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An inductive interaction in 4-cell stage *C. elegans* embryos involves APX-1 expression in the signalling cell

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SUMMARY

During the 4-cell stage of *C. elegans* embryogenesis, the P_2 blastomere provides a signal that allows two initially equivalent sister blastomeres, called ABa and ABp, to adopt different fates. Preventing P_2 signalling in wild-type embryos results in defects in ABp development that are similar to those caused by mutations in the *glp-1* and *apx-1* genes, which are homologs of the *Drosophila* genes *Notch* and *Delta*, respectively. Previous studies have shown that GLP-1 protein is expressed in 4-cell stage embryos in both ABa and ABp. In this report, we show that APX-1 protein is expressed in the P_2 blastomere and that a temperature-sensitive *apx-1* mutant has a temperature-sensitive period between the 4-cell and 8-cell stages. We propose that APX-1 is part or all of the P_2 signal that induces ABp to adopt a fate different than ABa.

Key words: APX-1, GLP-1, cell-cell interactions, cell-fate specification, *Caenorhabditis elegans*

INTRODUCTION

The *glp-1*/*lin-12*/*Notch* genes encode transmembrane proteins that function as receptors in many different cell-cell interactions in *C. elegans* and *Drosophila* and related genes have now been found in a large number of animal species (reviewed in Artavanis-Tsakonas et al., 1995). Activation of the receptor NOTCH in *Drosophila* appears to occur through cell signalling by a second transmembrane protein called DELTA (Vassin et al., 1987; Kopczynski et al., 1988; reviewed in Simpson, 1995). The *lag-2* gene of *C. elegans* encodes a transmembrane protein that is similar to DELTA; the LAG-2 protein appears to function as a ligand for both GLP-1 and LIN-12 in certain cell-cell interactions (Lambie and Kimble, 1991; Henderson et al., 1994; Tax et al., 1994; Wilkinson et al., 1994).

The cell-cell interactions mediated by these proteins can involve inductive signals between dissimilar cells, or lateral signals between initially equivalent cells. In *C. elegans* for example, *glp-1* mediates the response of proliferating germ cells to an inductive signal from the distal tip cell (DTC) (Kimble and White, 1981; Austin and Kimble, 1987). In this interaction, *glp-1* is expressed only in the responding germ cells and *lag-2* is expressed only in the DTC (Crittenden et al., 1994; Henderson et al., 1994). *lin-12* has been shown to mediate lateral signalling between two initially equivalent cells that then adopt distinct fates (AC or VU); the *lin-12* and *lag-2* genes initially are expressed in both of the interacting cells (Wilkinson et al., 1994).

During the first four cleavages of the *C. elegans* embryo, GLP-1 functions in at least two distinct cell-cell interactions (Priess et al., 1987; Hutter and Schnabel, 1994; Mello et al., 1994). The first interaction begins at the 4-cell stage, when there are two pairs of sister blastomeres called ABa and ABp, and EMS and P_2 (Fig. 1). In normal development, each of these blastomeres has a distinct pattern of cleavage and differentiation (Sulston et al., 1983). However, when ABa and ABp are born they initially are equivalent and interchangeable (Priess and Thomson, 1987). The P_2 blastomere appears to provide a signal during the 4-cell stage that causes ABp to adopt a fate that is different from ABa (Bowerman et al., 1992; Hutter and Schnabel, 1994; Mello et al., 1994; Mango et al., 1994; Moskowitz et al., 1994). A second interaction begins at the 12-cell stage when a daughter of EMS, called MS, provides a signal that influences the development of neighboring ABa descendants (Priess and Thomson, 1987; Hutter and Schnabel, 1994; Mango et al., 1994). We will refer to these stage-specific events as the 4-cell stage interaction and the 12-cell stage interaction.

GLP-1 is expressed on the surfaces of ABa and ABp and their descendants during the 4-cell stage and 12-cell stage interactions (Evans et al., 1994). Analysis of temperature-sensitive (ts) *glp-1* mutants has demonstrated requirements for *glp-1(+)* activity during each of the two interactions (Hutter and Schnabel, 1994; Mello et al., 1994). Thus, the P_2 and MS blastomeres could function as signalling cells by expressing ligands for the GLP-1 receptor. Alternatively, ABp and ABa, or their descendants, might express GLP-1 and a GLP-1 ligand simul-

taneously; lateral interactions between these blastomeres could be influenced by unrelated signalling molecules on P_2 or MS.

The *apx-1* gene was identified in screens for maternal-effect lethal mutants (Mango et al., 1994; Mello et al., 1994). Mutations in *apx-1* result in defects in ABp development that are very similar to those seen in wild-type embryos following the removal of P_2, or in *glp-1(ts)* mutants exposed to restrictive temperature during the 4-cell stage interaction (Mello et al., 1994). The *apx-1* gene encodes a transmembrane protein similar to the NOTCH ligand, DELTA, and the LIN-12/GLP-1 ligand, LAG-2 (Mello et al., 1994). These results have suggested that the APX-1 protein may function as a GLP-1 ligand during the 4-cell stage interaction. Only two mutations have been described that specifically affect the 12-cell stage interaction; these are both missense mutations in the *glp-1* gene itself (Priess et al., 1987; Kodoyianni et al., 1992).

In this paper, we address the possible roles of *apx-1* in the 4-cell stage and 12-cell stage interactions. We show a temperature-sensitive mutant has a single temperature-sensitive period (TSP) that coincides with the 4-cell stage interaction and the first embryonic TSP for *glp-1(ts)* mutants. We show that in 4-cell stage embryos *apx-1* RNA is present in all blastomeres, while the APX-1 protein accumulates only in the signalling cell, P_2. We have not detected APX-1 protein in the MS blastomere, which is the signalling cell for the 12-cell stage interaction. These results provide evidence that APX-1 is part or all of the inductive P_2 signal in the 4-cell stage interaction. These results also suggest that MS signalling at the 12-cell stage does not involve APX-1.

