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Time-dependent responses to *glp-1*-mediated inductions in early *C. elegans* embryos

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SUMMARY

In an embryo of the nematode *Caenorhabditis elegans*, two blastomeres at the 4-cell stage, ABa and ABp, are born with equivalent developmental potential. Subsequently, interactions with the P_2 blastomere at the 4-cell stage and the MS blastomere at the 12-cell stage generate differences in developmental fate among descendants of ABa and ABp. We have reproduced these inductions in vitro using embryonic blastomeres isolated in cell-culture medium. We show that during these inductions only the responding AB descendants require the activity of the *glp-1* gene, which is similar in sequence to *Drosophila Notch*, supporting models in which GLP-1 protein acts as a receptor for both the P_2 and MS signals. We also show that P_2 signaling requires the activity of the *apx-1* gene, similar in sequence to *Drosophila Delta*, and that MS signaling requires the putative transcription factor SKN-1. We present evidence that the primary factor determining the different responses to these two signals is the age of the AB descendants, not the identity of the signaling cell or ligand. Therefore, we suggest that time-dependent changes in factors within AB descendants are responsible for their different responses to inductive signals that use a common receptor.

Key words: *glp-1*, *apx-1*, *skn-1*, *Caenorhabditis elegans*, embryogenesis, cell signaling

INTRODUCTION

During animal development, cells that initially have identical developmental potential often produce progeny with very different patterns of cell fate. For example, in a 2-cell-stage *C. elegans* embryo, division of the anterior blastomere, called AB, produces two daughters, ABa and ABp, that eventually give rise to very different cell types. Certain descendants of ABa form the anterior portion of the pharynx, the neuromuscular feeding organ of *C. elegans*. In contrast, descendants of ABp do not contribute to the formation of the pharynx; instead, ABp descendants produce a number of unique cell types, including intestinal-rectal valve cells and muscle cells associated with the anus and rectum (Sulston et al., 1983). Despite these differences, ABa and ABp can be experimentally switched at the 4-cell stage without disrupting embryogenesis, demonstrating that these blastomeres are developmentally equivalent (Priess and Thomson, 1987). Thus, the different fates of ABa and ABp descendants depend on external influences from neighboring blastomeres.

Two interactions that specify, in part, the different developmental fates of ABa and ABp descendants occur at the 4-cell and 12-cell stages of embryogenesis (Fig. 1). Production of ABp-specific cell types requires a signal from the P_2 blastomere at the 4-cell stage. Removal of P_2 early in the 4-cell stage prevents the production of intestinal-rectal valve cells and increases the production of pharyngeal cells (Mango et al., 1994a; Moskowitz et al., 1994), suggesting that, in the absence of P_2 signaling, ABp adopts a fate like that of ABa. At the 12-cell stage, the MS blastomere signals two granddaughters of ABa to produce pharyngeal cells (Fig. 1); when MS is killed by laser ablation before the ABa granddaughters are born, pharyngeal cells are no longer made (Hutter and Schnabel, 1994; Mango et al., 1994b).

Genetic analyses have identified some of the molecules required for the 4-cell and 12-cell-stage inductions. Maternal expression of *apx-1* is required for the P_2 induction at the 4-cell stage. In embryos from homozygous *apx-1* mutant mothers, ABp appears to adopt an ABa-like fate, resulting in the loss of ABp-specific cell types and the production of pharyngeal cells by ABp in response to the MS signal (Mango et al., 1994a; Mello et al., 1994). The sequence of *apx-1* is similar to a group of genes, exemplified by *Drosophila Delta*, that function in many inductive events and encode membrane-bound ligands for a related family of receptors, represented in *Drosophila* by Notch (Fehon et al., 1990; Fortini and Artavanis-Tsakonas, 1994; Mello et al., 1994; reviewed in Artavanis-Tsakonas et al., 1995 and Muskavitch, 1994). Thus, *apx-1* may encode the ligand used by P_2 to signal ABp.

The MS signal is unaffected by mutations in *apx-1*, implying the use of a different ligand. Though a genetic candidate for the MS ligand has not been identified, the production of pharyngeal cells by ABa descendants depends on the activity of the *skn-1* gene (Bowerman et al., 1992a). *skn-1* encodes a putative transcription factor required for the correct specification of the EMS blastomere, the progenitor of MS (Blackwell

et al., 1994; Bowerman et al., 1992a). SKN-1 protein is found in both EMS and its daughter, MS (Bowerman et al., 1993). Consequently, SKN-1 might regulate the ability of MS to express an inductive signal, perhaps by activating transcription of a ligand-encoding gene. However, SKN-1 is also present, albeit at a lower level, in descendants of the AB blastomere. Thus, it also is possible that SKN-1 is required within AB descendants for responding to the MS signal.

The *glp-1* gene appears to encode a receptor for both the P_2 and MS signals. *glp-1* is required for multiple signaling events during development in *C. elegans*. Genetic mosaic studies and the distribution of the GLP-1 protein indicate that GLP-1 acts as a receptor in the regulation of germ-line proliferation during postembryonic development (Austin and Kimble, 1987; Crittenden et al., 1994). Certain mutant alleles of *glp-1* also demonstrate a maternal requirement during embryogenesis, eliminating both the P_2 induction of ABp-specific cell types and the MS induction of ABa-derived pharyngeal cells (Hutter and Schnabel, 1994; Mello et al., 1994). Furthermore, temperature-shift experiments with conditional alleles of *glp-1*, *e2141ts* and *e2144ts*, show that *glp-1* activity is required at both the 4-cell and 12-cell stages for the P_2 and MS inductions (Hutter and Schnabel, 1994; Mello et al., 1994). Finally, GLP-1 is similar in sequence to *Drosophila* Notch (Yochem and Greenwald, 1989), and antibody staining in the early embryo shows that GLP-1 is present at the plasma membranes of ABa and ABp, and their descendants, until the 28-cell stage (Evans et al., 1994). Therefore, GLP-1 and the predicted APX-1 protein may act as a receptor/ligand pair for the P_2 induction, and GLP-1 also may act as a receptor for an as yet unidentified MS ligand.

