LET-23-Mediated Signal Transduction During Caenorhabditis elegans Development

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ABSTRACT We are using Caenorhabditis elegans vulval induction to study intercellular signaling and its regulation. Genes required for vulval induction include the LIN-3 transforming α-like growth factor, the LET-23 epidermal growth factor (EGF)-receptor-like transmembrane tyrosine kinase, the SEM-5 adaptor protein, LET-60 Ras, and the LIN-45 Raf serine/threonine kinase. Inactivation of this pathway results in a failure of vulval differentiation, the "vulvaless" phenotype. Activation of this pathway either by overexpression of LIN-3, a point mutation in the LET-23 extracellular domain, or hyperactivity of LET-60 Ras results in excessive vulval differentiation, the "multivulva" phenotype. In addition to searching for new genes that act positively in this signaling pathway, we have also characterized genes that negatively regulate this inductive signaling pathway. We find that such negative regulators are functionally redundant: mutation of only one of these negative regulators has no effect on vulval differentiation; however, if particular combinations of these genes are inactivated, excessive vulval differentiation occurs. The LIN-15 locus encodes two functionally redundant products, LIN-15A and LIN-15B, that formally act upstream of the LET-23 receptor to prevent its activity in the absence of inductive signal. The LIN-15A and B proteins are novel and unrelated to each other. The unc-101, sli-1, and rok-1 genes encode a distinct set of negative regulators of vulval differentiation. The unc-101 gene encodes an adaptin, proposed to be involved in intracellular protein trafficking. The sli-1 gene encodes a protein with similarity to c-cbl, a mammalian proto-oncogene not previously linked with a tyrosine kinase-Ras-mediated signaling pathway. LIN-3 and LET-23 are required for several aspects of C. elegans development—larval viability, P12 neuroectoblast specification, hermaphrodite vulval induction and fertility, and three inductions during male copulatory spicule development. Fertility and vulval differentiation appear to be mediated by distinct parts of the cytoplasmic tail of LET-23, and by distinct signal transduction pathways.

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Key Words: Growth factor, Receptor tyrosine kinase, Nematode, Induction, Tissue specificity

INTRODUCTION

We have been studying a receptor-tyrosine kinase/Ras pathway of signal transduction in the context of Caenorhabditis elegans development. Activity of this pathway promotes vulva differentiation at the expense of nonspecialized epidermal differentiation. Our approach has been to identify the main components of the trunk of this pathway and to explore its branches. Many of the proteins that have been identified as members of signal transduction pathways are clearly more complicated and larger than they need to be for their currently identified catalytic function, suggesting that they have other regulators or targets. Our goals are 1) to define how these pathways function in different tissues, so that we can understand how, in different cellular contexts, the same pathway can produce diverse phenotypes, and 2) to identify regulatory inputs. To study these questions, we have used a molecular genetic approach.

The proteins that participate in this pathway were first identified genetically, as mutations that perturb development (Fig. 1). The genes defined by those mutations were then cloned and found to have similarities to mammalian oncoproteins. The receptor-tyrosine kinase/Ras pathway involves a growth factor of the epidermal growth factor (EGF) family encoded by the lin-3 gene. LET-23, the presumed receptor for LIN-3, is a nematode homolog of the EGF receptor, a transmembrane tyrosine kinase (Aroian et al., 1990). LET-23 acts via SEM-5, an adaptor protein that has Src homology 2 (SH2) and Src homology 3 (SH3) domains (Clark et al., 1993; Lackner et al., 1994; Wu and Han, 1994). One outcome of the pathway is to stimulate vulval differentiation.

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C. elegans hermaphrodites are females that make approximately 320 sperm before they switch over during development to making oocytes. They are very useful for genetics because they do not need to mate to reproduce (Brenner, 1974). If the vulva is not formed, the eggs are fertilized internally and the animals hatch inside the mother (Horvitz and Sulston, 1980). Thus, mutants that result in vulvaless or multivulval animals can be detected by observing adult animals under a dissecting microscope. Nomarski optics allow examination of the differentiation of individual precursor cells during larval development (Sulston and Horvitz, 1981). In wild-type hermaphrodites only three precursor cells are involved in vulval development. Any deviation from this number can be readily seen.