MATERIALS AND METHODS

Strains and alleles

Strain N2 was used as the standard wild-type strain (Brenner, 1974). Genetic markers and balancers used were: linkage group III (LGIII): *pie-1(zu154)*, *unc-25(e156)*, *qC1*. LGIV: *unc-5(e53)*, *him-8(ec56)*. LGV: *apx-1(zu183)*, *apx-1(zu347ts)*, *dpy-11(e224)*, *nT1*. LGX: *lon-2(e678)*.

Isolation of *apx-1(zu347ts)*

apx-1(zu347ts) was identified as a mutant with defective pharyngeal development and body morphogenesis in a collection of conditional embryonic lethals generated by K. Harris, M. Morrison and M. Roth (personal communication). *apx-1(zu347ts)* was mapped to LGV and was shown not to complement the transposon-induced allele *apx-1(zu183::Tc1)* using the strain *apx-1(zu183) dpy-11(e224)/nT1*. For the complementation test, *apx-1(zu183) dpy-11/nT1* males were mated to homozygous *apx-1(zu347ts)* hermaphrodites at the permissive temperature. F_1 progeny were shifted individually to 26°C at the L4 stage and scored for the production of viable progeny. Approximately one half of the F_1 animals produced dead eggs with an *apx-1* phenotype. To confirm that these F_1 animals were cross-progeny, animals that segregated *apx-1* dead eggs at 26°C were shifted down to the permissive temperature and allowed to lay viable progeny; about 1/4 of these F_2 progeny were DPY, indicating that their parents were heterozygotes for *dpy-11(e224)*.

Temperature-shift analysis

The temperature-sensitive period of *apx-1(zu347ts)* was determined by placing hermaphrodites homozygous for *apx-1(zu347ts) dpy-11(e224)* at the permissive (23°C) or non-permissive temperature (26°C) at the L4 stage for about 18 hours, during which time the larvae became adults and produced embryos. Embryos were then isolated and mounted on microscope slides, scored for cell number, then temperature-shifted to 23°C or 26°C. The embryos were allowed to develop and then scored for hatching.

The postembryonic temperature-sensitivity of *apx-1(zu347ts)* was examined by shifting newly hatched larvae to the permissive or non-permissive temperature on plates and then scoring them for the ability to produce eggs.

In situ hybridization

Whole-mount in situ hybridizations on embryos were performed as described (Seydoux and Fire, 1994, 1995). Digoxigenin (DIG)-labeled single-stranded DNA probes were synthesized by multiple cycles of primer extension in the presence of DIG-dUTP using an *apx-1* cDNA clone (pJP606) as template as described by Patel and Goodman (1992) with the following modifications. Following synthesis, sense and anti-sense probes were purified using a Qiagen nucleotide removal kit and were eluted in 50 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before dilution in 300 μl hybridization buffer (see Seydoux and Fire, 1995). No signal was detected with the sense probe (data not shown).

Generation of APX-1 antiserum

A full-length *apx-1* cDNA was cloned into pET16B (Novagen) and transformed into BL-21 (Novagen) cells. The APX-1 fusion protein was isolated on a polyacrylamide gel and the induced APX-1 band was gel purified. Rabbits from the Jackson laboratory were immunized with the purified APX-1 fusion protein, boosted monthly and bled 2 weeks after each boost. The affinity purification was achieved using nitrocellulose-bound antigen. Briefly, the APX-1 fusion protein was isolated on a polyacrylamide gel and blotted to nitrocellulose. The region of nitrocellulose containing the APX-1 fusion protein was identified by Ponceau S staining. The APX-1 antiserum was absorbed onto the nitrocellulose overnight at 4°C. The APX-1-specific antibodies were removed from the nitrocellulose using 100 mM glycine, pH 2.5, and then dialyzed against phosphate-buffered saline (PBS), pH 7.4.

Immunofluorescence

Embryos were processed for staining with APX-1 antiserum in the following manner. Adult hermaphrodites were cut open on polylysine-coated slides in 20 μl of PBS to release the gonads and embryos. The PBS was then replaced with 20 μl of fixative (4% paraformaldehyde, 60 mM Pipes, 25 mM Hepes [pH 6.8], 10 mM EGTA, 2 mM $MgCl_2$) and the embryos were squashed with a coverslip as described previously (Bowerman et al., 1993). After 5 minutes in a moist chamber, the slides were frozen on a block of dry ice and left for 10 minutes. The coverslips were then removed and slides were placed in methanol at –20°C for 5 minutes, followed by 5 minutes in acetone at –20°C. The embryos were air dried and then blocked in Tris-Tween (100 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.1% Tween), 3% BSA (bovine serum albumin) for 30 minutes at room temperature. Affinity-purified APX-1 antiserum was diluted 1:20 in the blocking solution and 10 μl was added to the embryos. Slides were incubated with the primary antibody for 2-4 hours at room temperature followed by 6 hours or overnight at 4°C. The embryos were then incubated with rhodamine-conjugated goat anti-rabbit secondary antibody for 1-2 hours at room temperature. Following each antibody incubation, embryos were washed in Tris-Tween 3 times, 5 minutes each; the last wash contained 20 ng/mL DAPI (4′, 6-diamidino-2-phenylindole) to stain chromosomal DNA. Embryos were mounted in 70% glycerol for viewing with epifluorescence.