The requirement for *glp-1* in both the P_2 and MS inductions suggests a common molecular mechanism, despite the fact that the two signals result in very different responses. Though genetic studies have identified key regulatory molecules, it is not known how the P_2 and MS signals effect their specific responses. Furthermore, the responding cells are derived from two blastomeres, ABa and ABp, that are born with equivalent developmental potential. The inductions do, however, originate from different cells and occur at different times. Two models can explain how the P_2 and MS signals elicit different responses from AB descendants at the 4-cell and 12-cell stages. First, P_2 and MS might express different signals, perhaps by using different ligands or by expressing additional molecules that would exert specific effects on the responding cells. Alternatively, factors expressed by the responding AB descendants might change over time, causing qualitatively similar signals to be interpreted differently.

To test the postulated roles for the maternally expressed *glp-1*, *apx-1* and *skn-1* gene products within individual blastomeres and to understand how the P_2 and MS signals generate specific responses, we have used cell culture techniques to reproduce these embryonic inductions in vitro. Our results show that *glp-1* function is required only in the responding blastomeres, supporting a model in which GLP-1 functions as a receptor for both the P_2 and MS signals. *apx-1* function is required in P_2, consistent with the predicted APX-1 protein functioning as a P_2-specific ligand. We also show that the putative transcription factor SKN-1 is required to activate the MS signal but is not required for ABa descendants to respond. Finally, we demonstrate that the specific responses depend not on the identity of the inducing blastomere, but on the age of the AB descendants when the signal is received. We suggest that factors regulated in a time-dependent manner within AB descendants determine how *glp-1*-mediated inductive signals are interpreted.

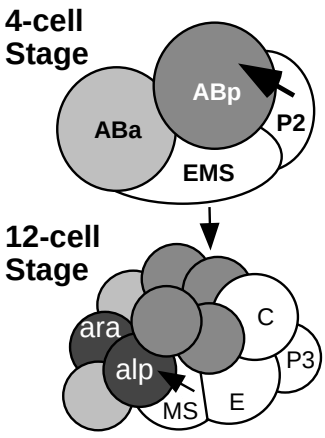

Fig. 1. Schematic of sequential inductions acting on the ABa and ABp cells at the 4-cell stage and their descendants at the 12-cell stage in a *C. elegans* embryo. ABa, ABp and their descendants are shaded to indicate their responses to signaling from P_2 and MS. Early in the 4-cell stage, a signal from the posterior-most blastomere, P_2, induces the production of ABp-specific cell types, including intestinal-rectal valve cells and muscle cells. A second signal occurs at the 12-cell stage when the ventrally positioned MS blastomere signals ABara and ABalp to produce anterior pharyngeal cells. Additional signals, not shown here, also specify anterior/posterior and left/right differences in cell identity among 12-cell-stage AB descendants (Hutter and Schnabel, 1995a,b).

MATERIALS AND METHODS

Isolation and manipulation of blastomeres

Nematode culture methods are described in Brenner (1974) and the *C. elegans* embryonic lineage is described in Sulston et al. (1983). Embryonic cells were obtained by removal of the eggshell and vitelline membrane as described in Edgar (1995) and Goldstein (1992) with the following modifications. Cells were incubated in a modified cell culture medium (see below) in a sealed chamber: a 1 mm thick plastic spacer with a 1 cm square hole in the center was sealed to a siliconized slide using Vaseline. An approximately 40 μl drop of medium was placed in the hole of the spacer and sealed by placing a coverslip on top of the spacer.

Hypochlorite treatment, which is used to weaken the eggshell (Edgar, 1995), damages very young embryos, precluding the isolation of properly differentiating blastomeres from early 2-cell embryos. Consequently, blastomeres were obtained from late 2-cell and 4-cell-stage embryos. AB descendants were obtained by isolating the AB blastomere and allowing divisions to occur in vitro in drops of culture medium. P_2 was obtained in two ways: allowing an isolated P_1 to divide in vitro or isolating P_2 directly from a 4-cell embryo. MS and E were obtained by taking EMS, the parent of MS and E, after an isolated P_1 divided. P_1, AB, P_2 and EMS all can be distinguished by their different sizes. Blastomeres were placed in contact by directing a stream of medium using a fine glass capillary and a mouth pipette at the blastomere to be moved. Once in contact, the blastomeres adhere quickly. To separate adhering blastomeres, a glass capillary held in a micromanipulator was slowly lowered between the contacting cells, forcing them apart.

To make drawn capillaries for manipulating blastomeres, 1.2 mm outer diameter and 0.69 mm inner diameter borosilicate capillaries were pulled using a Sutter Instruments P-2000 needle puller with

settings of heat 650, filament 10, velocity 30, delay 200 and pull 150. The drawn capillaries were then broken to an appropriate diameter opening using forceps under a dissecting microscope. Capillaries used to separate adhered blastomeres were not broken.

