The PIE-1 protein and germ line specification in *C. elegans* embryos

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TOTIPOTENT germ line blastomeres in *Caenorhabditis elegans* contain, but do not respond to, factors that promote somatic differentiation in other embryonic cells12. Mutations in the maternal gene pie-1 result in the germ line blastomeres adopting somatic cell fates4. Here we show that pie-1 encodes a nuclear protein, PIE-1, that is localized to the germ line blastomeres throughout early development. During division of each germ line blastomere, PIE-1 initially associates with both centrosomes of the mitotic spindle. However, PIE-1 rapidly disappears from the centrosome destined for the somatic daughter, and persists in the centrosome of the daughter that becomes the next germ line blastomere. The PIE-1 protein contains potential zinc-finger motifs also found in the mammalian growth-factor response protein TIS-11/NUP475 (ref 4–7). The localization and genetic properties of pie-1 provide an example of a repressor-based mechanism for preserving pluripotency within a stem cell lineage.

In the development of all animal embryos, certain cells must remain totipotent to form the reproductive cells, or germ cells, for the next generation. The germ cells of the nematode *C. elegans* arise from a sequence of unequal divisions during early embryogenesis8 (Fig. 1). After each of these divisions, one daughter will produce only somatic cell types, such as muscle cells or neurons; this daughter can be described as a somatic blastomere. The other daughter will produce germ cells in addition to somatic cells, and thus can be described as a germ line blastomere.

Studies on cell-fate determination in *C. elegans* embryos have shown that some maternally expressed factors that function in somatic development also are present in the germ line blastomeres12. For example, development of the somatic blastomere in a four-cell embryo, EMS, but not the sister germ line blastomere, P2, requires the maternally expressed transcription factors SKN-1 (refs 9, 10) and POU-1 (ref 2), although these proteins are present in P2 at the same levels as in EMS12. P3 seems to be prevented from responding to these factors by pie-1(+) activity23. The pie-1 gene is expressed maternally; in pie-1 mutant embryos, the P3 blastomere does not produce germ cells, and instead undergoes a pattern of somatic differentiation similar to a wild-type EMS blastomere8. In pie-1, skn-1 double mutants, the germ line blastomere in eight-cell stage embryo, Pn, does not produce germ cells and instead undergoes somatic differentiation similar to a wild-type somatic C blastomere8. These results suggest that pie-1(+) activity in wild-type development prevents P3 and P4, from responding to factors that determine the EMS and C fates, respectively.

In initial experiments to clone the pie-1 gene, we found that the genetic position of pie-1 coincided with a region of the physical map of the *C. elegans* genome for which there were no available genomic clones or sequences. We therefore screened for rare, spontaneous pie-1 alleles in a strain with a high frequency of transposon mutagenesis. A single, transposon-induced allele, pie-1(zu177::Tc1), was obtained from a screen of ~500,000 animals13, and was used to clone the pie-1 gene (Fig. 2). A full-length pie-1 complementary DNA sequence was used to search for related products in the protein and nucleic acids databases (Fig. 2). The pie-1 gene encodes a novel protein, but has two copies of a motif originally described in the TIS-11/NUP475 family of proteins2,4,7 (Fig. 2). This motif consists of a pattern of three cysteine and one histidine residues (Fig. 2b) and has been proposed to form a zinc-binding domain, or finger4. This motif may have an ancient origin; similar sequences are found in many animal, plant and fungal proteins, and are present in the predicted products of at least 10 genes identified by the *C. elegans* genome sequencing project (C.C.M., unpublished observations). Examples include the mammalian protein U2AF35 (ref. 12) and the *Drosophila* proteins Suppressor of Sable13 and Unkempt14. Although U2AF35 and Suppressor of Sable have been implicated in pre-messenger RNA splicing, a biochemical function has not been established for this motif.

The pie-1 mRNA is expressed maternally and is detected in gonads, oocytes and in all blastomeres until the four-cell stage of embryogenesis; during later stages the pie-1 mRNA is degraded in somatic blastomeres but retained in the germ line blastomeres (G. Seydoux, personal communication). This distribution has been reported previously for several other unrelated mRNAs in the early *C. elegans* embryo, and may represent the general pattern of degradation for non-localized maternal mRNAs15.

