu186), aph-2(zu190), srp-2(yj262), unc-75(e950), hd9(h601), qDf5, qDf6, hT2(101-4(e937) let-7(h661))1(f131); LGII, unc-32(e189), lin-12(n137n720); LGIV, him-8(e1489); LGV, him-5(e1490).

The qDf5 and qDf6 chromosomal deficiencies were provided by R. Ellis. The strain PD8260 was provided by Pete Okkema, and is homozygous for an integrated copy of a ceh-22::lacZ fusion (Okkema and Fire, 1994).

Genetic analysis

aph-2 alleles were isolated in a previously described screen for non-conditional, maternal-effect, embryonic lethal mutations using a transposon-mobilized strain, RW7096 [mut-6(st702) unc-22(st192);Tcl] (Mello et al., 1994). All alleles failed to complement aph-2(zu172) and were outcrossed to the N2 strain ten times before further analysis. aph-2(zu181) was mapped using standard techniques; data for these crosses are available from the C. elegans Genetic Stock Center. The chromosomal deficiency qDf6 fails to complement the aph-2(zu181) allele as demonstrated by the following cross: males from the strain unc-29 aph-2(zu181)/ht2; him-8 were crossed with qDf6[dpy-5 srf-2 unc-75] hermaphrodites. The resulting fertile aph-2/qDf6 heterozygotes were viable and produced embryos indistinguishable from those of aph-2(zu181) homozygotes (little differentiation, posterior half-pharynx only and no body morphogenesis; n=56 embryos from 11 heterozygotes) and displayed the aph-2 egg-laying defect. The chromosomal deficiencies qDf5 and hd9 were found to complement aph-2(zu181).

The following experiments demonstrate that maternal expression of the aph-2 gene is necessary for embryogenesis. No viable self-progeny were produced by hermaphrodites homozygous for unc-29 aph-2(zu181) (0%; n=>5000). No viable cross progeny were produced by unc-29 aph-2(zu181) homozygotes that were mated to wild-type males (0%; n=>250 embryos from 12 different crosses) indicating that the embryonic lethality cannot be rescued by paternal or embryonic expression of the aph-2 gene. Furthermore, virtually all self-progeny from unc-29 aph-2(zu181)+ hermaphrodites are viable, including one quarter that are homozygous for aph-2(zu181) (98.6%, n=800).

Analysis of embryos

Embryos were prepared for microscopy as described by Sulston et al. (1983), and for immunofluorescence microscopy as described by Albertson (1984) and Bowerman et al. (1992a). Tissues were identified as follows: Intestinal cells by birefringent gut-specific granules; pharynx by staining with mAb3NB12 (Priess and Thomson, 1987); hypodermal cells by staining with mAbMH27 (Priess et al., 1987). The presence of the intestinal-rectal valve cells was scored by using mAb ICB4; because this antibody also recognizes intestinal cells, the EMS blastomere was killed by laser microbeam before analysis as in Bowerman et al. (1992b); using this protocol, 12 out of 12 wild-type embryos and 1 out of 9 aph-2(zu181) embryos showed valve cell staining. All photographs shown in Figs 2, 3, 5 and 7 were taken using Kodak Technical Pan film and developed in Kodak HC110 developer. Photographs shown in Fig. 4 were captured with a Synsys digital camera (Photometrics).

Lineage analysis was performed on embryos collected from aph-2(zu181) homozygous hermaphrodites according to Sulston et al. (1983). One embryo was used for each lineage. Lineage patterns were followed for the following blastomeres: ABPrpapp, ABPrpapp, ABPbpapp and ABAlbpapp. In each case, the indicated cell did not show the behavior expected in a wild-type embryo, but instead behaved more like the lineally equivalent daughter of ABarp. For example, in wild type, the cell ABPrpapp divides three times to produce eight small neuronal cells while the cell ABarpapp divides twice to produce four hypodermal cells. The aph-2 mutant ABPrapp cell divided twice (observed for 3.5 additional hours) and produced a group of cells resembling hypodermal cells.

Analysis of aph-2 phenotypes

The percentage of aph-2(zu181) and aph-2(zu179) embryos that appear defective in only the 12-cell-stage interaction was determined by counting the number of embryos that were fully enclosed by hypodermis and that had elongated at least 1.5-fold. The percentage of aph-2(zu181) and aph-2(zu186) embryos that appear defective in only the 4-cell-stage interaction was determined by counting the number of embryos that displayed an abnormally large amount of pharynx when immunostained with mAb3NB12.