In some experiments, blastomeres were killed using a laser microbeam (Laser Science, Inc.; Avery and Horvitz, 1989) after mounting chimeras under a glass coverslip in a thin layer of medium on a gasket slide (30 μm thickness, Cel-Line Associates). The irradiated cells were monitored for several hours to insure that they no longer divided.

Growth medium for blastomeres was made as follows. 1 ml of 5 mg ml^{-1} inulin (Sigma; dissolved with brief autoclaving), 50 mg Tissue Culture Grade PVP powder (Sigma) and 100 μl each of BME Vitamins (Gibco BRL), chemically defined lipid concentrate (Gibco BRL) and 100× concentrated Penicillin-Streptomycin (Gibco BRL) were added to 9 mls of *Drosophila* Schneiders' Medium (Gibco BRL). Final volume was 10.3 ml. Prior to use, bovine fetal calf serum (FCS; Gibco BRL, heat-treated 30 minutes at 56°C) was added; the tonicity of the medium was adjusted by varying the percentage of FCS (volume/volume) added. Addition of 35% FCS resulted in embryonic blastomeres producing differentiated descendants. The osmolality was 307 milliosmoles as measured using a vapor pressure osmometer (Wescor).

Immunostaining of cultured blastomeres

Once blastomere manipulations were finished, the blastomeres were incubated for approximately 24 hours at 15°C. The resulting tissue was fixed and stained as described in Goldstein (1995). Briefly, the tissue was fixed for 45 minutes in 2% (w/v) paraformaldehyde in growth medium lacking bovine fetal calf serum. Immunostaining was then performed by pipetting the fixed tissue through drops of antibody solutions and wash solution (100 mM Tris-HCl, pH 7.5, 162 mM NaCl and 0.1% Tween [vol/vol]) on siliconized slides. Antibody incubations were done in a humidity chamber. For the P_2/ABp interaction, intestinal-rectal valve cells were detected by monoclonal antibody (mAb) J126 (Mango et al., 1994a), and ABp-derived muscle cells were detected by mAb 5.6 (Miller et al., 1983). J126, like mAb 2CB7 (Bowerman et al., 1992b), also stains processes within pharyngeal gland cells, derived from MS, and faintly stains some ABa- and ABp-derived neurons. Valve cells can be scored by their characteristic small round morphology and because they are typically found paired (see Figure 2B). J126 and 2CB7 are similar in specificity to mAb ICB4 (Kemphues et al., 1988) but stain neuronal cells much less brightly (B. Bowerman, unpublished data). Pharyngeal muscle cells, a marker for MS induction, were detected with mAb 3NB12 (Priess and Thomson, 1987). Double antibody labeling experiments were performed using secondary antibodies conjugated to rhodamine and FITC (Tago, Inc.). Antibody staining was examined using a Zeiss Axioskop equipped for epifluorescence.

Genetic manipulations

N2 Bristol was used as the wild-type strain. Mutant embryos were obtained from mothers homozygous for maternal-effect embryonic-lethal mutations using the following strains: *apx-1(or3) dpy-11(e224)/DnT1 V, glp-1(e2141ts) III and skn-1(zu67)/DnT1 IV. glp-1(e2141ts)* mothers were grown at 15°C. 1-2 hours prior to obtaining embryos, they were shifted to 25°C, at which temperature the *glp-1* embryonic loss-of-function phenotype is expressed by *glp-1(e2141ts)* embryos (Kodoyianni et al., 1992; Priess et al., 1987).

RESULTS

The P_2 and MS inductions can be reproduced with isolated blastomeres in culture medium

To reproduce the 4-cell-stage induction of ABp-specific cell types, we brought P_2 in contact with the two cells produced by the division of an isolated AB blastomere (Fig. 2A). Because these two AB-derived blastomeres are born equivalent and are indistinguishable in cell culture, we refer to them simply as 4-cell-stage AB descendants. After incubating these partial

