SIGNALING BY *LET-23*, A *CAENORHABDITIS ELEGANS* EPIDERMAL GROWTH FACTOR RECEPTOR HOMOLOG

Thesis by

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I would like to dedicate this thesis

to my father Francesco, who died on 5 September 1995,

and

to Bino Palmer, who died on 21 May 1997.

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Abstract

The *Caenorhabditis elegans* gene *let-23* encodes a tyrosine kinase of the Epidermal Growth Factor Receptor (EGFR) family. Ligand-independent activation of EGFR tyrosine kinases is involved in many types of cancer. A ligand-independent activating mutation of *let-23*, *sa62*, has been characterized. *sa62* maps to the extracellular domain of the LET-23 protein and results in excessive proliferation of the cells which forms the hermaphrodite vulva. Analysis of *sa62* has suggested that the region to which it maps is important for transmembrane activation of LET-23 signaling.

let-23 mediates multiple functions in *C. elegans* including viability, vulval differentiation, and fertility. Analysis of tissue specific and loss-of-function mutations of *let-23* suggested that the C-terminus of LET-23 can be divided into at least three domains, each mediating a subset of *let-23* functions. The EGFR family of tyrosine kinases contain potential C-terminal phosphotyrosines whose role is still unclear. Using *in vitro* mutagenesis and transgenic technology, this thesis shows that *in vivo* the LET-23 C-terminal tyrosines are required for wild-type activity and that they are differently used to mediate cell specific, positive, and negative regulation. One tyrosine is necessary and sufficient for wild-type fertility. Three other tyrosines are involved in viability and vulval differentiation. Another tyrosine appears to mediate tissue specific negative regulation. Two mechanisms are proposed for receptor tyrosine kinase signaling: a positive mechanism, which promote, and a negative mechanism, which inhibits receptor tyrosine kinase activity. It is also shown that LET-23 activates at least two pathways: the Ras pathway to mediate viability and

vulval differentiation, and another pathway to mediate fertility. A genetic screen to identify genes acting positively in the *let-23*-mediated fertility pathway is described. In addition, the initial characterization of a gene acting in this pathway is reported.

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Chapter I

Signal transduction by Receptor Tyrosine Kinases

A central problem in biology is to understand the cues and mechanisms that a cell uses for its developmental decisions. When and how does a cell decide to proliferate or to stop dividing, to become one cell type or another? These decisions are important during development as well as during survival and, when altered, can result in death of an organism. The making of an organism is a complex task that requires a great deal of regulation and coordination among cells. Cell-cell communication and signal transduction must be tightly controlled to allow development. In addition, they have to be highly reproducible so that near identical organisms or tissues are produced and the species can be maintained. In an adult organism dying cells are constantly replaced; therefore, the developmental information must be transmitted also to the new cells. For example, a newborn cell must be instructed to stop dividing and to localize at a specific position so that it can correctly replace the dead cell.

Signal transduction is normally performed by receptors coupled to intracellular effectors. Then the signal must be processed in the cytoplasm so that only the appropriate effectors are activated in the right way and at the right time. The complex set of proteins involved in transduction of signals from the cell membrane to the nucleus defines a signal transduction pathway. Since many types of cancer have been attributed to misregulation of specific signal transduction pathways (Hunter, 1997), understanding these pathways in detail may have important therapeutic applications. For example, it could be helpful for the development of drugs specifically targeted against the altered component(s) of the signal transduction pathway. Such drugs would be specific for malignant cells. Presently, all cancer drugs are toxic to both normal and cancerous cells.

Two major approaches have been used to study signal transduction, the "biochemical" and the "genetic" approach. The biochemical approach makes use of cultured cells and is particularly useful to detect phosphorylation, protein-protein interactions and to determine the specific protein domains involved. Since biochemical studies are not carried out in a true physiological background, their results should be interpreted with caution. The cell lines used are distant from their progenitors, they mutate very easily, grow in an anchorage-independent manner, and often do not even normally express the molecule under study. The genetic approach requires a model organism employing a signal transduction pathway for a known developmental process. Mutagens are then used to disrupt this developmental process. Genetic techniques can then be used to find the genes involved. In addition, suppressors of the phenotype(s) associated with loss of signaling can be isolated and the genes can be ordered using epistasis tests (Avery and Wasserman, 1992). The genetic approach has been very successful to dissect several signal transduction pathways both in *Drosophila* (for example, see Duffy and Perrimon, 1994; Wassarman *et al.*, 1995; Perrimon *et al.*, 1995) and *C. elegans* (for example, see Horvitz and Sternberg, 1991; Sternberg, 1993; Stern and DeVore, 1994). The limitation of the genetic approach is that it does not give information on the state, location, or structure of proteins. Therefore, the ideal would be to complement genetic studies with molecular biology and biochemistry.

