pha-4, an HNF-3 homolog, specifies pharyngeal organ identity in Caenorhabditis elegans

Michael A. Horner,1 Sophie Quintin,2 Mary Ellen Domeier,1 Judith Kimble,3 Michel Labouesse,2 and Susan E. Mango1,4

1Huntsman Cancer Institute, Center for Children, Department of Oncological Sciences, University of Utah, Salt Lake City, Utah 84112 USA; 2Institut de Genetique et de Biologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique/Institut National de la Santé et de la Recherche Medicales/Universite Louis Pasteur (CNRS/INSERM/UHP), 67404 Illkirch Cedex, France; 3Howard Hughes Medical Institute, Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706 USA

To build complex organs, embryos have evolved mechanisms that integrate the development of cells unrelated to one another by cell type or ancestry. Here we show that the pha-4 locus establishes organ identity for the Caenorhabditis elegans pharynx. In pha-4 mutants, pharyngeal cells are transformed into ectoderm. Conversely, ectopic pha-4 expression produces excess pharyngeal cells. pha-4 encodes an HNF-3 homolog selectively expressed in the nascent digestive tract, including all pharynx precursors at the time they are restricted to pharyngeal fate. We suggest that pha-4 is a key component of a transcription-based mechanism to endow cells with pharyngeal organ identity.

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The digestive tract consists of an epithelial tube that is spatially and functionally regionalized. In simple animals, such as Hydra, the gut contains only a few specialized cells and is divided into only two regions: the mouth and gut sac. In more complex animals, the digestive tract is composed of many different cell types and compartments. How is this complicated organ built? To tackle this question, we are studying foregut, or pharynx, development in the experimentally tractable organism Caenorhabditis elegans.

The nematode pharynx is a large neuromuscular organ used for feeding. Like the foregut compartments of more complex animals, the pharynx is composed of many different cell types such as muscles, glands, neurons, epithelia, and valves (Albertson and Thomson 1976). Two early blastomeres, ABa and MS, generate the pharynx (Sulston et al. 1983). Each blastomere gives rise to multiple cell types within the pharynx, and each uses a different genetic program to produce pharyngeal cells (for review, see Schnabel and Priess 1997). The ABa-derived pathway depends on intercellular signaling mediated by the GLP-1 receptor, whereas pharynx production from MS appears to be cell autonomous. Two predicted transcription factors, skn-1 and pop-1, are necessary for the MS developmental program, including the production of pharyngeal cells. Recent studies have begun to elucidate the gene networks controlling glp-1, skn-1, and pop-1 (for review, see Han 1997); however, the mechanisms that act downstream of these genes, to mediate pharyngeal organogenesis, are largely unknown.

The genetic pathways that generate the ABa and MS cell lineages converge on the pha-4 locus. Animals lacking zygotic pha-4 activity fail to produce pharynx cells from either ABa or MS (Mango et al. 1994a). In addition, these mutants lack a rectum. The rectum is descended from a third blastomere called ABp and does not derive from the pharyngeal cell lineages. Most other cells appear to be generated normally in pha-4 mutant embryos, including cells from ABa and MS that are not part of the pharynx. Thus, the pha-4 locus is critical to generate a group of cells related by function (the digestive tract) rather than by cell lineage (ABa, MS) or cell type (pharynx neuron, pharynx muscle, etc.). This phenotype reflects a transition during embryogenesis from maternal genes, like glp-1, skn-1, and pop-1, that control entire cell lineages and broad domains of axial patterning, to zygotic genes, like pha-4, that regulate the formation of specific organs, tissues, and cell types.

In the present study we address two potential mechanisms by which pha-4 could function. First, pha-4 could establish an organizing center that is critical for development of the entire pharynx. By analogy, loss of the anchor cell during C. elegans vulval development results in no vulva being formed (Greenwald 1997). Similarly, mutations in WT-1 or c-ret lead to the absence of the kidney in mouse embryos (Kreidberg et al. 1993; Schuchardt et al. 1994). Each of these phenotypes reflects a loss in cell signaling. For vulval development, signaling occurs between cells in two different organs, whereas in kidney formation WT-1 and c-ret are necessary for epithelialmesenchymal interactions among different tissues within the developing kidney primordium.