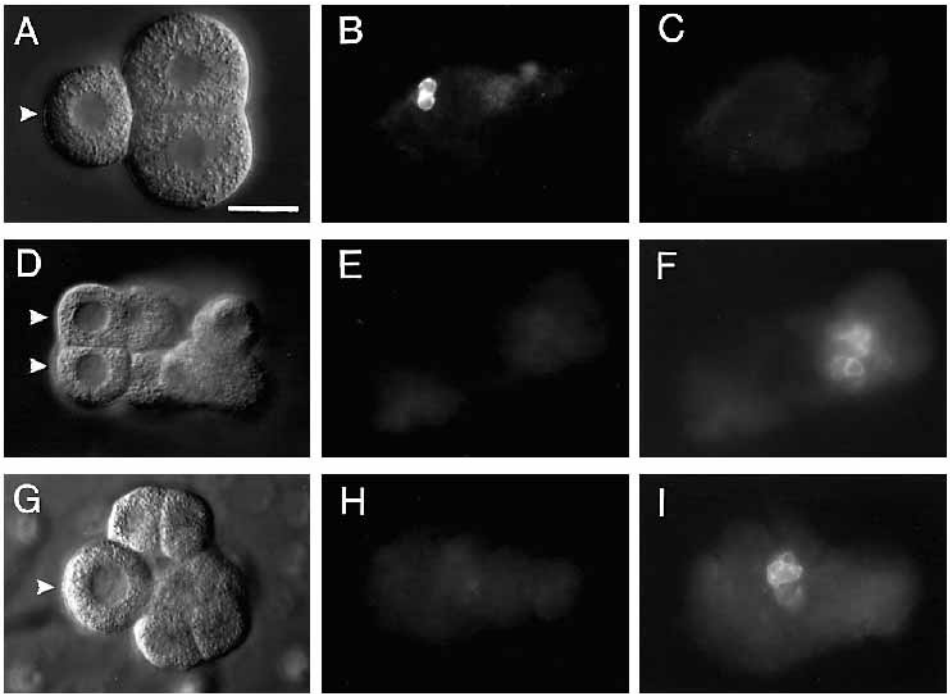

Fig. 2. Reproduction of the 4-cell and 12-cell-stage inductions using reassociated blastomeres. (A,D,G) Nomarski photomicrographs of reassociated blastomeres. Differentiated tissues produced by the partial embryos after overnight incubation in culture medium were fixed and immunostained with double labeling (see Materials and Methods) to assay induction of the ABp-derived rectal intestinal valve cells (B,E,H) and of the ABa-derived pharyngeal muscle cells (C,F,I). Reproduction of the P_2 induction of ABp-specific cell types: (A) P_2 (white arrowhead) was placed in contact with 4-cell-stage AB descendants, inducing the production of intestinal-rectal valve cells (B) but no pharyngeal muscle cells (C). Reproduction of the MS induction of anterior pharyngeal cells: (D) MS and E (white arrowheads) were placed in contact with the eight AB descendants that would be present in a 12-cell-stage embryo, inducing production of pharyngeal muscle cells (F) but no intestinal-rectal valve cells (E). MS and E were killed after 10-15 minutes of contact with AB descendants to eliminate production of MS-derived pharyngeal muscle cells. Substitution of P_2 for MS in the induction of anterior pharyngeal cells: (G) P_2 (arrowhead), was placed in contact with the eight AB descendants normally present in a 12-cell-stage embryo, resulting in production of pharyngeal muscle cells (I) but no intestinal-rectal valve cells (H). Additional experiments are summarized in Tables 1, 2 and 4. Panels are the same magnification and the bar in A represents 10 μm.

Table 1. Induction of intestinal-rectal valve cells in genetic chimeras cultured in vitro

Maternal genotype of P_2	Maternal genotype of the responding 4-cell-stage AB descendants (ABa and ABp)		
	Wild type	*apx-1(or3)*	*glp-1(e2141)*
Wild type	15/21†	0/13*†	0/5
Wild type, killed**	3/3		
apx-1(or3)	0/14		
glp-1(e2141)	3/7		

Mutant or wild-type P_2 blastomeres were placed in contact with mutant or wild-type 4-cell-stage AB descendants (an example is shown in Fig. 2A-C). Maternal genotypes are indicated by column and row descriptions. Incubations and antibody staining were carried out as described in the Materials and Methods. The results are presented as the number of experiments that produced positively staining valve cells/the number of differentiated chimeras that were fixed and stained. In all experiments, double-labeling was used to detect MS-induced pharyngeal cells (as in Fig. 2); in no cases were pharyngeal cells seen.

*Valve cells were not detected in an additional 6 cases in which AB daughters were from embryos obtained by mating wild-type males with homozygous *apx-1(or3)*; *fem-1(e1965)* mothers. Such blastomeres lack maternal *apx-1* function but carry a zygotic *apx-1(+)* allele.

†Similar results were obtained by detection of ABp-specific muscles (Materials and Methods; data not shown).

**P_2 was killed with a laser microbeam after 15 minutes of contact

embryos overnight, we fixed their differentiated descendants and used antibodies to detect two cell types normally made by ABp in response to interaction with P_2: the two intestinal-rectal valve cells, hereafter called valve cells, and several muscle cells (Mango et al., 1994a). As in an intact embryo, P_2 can induce the production of valve cells and muscle cells in this experiment (Fig. 2A-C, Table 1). Valve cells were made after killing P_2 with a laser microbeam following several minutes of contact (Table 1). In addition, because neither AB (n=10) nor P_2 (n=9) produce valve cells when cultured in isolation, we conclude that these ABp-specific cell types are made by the descendants of AB specifically in response to interaction with P_2.

To reproduce in vitro the MS induction of ABa-specific cell types, we placed MS and its sister, E, in contact with 'naive' 12-cell-stage AB descendants (Fig. 2D-F; Table 2). Initially, both MS and E were used because they are similar in size and therefore are not readily distinguishable after an isolated EMS blastomere divides in vitro (see Fig. 2D). Naive 12-cell AB descendants were obtained by allowing AB blastomeres from 2-cell embryos to divide three times in isolation. Such blastomeres are naive in that they have not received the P_2 signal or the MS signal. Consequently, all eight 12-cell-stage AB descendants should be capable of producing ABa-specific cell types in response to the MS signal (see Introduction). To assay their response to the MS signal, we used an antibody that recognizes pharyngeal muscle cells, which in an intact embryo are made by descendants of ABa that touch MS (Fig. 1). Because MS also produces pharyngeal muscle cells, both MS and E were killed with a laser microbeam after 15 minutes of contact. The production of pharyngeal muscle cells by these partial embryos demonstrates that the MS induction of ABa-specific cell types can be reproduced in vitro.