In C. elegans, the anchor cell, which expresses the LIN-3 growth factor, normally induces three precursor cells to divide and make vulval tissue (Kimble, 1981; Hill and Sternberg, 1992). Three other cells are competent to respond but normally do not, and instead undergo only a single round of division. We know that the cells are competent to respond to LIN-3 because overexpression of LIN-3 results in all six of the vulval precursor cells (VPCs) generating vulval tissue (Hill and Sternberg, 1992, 1993). During normal development, after the initial signaling event, the three induced precursor cells undergo three rounds of mitosis and then begin characteristic patterns of differentiation and morphogenesis (Sulston and Horvitz, 1977; reviewed by Horvitz and Sternberg, 1991). The end result of vulva induction is the formation of a hole in the ventral surface of the animal. This opening connects the developing uterus to the exterior and allows for transit of eggs out and sperm in.

**A MULTIVULVA MUTANT OF LET-23**

We have found a mutation that activates the LET-23 protein. We isolated a mutation, called *sa62*, that results in a multivulva phenotype. (Note that there is only one vulva with a connection to the uterus because there is only one anchor cell in these animals; the rest of the induced vulval tissue forms protrusions on the ventral surface of the adult.) The *sa62* mutation is semidominant: only 7% of animals heterozygous for *sa62* have excessive vulval differentiation, while 90% of homozygotes do. If we remove the gonad from a wild-type animal, the source of the LIN-3 growth factor is removed and none of the VPCs make vulval tissue. If we remove the gonad from an *sa62* animal, about four of the six precursor cells make vulval tissue. Thus, this mutation renders the cells partially constitutive for vulva differentiation. In an animal heterozygous for *sa62*, there is still some vulva differentiation in the absence of the gonad.

The *sa62* mutation maps to the same region of chromosome II as the *let-23* locus. We carried out a series of genetic experiments to demonstrate formally that the *sa62* mutation is in the *let-23* gene (Katz et al., in preparation). We also completely sequenced the *let-23* coding region from the *sa62* mutant and discovered a single point mutation. When the sequence of *LET-23* was aligned with members of the EGF receptor family in other species, the point mutation was found to be a Cys to Tyr codon change located in a Cys-rich region in the extracellular domain of the protein. When the intact wild-type gene was engineered to contain the same point mutation as found in *sa62* and used to make transgenic nematodes, the resulting nematodes displayed the multivulva phenotype. Thus, although we cannot rule out other changes in the *let-23* promoter, these results demonstrate that the point mutation alone will give rise to this phenotype.

The point mutation is just at the carboxyl edge of the first extracellular cysteine-rich domain of the receptor (Katz et al., in preparation). This is the first point mutation in the extracellular domain of any of the members of the EGF receptor family that activates the protein. Thus, the existence of this mutant may indicate a region of the molecule that is important for dimerization or conformational changes upon ligand binding.

**LET-23 TYROSINE KINASE RECEPTOR SIGNAL TRANSDUCTION PATHWAY**

We used *sa62* and other mutants for genetic pathway analysis. In this approach the phenotypes of double mutants are observed. Loss of function *sem-5* and *lin-3* mutants are vulvaless. The double mutants containing *sa62* and a defective *sem-5* gene are also vulvaless. By contrast, double mutants containing *sa62* and a defective *lin-3* gene are multivulva rather than vulvaless.
Ras in the pathway (Han et al., 1993) and allowed us to place LIN-45 Raf downstream of LET-60 (Clark et al., 1992) to place SEM-5 upstream of LET-60. Similar double mutant analysis had allowed us to place LIN-35 Raf downstream of LET-60 and the biochemistry of their mammalian counterparts. The conclusion from these experiments is that the pathway is involved in making a choice between two cell fates rather than in controlling the specific aspects of cell fates such as cell division, differentiation, or apoptosis. We think that this pathway is being used in different cells as a conserved set of mutations in the let-23 gene which diminished or provided only partial function; provided complete function; provided no function; provided intermediate level of function; provided only partial function; provided no function; provided complete function; provided intermediate level of function; provided only partial function. Data from Aroian and Sternberg (1991) and Aroian et al. (1993, 1994).

MULTIPLE ROLES OF THE LET-23 TYROSINE KINASE RECEPTOR SIGNAL TRANSDUCTION PATHWAY

From our studies of the Ras-mediated pathway, we believe that this pathway is involved in making a choice between two cell fates rather than in controlling specific aspects of cell fates such as cell division, differentiation, or apoptosis. We think that the same set of genes is being used in different cells as a conserved signaling module to control the cell's fate at different times and in different places in development. The output of the pathway forces a cell to choose between two alternative cell fates, and the choices depend on the context in which the cell finds itself. In the hermaphrodite vulva, one fate is to proliferate and differentiate as vulva and the other fate is to divide once and differentiate into nonspecialized epidermis.