The free-living nematode *C. elegans* offers many advantages to study signal transduction *in vivo*. It is relatively simple (it contains about 1,000 somatic cells) and can be studied using genetics, cell biology, and molecular biology. *C. elegans* comes in 2 sexes, hermaphrodites and males,

its generation time is only ~3.5 days, and about 60% of its 80 million base-pair genome is sequenced. For these reasons genetics are easier than in other organisms. In addition, the timing, number, and location of all cell divisions are known and invariant (Epstein and Shakes, 1995; Riddle, D. L. *et al.* 1997). Therefore, *C. elegans* offers a unique opportunity to study cell-cell interactions and signal transduction. A laser microbeam can be used, in association with genetic mutations, to perturb the environment around particular cells. Analysis of development of those cells can then be evaluated. Molecular genetic techniques can also be employed to clone the genes of interest. These same genes can be reintroduced in transgenic animals and studied in detail. This is extremely useful because it allows to study the effects of a protein directly *in vivo*, in an intact organism, where all physiological controls and interactions are maintained.

I have used the features that *C. elegans* offers to study signal transduction by LET-23, an Epidermal Growth Factor Receptor (EGFR) homolog. EGFR has been extensively studied in cultured cells but the role of its C-terminus in signaling is still unclear. LET-23 mediates several functions in the worm including viability, vulval differentiation and fertility. Using a genetic and molecular biological approach, I have dissected the role of LET-23 C-terminus for these functions. I have shown that LET-23 C-terminal tyrosines are essential for wild-type signaling and that they are used to mediate positive, negative, and tissue specific regulation. Finally, I have performed a genetic screen to find additional components of the tissue-specific *let-23* pathway that mediates fertility. My systematic analysis is one of the few carried out in an intact organism and is the most complete available.

Receptor Tyrosine Kinase signaling

Overview

Several growth factors and hormones mediate their diverse biological functions by activating Receptor Tyrosine Kinase (RTK) signaling pathways. RTKs influence many cellular processes including, mitogenesis, differentiation, metabolism, migration, and shape (Carpenter and Wahl, 1990; Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993). Mutations that activate RTKs lead to oncogenesis and other disorders associated with excessive cell proliferation. In addition, studies in model organisms like *C. elegans* and *Drosophila* have demonstrated that RTKs are required for specific developmental processes. Correct RTK activity is essential for normal development and mutations that inactivate RTKs result in a variety of developmental disorders (Dickson and Hafen, 1994; Perrimon, 1997).

RTKs are transmembrane proteins with an extracellular and a cytoplasmic portion (Fig. 1). The extracellular portion contains the ligand binding domain. The cytoplasmic portion contains a conserved tyrosine kinase domain. In addition, it carries regulatory sequences that are subject to autophosphorylation as well as phosphorylation and dephosphorylation by cytoplasmic kinases and phosphatases (Lemmon and Schlessinger, 1994). Upon ligand binding, RTKs undergo conformational changes, oligomerize, and become catalytically active. As a result, in addition to phosphorylating other substrates, they phosphorylate specific tyrosine residues in their own cytoplasmic region generating short sites that bind to non catalytic domains referred to as Src Homology 2 (SH2) domains. Many cytoplasmic proteins have been shown to associate with RTKs via their SH2 domains. Different SH2 domains bind different tyrosine-phosphorylated

regions depending on the amino acids immediately surrounding the phosphotyrosine (Cohen *et al.*, 1995; Pawson, 1995). It is thought that activation of the more downstream effectors by RTKs requires localization at the receptor level of several SH2-containing proteins. Some of these proteins, for example phospholipase C-γ (PLC-γ) (Hernandez-Sotomayor and Carpenter, 1992), become activated upon phosphorylation. Some others, for example GRB-2, seem to function only as adapters (Cohen *et al.*, 1995; Pawson, 1995).

What we know about the mechanism of RTK signaling at the receptor level comes from complementary studies in mammalian cells, *Drosophila*, and *C. elegans*. Genetically tractable organisms have been very useful in identifying new molecules involved in RTK signaling pathways, in relating them to specific developmental processes, and in ordering them in their pathways. Surprisingly, in fact, organisms so distantly related as mammals, *Drosophila*, and *C. elegans* share conserved cassettes of genes activated by RTKs. Studies in *Drosophila* and in *C. elegans* are, for example, clarifying the role of negative regulators in RTK signaling (Sternberg, 1993; Lee *et al.*, 1994; Sternberg *et al.*, 1994; Jongeward *et al.*, 1995; Schweitzer *et al.*, 1995; Yoon *et al.*, 1995; Freeman, 1996; Golembo *et al.*, 1996; Perrimon, 1997; Yoon, 1997). Moreover, molecular and genetic analyses in *C. elegans* has shown that an EGFR tyrosine kinase is able to activate at least two independent pathways demonstrating for the first time in a clean way that branching happens also at the RTK level (Aroian *et al.*, 1994; Clandinin, 1997; Lesa and Sternberg, 1997). Below I review positive signaling by RTKs at the level of the cytoplasmic portion of the receptor. In particular, I start by introducing the Ras pathway, which is the most