A second possible explanation for pha-4 function is that the protein could act within individual pharyngeal cells to establish pharynx identity. That is, pha-4 might be analogous to pax-6 and the network of transcription factors that initiate eye formation (Desplan 1997). Loss-of-function mutations in either eyeless, sine oculis, eyes absent, or dachshund leads to a complete absence of the eye in Drosophila. Conversely, each of these genes, either alone or in combination, is capable of inducing ectopic eyes. These phenotypes suggest that this group of factors synergize to define eye identity during development.

[Key Words: C. elegans; fork head; HNF-3; pha-4; organogenesis; foregut development]

*Corresponding author.
E-MAIL smango@genetics.utah.edu; FAX (801) 581-7796.
In this report we present evidence that pha-4 encodes an HNF-3 homolog that is expressed in all pharyngeal precursors and establishes their fate. We suggest that pha-4 and the Drosophila eye genes represent a new class of developmental regulator that specifies organ identity. Given that HNF-3 genes have been implicated in gut development in other organisms (Kaufmann and Knochel 1996), we propose that specification of organ identity within the digestive tract may be a conserved feature of HNF-3 proteins.

Results and Discussion

pha-4 encodes the HNF-3 homolog Ce-fkh-1 and is expressed in the developing digestive tract

To understand the molecular mechanism of pha-4 function, we identified the gene by a combination of positional cloning and candidate gene approaches. Previous analyses showed that pha-4 is located within the YAC Y79B4 (S. Mango, unpubl.). YAC Y79B4 contains the predicted winged helix transcription factor Ce-fkh-1, which was an excellent candidate to encode pha-4 based on map position and mRNA expression (Azzaria et al. 1996; S. Mango, unpubl.). Three lines of evidence demonstrate that pha-4 corresponds to Ce-fkh-1. First, extrachromosomal arrays carrying either the cosmids F38A6, which carries Ce-fkh-1, or a 16.7-kb PCR fragment spanning the Ce-fkh-1 locus can rescue homozygous pha-4 mutants (Fig. 1A). Second, antisense Ce-fkh-1 RNA injected into wild-type hermaphrodites causes embryos and larvae to arrest with a weak pha-4 phenotype (see Materials and Methods). Third, three EMS-induced pha-4 alleles harbor mutations within Ce-fkh-1 (Fig. 1B). The Ce-fkh-1 locus generates three mRNAs, which are predicted to encode three proteins of 506, 441, and 410 amino acids (Azzaria et al. 1996). The allele pha-4(q490) carries a nonsense mutation at amino acid 99 relative to the largest pha-4 isoform and is predicted to truncate all three proteins severely. Therefore, this allele, which behaves like a null allele in genetic tests (Mango et al. 1994a), is likely to be a molecular null as well. The other two sequenced alleles, n2498 and q487, encode nonsense mutations at positions 250 and 310, respectively. These data show that pha-4 is encoded by Ce-fkh-1.

To determine when and where PHA-4 protein is expressed, we designed a polyclonal antibody that recognizes the PHA-4 carboxyl terminus. This antibody is specific, as no staining is observed in pha-4(q490) null embryos (Fig. 2A) or after preincubation with the immunizing peptide (Materials and Methods). We observe PHA-4 in the nuclei of cells destined to form the digestive tract. At the 4E stage, weak expression is detected in 8 MS great-granddaughters and 10 ABa descendants (ABaraaa/p, ABarapa/p, ABarapa/p, ABarapa/p, all MS great-granddaughters, midgut). (C) 8E stage embryo (expression in MSaapa/p, MSapa, MSapa/p, MSapa/p, MSapa, ABa descendants, midgut). (D–E) Midstage embryos [97 ± 3 PHA-4+ cells in the head (expression in all pharyngeal cells, pharynx–intestinal valve cells, and arcade cells, based on cell position and double staining (n = 3)], rectal cells (vir, rep, KK), and midgut. (F) Terminal embryo (expression in entire pharynx, midgut, and rectum). (Arrow) Pharynx; (arrowhead) midgut; (*) rectum. Nuclear positions in pha-4(+)(G) and pha-4(−)(H) embryos at the 8E stage, ventral view. Pharyngeal cells are colored according to their lineal ancestry (yellow, ABalp; pink, ABara; green, MS). Midgut cells are red and rectal cells are blue. The embryos in B and C are rotated relative to the embryos shown here. Anterior is left (A–H); each embryo is ~50 µm.
defines the digestive tract during embryogenesis. The initiation of bright expression at the 8E stage corresponds to the time when the pharyngeal precursors are born. These cells are destined to produce only pharyngeal cells. However, they are not restricted to any particular cell type within the pharynx, consistent with pha-4's proposed global role in specifying pharynx identity (Sulston et al. 1983; Mango et al. 1994a).