Sterility in aph-2(zu181) homozygotes was assayed by plated out individual Unc self-progeny from the strain unc-29 aph-2(zu181) +/- and scoring the animals for production of embryos. Control unc-29 aph-2(+) homozygous worms showed 0.2% sterility (n=1109). In a separate experiment, hermaphrodites that were heterozygous for unc-29 aph-2(zu181) and the qDf6 chromosomal deficiency were analyzed. qDf6 on its own causes a semidominant sterility of variable penetrance; nevertheless, fertile unc-29 aph-2(zu181) qDf6 hermaphrodites were recovered readily in three separate crosses between a qDf6 heterozygous hermaphrodite and unc-29 aph-2(zu181)/balancer males (n=11).

GLP-1 and APX-1 immunostaining

Embryos were processed for staining with APX-1 antisera as described previously (Mickey et al., 1996). For GLP-1 immunolocalization, embryos were processed in the following...
Molecular analysis of aph-2

Interaction. Reconstituted embryos that showed detectable levels of fates are dependent upon induction at the 12-cell stage, N2 hermaphrodites or $\textit{unc-29}(e1072)$ $\textit{aph-2}(zu181)$.

A single Tc1 element was found closely linked to $\textit{aph-2}(zu181)$ to release embryos. The water was replaced with fixative (2% formaldehyde, 60 mM Pipes, 25 mM Hepes [pH 6.8], 10 mM EGTA, 2 mM MgCl$_2$) and the embryos were squashed with a coverslip. After 10 minutes in a moist chamber, the embryos were frozen on dry ice for 5 minutes. The coverslips were then removed and the slides were placed in −20°C methanol for 5 minutes, washed twice in phosphate-buffered saline-Tween (PBS-T) and blocked with 10% normal goat serum (GIBCO) for 30 minutes. The GLP-1 polyclonal rabbit antibody (kindly provided by S. Crittenden) was diluted 1:20 in the blocking solution and 10 µl was added to each slide and incubated overnight at room temperature. Secondary antibody incubation, washing and mounting were as described for APH-2 immunostaining below.

Blastomere recombination assays

Blastomeres were isolated and cultured according to the method described by Shelton and Bowerman (1996). AB and P1 blastomeres of different genotypes were recombined immediately after they had each divided in culture once. To ensure that signaling cells and responding cells would be in contact at the 12-cell stage, the P1 daughter cells were oriented towards the AB daughters such that the EMS blastomere contacted one or both of the AB cells. Both daughter cells of EMS (E and MS) can function as signaling cells in the 12-cell-stage interaction if they contact competent AB descendants (Lin et al., 1995; Shelton and Bowerman, 1996). In some reconstituted embryos, the P2 cell did not contact either of the AB daughter cells, presumably preventing induction of the ABp cell fate at the 4-cell stage, and leading to more cells in the embryo capable of responding to the 12-cell-stage induction (see Mello et al., 1994). Reconstituted embryos were allowed to develop in modified cell culture medium to the 12-cell-stage induction (see Mello et al., 1994). Reconstituted embryos that had reached the 12-cell stage, and leading to more cells in the embryo capable of responding to the 12-cell-stage induction (see Mello et al., 1994). Reconstituted embryos were allowed to develop in modified cell culture medium to the 12-cell-stage induction (see Mello et al., 1994). Reconstituted embryos were allowed to develop in modified cell culture medium to the 12-cell-stage induction (see Mello et al., 1994).

Molecular analysis of aph-2

A single Tc1 element was found closely linked to $\textit{aph-2}(zu181)$ by the following analysis. Recombinants were collected from the strain $\textit{aph-2}(zu181)$ $\textit{lin-11}$ $\textit{unc-75}$; three recombinants in the $\textit{lin-11}$ – $\textit{aph-2}$ interval (0.15 m.u.), and 16 recombinants in the $\textit{aph-2}$ – $\textit{unc-75}$ interval (4.5 m.u.). Genomic DNA from these strains was restriction-digested and probed with a Tc1 DNA fragment. A 2.2 kb HindIII fragment was found linked to $\textit{aph-2}(zu181)$ and 600 bp of genomic DNA contained in this fragment was recovered by inverse PCR, as described by Hill and Sternberg (1992). This fragment was used as a probe against genomic DNA prepared from strains heterozygous for the $\textit{hT2}$ balancer and either $\textit{aph-2}(zu172)$, $\textit{aph-2}(zu186)$, $\textit{aph-2}(zu190)$ or $\textit{aph-2}(zu192)$, and polymorphic restriction fragments were identified for all strains except $\textit{aph-2}(zu192)$. The 600 bp fragment hybridized to two overlapping YACs in the $\textit{aph-2}$ region on LGI: Y33G3 and Y0D5 on a polytene YAC grid provided by the $\textit{C. elegans}$ Genome Project. The 600 bp fragment was used to screen a $\textit{C. elegans}$ genomic library (Stratagene) and a mixed stage cDNA library (provided by Andy Fire). DNA sequence was obtained from three different cDNA clones and corresponding fragments of a genomic clone. The 5’ end of the $\textit{aph-2}$ transcript was determined by reverse transcription of total mixed-stage N2 RNA using a random hexamer primer mix (Boehringer Mannheim) followed by PCR using an $\textit{aph-2}$-specific primer paired with a primer specific for one of the two trans-spliced leader sequences, SL1 or SL2, as described by Spiehl et al. (1993). A product of the expected size was produced only when the SL1 primer was used and the sequence of this product corresponds to the 5’ end of the cloned $\textit{aph-2}$ cDNA. A probe corresponding to the entire $\textit{aph-2}$ cDNA was used on low-stringency Southern blots of genomic N2 DNA and revealed no hybridizing species other than the $\textit{aph-2}$ gene. The $\textit{aph-2}$ cDNA is identical to the partial $\textit{C. elegans}$ cDNA clone wEST01925. The $\textit{C. elegans}$ genome sequencing project has since sequenced through the $\textit{aph-2}$ gene. Our $\textit{aph-2}$ sequence corresponds to the open reading frame, ZC434.6 of cosmID ZC434.