The copulatory tail of the C. elegans male is a specialized sensory structure innervated by an additional 87 neurons that allow it to locate the hermaphrodite’s vulva and transfer sperm (Salston et al., 1986; Liu and Sternberg, 1995). The tail consists of two copulatory spicules. In a male with a defect that diminishes signaling through the Ras pathway, such as a mutation in the let-23 gene, the spicules are distorted, short, and nonfunctional. The cells that are affected by this mutation are intermediate precursor cells that will divide one to three rounds and make different types of differentiated progeny. There are three types of pairs of intermediate precursor cells. The anterior and posterior cell in each pair generate different sets of cells. The distinction between the anterior and posterior cell in each pair (e.g., α and β, respectively) depends on at least three signals (Chamberlin and Sternberg, 1993). One important signal is an inductive signal from the anterior and dorsal F and U sister cells which produce a redundant inductive signal. F and U induce one cell in each pair to differentiate to the α subtype. Defective mutations in any one of the genes that contribute to the Ras signaling pathway result in deformed spicules and lack α cells (Chamberlin and Sternberg, 1994). In the inverse experiment, in which one of the members of the Ras signaling pathway is activated or overexpressed, the β cells behave like α cells. Thus, the Ras pathway is one determinant of whether cells have the α and β fates.

Other fates are also determined by the Ras signaling pathway. These can be identified in the absence of the gene product LET-23. let-23 gained its name because it was originally identified by mutations that result in lethality (Herman, 1978). Homozygous animals die as larvae, as do animals completely defective in LIN-3, SEM-5, LET-60, or LIN-45. Second, in addition to males being infertile, hermaphrodites are infertile because of a problem in gonadal development (Aroian and Sternberg, 1991). From a number of genetic screens we isolated a set of mutations in the let-23 gene which diminished or activated LET-23 activity, or which had tissue-specific effects (Table 1).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Change</th>
<th>Ess.</th>
<th>Vulva</th>
<th>Fert.</th>
<th>Male</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>sy17</td>
<td>5' splice donor, exon 4-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>sy1</td>
<td>stop 6 aa from C-terminus</td>
<td>+</td>
<td>(-)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sy97</td>
<td>Splicing-removes last exon</td>
<td>(-)</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>sy10</td>
<td>C368Y, extracellular domain</td>
<td>±</td>
<td>±</td>
<td>±</td>
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<tr>
<td>n1045</td>
<td>Splicing-variable deletions</td>
<td>±</td>
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</table>

*Ess., essential function; vulva, vulval differentiation; fert., hermpahrodite fertility; male, male spicule development. +, provides complete function; -, provides no function; ±, provides intermediate level of function; nd, not determined.

†Data from Aroian and Sternberg (1991) and Aroian et al. (1993, 1994).

TABLE 1. Mutations of let-23 With Tissue-Specific Defects

BRANCHING FROM LET-23

The let-23 mutation sy97 is a G to A point mutation located in the splice acceptor of the last exon encoding the C-terminal tail of the LET-23 (Aroian et al., 1993, 1994). The RNA transcripts of this mutant DNA are spliced to a cryptic acceptor site that is two nucleotides downstream, with the result that the reading of the last exon is out of frame. We have also obtained an intragenic revertant of this mutation in which the cryptic mutation is removed with a second G/A conversion (Jongeward et al., 1995; Aroian et al., 1993). Unexpectedly, in this revertant, splicing has returned to normal even though the revertant still lacks the conserved AG at the 3' splice junction. In the sy97 mutant most if not all of the protein product lacks the last exon, the part which encodes the C-terminal tail and contains three of the eight tyrosines in the C-terminal domain. The phe...
nototypic results of the sy97 mutation are that 90% of the animals die and both vulva differentiation and male spicule development are completely defective (Aroian and Sternberg, 1991). However, the 10% of sy97/sy97 hermaphrodites that live are completely fertile. These observations lead us to the simple hypothesis that the functions of the C-terminal domain of LET-23 are partitioned, with the fertility functions located towards the N-terminus and the functions which promote vulva differentiation, male spicule development, and viability located at the C-terminus.

We have established a means of analyzing the involvement of various parts of the C-terminal domain of LET-23 in cellular functions (G. Lessa and P. Sternberg, unpublished observations). The C-terminal tail contains 10 tyrosines that constitute eight potential sites for phosphorylation and interaction with SH2-containing proteins. We used a genetic instead of a biochemical approach to analyze the involvement of these various sites in biological responses. We inserted double stop codons at two sites in the C-terminus of LET-23. The construct, STX, produces a truncated protein retaining the first five tyrosine sites. With STX we created the equivalent of the sy97 mutation. By using a double stop codon to truncate the protein after the first five tyrosines we avoided using the splicing defect because it would have been possible to obtain wild-type splicing without the correct acceptor.