pha-4 is required to establish the pharyngeal precursors

We used lineage analysis to show that pha-4 function is required by the 8E stage in development. Previous studies demonstrated that pha-4 mutants lack a pharynx (Mango et al. 1994a). Here we investigate the pha-4 mutant phenotype during early developmental stages and show that pha-4 function is required for the earliest signs of pharyngeal development during embryogenesis. In the wild type, the pharyngeal precursors congregate at the ventral midline, where they ingress, beginning at the 4–8E stage (Fig. 2G). In pha-4 embryos, ingressation is delayed or aborted so that most of these cells remain on or near the ventral surface. In addition, whereas wild-type pharyngeal precursors cluster into a column as they gastrulate, this process usually fails in pha-4 mutants (Fig. 2H). All other embryonic blastomeres behave normally at this time, including midgut cells (4/4 embryos lineaged completely to the 8E stage). Thus, pha-4 activity is required for the proper migration and possibly adhesion of the pharyngeal precursors by the 4–8E stage.

In the absence of pha-4 function, pharyngeal cells adopt an ectodermal fate. Staining with an antibody that recognizes LIN-26, a zinc-finger protein expressed in epidermis and neuronal support cells (Labouesse et al. 1994, 1996), shows that excess numbers of LIN-26+ cells are made in pha-4 mutants (M. Labouesse and S. Mango, unpubl.; see also Chanal and Labouesse 1997). To determine the source of these cells, we allowed individual blastomeres to develop after all other blastomeres were killed with a laser microbeam. This experiment demonstrated that excess LIN-26+ cells were made by isolated ABA and MS blastomeres, whereas their sister cells, ABp and P2, were unaffected (Fig. 3). Therefore, cells that would become part of the pharynx in a wild-type embryo, appeared to be transformed into LIN-26+ ectodermal cells in a pha-4 mutant. We confirmed this conclusion by following the late cell lineages of ABA and MS in pha-4 embryos. The terminal cell divisions and programmed cell deaths were altered in the cell lineages that would normally generate pharyngeal cells, suggesting that these cells no longer had pharynx identity. For example, in wild-type embryos, ABaraapppaa and ABaraappppaa divide to generate one daughter that dies and one pharyngeal neuron (Sulston et al. 1983). In pha-4 embryos, ABaraappppaa and ABaraappppaa arrested without undergoing the terminal cell division (2/2 embryos followed). In addition, no programmed cell deaths were observed for ABalppapp, ABarapapp, or Mppapp (2/2 embryos examined for each cell).

The new cell lineage patterns observed in pha-4 embryos did not resemble a different branch of the wild-type lineage. Normally, individual sublineages can be identified by the total number of cell divisions within the lineage (e.g., 8, 9, or 10 for AB-derived blastomeres), the pattern of programmed cell deaths and the morphology of individual cells. In pha-4 embryos, AB-derived cells divided nine times and had few programmed cell deaths, unlike any specific sublineage in wild-type embryos. Thus, pha-4 mutant cells lost their pharyngeal lineage characteristics without adopting a pattern that mimicked another wild-type lineage. This result is consistent with pha-4's involvement in establishing pharyngeal fate rather than a particular cell lineage.