Generation of APH-2 antisera and immunostaining

The $\textit{aph-2}$ bacterial expression plasmid, JP654, was constructed by cloning a 1.5 kb $\textit{BamHI}$ fragment encoding the carboxy-terminal 436 amino acids of $\textit{APH-2}$ (APH-2:2286-721) into the $\textit{BamHI}$ site of the expression vector pET-16b (Novagen). Upon induction in BL21(DE3) cells, JP654 expresses a 48 kd fusion protein consisting of 26 amino acids encoded by the expression vector, followed by APH-2:2286-721.

The fusion protein was partially purified from bacterial proteins and injected subcutaneously into three New Zealand white rabbits (R and R Rabbity, Stanwood, WA). Initial immunizations contained Freund’s complete adjuvant, and subsequent immunizations contained Freund’s incomplete adjuvant (Cappel Research Products). Rabbits were boosted every 4-5 weeks and bled for serum 10 days after each boost. Serum was tested for immunoreactivity by staining wild-type $\textit{C. elegans}$ embryos. Specificity of immunoreaction was determined by comparing the staining pattern in wild-type and $\textit{aph-2}$ mutant embryos. Serum was affinity purified according to Robinson et al. (1988).

Embryos and adult gonads were prepared for immunostaining following the general procedures of Albertson (1984). After fixation, embryos were blocked with 2% bovine serum albumin in Tris-Tween for 30 minutes, and then incubated with affinity-purified APH-2 antisemur diluted 1:30 in Tris-Tween for at least 6 hours at room temperature. Slides were washed three times with Tris-Tween, then incubated with rhodamine-conjugated goat anti-rabbit or FITC-conjugated donkey anti-rabbit antibodies (Jackson Laboratories) at room temperature for 1-2 hours Slides were washed as above with a final wash in 5 ng/ml of 4’6-diamino-2-phenylindole (DAPI) for visualization of DNA.

RESULTS

Background

The receptor GLP-1 has been shown to function during the 4-
cell and 12-cell stages of C. elegans embryogenesis in two critical, cell-cell interactions (for reviews see Kimble and Simpson, 1997; Schnabel and Priess, 1997; Greenwald, 1998). Specific defects in one, or both, of the interactions lead to diagnostic patterns of developmental abnormalities that are summarized in Fig. 1 and described here briefly. In wild-type embryos, all descendants of the AB blastomere (ABa, ABp, etc) express the receptor GLP-1 during the first few rounds of cell division (Evans et al., 1994). At the 4-cell stage, ABp contacts the P2 blastomere, and P2 expresses the ligand APX-1 (Mickey et al., 1996). In response, ABp adopts a pattern of development that is distinct from its sister, ABa. For example, ABp uniquely acquires the ability to produce the pair of valve cells that connect the intestine to the rectal epithelia (Bowerman et al., 1992b). At the 12-cell stage, two of the ABa descendants contact the MS blastomere, and MS appears to express a second ligand equivalent to APX-1 (Shelton and Bowerman, 1996). In response, these ABa descendants change their pattern of development and produce, among other things, the anterior half of the pharynx.