The STX mutant had the same characteristics of the sy97 mutation: low viability and complete fertility. However, whereas vulva differentiation was completely lacking in sy97, it was observed in 25% of the animals in which the STX mutation was overexpressed. Therefore, some of the remaining tyrosines other than the ones in the extreme C-terminus are able to mediate, in part, vulva differentiation. The last three tyrosines in LET-23 are each in a YXXN context, and are the most likely of the 10 to interact with SEM-5/Grb2 (e.g., Songyang et al., 1993). Genetic evidence shows that this interaction is likely to be an essential component of the signaling pathway (Clark et al., 1992). We believe that one explanation of the leakiness of the STX mutant with respect to vulva differentiation might result from SEM-5 binding to other tyrosines. We are now doing experiments to add back particular tyrosines to a mutant that lacks all the C-terminal tyrosines to determine which ones are sufficient for each function of LET-23.

Our results suggested that different portions of the receptor tail mediate different biological effects such as viability and fertility. To test this hypothesis we tested whether activated LET-60/Ras could rescue let-23 defects (Han et al., 1990; Jongeward et al., 1995). From these experiments we found that vulva differentiation and viability were rescued by activated Ras but fertility was not. These results were confirmed using a mutation in the lin-1 gene, which acts downstream of LIN-45 to negatively regulate vulval differentiation. Thus, we think that activity of the Ras signaling pathway only stimulates vulva differentiation and viability, and that there must be another signaling pathway for fertility. We are now working on an approach to identify the components of this signaling pathway.

NEGATIVE REGULATORS OF THE LET-23 TYROSINE KINASE RECEPTOR SIGNALING PATHWAY

To search for negative regulators of the pathway we mutagenized animals that already had mutations in LET-23 (such as the sy97 mutation) and searched for evidence of restoration of the signaling pathway in the form of animals which had a vulva and that could lay eggs (Jongeward et al., 1995). From this screen we found several mutations including a mutation that corrected the splicing defect of sy97, a mutation that activated LET-60/Ras, and mutations in new genes such as unc-101 and sli-1. These genes are defined by mutations that would not have indicated their involvement in vulva differentiation. unc-101 had been identified previously because the mutant animals had low viability, and defects in moving, defecating, eating, and mating (see references in Lee et al., 1994). The sli-1 gene had not previously been identified because it displays no vulval phenotype when deleted. Animals expressing either unc-101 or sli-1 mutations have wild-type vulval phenotypes. Only when the two mutations are combined does the animal display a multivulva phenotype (Yoon et al., 1995). Thus, this is an example of apparent redundancy. These genes apparently are not targets that are negatively regulated by LET-23 because they do not compensate for a loss of the receptor. However, sy97 mutants, which have a defective but still partially active LET-23, can be rescued by decreasing activity of sli-1 or unc-101. For example, an animal defective in sli-1 has a wild-type phenotype whereas an animal defective in let-23 has no vulva differentiation. An animal containing both mutations has an almost normal vulva. Similar results are found with combinations of sli-1 and unc-101 with SEM-5/Grb2. However, mutants of lin-45 raf were not affected. The simplest interpretation of these results is that unc-101 and sli-1 act at or near the step of receptor-adaptor interaction (Jongeward et al., 1995).

sli-1, which acts only on vulva differentiation and viability, seems to be a negative regulator of the LET-60 signaling pathway because, in its absence, the pathway works better. We have also found mutations that can restore the fertility pathway but do not restore vulva differentiation or viability to let-23 mutants (T. Clandinin and P. Sternberg, unpublished). Thus, we think that there is a branch in the LIN-3/LET-23 pathway from LET-23, most likely involving distinct tyrosine residues in the cytoplasmic tail, that acts via distinct signal transduction pathways.

In addition to unc-101 and sli-1, other genes encoding negative regulators of the LET-23 tyrosine kinase pathways have been found. One set of negative regulators are the lin-15A and lin-15B genes that are in the lin-15 locus which was discovered in Bob Horvitz's lab (Ferguson and Horvitz, 1985, 1989, Ferguson et al.,
1987). We have tentatively called the fifth suppressor generok-1for regulator of kinase-mediated signaling (J. Lee, Neil Hopper, and P. Sternberg, unpublished observation).