pha-4 can specify pharyngeal fate

Expression of ectopic pha-4 is sufficient to confer pharynx identity to embryonic blastomeres. We used a heat shock promoter (Jones et al. 1986; A. Fire, pers. comm.) to express PHA-4 throughout the embryo. By Nomarski optics, all manipulated embryos arrested with a large pharynx-like organ, bounded by a basement membrane. In addition, these embryos failed to undergo normal morphogenesis and contained fewer epidermal cells or body wall muscles. We confirmed these observations by staining for pharyngeal muscles and marginal cells, which are generated by multiple branches of the lineage tree in wild-type animals (Sulston et al. 1983). Most heat-treated embryos contained at least 50% more pharyngeal muscle cells (Fig. 4A,D) and three times more pharyngeal marginal cells (Fig. 4B,E) compared to the wild type. Conversely, the number of LIN-26+ epidermal and neuronal support cells was strongly reduced after heat shock, as was the number of body wall muscles (Fig. 4C,F; Materials and Methods). These effects were only observed with an intact pha-4 gene and were not seen.
Interestingly, there are two consensus binding sites for PHA-4 encoding a control plasmid expressing a modified pha-4 construct that lacks the DNA binding domain (Fig. 4).

Not all embryonic cells were transformed into pharyngeal cells by ectopic pha-4 (Materials and Methods; Fig. 4). The incomplete transformation probably reflects two phenomena. First, cells may only be competent to respond to exogenous pha-4 for a brief period of time. For example, we observed the strongest effects when pha-4 was induced by the mid-4E stage. Induction after the 6E stage, on the other hand, gave rise to healthy L1 larvae with no obvious defects (n > 25 embryos for each stage; data not shown). A second reason for the partial transformation is that PHA-4 activity may be modulated by positive and negative cofactors that are each present in only a subset of embryonic blastomeres. Existence of cofactors could also explain the apparent paradox that ectopic pha-4 can confer pharyngeal identity, but the endogenous gene is normally expressed in the midgut and rectum. We propose that one or more cofactors cooperate with PHA-4 to promote pharynx development, another dampens PHA-4 activity in midgut precursors, and a third functions with PHA-4 to establish rectal cells. This hypothesis is consistent with our observation that midgut development is not inhibited by ectopic pha-4.

Exogenous pha-4 is sufficient to down-regulate LIN-26 expression. During normal pharynx development, PHA-4 and LIN-26 are not found in the same cell or cell lineage (Labouesse et al. 1996; this work). Therefore, it is unlikely that PHA-4 initiates pharynx formation by repressing LIN-26, which is essential for epidermal and glial cell development (Labouesse et al. 1994, 1996). To test this idea, we examined the phenotype of embryos lacking both pha-4 and LIN-26. Pharynx development was not restored in mutants carrying null mutations in both genes, suggesting that repression of LIN-26 by PHA-4 is not sufficient for pharynx development (see Materials and Methods). One facet of maintaining pharynx identity, however, may be the continual repression of LIN-26. Because pha-4 encodes a DNA-binding protein, it is possible that PHA-4 represses LIN-26 transcription directly. Interestingly, there are two consensus binding sites for HNF-3 within the LIN-26 promoter, and these sites are conserved in LIN-26 from Caenorhabditis briggsae (P. Chanal and M. Labouesse, unpubl.).

How might pha-4 govern pharynx fate? One idea is that pha-4 regulates the proper positioning and migration of cells, and the new location of cells in pha-4 mutants interferes with normal pharyngeal development. Alternatively, pha-4 might specify pharynx fate directly, and the loss of pharyngeal identity in pha-4 mutants lead to altered behaviors. Two lines of evidence support the latter hypothesis. First, most embryos with severe defects in morphogenesis and cell placement still form a pharynx (e.g., Ahnn and Fire 1994; Labouesse 1997; Schnabel and Priess 1997). Second, the ectopic pharyngeal cells seen in our heat shock experiments are often located in regions of the embryo that do not normally produce pharyngeal cells. Thus, normal patterns of cell migration, adhesion, and ingression depend on correct pharynx fate specification by pha-4.