In addition to the APX-1-specific staining, APX-1 antiserum also appeared to stain chromosome-associated structures in all embryonic cells in a cell-cycle-dependent manner (data not shown). This staining pattern persists in *apx-1(zu183)* mutant embryos, which lack all other staining. The *zu183* mutation is an insertion of the transposon Tc1 into the 3′UTR of the *apx-1* transcript and thus does not affect the *apx-1*-coding sequences.

Cortical granule tracings

To characterize the segregation of membranes during the early cleavages, granules associated with these membranes were observed in living embryos. N2 embryos were mounted on microscope slides at the 2-cell stage and video-recorded under Nomarski differential interference optics using a Hamamatsu CCD C2400 camera. Recordings were played back in real time and individual cortical granules were marked at the 4-cell stage. These granules were traced back to the 2-cell stage on acetate sheets mounted to the video screen. Granules that remained visible throughout the 2-cell to 4-cell cleavages were chosen for tracing.

RESULTS

An *apx-1 (ts)* mutant has a TSP between the 4-cell and 8-cell stages

apx-1(zu347ts) is a temperature-sensitive allele of *apx-1* identified in a screen for conditional embryonic lethal mutations. At 23°C, the permissive temperature, adults homozygous for *apx-1(zu347ts)* produce viable embryos. At 26°C, the non-permissive temperature, these adults produce all inviable embryos. These inviable embryos are morphologically indistinguishable from those produced by non-conditional *apx-1* mutants described previously (Mello et al., 1994; Mango et al., 1994).

Because APX-1 has been proposed to be a ligand for the GLP-1 receptor, we wanted to compare the embryonic TSP of the *apx-1(zu347ts)* mutant with the previously determined TSPs of *glp-1(ts)* mutants. *glp-1(ts)* mutants show two TSPs during embryogenesis, one between the 4-cell and 8-cell stages (corresponding to the 4-cell stage interaction) and a second between the 12-cell and 28-cell stages (corresponding to the 12-cell stage interaction) (Mello et al., 1994). To determine the embryonic TSP for *apx-1(zu347ts)*, embryos from homozygous mothers were up-shifted (permissive to non-permissive) or down-shifted (non-permissive to permissive) at various times between the 1-cell and 28-cell stages of embryogenesis and later scored for hatching (Fig. 2; Table 1). We found that none of the embryos up-shifted at the 2-cell stage hatched and that these embryos had morphological abnormalities similar to those of non-conditional *apx-1* mutant embryos. In contrast, 94% of the embryos that were up-shifted at the 12-cell stage hatched. In reciprocal experiments, 82% of the embryos down-shifted at the 2-cell stage hatched, while only 3% of the embryos down-shifted at the 12-cell stage hatched. These results define a single TSP for *apx-1 (zu347ts)* between the 4-cell and 8-cell stages of embryogenesis (Fig. 2). The TSP of *apx-1(zu347ts)* thus coincides with the first, but not the second, embryonic TSP of *glp-1(ts)* mutants.

After hatching, *C. elegans* undergoes four distinct larval stages before becoming an adult. Each larval stage involves cell division and differentiation (Sulston and Horvitz, 1977), and important cell-cell interactions are known to occur during postembryonic development (reviewed in Katz and Sternberg, 1992). We asked whether *apx-1(zu347ts)* had effects on post-embryonic development by shifting newly hatched larvae to the restrictive temperature. We found that 100% (n=49) of these shifted larvae developed into apparently normal adults that produced eggs with an *apx-1* phenotype.

apx-1 RNA is not spatially localized in early embryos

An understanding of *apx-1* expression patterns is essential in evaluating its roles in the 4-cell and 12-cell stage interactions. We examined the distribution of *apx-1* RNA in embryos by in situ hybridization. *apx-1* RNA is detected in newly fertilized eggs, consistent with previous genetic results demonstrating that *apx-1* must be expressed maternally. Between the 1-cell and 8-cell stages, *apx-1* RNA is present in all blastomeres at apparently equivalent levels (Fig. 3). After the 8-cell stage, *apx-1* RNA rapidly disappears from all somatic blastomeres in a pattern that has been described previously for several unrelated maternal RNAs (Seydoux and Fire, 1994). In 12-cell stage embryos, *apx-1* RNA is visible in the P_3 blastomere, but disappears rapidly from MS and all other blastomeres (Fig. 3G). In 36-cell stage and later embryos, *apx-1* RNA can be detected in one to five unidentified nuclei as double spots of staining (Fig. 3I). This type of nuclear staining has been reported in the analysis of other RNAs and is thought to result from embryonic, rather than maternal, transcription (Seydoux and Fire, 1994). We conclude that in early embryos maternal *apx-1* RNA is present in both the signalling and responding cells during the 4-cell stage interaction and that embryonic transcription of *apx-1* is not detected in either the signalling or responding cells during the 12-cell stage interaction.

APX-1 protein is localized to the signalling cell

Rabbit polyclonal antiserum was generated against a bacterially expressed, full-length *apx-1* fusion protein and affinity-purified. The staining patterns that we describe below were not observed with preimmune serum, nor were they observed in embryos from mothers homozygous for *apx-1(zu183::Tc1)*, a transposon-induced allele. We thus believe that these staining patterns represent the distribution of the APX-1 protein.