Mutation embryos defective only in the 4-cell interaction, such as apx-1 mutants or temperature-pulsed glp-1(ts) mutants, lack the intestinal-rectal valve cells. Because ABp descendants in these embryos remain competent to respond to subsequent signaling at the 12-cell stage, these embryos overproduce anterior pharyngeal cells (Mango et al., 1994; Mello et al., 1994; Moskowitz et al., 1994; Hutter and Schnabel, 1994). Mutant embryos defective only in the 12-cell interaction, or in both the 12-cell and 4-cell interactions, lack the anterior pharynx but contain a posterior half-pharynx; the posterior half of the pharynx is produced by the MS blastomere through a GLP-1-independent pathway (Priess et al., 1987). Mutant embryos defective in either the 4-cell or 12-cell interactions also have characteristic defects in body morphogenesis. Morphogenesis is controlled by a group of epithelial cells called hypodermal cells, and defects in the 4-cell and 12-cell interactions alter the number, types and positions of hypodermal cells (Hutter and Schnabel, 1994; Mello et al., 1994; Moskowitz et al., 1994). Thus the two GLP-1-mediated interactions can be evaluated by scoring body morphogenesis and the production of intestinal-rectal valve cells and pharyngeal tissue (Fig. 1).

**aph-2 mutant embryos are defective in the 4-cell and 12-cell interactions**

To identify genes that act in the GLP-1 pathway, we collected and analyzed maternal-effect lethal mutants that produce embryos lacking anterior pharyngeal cells (see Materials and Methods). These mutants were isolated in several genetic screens described previously that involved chemical (Draper et al., 1996) or transposon mutagenesis (Mello et al., 1994). Genetic tests on 26 such mutants showed that they corresponded to three complementation groups. 18 mutants were new alleles of glp-1 and two mutants were defective in a new gene that we call *aph-1* and will be described elsewhere. Six mutants were defective in a second new gene that we call *aph-2* (anterior pharynx defective) and will be described here.

Hermaphrodites that are homozygous for any one of the six *aph-2* alleles produce only dead embryos. Genetic mapping experiments indicate that *aph-2* is located 0.15 map units to the right of the *lin-11* gene on LGI (see Materials and Methods). Unless otherwise noted, our analysis was performed using the *aph-2(zu181)* allele, which has a transposon insertion in the *aph-2* coding region and does not produce detectable APH-2 protein (see below); the embryos produced by homozygous *aph-2(zu181)* hermaphrodites will be referred to simply as *aph-2* embryos in this paper. In several experiments, *aph-2* embryos were compared with, and found identical to, progeny of hermaphrodites that were heterozygous for *aph-2(zu181)* and a chromosomal deficiency that removes the *aph-2* locus (see Materials and Methods).

In the light microscope, most *aph-2* embryos bear a striking resemblance to glp-1 embryos that are defective in both the 4-cell and 12-cell interactions. The *aph-2* embryos fail to undergo morphogenesis and contain dorsal clumps of contracted hypodermal tissue (Fig. 2D; Mello et al., 1994).

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**Fig. 1.** Summary of the 4-cell- and 12-cell-stage cell interactions. (Left) Schematic drawings of 4-cell- and 12-cell-stage embryos; the receptor GLP-1 is present on all AB descendants (bold outline). Cell signaling is indicated by bold arrows. Signaling induces otherwise equivalent AB descendants to have different fates (shaded). (Right) Summary of some of the phenotypes resulting from defects in either, or both, of the interactions. *glp-1(ts)* mutant embryos exposed to high temperature only during the 4-cell or 12-cell interactions resemble mutant embryos that lack the anterior pharynx. Both the 12-cell and 4-cell interactions are critical, cell-cell interactions (for reviews see Kimble and Simpson, 1997; Schnabel and Priess, 1997; Greenwald, 1998). Specific defects in one, or both, of the interactions lead to diagnostic patterns of developmental abnormalities that are summarized in Fig. 1 and described here briefly. In wild-type embryos, all descendants of the AB blastomere (ABa, ABp, etc) express the receptor GLP-1 during the first few rounds of cell division (Evans et al., 1994). At the 4-cell stage, ABp contacts the P2 blastomere, and P2 expresses the ligand APX-1 (Mickey et al., 1996). In response, ABp adopts a pattern of development that is distinct from its sister, ABa. For example, ABp uniquely acquires the ability to produce the pair of valve cells that connect the intestine to the rectal epithelia (Bowerman et al., 1992b). At the 12-cell stage, two of the ABa descendants contact the MS blastomere, and MS appears to express a second ligand equivalent to APX-1 (Shelton and Bowerman, 1996). In response, these ABa descendants change their pattern of development and produce, among other things, the anterior half of the pharynx.

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APH-2 is required for GLP-1 signaling

They lack the anterior pharyngeal cells that normally require GLP-1(+) activity, such as the epithelial cells that make the buccal cavity (Fig. 2D,F). However, aph-2 embryos produce the GLP-1-independent, posterior pharyngeal cells, such as the cells that form the grinder of the pharynx (Fig. 2D,F). Immunostaining experiments showed that aph-2 embryos lack the intestinal-rectal valve cells (Fig. 2H; Materials and Methods) that normally require the 4-cell-stage interaction. All embryos shown are about 12 hours after fertilization (corresponding to the end of wild-type embryogenesis), and are oriented with anterior to the left. All images are at the same magnification; embryos are approximately 50 μm in length.