Compartmentalization of the gut during development

Our studies suggest that the subdivision of the digestive tract into foregut, midgut, and hindgut is evolutionarily ancient and relies on conserved molecular mechanisms. We have shown that pha-4 encodes an HNF-3 homolog necessary to specify pharyngeal and rectal precursors. In other organisms, HNF-3 proteins have been implicated in foregut and hindgut development, the equivalent of the nematode pharynx and rectum. In Drosophila fork head mutants, for example, foregut and hindgut cells are transformed into head sclerite structures, whereas in vertebrates, the absence of HNF-3 leads to severe abnormalities in the foregut (for review, see Kaufmann and Knochel 1996). Moreover, the nematode nfxc2.5 homolog ceh-22 is expressed in foregut muscle, similar to its murine and Drosophila counterparts (Oikkema and Fire 1994; Harvey 1996; Lane et al. 1997; Schnabel and Schnabel 1990). The conserved patterns of expression and phenotype for these three genes strongly support the notion that the mechanisms that compartmentalize the digestive tract have been phylogenetically conserved.

The anatomy of the foregut, midgut, and hindgut differs substantially from animal to animal. This observation implies that homologs of pha-4 and end-1 regulate different target genes in different animals, either directly or indirectly. Few targets have been identified, so it is unclear at what point the pathways diverge. Genes such as ceh-22 (Mango et al. 1994a; J. Kalb, K. Lau, B. Goshczynski, T. Fukushima, D. Moons, P. Oikkema, J. M. Gheee, pers. comm.) and K07C11.10, a paired-box homolog (A. Chisolm, pers. comm.; M. Domeier, and S. Mango, unpubl.), are good candidates to be regulated by pha-4/HNF-3 proteins in many animals. On the other hand, no obvious match to LIN-26 from another species, nor to pha-1, another gene required for C. elegans pharynx formation and potential PHA-4 target (Schnabel and Schnabel 1990). In Drosophila, the
HNF-3 homolog fork head represses trachealless to promote salivary gland development (Kuo et al. 1996). At least two trachealless homologs exist in C. elegans, one of which we have inactivated by RNA interference. No pharyngeal phenotype was observed (M. Domeier and S. Mango, unpublished). From this short list of genes it is impossible to generalize about how HNF-3 proteins produce different specialized structures in different animals. However, it is interesting to note that both trachealless and lin-26 appear to be repressed by HNF-3 homologs. In rats, HNF-3β blocks activation of aldolase B transcription directly, by binding to the promoter (Gregori et al. 1993, 1994). These data suggest that PHA-4 and its homologs may function as transcriptional repressors, as well as activators, to control developmental fates.

Experiments performed half a century ago described the ability of dissociated organ rudiments to selectively recognize and adhere to cognate organ cells (Townes and Holtfreter 1955; Moscona 1957). This behavior constitutes a cellular description of organ identity. However, the underlying molecular mechanisms were unknown. We propose that pha-4 is an essential component of a transcription-based process that endows pharyngeal cells with organ identity. Regulators of organ identity may act combinatorially with factors that control positional, blastomere, or cell type identity to build complex organs and tissues.

Materials and methods

Genetics

C. elegans strains were maintained as described (Brenner 1974). Mutations and chromosomal balancers used were dpy-2(e489)lin-26(mcl), unc-4(e120), mcn1 LGII, unc-101(mu108)LGIV, fog-2(q71), stz-3(q265), rol-9(sc148), pha-4(q490), pga-4(q487), pga-4(q500), and pga-4(n2498) LGV.

For the lin-26pha-4 double mutant analysis, embryos were collected from dpy-2 lin-26 unc-4/mc1; fog-2 pga-4(q490)/stz-3 rol-9 embryos, were recorded until the 1.5-fold stage. We followed all cells to the 350-cell stage in two N2 embryos, three Pha-4 embryos, and one non-Pha-4 embryo (i.e., pha-4+/ or +/+) for 15–20 min using SIM 1 BioCell software, which can reconstitute the tridimensional aspect of the embryo (Schnabel et al. 1997).

Individual cells were destroyed with a laser microbeam as described (Sulston and White 1980; Avery and Horvitz 1987; Mango et al. 1991) except that the Micropoint laser system (Photonic Instruments) was used. After ablation, embryos were allowed to mature and then stained. The genotype of individual operated embryos was determined by staining for pharyngeal muscle (ABA or MS; 3NB12) or for rectal valve cells (ABp; J126). The genotype of P2-derived cells could not be determined; however, 25% of these embryos are predicted to be pha-4.

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