APX-1 protein is detected first in late 2-cell stage embryos at the anterior periphery of P_1, where P_1 contacts the AB blastomere (Fig. 4A). Because APX-1 has a predicted membrane-spanning domain and is closely related to the membrane protein DELTA in *Drosophila*, we will describe this peripheral staining as representing plasma membrane localization. In approximately 75% of the embryos, APX-1 appears to be present uniformly across the anterior membrane of P_1. However, the remainder of embryos show a gap in the staining pattern in one sector of the anterior P_1 membrane. We do not know the significance of this non-uniform staining, nor whether the position of the gap is the same in all embryos, relative to the future position of blastomeres at the 4-cell stage. In addition to the peripheral, presumably membranous, staining pattern, APX-1 also is detected in small, numerous, punctate structures underlying the P_1 membrane (Fig. 4A). We will describe this as cortical staining. A few punctate structures also are visible throughout the P_1 cytoplasm. Similar punctate structures have been observed in *Drosophila* embryos stained with an antibody that recognizes the DELTA protein and may represent ligand-containing vesicles going to or from the membrane (Kooh et al., 1993).

In embryos where AB has completed division into ABa and ABp, and P_1 is in the middle of division, the punctate APX-1-containing structures remain visible near the surface where P_1 contacts ABa and ABp. However, membrane staining is visible only where P_1 contacts ABp (Fig. 4D). In early 4-cell stage embryos, APX-1 staining becomes prominent in P_2 at the anterior membrane where P_2 contacts ABp and in punctate structures underlying this membrane (Fig. 4G). APX-1 also is detected as punctate structures in the cytoplasm of EMS (data not shown); these structures are no longer detected in slightly

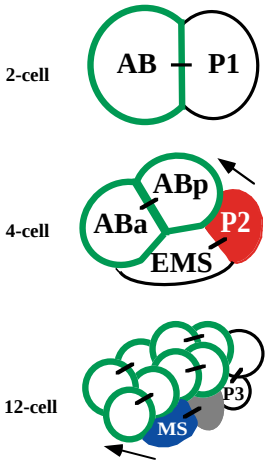

Fig. 1. Early embryonic stages. The 2-cell stage embryo consists of an anterior blastomere, called AB, and a slightly smaller, posterior blastomere, called P_1. At the next cleavage cycle, AB divides before P_1, such that the AB daughters have separated by the time P_1 is in metaphase. The AB spindle initially is oriented perpendicular to the long axis of the egg. As the AB spindle elongates, it pushes against the eggshell surrounding the embryo and is forced to rotate. This rotation causes the AB daughters, ABa and ABp, to make asymmetrical contacts with the two P_1 daughters, EMS and P_2; at the 4-cell stage, EMS contacts both ABa and ABp, while P_2 (shown in red) contacts only ABp. By the 12-cell stage, ABa and ABp have undergone two synchronous divisions to produce the eight AB great-granddaughters. The anterior daughter of EMS is the MS blastomere (shown in blue) and the anterior daughter of the P_2 blastomere is P_3. Arrows indicate the GLP-1-mediated 4-cell and 12-cell interactions. AB and its descendants are outlined in green to represent the localization of the receptor GLP-1 (Evans et al., 1994).

later 4-cell stage embryos (Fig. 4G). APX-1 is not detected in either ABp or ABa.

At the 12-cell stage, APX-1 is detected faintly in the cytoplasm of P_3, but is not detected in MS, or any of the ABp

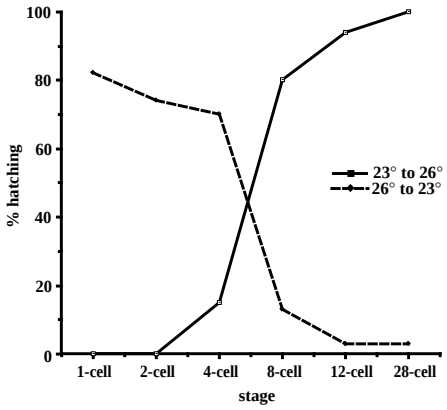

Fig. 2. The temperature-sensitive period of *apx-1(zu347ts)*. The temperature-sensitive period of *apx-1(zu347ts)* was determined by up-shift (23°C to 26°C, solid line) and down-shift (26°C to 23°C, dashed line) experiments on embryos from mothers homozygous for *apx-1 (zu347ts)* (See also Table 1). The TSPs for *glp-1(ts)* mutants are between the 4-cell and 8-cell stages and between the 12-cell and 28-cell stages (Hutter and Schnabel, 1994; Mello et al., 1994). *apx-1(zu347ts)* has a TSP very similar to the first TSP for *glp-1(ts)*.

Table 1. Temperature-shift analysis of *apx-1* (*zu347ts*)

	23°C→26°C	26°C→23°C
Stage	Hatching/total	Hatching/total
1-cell	0/27	27/33*
2-cell	0/34	26/35*
4-cell	7/48	30/44
8-cell	40/50	5/39
12-cell	30/32	1/36
28-cell	31/31	1/35

Embryos were isolated from mothers homozygous for *apx-1*(*zu347ts*) at 23°C (permissive) or 26°C (non-permissive), scored for embryonic stage, and shifted to 26°C or to 23°C, respectively. The embryos were allowed to develop, then scored for hatching.

*The few down-shifted 1-cell and 2-cell stage embryos that did not hatch did not resemble previously described *apx-1* mutants. These embryos were nearly normal but appeared to have morphogenesis defects late in embryogenesis. The basis for this defect is not known.

or ABa descendants (Fig. 4J). In later embryonic stages, faint APX-1 staining could be detected in a few cells whose identities were not determined in this study. We conclude that during the 4-cell stage interaction, APX-1 is present in the signalling cell, P_2, but that during the 12-cell stage interaction there is no evidence of APX-1 expression in either the signalling or responding blastomeres.