Both E and MS can signal after division of their parent blastomere, EMS

Recent studies using *apx-1* mutant embryos indicate that both MS and its sister E can induce the production of pharyngeal cells (Lin et al., 1995). The signaling ability of E is not apparent in wild-type embryos because E contacts only ABp and not ABa descendants (Fig. 1), and the 4-cell-stage signal from P_2 prevents ABp descendants from producing pharyngeal cells in response to the 12-cell-stage inductive signal. However, in *apx-1* mutant embryos, the P_2 signal is inactivated and E can induce ABp descendants to make pharyngeal cells (Lin et al., 1995). To determine if a wild-type E blastomere can signal like MS, we directly tested the individual signaling abilities of MS and E. In each experiment only one of the two sisters was placed in contact with naive 12-cell-stage AB descendants and the partial embryos allowed to develop without killing E or MS. Those that produced birefringent granules characteristic of E-derived intestinal cells were analyzed for the induction of pharyngeal muscle cells. We found that E from wild-type embryos can induce production of pharyngeal cells (Table 3).

To test the inductive ability of MS, we allowed isolated P1 blastomeres to divide and then took advantage of the observation that P_2 polarizes the division axis of EMS (Goldstein, 1995). The EMS daughter that remains in contact with P_2 assumes an E identity while the other daughter assumes an MS identity. A fine glass fiber was used to separate MS from the E and P_2 blastomeres. MS blastomeres isolated in this fashion were able to induce 12-cell-stage AB descendants to produce pharyngeal cells (Table 3).

Because both E and MS can signal, we asked if their parent blastomere, EMS, can induce production of pharyngeal cells. EMS was separated from P_2 and placed in contact with older, naive 12-cell-stage AB descendants. Just prior to its division, EMS was killed with a laser microbeam. EMS has little inductive ability; only a single pharyngeal cell was seen in nine experiments (Table 3). Thus, the signaling abilities of E and MS are expressed following division of their parent, EMS.

glp-1 function is required in responding but not signaling blastomeres

All genes currently known to be required for the P_2 and MS induction in *C. elegans* are maternally expressed. Because

Table 2. Induction of pharyngeal muscle cells by MS and E in genetic chimeras cultured in vitro

Genotype of MS and E	Maternal genotype of 12-cell-stage AB descendants		
	Wild type	*glp-1(e2141)*	*skn-1(zu67)*
Wild type	7/7	0/8	2/3
apx-1(or3)	2/3		
glp-1(e2141)	3/5		
skn-1(zu67)	0/6		

Mutant or wild-type MS and E blastomeres were placed in contact with mutant or wild-type 12-cell-stage AB descendants (an example is shown in Fig. 2D-F). Maternal genotypes are indicated by column and row descriptions. Incubations and antibody staining were carried out as described in the Materials and Methods. The results are presented as the number of experiments that produced positively staining pharyngeal muscles/the number of differentiated chimeras that were fixed and stained. All chimeras also were stained for intestinal-rectal valve cells (as in Fig. 2) and found to be negative. Because MS also produces pharyngeal cells, both MS and E were always killed with a laser microbeam after approximately 15 minutes of contact (Materials and Methods).

Table 3. Induction of pharyngeal muscle cells by EMS or descendants of EMS

Inducing cell	Pharyngeal staining (number positive/total number stained)
EMS	1/9*
MS	2/4
E	2/3

The EMS, MS and E blastomeres were isolated from wild-type embryos as described in Materials and Methods. Each was tested for the ability to induce pharyngeal cell production by being placed in contact with wild-type 12-cell-stage AB descendants. Except for experiments involving the E blastomere, the inducing cell was killed with a laser microbeam after approximately 15 minutes of contact and the AB descendants were incubated and immunostained for pharyngeal cells (Materials and Methods). Unlike EMS and MS, E does not produce pharyngeal cells and was not killed. After incubation, descendants of E could be identified by the presence of birefringent granules typical of E-derived intestinal cells. The data is presented as the number of experiments producing at least one pharyngeal cell/the total number of experiments.

*A single pharyngeal cell was detected in one experiment. For MS and E inductions, three to four pharyngeal cells typically were detected in positive experiments.

Table 4. Induction of pharyngeal muscle cells by P_2 in genetic and heterochronic chimeras cultured in vitro

	Phenotype of the responding 12-cell-stage AB descendants	
Genotype of P_2	Wild type	*apx-1(or3)*
Wild type	9/12	3/6
apx-1(or3)	0/7	

P_2 was placed in contact with 12-cell-stage descendants of an isolated AB blastomere (an example is shown in Fig. 2G-I). Maternal genotypes are indicated by column and row descriptions. The results are presented as the number of experiments that produced positively staining pharyngeal muscles/the number of differentiated chimeras that were fixed and stained. All chimeras also were stained for intestinal-rectal valve cells (as in Fig. 2) and found to be negative.

maternal gene products are put into oocytes prior to fertilization, it is not possible to use, for example, genetic recombination to generate mosaic embryos. Therefore, models of gene function within specific blastomeres have not been directly tested. Our reproduction of these inductions in vitro made it possible to functionally test gene requirements within individual blastomeres.