A small percentage of aph-2 embryos showed characteristics of embryos that are defective only in the 12-cell-stage interaction (1.5% (n=318) for aph-2(zu181); 14% (n=555) for aph-2(zu179)). These embryos lacked the anterior pharynx, but had partial body morphogenesis (Fig. 3A) and produced the valve cells (Fig. 3B). These embryos had extra lateral hypodermal cells (Fig. 3C) with a pattern similar to that of glp-1 mutants defective only in the 12-cell-stage interaction (see Priess et al., 1987). A second subset of the aph-2 embryos appeared to be defective only in the 4-cell-stage interaction (0.4% (n=615) for aph-2(zu181); 0.3% (n=1178) for aph-2(zu186)). These embryos lacked body morphogenesis, but had more than the wild-type amount of pharyngeal tissue (Fig. 3E). Thus a small percentage of aph-2 mutant embryos appear capable of completing either the 4-cell or 12-cell interaction successfully, although most embryos fail in both interactions.

Defects in the 4-cell and 12-cell interactions cause reproducible abnormalities in the cell lineages of glp-1 and apx-1 mutants (Mello et al., 1994; Moskowitz et al., 1994). For example, in glp-1 mutants the ABa and ABp descendants named ABalp, ABplp and ABprp inappropriately adopt a lineage pattern that resembles that of a wild-type blastomere called ABarp (see Mello et al., 1994; Moskowitz et al., 1994). We followed the development of one or more representative descendants from the ABa, ABarp and ABprp blastomeres in aph-2 embryos and, in each case, observed a lineage that resembled that of a wild-type ABarp descendant (see Materials and Methods). Thus both the cell lineage and cell differentiation defects in aph-2 embryos appear identical to those of mutant embryos defective in the GLP-1-mediated, 4-cell-stage and 12-cell-stage interactions.

We wanted to determine if aph-2 embryos had defects in the expression or localization of GLP-1 or its ligand APX-1. In
wild-type 4-cell and 12-cell-stage embryos, GLP-1 is localized to the surfaces of all AB descendants (Fig. 4A; Evans et al., 1994). The APX-1 protein is localized to the interface between the ABp and P2 blastomeres in wild-type 4-cell embryos (Fig. 4C; Mickey et al., 1996). We found that 4-cell-stage aph-2 embryos appeared to have the normal levels, and membrane localization patterns, of both GLP-1 (Fig. 4B) and APX-1 (Fig. 4D). In addition, GLP-1 appeared to be localized normally to AB descendants in aph-2 embryos during the 12-cell stage (data not shown).

**aph-2 and other GLP-1 dependent/independent interactions**

At the same time that P2 and ABp undergo a GLP-1-mediated interaction, P2 and the EMS blastomere interact through a separate, WNT/WINGLESS-like signaling pathway (see Fig. 1 for blastomere positions; Goldstein, 1992; Rocheleau et al., 1997; Thorpe et al., 1997). The P2 blastomere appears to be the source of the ligand MOM-2 (WNT). Mutations in genes in the WNT-like pathway disrupt the interaction between P2 and EMS, preventing EMS descendants from producing intestinal cells (Rocheleau et al., 1997; Thorpe et al., 1997). To determine if aph-2 mutants were defective in the interaction between P2 and EMS, we used polarization optics to score embryos for the presence of intestine-specific, birefringent granules. Of 865 aph-2 embryos examined, 99.9% had intestinal cells. Thus aph-2 mutant embryos appear defective in the GLP-1-mediated interactions between P2 and ABp, but not in the WNT-mediated interactions between P2 and EMS.

During postembryonic development, GLP-1 activity controls germ line proliferation in the gonad (Austin and Kimble, 1987). Briefly, a somatic cell at one end of the gonad (called the distal tip cell) signals underlying germ cells to divide mitotically; cells that move away from the distal tip cell cease division and enter meiosis. GLP-1 is expressed on the dividing germ cells (Crittenden et al., 1994) and an APX-1-related ligand, LAG-2, is expressed by the distal tip cells (Fitzgerald and Greenwald, 1995); defects in either GLP-1 or LAG-2 cause germ cells to exit mitosis inappropriately, resulting in adult sterility (Austin and Kimble, 1987; Lambie and Kimble, 1991). We asked whether aph-2(+) function might also be required for this postembryonic GLP-1-mediated event by scoring aph-2 homozygous adults for sterility. Only 1% of aph-2(ze181) adults were sterile (n=214), a percentage only slightly higher than that of wild-type adults grown under similar conditions. Thus aph-2(+) function is not essential for germline proliferation in the larval and adult gonads.