Blastomere contacts during the 2-cell to 4-cell division

Several models could explain the relationship between APX-1 expression in the P_1 and P_2 blastomeres. For example, membrane-associated APX-1 in the P_1 blastomere could be segregated during division to the nascent P_2 blastomere. Alternatively, APX-1 in P_1 could be degraded during division and re-synthesized only in P_2. At present, we cannot follow the movement of APX-1 directly in the P_1 membrane during division. However, it is possible to characterize the movement of granules closely associated with the membrane using video light microscopy (Hird and White, 1993). We define a blastomere's cortex to be the cytoplasm underlying its surface membrane to a depth of approximately 1 μm, corresponding to a thickness of 2-3 cytoplasmic granules. We filmed dividing early embryos and numbered individual cortical granules at the 4-cell stage (Fig. 4C,F,I). Granules 1-6 in Fig. 4I correspond to the cortex of EMS that is adjacent to ABa and ABp, and granules 7-9 correspond to the cortex of P_2 that is adjacent to ABp. These granules were then traced back to the 2-cell stage embryo (Fig. 4C). We found that granules 1-6 were originally in the P_1 cortex adjacent to the AB blastomere and that granules 7-9 were originally in a region of the P_1 cortex that was not adjacent to AB. Because the relative positions of granules at the

Table 2. Detection of APX-1 in P2 at the 4-cell stage in *pie-1* mutants

Wild-type	2/30
Cytoplasmic only	8/30
Faint membrane	4/30
Staining absent	16/30

Embryos at the 4-cell stage from mothers homozygous for *pie-1*(*zu154*) were fixed and stained for APX-1.

AB and P_1 interface remain fairly constant during divison, we believe the cortical regions of AB and P_1 that were adjacent in the 2-cell embryo remain adjacent in 4-cell embryos as the junction between EMS and the AB daughters. Thus the cortical APX-1 staining in the P_2 blastomere appears to occur at a site on the cortex where no staining was visible in earlier, 2-cell stage embryos. In contrast, the cortical regions that stained positively for APX-1 in 2-cell stage embryos do not appear to stain in late 4-cell stage embryos. Therefore we suggest that most, if not all, of the APX-1 associated with the cortex of P_1 is partitioned to EMS at division and that APX-1 expression in P_2 may represent new synthesis or membrane targeting (see Discussion).

pie-1 mutations affect APX-1 expression

Previously described mutations in the *pie-1* gene have incompletely penetrant defects in ABp development, such that ABp can fail to produce the cell types that normally result from P_2 signalling (Mango et al., 1994; C. C. Mello, unpublished results). *pie-1*(+) activity could be required in ABp for the GLP-1-mediated response to P_2 signalling. However, *pie-1*(+) activity is known to be required for the correct specification of the fate of the P_2 blastomere and thus *pie-1* mutations could cause abnormal ABp development by disrupting P_2 signalling. Since our present study suggests that APX-1 is the P_2 signal, we used the APX-1 antiserum to stain embryos from mothers homozygous for *pie-1(zu154)* to determine if APX-1 was expressed and localized correctly at the 4-cell stage.

We found that in 93% (n=30) of the 4-cell stage embryos examined, APX-1 was either not detectable in P_2 or was present at reduced levels relative to wild-type embryos (Fig. 5, Table 2). In several of the 4-cell stage *pie-1(zu154)* mutant embryos (8 of 30), APX-1 was present in the cytoplasm of the P_2 blastomere in punctate granules similar to those observed in a wild-type EMS blastomere early in the 4-cell stage (Table 2). In most 2-cell stage *pie-1(zu154)* mutant embryos, APX-1 was not localized to the P_1 anterior membrane but could be detected faintly in the cytoplasm (data not shown). While it is unclear how *pie-1* affects the expression or localization of APX-1, we can conclude that the ABp defect in *pie-1(zu154)* mutants is likely to be the result of a disruption in the expression or maintenance of APX-1.

DISCUSSION

During the 4-cell stage of *C. elegans* embryogenesis, the ABp blastomere becomes different from its sister, ABa, through interactions with the P_2 blastomere. The specification of the ABp fate requires the wild-type activities of *glp-1*, which encodes a transmembrane receptor protein, and *apx-1*, which encodes a presumptive GLP-1 ligand (Austin and Kimble, 1989; Yochem and Greenwald, 1989; Hutter and Schnabel, 1994; Mango et al., 1994; Mello et al., 1994; Moskowitz et al., 1994). In 4-cell stage embryos, GLP-1 protein is present in both ABp and ABa (Evans et al., 1994). In this study, we have shown that the APX-1 protein is present only in the P_2 blastomere, which contacts ABp but not ABa. Our analysis of an *apx-1(ts)* mutant indicates that this mutant has a single TSP between the 4-cell and 8-cell stages of embryogenesis. This TSP is very similar to the first embryonic TSP of *glp-1(ts)* mutants and corresponds to the