To test the idea that GLP-1 functions as a receptor for both the P_2 and MS signals (see Introduction), we assembled partial embryos chimeric for *glp-1* function (Tables 1, 2). Wild-type P_2 blastomeres were unable to induce production of valve cells from *glp-1* mutant AB descendants (Table 1, column 3). In contrast, *glp-1* mutant P_2 blastomeres were able to induce the production of valve cells from wild-type AB blastomeres (Table 1, row 4). Similar results were obtained for the MS induction: *glp-1* function is required in AB descendants for responding but not in MS for signaling (Table 2, compare row 3 with column 2). These results support models in which GLP-1 functions as a receptor for both the 4-cell and the 12-cell-stage signals.

apx-1 function is required for the P_2 signal

The phenotype of *apx-1* mutant embryos and the similarity of *apx-1* to *Drosophila Delta*, suggest that *apx-1* encodes a P_2-specific ligand (see Introduction). Consistent with this hypothesis, *apx-1* mutant P_2 blastomeres do not induce production of valve cells by wild-type 4-cell-stage AB descendants in genetically chimeric partial embryos, indicating that P_2 requires *apx-1* function to signal (Table 1, row 3). However, we also found that *apx-1* mutant 4-cell-stage AB descendants failed to produce ABp-specific cell types in response to signaling from a wild-type P_2 (Table 1, column 2). One explanation for this result is that the production of ABp-specific cell types depends not only on maternal *apx-1* expression but also on subsequent zygotic expression. Indeed a zygotic defect has been noted in *apx-1* mutants (Mango et al., 1994a). To test this possibility, we mated feminized *apx-1* adults with wild-type males (see Table 1). Supplying *apx-1* zygotically did not restore the ability of 4-cell-stage AB descendants to produce valve cells. In contrast to models in which *apx-1* encodes a P_2-specific ligand for the 4-cell-stage induction, these results suggest that maternal expression of *apx-1* function may be required in both signaling and responding cells during the induction of ABp-specific cell types. Alternatively, the response of the 4-cell-stage AB descendants may require *apx-1* activity in P1, the precursor of P2 at the 2-cell stage. Because P_1 and AB cannot be isolated until the late 2-cell stage in these experiments (Materials and Methods), reproduction of P2 signaling in this case may not be possible when AB is derived from an *apx-1* mutant embryo. Indeed, two observations are consistent with an early requirement for *apx-1*: P_2 induction occurs early in the 4-cell stage (Mango et al., 1994a), and APX-1 is expressed at the boundary of P1 and AB in 2-cell-stage embryos (Mickey et al., 1996).

Finally, consistent with previous observations that *apx-1* function is required for only the P_2 and not the MS induction (Mango et al., 1994a; Mello et al., 1994), the MS induction can be reproduced using *apx-1* mutant blastomeres (Table 2, row 2; Table 4, column 2). Thus, the failure of *apx-1* mutant blastomeres to respond to the P_2 signal (see above) is not due to a general inability of *apx-1* mutant blastomeres to signal and respond in these in vitro experiments.

P_2 can functionally substitute for MS

Two models can explain how the same receptor, GLP-1, transmits two developmental signals with very different consequences. First, the response might be a function of the inducing cell. Alternatively, temporal changes in the cellular factors expressed by responding cells might account for differing responses to otherwise similar signals (Mango et al., 1994a). In the latter model, it might be possible to substitute one signaling blastomere for another without changing the inductive response. To distinguish between these two models, we asked if P_2 from a 4-cell-stage embryo can substitute for MS in the 12-cell-stage induction of ABa-specific pharyngeal cells. After placing P_2 in contact with naive 12-cell-stage AB descendants, we reproducibly observed induction of pharyngeal cells but never of valve cells (Fig. 2G-I, Table 4). The finding that P_2 can replace MS for the second induction suggests that P_2 and MS are functionally interchangeable and that the specific responses to each signal depend simply on the age of the AB descendants.

Another explanation for the ability of P_2 to substitute for MS is that specific ligands are in fact required for each signal, but P_2 expresses both ligands. Because P_2 requires *apx-1* function to signal at the 4-cell stage, we tested whether pharyngeal cell production by 12-cell-stage AB descendants in response to P_2 also depends on *apx-1* activity. We found that for pharyngeal