In the course of these experiments, we found that all six aph-2 alleles caused incompletely penetrant defects in the ability of adults to lay eggs. For example, 55% of hermaphrodites
homozygous for aph-2(zu181) were unable to lay their eggs, and another 22% became egg laying defective after laying less than 30 eggs (n=214). Although the animals cease to lay eggs, they continued to produce eggs and ultimately fill up with dead embryos. We did not detect gross abnormalities in the initial formation of a vulva in aph-2 homozygous hermaphrodites. Egg laying defects have not been described for mutants defective in GLP-1, however defects in the closely related receptor LIN-12 can cause defects in egg laying (see Discussion).

**aph-2(+) function can be supplied by either the signaling or responding blastomere**

To assay which embryonic blastomere(s) require aph-2(+) function, we analyzed the 12-cell interaction in which the MS blastomere induces ABa descendants to produce pharyngeal cells. Chimeric embryos were constructed with blastomeres isolated from 2-cell-stage embryos: The AB blastomere of a 2-cell-stage embryo produces the GLP-1 responsive cells while the P1 blastomere produces MS, the signaling cell. As a marker for pharyngeal cell differentiation, all AB blastomeres carried a transgene with a pharyngeal-specific promoter driving β-galactosidase (β-gal) expression (Okkema and Fire, 1994). AB and P1 blastomeres isolated from wild-type and/or aph-2 mutant embryos were combined, allowed to develop in culture, and scored for β-gal production as an indication of whether or not the 12-cell interaction had been successfully carried out (see Materials and Methods for details).

In control experiments, β-gal expression was not detected when either wild-type or mutant AB blastomeres were cultured in isolation (Fig. 5, lines 1 and 2). β-gal expression was detected in most of the chimeras where both AB and P1 were wild type (Fig. 5, line 6), but not when both AB and P1 were from aph-2 embryos (Fig. 5, line 3). Surprisingly, β-gal expression was observed in both classes of aph-2(+)/aph-2(−) chimeras (Fig. 5, lines 4 and 5). Thus, aph-2(+) function apparently can be supplied by either the signaling cell (MS), or by the responding cells (AB descendants).

**aph-2 encodes a novel protein**

The six aph-2 mutations were isolated as spontaneous mutants from a strain with a high frequency of transposon movement (see Materials and Methods). We cloned the aph-2 gene by identifying a novel Tc1 transposon in the aph-2(zu181) strain that co-mapped with the aph-2 mutant phenotype. The genomic DNA flanking this Tc1 insertion was isolated and used to identify genomic polymorphisms for three other independently isolated aph-2 alleles (see Fig. 6). The cloned DNA fragment was used to identify aph-2 genomic and cDNA clones from *C. elegans* phage libraries. Sequence analysis of the cDNA clones and partial analysis of genomic clones led to the identification of a region of 4.5 kb from which eight exons are spliced together to create an mRNA with one long open reading frame of 2166 bp. The aph-2(zu181) mutation corresponds to a Tc1 insertion in the fourth exon. The 5′ end of the message is transspliced to the SL1 splicing leader sequence (Krause and Hirsh, 1987) just before the beginning of the coding sequence (data not shown).

The aph-2 gene encodes a novel, predicted protein of 721 amino acids. Hydropathy analysis (Kyte and Doolittle, 1982) of this amino acid sequence reveals two strongly hydrophobic regions at positions 4-14 and positions 681-699. The first of these regions is predicted to function as a signal sequence according to the parameters established by von Heijne (1986). The C terminus contains a stretch of 19 predominantly hydrophobic residues that could function as a transmembrane domain. Alternatively, this region also meets the criteria for an attachment site of glycosyl phosphatidylinositol (GPI). GPI attachment sites are found near the C termini of proteins. The site consists of three small amino acids followed by a small hydrophilic spacer and a 15- to 20-residue hydrophobic sequence which can contain some hydrophilic amino acids (Englund, 1993). The sequence of the predicted APH-2 protein suggests that this protein is either secreted or attached to cell membranes, and there are six potential glycosylation sites within the protein.