To examine the distribution of the PIE-1 protein we raised antibodies against three different PIE-1-specific peptides (Fig. 3). Sera against all three peptides reveal a similar embryonic and mitotic distribution of PIE-1 protein. PIE-1 protein first becomes detectable at low levels in the posterior cytoplasm of one-cell stage embryos (data not shown). In two-cell stage embryos, PIE-1 is

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present in the nucleus and cytoplasm of the germline blastomerem P1, but at low or non-detectable levels in the somatic blastomerem AB (Fig. 3a). In four-cell stage embryos, PIE-1 is present in P1, but at low or non-detectable levels in its somatic sister EMS (Fig. 3b). As described previously, the SKN-1 protein is present in P1, but when P divides SKN-1 is present at equal levels in both of the P daughters, EMS and P2. Thus the asymmetrical localization of PIE-1 to P1 nucleus provides a simple explanation of the observation that pie-1(+1) activity seems to prevent the activity of the SKN-1 transcription factor in P2, but not in EMS. PIE-1 protein remains localized to the germline blastomeres at each of the subsequent cleavages: In 8-cell and 16-cell stage embryos PIE-1 is detected only in P1 and P2 respectively (Fig. 3c, d). When P divides, both of its daughters are germ-cell precursors, and PIE-1 is detected in the nuclei of both daughters (Fig. 3e).

PIE-1 appears to be associated with punctate structures in the cytoplasm of germine blastomeres (Fig. 3). Double-labeling experiments indicate that these structures are P granules56, which are germine-associated particles of unknown function and composition (data not shown). P granules are present at all stages of the C. elegans life cycle in either mitotic germ cells (larvae), mature germ cells (oocytes in adults), or in germine blastomeres (in embryos)56. In contrast, PIE-1 is found in P granules only in germline blastomeres, beginning with the P1 blastomere in the two-cell stage embryo.

Because the pie-1 mRNA is present in all blastomeres in two-cell- and four-cell-stage embryos, translational or post-translational mechanisms must control the asymmetrical distribution of PIE-1 protein during these stages. For example, the association of PIE-1 with P granules during the early cell divisions could serve to localize PIE-1 to the germline blastomeres. A second possible contribution to PIE-1 asymmetry is suggested by the finding that PIE-1 antisera specifically stain the centrosomes of dividing germine blastomeres (Fig. 4). We have examined the intracellular distribution of PIE-1 as each germline blastomere divides, and find a common sequence of events. When germline blastomeres begin to divide, the nascent mitotic spindle complex rotates by roughly 90° (ref. 17). Before rotation, PIE-1 accumulates at apparently equal levels around each centrosome of the spindle, and PIE-1 staining diminishes in the nucleus, cytoplasm and P

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FIG. 2 The pie-1 locus. a. Deduced amino-acid sequence from the nucleotide sequence of a 1,105-bp pie-1 C DNA clone. Nucleotides 529 to 746 are coding sequences deleted in pie-1(zu127) (data not shown). b. Alignment of the pie-1 zinc-finger motifs with sequences from nup745 (ref. 4), U2AF35 (ref. 12) and unkn68. METHODS. The spontaneous mutation pie-1(zu177:Tc1) was obtained in a previously described genetic screen32. The mutant strain was isolated after several times and placed over a chromosome marked with nob-1(c230) and unc-25(e156) which flank pie-1. Recombinant animals yielded a map order of nob-1 – 12 pie-1 – 17 unc-25, and in all recombinants a single novel Tc1 transposon co-segregated with the pie-1 mutation. The C. elegans genomic DNA transfection of this transposon was described as described42. Probes were prepared and used to isolate four full-length C DNA clones; each C DNA contained the trans-spliced leader sequence SL1 (ref. 25) at its 5' end and a poly(A) tail of varying length at its 3' end. Three lines of evidence indicate that the open reading frame encodes the PIE-1 protein: The pie-1(zu177:Tc1) and pie-1(zu127) mutations are a transposon insertion and a deletion, respectively, in the genomic DNA corresponding to this open reading frame; in vitro transcribed RNA from these sequences, prepared as described28, can induce a pie-1 phenocopy when microinjected into wild-type animals; and antibodies raised against peptides deduced from this sequence recognize a protein that is present in wild-type embryos but absent in pie-1 mutants (Fig. 3 and data not shown).
FIG. 4 Centrosomal localization of PIE-1 during division of the germline blastomere P1. All images except e are of embryos stained with an affinity-purified antibody against PIE-1 peptide A and viewed with either a standard fluorescence microscope (a, c, d, e) or an optical sectioning microscope (b).