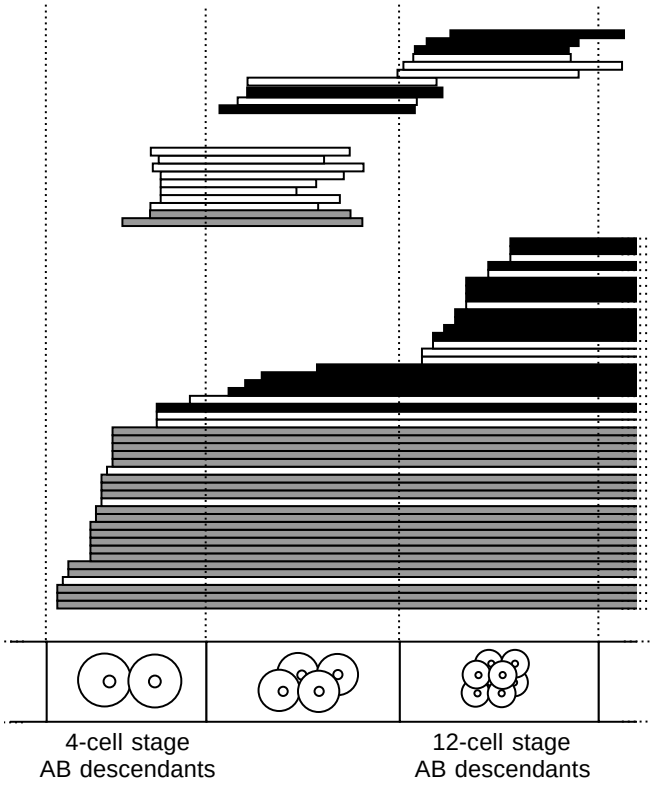

Fig. 3. Inductive response versus age of AB descendants when contacted by identically aged P_2 blastomeres. The age of the AB descendants is indicated at the bottom. Contact time between P_2 and the AB cells is represented by horizontal bars. The start of the bars represents the initial time of contact between P_2 and the AB descendants, and the lengths of the bars indicate the time period in which the inducing cells and responding cells remained in contact. In some experiments, P_2 remained in contact until fixation and staining. This is represented by the lower group of bars that extend to the right and end in dots. For the upper group of bars, which do not extend all the way to the right, the P_2 blastomere was removed using a fine glass fiber at the time shown by the termination of the bar. Embryos were stained for valve cells and pharyngeal cells as in Fig. 2. Gray bars represent those partial embryos that produced valve cells, black bars represent those embryos that produced pharyngeal cells and white bars represent those embryos that failed to produce either valve cells or pharyngeal cells. The respective cell cycle times of 4-cell, 6-cell and 12-cell-stage AB descendants were 14.5±1.5, 17.5±2.5 and 18.0±2.0 minutes.

cell induction *apx-1* function is required in P_2, but not in the 12-cell-stage AB descendants (Table 4). Thus, *apx-1* is required in P_2 for both the normal induction of valve cells and for the ability of P_2 to substitute for MS, suggesting that the putative ligand encoded by *apx-1* can function in both inductions.

To determine if MS and E can likewise substitute for P_2, we placed MS and E in contact with 4-cell-stage AB descendants for 15 minutes, and then killed both E and MS with a laser microbeam. In 9 cases, neither pharyngeal nor valve cells were induced, suggesting that the MS signal cannot functionally replace the P_2 signal. Because P_2 can replace MS, we suspect that this failure may reflect the small contact area between MS and E and the much larger 4-cell-stage AB descendants (compare cell sizes in Fig. 2). Alternatively, the amount of ligand expressed may be important for signaling and P_2 may produce more ligand than do MS and E.

AB descendants can respond early in the 4-cell stage and, following a refractory period, at the 12-cell stage

To further investigate the role of timing in the response of the AB descendants, we recombined similarly aged P_2 blastomeres with AB descendants of several different ages (Fig. 3). The division time of the AB descendants and the time of contact with the inducing P_2 blastomere were then correlated with inductive response. AB descendants responded by producing valve cells up until halfway through the cell cycle of ABa and ABp. If P_2 was placed in contact later than this, the AB descendants responded by producing pharyngeal cells.

We have found that P_2 can signal throughout its cell cycle and that, after dividing, the daughters of P_2 are still able to induce pharyngeal tissue from AB descendants (data not shown). Because P_2 was not removed and presumably continued to signal, the timing experiments above did not resolve when AB descendants begin to respond with pharyngeal cell production. To define the earliest time for pharyngeal cell induction, we performed the same experiments as above, except that P_2 was removed with a glass fiber at varying times after contact (Fig. 3, upper bars). We asked how late P_2 must remain in contact to induce pharyngeal cell production from AB descendants. If P_2 was removed shortly before the AB descendants divided to eight cells, pharyngeal cells were never produced. In contrast, if P_2 remained in contact with the AB descendants after they divide at this time, they often were induced to produce pharyngeal cells (Fig. 3, top bars). Therefore, AB descendants begin to respond by producing pharyngeal cells as they divide to produce 12-cell-stage descendants and are refractory to induction between this time and the midpoint of the ABa and ABp cell cycle.

SKN-1 activates the MS signal

Mutations in the maternal gene *skn-1* prevent the production of ABa-derived pharyngeal cell types (Bowerman et al., 1992a). In addition, *skn-1* is required for correctly specifying the identity of EMS, the parent of MS (Bowerman et al., 1992a). It is possible that SKN-1 might regulate the production of pharyngeal cells by acting autonomously within AB descendants, downstream of the MS signal. Alternatively, SKN-1 could regulate expression of the MS signal as part of its role in specifying EMS identity. By constructing chimeras, we have found that *skn-1* function is required for MS to signal but not for AB descendants to respond (Table 2, row 4 versus column 3), indicating that SKN-1 regulates expression of the MS signal.

DISCUSSION

During embryogenesis in *C. elegans*, the different identities of ABa and ABp descendants are specified, in part, by a sequence of two signals, one from P_2 at the 4-cell stage and another from MS at the 12-cell stage. By reproducing these two inductions in culture medium using isolated blastomeres, we have confirmed the suggestion from previous studies that the same receptor, GLP-1, is required for AB descendants to respond to both signals. Moreover, we have shown that P_2 signaling can activate either response, with the specificity depending not on the identity of the signaling cell or ligand, but on the age of

the AB descendants when they receive the signal. Thus, we suggest that time-dependent changes in factors present within the AB descendants specify the different responses to otherwise similar signals. Our results also support previous studies suggesting that the maternally expressed *apx-1* gene encodes a P_2-specific ligand for the first induction. Finally, we have shown that the putative transcription factor SKN-1 is required to activate expression of the MS signal.

Role of *apx-1* at the 2-cell stage

Laser ablation studies suggest that a signal at the 2-cell stage from P_1 polarizes the AB blastomere, resulting in anterior-posterior differences in fate among 12-cell-stage descendants of AB (Hutter and Schnabel, 1995a). Our observation that *apx-1* may be required prior to the 4-cell stage and the observation that APX-1 is present at the boundary of AB and P_1 (Mickey et al., 1996) raise the possibility that *apx-1* is required for the P_1/AB polarizing induction. However cell-lineage analyses have shown that anterior-posterior differences among 12-cell-stage AB descendants are maintained in *apx-1* mutant embryos (Mango et al., 1994a; Mello et al., 1994), indicating that *apx-1* is not required for polarizing AB. Alternatively, expression of APX-1 at the P_1/AB boundary and our analysis of *apx-1* function may indicate that there is a preliminary phase to the P_2 induction at the 2-cell stage, consistent with this induction being complete shortly after AB and P_1 have divided (Mango et al., 1994a).