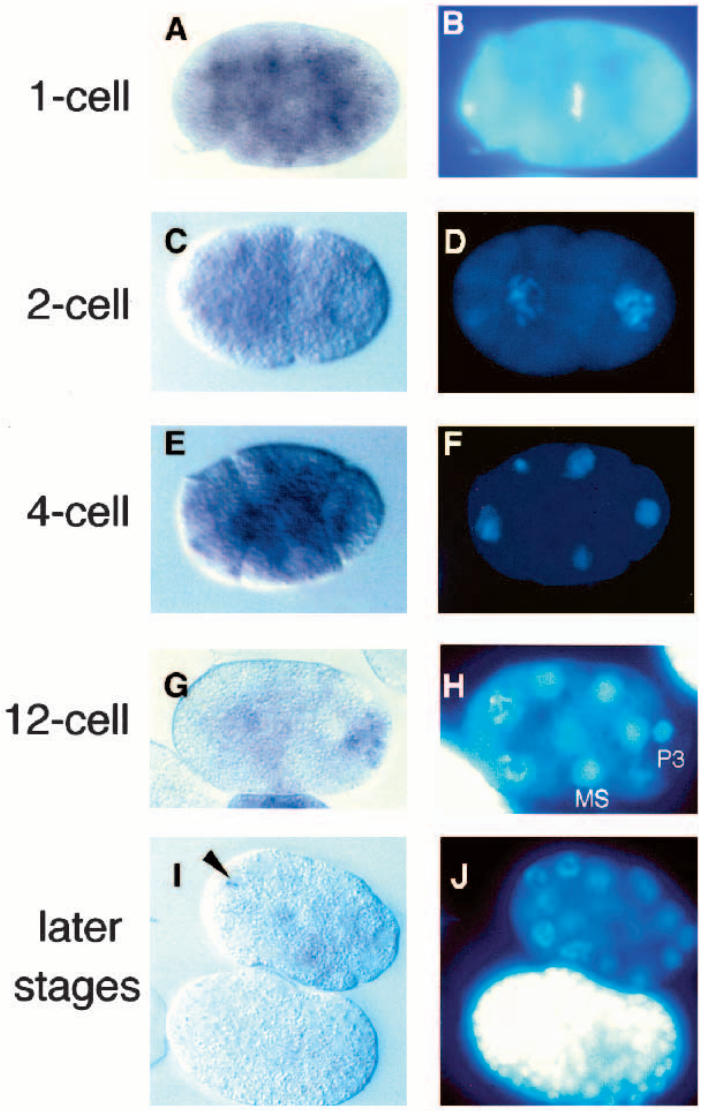

Fig. 3. Localization of *apx-1* mRNA in *C. elegans* embryos by whole-mount in situ hybridization. The left column shows embryos hybridized to *apx-1* antisense probe visualized using alkaline-phosphatase-mediated detection (purple color indicates presence of *apx-1* RNA). Micrographs of each embryo stained with the DNA-binding dye DAPI are shown at right. Maternal *apx-1* mRNA is uniformly distributed in early embryos (A-F). By the 12-cell stage (G,H), maternal RNA is being degraded rapidly in somatic blastomeres so that *apx-1* RNA is present in the germline precursor P_3, but little is detectable in other cells, including MS. (I,J) Beginning at approximately the 36-cell stage, double spots of nuclear staining (arrowhead), presumably the result of embryonic *apx-1* transcription (Seydoux and Fire, 1994), are evident in 1 (shown here) to 5 unidentified cells. No staining is detected in embryos containing more than 100 cells (bottom embryo). All embryos are oriented with anterior at left and dorsal at top. Embryos measure approximately 50 μm along their anterior-posterior axis. Images in the left column were obtained using Nomarski differential interference optics; images on the right were obtained using epifluorescence with UV illumination. The highly condensed DAPI-staining foci at the anterior tip of the 1-cell embryo (B) and in the upper-left of the 4-cell embryo (F) are polar bodies.