Preliminary analysis suggests that the five negative regulatory activities might have different properties. For example,lin-15A and lin-15B seem to act on the basal activity of the pathway. By contrast,rok-1seems to only act on the pathway when it has been activated (J. Lee and P. Sternberg, in preparation). unc-101and sli-1seem to act on both the basal and activated pathways (G. Jongeward and P. Sternberg, in preparation).

Now thatlin-15has been cloned we know that this locus has two products,lin-15A B, which encode novel proteins (Huang et al. 1994; Clark et al., 1994). LIN-15A and B do not necessarily interact directly with the receptor since there are a number of other genes that act with LIN-15A and B to regulate LET-23 (see Ferguson and Horvitz, 1989). Theunc-101gene encodes a clathrin-associated adaptor protein. These proteins couple transmembrane receptors, such as the EGF receptor, to clathrin. These proteins act either at the plasma membrane in endocytosis or at the trans-Golgi apparatus in protein sorting and lysosomal assembly. UNC-101 is more like the Golgi form and thus might not be involved in endocytosis. One of the chains of the clathrin adapters interact directly with the human EGF receptor (Sorkin and Carpenter, 1993). Peyrard et al. (1994) discovered a human gene of this class as a meningioma tumor suppressor.

The sli-1 gene encodes a nematode homolog of thecblviral oncogene (Yoon et al., 1995). Thus, it may be involved in regulating signal transduction. Studies of Cbl also indicate a means by which SLL-1 might interact with the EGF receptor. By way of an SH2-binding domainCblinteracts with some adaptor proteins that can interact with the EGF receptor. CBL and SLL-1 have putative SH3-binding domains and we believe that they function by interacting with other signal transduction components.

**FUTURE DIRECTIONS**

With our genetic approaches we are now facing the challenging problems of identifying the branches in the tyrosine receptor signaling pathways in the various tissues, determining how they are regulated, and determining whether actions of the negative regulators define the tissue specificity of the LET-23 signaling pathway.

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QUESTIONS AND ANSWERS

Q: Your phenotypes fit into a hierarchy. That is, either you inhibit everything or you inhibit only fertility. Could you say that fertility is the more sensitive and requires a larger quantity of signal than the others, and that there is no real specificity in the signals?

A: We also have other mutations that have complementary or very different tissue specificities. For example, a mutation that truncates six amino acids from the C-terminus only affects vulva differentiation. There are other mutations in which the effects are more quantitative. We only screened for vulvaless or lethal phenotypes. When Helen Chamberlin was trying to find genes for other functions, she screened for mutants that affect male spicule development. One mutation was in let-23 and to our knowledge this mutation only affects the male. Thus, I believe that there are differences which involve branches beyond the receptor.

Q: Have you identified any genes in the vulva signal transduction pathway that lie downstream of MAP kinase?

A: There are two candidates that have been cloned. The first is lin-1, cloned by Beitel and Horvitz (pers. comm.), which encodes a negative regulator of vulva differentiation and contains an Ets domain. It is still not clear that lin-1 is a direct target of the LET-23 pathway. Stuart Kim at Stanford has cloned lin-31 and found that it encodes a protein with a forkhead domain. Miller, L.M., Gallegos, M.E., Morrisseau, B.A., and Kim, S.K. (1993). lin-31, a Caenorhabditis elegans, HWF-3/fork head transcription factor homolog, specifies three alternative cell fetus in vulval development. Genes Dev. 7:933–947. Kim argues that this gene is part of the signaling pathway. The complication with this gene is that it also has a role earlier in development. Miller, L.M., Gallegos, M.E., Morrisseau, B.A., and Kim, S.K. (1993). lin-31, a Caenorhabditis elegans, HWF-3/fork head transcription factor homolog, specifies three alternative cell fetus in vulval development. Genes Dev. 7:933–947. Kim argues that this gene is part of the signaling pathway. The complication with this gene is that it also has a role earlier in development. It is very likely to be a transcription factor. However, it is still open as to whether the product of this gene is a target of the pathway or sets up a precondition for signaling.

Q: Are any of the negative regulators structurally similar to phosphatases?

A: No.

Q: You showed that a point mutation in the extracellular domain could affect LET-23 tyrosine kinase activity. Does it also affect the binding of LIN-3?

A: We don’t have a good assay for LIN-3 binding to LET-23. So, we are testing whether the mutation affects dimerization of the receptor. There is some effect when the ligand is present so LIN-3 seems to interact with LET-23 but we do not know if the affinity of the ligand for its receptor has been altered by the mutation.