a. Two-cell stage embryo (only the P1 blastomere is shown). P1 is at prophase; PIE-1 is not detected in the nucleus (not visible) but is detected prominently in the two centrosomes, which are shown here just before rotation of the centrosome–spindle complex (compare with the nuclear staining of the interphase P1 blastomere in Fig. 3a).
b. High-magnification image of PIE-1 staining at the P1 centrosomes. c. The P1 blastomere after rotation of the centrosome–spindle complex. The anterior (left) centrosome stains less intensely than the posterior (right) centrosome. d. Division of the AB and P1 blastomeres (compare with DAPI image shown in e). The mitotic spindles of AB and P1 have elongated; both centrosomes associated with the P1 spindle have detectable PIE-1 staining (arrows), although the posterior centrosome (destined for the germline blastomere P2) shows a much higher level of staining than the anterior centrosome (destined for the somatic blastomere EMS). Neither of the centrosomes associated with the dividing AB blastomere have detectable PIE-1 staining, e. DAPI image of embryo shown in d. The two nascent daughter nuclei of the dividing AB blastomere are visible on the left, and the two nascent daughter nuclei of the dividing P1 blastomere are visible on the right.

METHODS. Identification of the PIE-1-containing structures shown here as centrosomes is based on co-staining experiments with an antibody against beta-tubulin: in such experiments the PIE-1-containing structures are localized at the center of each spindle aster (data not shown). Embryos were prepared and stained as described in Fig. 3. Image in b was collected on a DeltaVision S3A.1 wide-field deconvolution optical sectioning microscope (Applied Precision). The image was deconvolved using the iterative constrained method.

granules (Fig. 4a, b). After spindle rotation, the level of PIE-1 staining in one of the two centrosomes diminishes rapidly and becomes undetectable (Fig. 4c, d). Thus at the completion of cell division, only one daughter cell contains PIE-1 at its centrosome, and that daughter invariably is the new germline blastomere (P1, P2, or P2). When the P1 blastomere divides, PIE-1 staining persists in both centrosomes (data not shown).

Although we do not know the behaviour of individual PIE-1 molecules during the cell cycle, one model is that the PIE-1 protein translocates to centrosomes at cell division. Other proteins, such as NuMA (ref. 18) and CP190 (ref. 19), have been described previously that seem to cycle between the nucleus and centrosomes. We are not aware of proteins other than PIE-1 that disappear from one of the two centrosomes, and thus become distributed asymmetrically after cell division.

What might distinguish the two centrosomes in a dividing germline blastomere? In a study on the control of spindle orientation in C. elegans, the relative positions of the two centrosomes of a germline blastomere were interchanged before rotation of the mitotic spindle was complete. These manipulated embryos developed normally, suggesting that both centrosomes initially are equivalent. We thus propose that rotation of the mitotic spindle in the germline blastomeres may bring the centrosomes into different intracellular environments that affect PIE-1 stability or binding. The idea that asymmetry exists within each germline blastomere is supported by the recent finding that P granules also seem to be degraded or unstable in the pre-somatic ‘half’ of a germline blastomere before division.

The early divisions of the C. elegans embryo represent a classic stem-cell-like lineage in which one pluripotent cell gives rise sequentially to daughters with distinct, and more restricted, developmental potentials. In C. elegans, the fate of the first somatic blastomere, AB, is determined in part by responses to external cell signals.21,22 As described above, the fate of the second somatic blastomere, EMS, is determined at least in part by two transcription factors that are present in both P2 and its sister cell EMS.23 It is likely that differentiation in stem cell lineages in other animals is also determined by different signals or intracellular signals, and that the pluripotent cells must either not contain, or not respond to, these signals.

The PIE-1 protein in C. elegans provides an example of how the pluripotent cell in a stem cell lineage might be protected from the signals that promote differentiation in other cells. We have shown here that the PIE-1 protein is localized to the totipotent germline blastomere after each division in the early embryo. This localization correlates with the repression of somatic cell fates, and also seems to correlate with a general repression of transcription within the germline.23 Thus PIE-1 may represent a localized general repressor that serves to antagonize the activity of a more broadly expressed set of transcriptional activators. This may provide an efficient means for generating diversity within a single cell lineage: if cells in a stem cell lineage express determinative molecules at different times or in response to different signals, there need not be separate mechanisms for segregating these molecules away from the pluripotent stem cell. Instead, a single mechanism for localizing a general repressor at each division could maintain the pluripotent state of the cell.