Two predicted proteins that show sequence similarity to APH-2 are present in the current databases. An uncharacterized human cDNA clone, KIAA0253 (GenBank accession number D87442) is predicted to encode a protein of at least 708 amino acids (the 5′ end of the cDNA is missing) with 19% identity to APH-2. Like APH-2, the carboxy terminus of the predicted KIAA0253 protein contains a cluster of hydrophobic amino acids that is predicted to serve as a transmembrane domain. A recently sequenced region of the *Drosophila* genome has the potential to encode a protein that shows 18% identity with APH-2 for the region of overlap, and 25% identity with the putative human protein in the same region. The similarity
Fig. 6. Sequence of the *aph-2* gene. (A) Physical map of the *aph-2* gene showing the deduced exon structure of the *aph-2* transcript relative to the genomic region of the gene. The 5′ end of the *aph-2* mRNA is spliced to the SL1 leader sequence (Krause and Hirsh, 1987). The positions of the Tc1 insertions corresponding to four different *aph-2* alleles are shown; only the general region of insertion is known for *zu186*: Tc1 and *zu192*: Tc1. The line representing the genomic DNA is dashed after the 5′ KpnI restriction site to indicate that sequences for this region were determined from the cDNA clone only. (B) The nucleotide and predicted amino acid sequence of the *aph-2* cDNA. Numbers on the left correspond to nucleotide positions, and numbers on the right correspond to amino acid positions. Start and stop codons are indicated in bold. Shaded boxes indicate a putative signal sequence at the amino terminus and a putative transmembrane domain in the carboxyl terminus. Additional residues that could contribute to a GPI linkage site are underlined. There are six potential N-glycosylation sites at residues 40, 181, 271, 328, 409 and 625, and a potential GAG attachment site at residue 435. The position of the *zu181*: Tc1 insertion is indicated with an asterisk at nucleotide position 1331. The *C. elegans* Genome Sequencing Project as part of cosmid ZC434 (accession #Q23316). (C) Alignment of the 31 amino acid region that shows the highest degree of similarity between APH-2 (residues 332-362) and the predicted human KIAA0253 protein and *Drosophila* AC014104 protein.
between these proteins is most striking in a 31 amino acid region (position 332-362 of APH-2) in which 16 amino acids are identical in all three proteins (Fig. 6C).

**APH-2 protein localizes to the cell membranes of embryos**

In order to determine the cellular location of the APH-2 protein, we raised polyclonal antisera against a bacterially expressed APH-2-fusion protein. Affinity-purified antisera from each of three different rabbits stain wild-type embryos in a similar manner, but show no staining of aph-2 mutant embryos (Fig. 7E; data not shown). We conclude that the immunostaining pattern described below is that of the APH-2 protein.

APH-2 protein is first detected during oogenesis. APH-2 is associated with the plasma membranes that partially surround the developing oocyte nuclei and with the surface membranes of mature oocytes (Fig. 7A). In newly fertilized eggs and in early cleavage stage embryos, APH-2 is associated with the peripheral membranes of all blastomeres (Fig. 7B-D; data not shown). The APH-2 protein can be detected on, or near, plasma membranes throughout the first 5-6 hours of embryogenesis. The APH-2 protein that is observed after the 28-cell stage may represent protein that persists from maternal expression of APH-2 protein. We have not been able to detect embryonically expressed APH-2 protein in early embryos that were obtained from aph-2 mutant hermaphrodites mated with aph-2(+) males (data not shown).

**DISCUSSION**

We have described here a new gene, *aph-2*, that is required for two cell-cell interactions in the early embryo that are mediated by a GLP-1/LIN-12/NOTCH signaling pathway. In every aspect analyzed, *aph-2* mutant embryos appear identical to mutants with specific defects in the GLP-1 pathway. For example, *aph-2* mutant embryos do not appear to have defects in a WNT-like interaction that occurs simultaneously with the GLP-1-mediated interaction, and that involves the same signaling blastomere. We have shown that the membrane-localization of the receptor GLP-1 and the ligand APX-1 do not appear altered in *aph-2* mutant embryos, suggesting that APH-2 is not required for the synthesis or localization of these proteins.

**Possible roles for APH-2 in cell interactions**

The APH-2 protein contains a sequence that is strongly predicted to be a functional signal sequence, suggesting that the protein is either membrane-associated or secreted. By immunostaining, APH-2 appears to be associated with surface membranes, and the carboxyl terminus of APH-2 contains a potential transmembrane domain or a glycosyl phosphatidylinositol (GPI)-linkage site. Our experiments on chimeric embryos demonstrate that APH-2 can be expressed by either the signaling or responding blastomere in GLP-1-mediated cell interactions. These results are consistent with the view that APH-2 functions at, or through, the cell surface, but do not resolve where the targets of APH-2 are localized. For example, secreted proteins could have targets on either the expressing or non-expressing blastomeres in the chimera. Similarly, some proteins that are associated with membranes through a GPI linkage have been shown to translocate between adjacent cells (Kooyman et al., 1995).