characterized RTK-activated pathway. I then introduce the SH2 and PTB phosphotyrosine binding domains that have been implicated in many interactions between RTKs and their cytoplasmic effectors. I continue by examining some representative RTKs and I discuss the evidence for activation of multiple pathways via SH2 binding sites. Finally, I analyze the function of RTK-activated pathways other than the Ras pathway. I do not discuss an important issue of RTK signaling, negative regulation. For reviews of this topic, see (Sternberg, 1993; Sternberg *et al.*, 1994; Perrimon, 1997; Yoon, 1997).

The Ras pathway

The Ras protein was originally studied in mammalian cells because, when mutated, it is oncogenic and thus forces a cell to become cancerous. It subsequently became clear that many other oncogenes are normal components of signal transduction pathways and a great deal of effort has been employed to characterize additional components of these pathways. The Ras pathway (Fig. 2) is activated by several RTKs and can control different processes. For example, the *Drosophila* RTK Torso controls pattern formation of the tail and the most anterior part of the head (Perkins *et al.*, 1992; Duffy and Perrimon, 1994). In addition, the RTK Sevenless is required for the correct specification of the R7 photoreceptor in the *Drosophila* eye (Banerjee *et al.*, 1987; Hafen *et al.*, 1987). The Ras pathway in *C. elegans* controls several functions including vulva development (see below) (Horvitz and Sternberg, 1991; Sternberg, 1993).

Briefly, activation of RTKs leads to relocation of the Guanine Exchange Factor (GEF) SOS (which is bound to the adapter GRB-2) to the

plasma membrane, where it can activate Ras by exchanging GDP for GTP (Downward, 1994). In turn, Ras activates Raf, a Mitogen Activated Protein Kinase Kinase Kinase (MAPK3), by direct association and by phosphorylation (Leevers *et al.*, 1994; Shirouzu *et al.*, 1994; Stokoe *et al.*, 1994). Raf typically activates MAPK2s (or MAPKKs or MEKs) by serine/threonine phosphorylation. MAPK2s phosphorylate specific threonine and tyrosine residues of MAPKs leading to their activation. This simple description of the Ras pathway is probably not accurate. Many branches and controls seem to exist. In particular, the following findings indicate a higher complexity than previously anticipated (Denhardt, 1996). 1) Many proteins interact with the C-terminus of activated RTKs, for example the p85 subunit of Phosphatidyl Inositol 3-OH Kinase (PI3K), the tyrosine phosphatase SHP-2, PLC-γ, the adapter proteins GRB-2, Shc, Nck, Crk, the GTPase Activating Protein of Ras, and others. These interactions suggest that each of these proteins has a specific role in signal transduction. Nonetheless, their function and their physiological significance is largely unknown. 2) Ras has been shown to interact with other Ras-like molecules such as Rac, Rho, (involved, among other functions, in regulation of the cytoskeleton) and Ral proteins (whose function is not known). Moreover, it has been demonstrated that for a complete transformation state, Ras requires Rac-1 and Rho-A which are thought to activate independent pathways (Khosravi-Far *et al.*, 1995). It is, therefore, probable that Ras activates other pathways in addition to Raf/MAPK (Rodriguez-Viciana *et al.*, 1997). 3) Studies in *Drosophila* and *C. elegans* have identified several regulators acting at different levels in the Ras pathway. For example, Ksr acts near Ras (Downward, 1995; Kornfeld