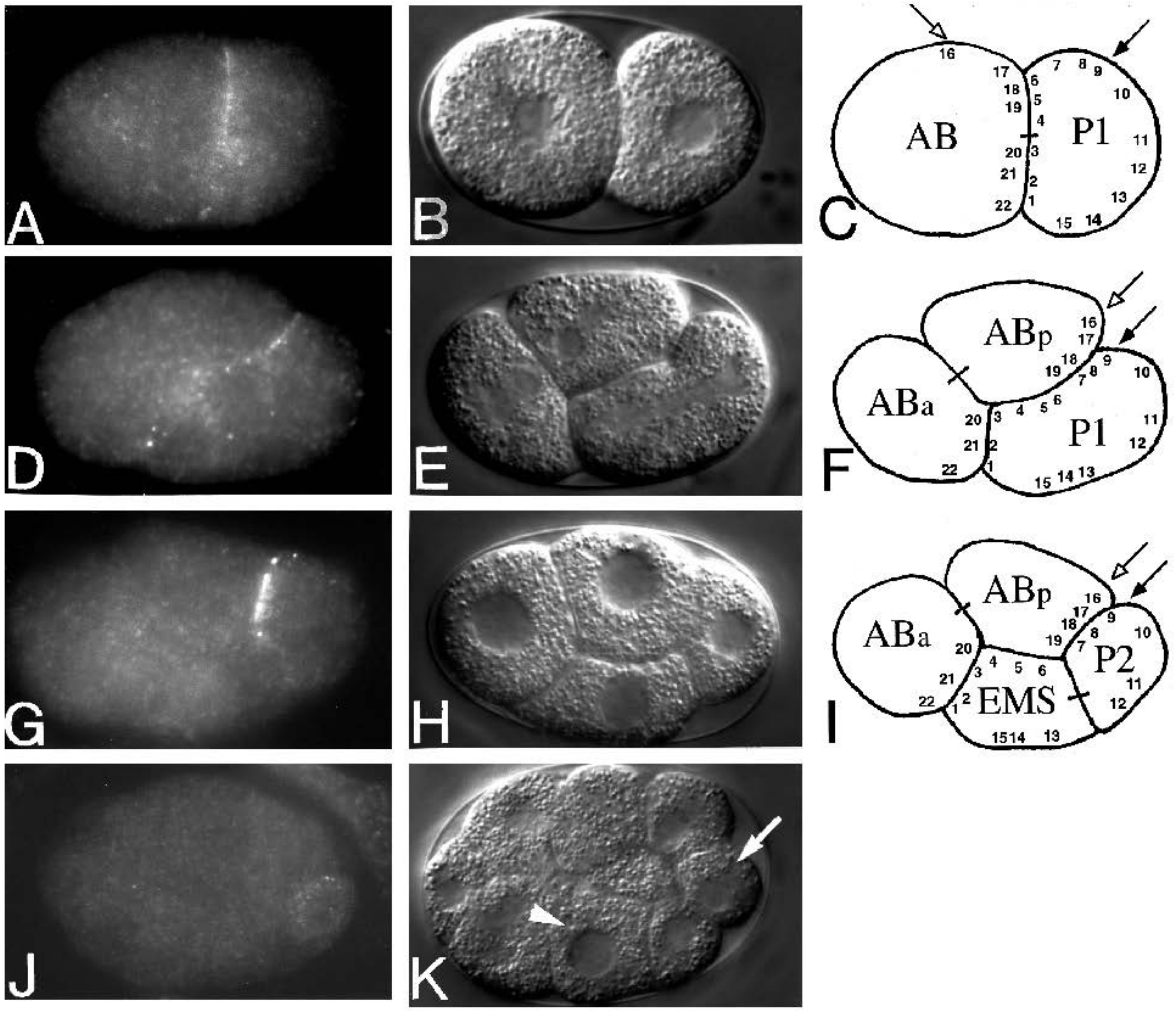

Fig. 4. APX-1 is localized to the P lineage. Each row represents embryos at a similar stage in development. (A-C) 2-cell stage. (D-F) 3-cell stage, with P_1 in the middle of division. (G-I) 4-cell stage. (J,K) 12-cell stage. The leftmost column (A,D,G,J) shows immunofluorescence micrographs of embryos stained for APX-1 protein. APX-1 first appears in 2-cell stage embryos (A) in the P_1 blastomere, predominately at the junction of P_1 and AB. During the division of P_1 (D), APX-1 appears in numerous granules in the region of P_1 that becomes the EMS blastomere. In late 4-cell stage embryos (G), APX-1 staining is restricted to the junction between the P_2 and ABp blastomeres. In 12-cell stage embryos (J), APX-1 can be detected in the P_3 blastomere (see arrow in K) but not in the MS blastomere (see arrowhead in K). The middle column (B,E,H,K) shows Nomarski micrographs of living embryos at each stage. The rightmost column (C,F,I) shows successive tracings from video images of a single living embryo between the 2-cell and 4-cell stages. The names of the blastomeres are indicated and sister blastomeres are connected by short dashes. Numbers represent the approximate location of individual cortical granules whose positions were followed during cell division. The division of P_1 appears to juxtapose cortical regions of AB (open arrows) and P_1 (closed arrows) that were not in contact previously. Note that granules 1-6 in P_1 at the 2-cell stage (C) are located near the region of APX-1 staining (A) and that each of these granules is in the EMS cortex at the 4-cell stage (I). In contrast, granules 7-9, which are in the cortex of P_1 that does not contact AB (C) are localized to the junction of ABp and P_2 in 4-cell stage embryos (I) where APX-1 is localized (G).

time during which P_2 signals ABp. Finally, we have shown that *pie-1* mutants, which can fail to specify the fate of ABp, have defects in the expression and localization of APX-1. These results together provide strong evidence that APX-1 is part or all of the P_2 signal.

APX-1 localization

At the 2-cell stage, APX-1 protein is present predominantly at the anterior periphery of the P_1 blastomere, which is the parent of both P_2 and EMS. We propose that most or all of this protein is partitioned to EMS and subsequently is degraded during the 4-cell stage. We have observed the anterior cortex of P_1, and the adjacent posterior cortex of AB, in living embryos during the division of P_1. We find that this region of contact appears to be maintained as P_1 divides, and that it becomes the junction between EMS and the AB daughters in 4-cell stage embryos. This model of cortical segregation is consistent with previous observations on the division of AB and P_1. During division of the AB blastomere, the confining eggshell forces ABp posteriorly, altering the cleavage plane of P_1. If the eggshell is removed, AB divides transversely and P_1 undergoes a simple anterior-posterior division; in such embryos, the anterior half of P_1 becomes EMS and the posterior half becomes P_2 (Hyman and White, 1987). Many of these initially ‘T-shaped’ embryos

eventually establish contact between the ABp and P_2 blastomeres, and develop normally (Wood and Kershaw, 1991; Schierenberg and Junkersdorf, 1992). Thus we consider it likely that most, or all, of the APX-1 protein at the anterior cortex of P_1 can be partitioned to the EMS blastomere without affecting development. We observe numerous, punctate APX-1-containing granules in EMS that disappear during the 4-cell stage, consistent with the hypothesis that APX-1 is degraded in EMS.

During the 4-cell stage the level of APX-1 staining in the P_2 blastomere increases although *apx-1* RNA is present uniformly in all 4-cell stage blastomeres. Therefore the asymmetric distribution of APX-1 protein may involve regulation at the level of translation, membrane targeting, or protein stability. It is possible that *apx-1* mRNA is not translated in the EMS, ABa and ABp blastomeres, or that APX-1 protein is synthesized but degraded in these blastomeres before it accumulates to detectable levels.

Maternally expressed *glp-1* mRNA also is present in all 2-cell and 4-cell stage blastomeres, although GLP-1 protein is detected only in AB and its daughters, ABa and ABp (Evans et al., 1994). Thus GLP-1 and APX-1 have nearly reciprocal patterns of expression. GLP-1 protein has been shown to be restricted to AB and AB descendants, by specific elements of the *glp-1* mRNA (Evans et al., 1994). It will be of interest to determine how APX-1 is restricted to P_1 descendants and whether this mechanism is coupled to, or independent from, the factors that regulate GLP-1 expression.