Inductive specificity determined by factors within the responding cells

Studies of Notch signaling in *Drosophila* suggest a molecular mechanism for specificity in responding to signals using the same receptor. The *Suppressor of Hairless* [*Su(H)*] and *Deltex* proteins have been recently identified as interacting with the intracellular portion of the Notch receptor in *Drosophila* (Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). Cell-culture studies with Delta, Notch and Su(H) show that Su(H) is colocalized with Notch at the cell membrane and that, upon binding of Delta, Su(H) translocates to the nucleus (Fortini and Artavanis-Tsakonas, 1994) where it may activate target genes (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). However, mutations in *Su(H)* show complex genetic interactions with *Delta* and *Notch* (Fortini and Artavanis-Tsakonas, 1994) and some processes requiring *Notch* are independent of *Su(H)* activity (Lecourtois and Schweisguth, 1995), suggesting that different downstream factors are necessary in different developmental contexts.

In the early *C. elegans* embryo, factors similar to Deltex and Su(H) could be expressed at different times, specifying the different responses of AB descendants at the 4-cell and 12-cell stages of embryogenesis. At the 4-cell stage, one factor might interact with GLP-1, regulating a group of genes that would specify ABp fate upon receipt of the P_2 signal. This protein could be degraded during subsequent divisions and replaced by another factor that would regulate a different group of genes when the MS signal is received. Alternatively, differences in the response could be brought about by the presence or absence of other co-factors. In either case, temporal changes in cellular factors present in AB descendants appear to modulate the response to GLP-1 stimulation by functionally similar signals.

Functional interchangability of receptor and ligand molecules

The hypothesis that differences within responding cells determine the specific responses to signals that use the same receptor is supported by observations that *Notch* and *Delta* homologs can functionally replace one another. In *Drosophila*, expression of the *Serrate* gene, similar to *Delta*, can replace *Delta* in the process of neuroblast segregation in the early embryo (Gu et al., 1995). In *C. elegans*, the *lag-2* gene encodes the putative ligand that activates GLP-1 to regulate germ-line proliferation postembryonically and, like *apx-1*, is similar in sequence to *Drosophila Delta* (Henderson et al., 1994; Tax et al., 1994). Recent studies have shown that the *apx-1*-coding sequence under control of the *lag-2* promoter will fully complement mutations in *lag-2* (Gao and Kimble, 1995; Fitzgerald and Greenwald, 1995). In addition to the interchangability of ligands, two *Notch* homologs in *C. elegans*, *lin-12* and *glp-1*, have overlapping function (Lambie and Kimble, 1991) and *glp-1* can functionally substitute for *lin-12* (Fitzgerald et al., 1993). These results suggest that the functional roles of *Notch* and *Delta* homologs are not discrete and can be interchanged without affecting the specificity of particular signaling events.

Regulation of inductive signals during *C. elegans* embryogenesis

Though related signaling molecules are functionally interchangeable, the use of different genes to express related molecules makes it possible to generate unique temporal and spatial patterns of gene expression that control patterning during development. The P_2 and MS signals appear qualitatively similar, but they must be expressed at the right times and places to properly specify the fates of ABa and ABp descendants. For example, P_2 touches only ABp and not ABa, making it possible for an early signal from P_2 to break the initial equivalence of ABa and ABp (see Fig. 1). In addition, because EMS touches both ABa and ABp, delay of the second signal is required for induction of the appropriate 12-cell-stage AB descendants. The use of different genes for the P_2 and MS signals may allow the proper spatial and temporal regulation of two closely spaced signals.

Consistent with a requirement for two different genes to encode functionally similar ligands, the P_2 and MS signals are likely regulated by different mechanisms. Post-transcriptional regulation of the maternally expressed *apx-1* gene appears to localize the first signal to P_2 (Mickey et al., 1996). In contrast, the second signal from MS requires the maternally supplied transcription factor SKN-1. Thus it is possible that SKN-1 transcriptionally activates a zygotically expressed ligand within the MS blastomere.

Proper spatial and temporal regulation of both the P_2 and MS signals also requires the maternal gene *pie-1*. Genetic studies indicate that *pie-1* is required to prevent *skn-1* from specifying EMS identity in P_2 (Mello et al., 1992). Consequently, in *pie-1* mutant embryos, P_2 develops identically to EMS. Indeed, P_2 fails to signal ABp in *pie-1* mutant embryos (Mango et al., 1994a), and APX-1 protein is not expressed in *pie-1* mutant embryos (Mickey et al., 1996). Furthermore, the C daughter of P_2 in *pie-1* embryos signals ABp descendants to produce pharyngeal cells, much like the MS daughter of EMS signals ABa descendants (Mango et al., 1994a). Thus, *pie-1* function permits expression of the P_2 signal and prevents

expression of the MS signal in P_2 descendants. Genetic studies in *C. elegans* promise to clarify our understanding of how closely related molecules are differentially regulated to control pattern formation during animal embryogenesis and of how time-dependent changes within responding cells can determine the specific responses to signals that use the same receptor.

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