The amino acid sequence of APH-2 does not provide any obvious insight into the function of this protein. APH-2 is localized to the cell surface, and our chimera experiments suggest that it might act at, or through, the cell surface. Thus, it is possible that APH-2 might be required for processing or modifying the GLP-1 receptor or its ligand. For example, the NOTCH protein appears to be cleaved at the cell surface both before, and after interaction with ligand (see Introduction). A second general possibility is that APH-2 could stabilize the receptor-ligand complex, or be necessary to inhibit other extracellular components that would otherwise impede receptor-ligand interactions. The SCABROUS protein in *Drosophila* has characteristics of an extracellular membrane protein, and mutations in the *scabrous* gene affect the NOTCH signaling pathway (Ellis et al., 1994). In mammalian cells, TGF-β signaling has been shown to involve GPI-linked proteins and transmembrane proteins that are thought to act at the cell surface to regulate ligand access to receptor (Massague, 1992; Capdevila and Belmont, 1999). Similarly, extracellular heparin sulfate proteoglycans in *Drosophila* may function in Fig. 7. Immunolocalization of APH-2 protein. All panels are immunofluorescence micrographs after staining with the APH-2 antisera. (A) Wild-type gonad; cells on lower branch of the gonad are marked with an asterisk. (B) 2-cell wild-type embryo. (C) 4-cell wild-type embryo; the signaling blastomere P2 is marked with an asterisk. (D) 12-cell embryo; the signaling blastomere P2 is marked with an asterisk. (E) 4-cell *aph-2* mutant embryo.
the WINGLESS signaling pathway by enhancing or preventing ligand interactions (Hacker et al., 1997; Binari et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999).

**APH-2 and late embryonic/postembryonic cell interactions**

We have shown that APH-2 is present in all of the early blastomeres. In contrast, the ligand APX-1 and the receptor GLP-1 are expressed in only a subset of blastomeres during the 4-cell and 12-cell interactions. GLP-1, and its closely related homolog LIN-12, have complex and dynamic patterns of expression during subsequent embryogenesis, and one or both receptors ultimately are expressed in descendants of every one of the 12-cell-stage blastomeres (Evans et al., 1994; Moskowitz and Rothman, 1996; our unpublished results). Experimental studies have demonstrated that GLP-1(+) and/or LIN-12(+) functions are required in almost all of those lineages (Schnabel, 1995; Hutter and Schnabel, 1995; Moskowitz and Rothman, 1996; our unpublished observations). Defects in the 4-cell and 12-cell interactions alter the fates of numerous embryonic blastomeres, and make it difficult to assay later GLP-1 or LIN-12 functions. If APH-2 has functions in other GLP-1- and/or LIN-12-mediated interactions in subsequent embryogenesis, then the ubiquitous expression of APH-2 in the early embryo is not surprising.

APH-2 has a role in postembryonic development that does not appear to involve GLP-1, but may instead involve LIN-12. Mutations in the *aph-2* gene cause an incompletely penetrant defect in egg laying. Proper formation and function of the *C. elegans* vulva requires a series of cell interactions that are mediated by both a LIN-12 pathway and a receptor tyrosine kinase pathway during larval and adult life (for reviews see Kornfield, 1997; Greenwald et al., 1984). Formation of the first cleavage spindle in nematode embryos (Austin and Kimble, 1987). However, homozygous hermaphrodites and vulval mutant adults appear to have a normal gonad. We have found evidence for such genes in low-stringency hybridization experiments (our unpublished results).

One possible difference between the early embryonic interactions and the interactions required for gonad development is in the ligand; the APX-1 ligand, which acts at the 4-cell-stage interaction, is only 25% identical with the LAG-2 ligand, which acts in gonad development. While APX-1 can substitute for LAG-2 function when expressed under the control of lag-2 regulatory sequences, it is not yet known whether LAG-2 can substitute for APX-1 (Fitzgerald and Greenwald, 1995). A second difference is in the extracellular matrix associated with the interacting cells. The gonad has a prominent basement membrane, while the early embryonic cells lack a visible basement membrane. Although the basement membrane appears to surround, rather than separate, the interacting cells, previous studies have suggested that the basement membrane of the gonad might affect the GLP-1 signaling pathway; mutations in several *dpy* genes that encode collagens can suppress certain *gpl-1* alleles (Maine and Kimble, 1989). Thus an intriguing possibility is that gonadal basement membrane components may obviate the need for APH-2 in at least some GLP-1- or LIN-12-mediated interactions.

In summary, we have demonstrated that the APH-2 protein is a novel and essential component of the GLP-1 signaling pathway in early embryos. The association of APH-2 with plasma membranes in the embryo suggests that APH-2 may facilitate receptor-ligand interaction. Future work will distinguish whether the primary targets of APH-2 are the receptor or ligand, or other components on the cell surface. Finally, the existence of *Drosophila* and human proteins that are related to APH-2 raises the possibility that the role of APH-2 has been conserved in NOTCH pathways in other organisms.

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