APX-1 asymmetry within the P lineage

APX-1 is localized asymmetrically at the anterior peripheries of the P_1 and P_2 blastomeres. This asymmetry may result from an intrinsic anterior-posterior polarity in these blastomeres. The *par* genes play a role in establishing the anterior-posterior polarity of the embryo and encode gene products that are asymmetrically distributed in the embryo (Cheng et al., 1995; Kemphues et al., 1988; Levitan et al., 1994; Morton et al., 1992). PAR-3 protein has been shown to be localized to the anterior cortex of the 1-cell embryo and to the anterior cortices of the P_1 and P_2 blastomeres in 2-cell and 4-cell stage embryos, respectively (Etemad-Moghadam et al., 1995). In contrast, the PAR-1 protein is localized to the posterior cortex of the 1-cell embryo and at the posterior cortices of P_1 and P_2 (Guo and Kemphues, 1995). APX-1 localization in P_1 and P_2 is similar to that of the PAR-3 protein and could be controlled by similar mechanisms.

Alternatively, the anterior localization of APX-1 within the P_1 and P_2 blastomeres could result from interactions with neighboring blastomeres. For example, GLP-1 protein in AB could play a role in localizing APX-1 toward the side of P_1 that contacts AB. It should be possible to discriminate between these models by analyzing APX-1 localization in isolated P_1 or P_2 blastomeres or in *glp-1* mutants.

The MS signal

At the 4-cell stage, the P_2 blastomere functions as a signalling cell but its sister, EMS, does not. However, when EMS divides, one of its daughters, MS, becomes a signalling cell. Since both the P_2 and MS signals activate GLP-1 in AB descendants, it seemed possible that the P_2 and MS signals were identical or related proteins. Indeed, recent studies with chimeric embryos suggest that P_2 can substitute for MS as a signalling cell in the 12-cell stage interaction (C. Shelton and B. Bowerman, personal communication). Although our results strongly suggest that APX-1 is the P_2 signal, we find no evidence for APX-1 being the MS signal. Previous work suggested that the MS signal may require an embryonically transcribed component (Mello et al., 1994); we did not detect embryonic transcription of *apx-1* in MS, though transcription was detected in other cells later in development. While APX-1 protein is present at high levels in P_2 at the 4-cell stage, we have not been able to observe any APX-1 protein in the MS blastomere. Finally, we also have shown that *apx-1(ts)* mutants have only one temperature-sensitive period, corresponding to the 4-cell stage interaction. This *apx-1(ts)* mutation does not result in any apparent defects in MS signalling, nor do any of the non-conditional *apx-1* mutations isolated to date (Mango et al., 1994; Mello et al., 1994).

The *C. elegans* genes *apx-1*, *lag-2* and *arg-1* can all encode closely related proteins, and appear to be functionally interchangeable (Mello et al., 1994; Henderson et al., 1994; Tax et al., 1994; Fitzgerald and Greenwald, 1995; the Genbank accession number for *arg-1* is U50143). LAG-2 has been shown to function as a GLP-1 ligand in some cell-cell interactions in late embryonic and in postembryonic development, and the function of *arg-1* has not been established (Lambie and Kimble, 1991; Henderson et al., 1994; Tax et al., 1994; Wilkinson et al., 1994). We have constructed a strain that produces embryos homozygous for chromosomal deficiencies deleting the *apx-1*, *lag-2* and *arg-1* genes simultaneously. Such embryos appear to have the normal 12-cell stage interaction, indicating that embryonic expression of these genes is not required for MS signalling (C. C. M., unpublished). It remains possible that maternal *lag-2* or *arg-1* functions in MS, or that MS signalling occurs through a fourth APX-1-related protein, or through a novel protein.

In summary, the fates of AB descendants are diversified rapidly during the first few cleavages of the *C. elegans* embryo. We have shown here that the 4-cell stage interaction involves two asymmetries. The first asymmetry is that the receptor GLP-1 and the ligand APX-1 are not expressed in the same blastomeres, preventing the types of lateral interactions observed with the AC/VU cells during larval development in *C. elegans*. The second asymmetry is that APX-1 is present in only one P_1 daughter, in contrast to GLP-1 which is expressed in both AB daughters. This asymmetrical pattern of expression results in only one of the GLP-1-expressing daughters coming

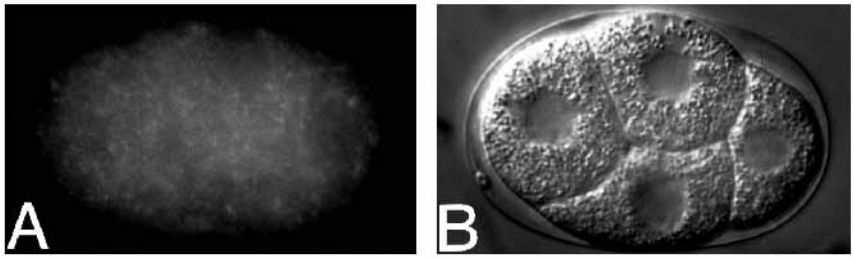

Fig. 5. APX-1 is reduced or absent in the P_2 blastomere of *pie-1(zu154)* mutants. Fluorescence micrograph of a 4-cell embryo stained for APX-1 (A) and a Nomarski micrograph of a similar stage to visualize cell membranes (B). APX-1 is not detected in the P_2 blastomere of a *pie-1(zu154)* 4-cell embryo, consistent with the *pie-1* phenotype of a loss of ABp-specific fates. (See also Table 2.)

into contact with ligand at the 4-cell stage, thus allowing the AB daughters to adopt different fates.

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