et al., 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995). Other positive and negative regulators have been found at the level of LET-23 (Ferguson and Horvitz, 1989; Kim and Horvitz, 1990; Huang *et al.*, 1994; Lee *et al.*, 1994; Sternberg *et al.*, 1994; Jongeward *et al.*, 1995; Yoon *et al.*, 1995; Hoskins *et al.*, 1996; Simske *et al.*, 1996; Perrimon, 1997). These findings suggest that there are several points in the pathway that can be individually regulated. 4) Many different GAPs have been isolated (GAPs inactivate the GTP-bound Ras by stimulating its intrinsic GTPase activity leading to the inactive GDP-Ras). Some of them are expressed only in certain tissues. For example, NF1 is found only in neural and gonadal tissues (Gutmann *et al.*, 1995) suggesting that it is required for a specific activity in those tissues. In addition, there are multiple GEFs specific for Ras and for distinct Ras-like molecules implying that also at this level there is specific regulation (Denhardt, 1996). 5) Several MAPK3 have been identified: at least three different Raf proteins (Raf-1, A-Raf, and B-Raf) and other proteins activated by the Ras family (for example, PAK and TAK). These proteins can specifically activate certain members of the MAPK2 family (Minden *et al.*, 1994; Wu *et al.*, 1996). 6) The MAPKs constitute a family of proteins that include the Extracellular-signal Regulated Kinases (ERKs), Jun N-terminal Kinases (JNKs), Fos-Related Kinase (FRK), Stress-Activated Protein Kinases (SAPKs), and others. Each of these proteins can potentially target different effectors. Thus, activation of a single RTK involves many downstream effectors, potentially leading to the activation of multiple signaling pathways.

The SH2 and PTB modular domain

Many proteins with SH2 and PTB domains have been implicated in activation of specific signaling pathways downstream of RTKs. SH2 and PTB domains are small protein modules (about 100 and about 200 amino acids, respectively) able to bind with high affinity phosphorylated, but not unphosphorylated, tyrosines. Both domains appear to localize cytoplasmic effectors at the level of RTKs (or of other cytoplasmic proteins) phosphorylated on specific tyrosine sites.

The SH2 domain was first shown to be involved in signaling in 1986 by Tony Pawson and co-workers (Sadowski *et al.*, 1986). Subsequently, it was found in a large variety of cytoplasmic proteins involved in Ras signaling, phosphatidyl inositol metabolism, tyrosine phosphorylation and de-phosphorylation, intracellular trafficking, gene expression and cytoskeletal organization (Pawson, 1995). Therefore it appears that the SH2 modular domain is largely used in response to tyrosine phosphorylation by different classes of molecules and signaling pathways.

Distinct SH2 domains bind distinct phosphotyrosine-containing regions. Specificity of binding is determined by the 3 amino acids immediately C-terminal to the phosphotyrosine. To clarify the binding specificities of different SH2 domains, (Songyang *et al.*, 1993; Songyang *et al.*, 1994) constructed a 12-amino acid phosphopeptide library with the positions +1 to +3 C-terminal to the phosphotyrosine randomized (pYXXX, where X is any amino acid). Twenty-two different SH2 domains were used to select from the library the phosphopeptides to which they bind with higher affinity. Songyang *et al.* found two classes of SH2 domains: one class preferred sequences pY-hydrophilic-hydrophilic-Ile/Pro; another class

preferred sequences pY-hydrophobic-X-hydrophobic, but each member of these classes (except the ones from the Src family of proteins) bound with affinity 30-100-fold higher (1-50 nM) to a single phosphopeptide. For example, the SH2 domain from the *C. elegans* adapter protein SEM-5 bound with highest affinity to the sequence Y-hydrophobic-N-(V/P). It is important to bear in mind that the specificity of SH2 domains is not absolute and that a single SH2 domain might be able to bind multiple sites with reasonably high affinity. Therefore, *in vivo*, the cytoplasmic protein carrying that particular SH2 domain might be targeted to different cellular locations and might be involved in different signaling pathways. Moreover, its targeting might be regulated by SH2 domains of other proteins.

The Phospho Tyrosine Binding (PTB) binding domain was recently identified in the adapter protein Shc (Blaikie *et al.*, 1994; Kavanaugh and Williams, 1994) and subsequently shown to bind to LXNDXY motifs (Batzer *et al.*, 1995; Prigent *et al.*, 1995; Songyang *et al.*, 1995). Until now only two proteins have been found to contain the PTB domain: Shc and the Insulin receptor Substrate-1 (IRS-1). In Shc the PTB domain mediates association with EGFR and with SHIP, a 145 kD SH2-containing Inositol Phosphatase whose function is not known (Kavanaugh and Williams, 1994). At present, the physiological significance of the PTB domain is unclear.

Role of SH2 domains in RTK signaling

The most successful approach to determine the role of SH2 domains in mediating specific RTK functions has been to mutate single RTK autophosphorylation sites. When the tyrosine in the site is changed to phenylalanine, SH2 binding is abolished (Kazlauskas *et al.*, 1990; Margolis

et al., 1990; Fantl *et al.*, 1992). Using this technique SH2 binding sites have been shown to mediate, in some cases, different RTK functions. In this section I will give some examples of successful and unsuccessful dissection of the function of SH2 binding sites.

Analysis of SH2 binding sites as mediators of specific pathway activation has been principally carried out for mammalian RTKs using cultured cells. The Platelet-Derived Growth Factor Receptor (PDGFR) mediates many biological responses in mammalian cells including turnover of phosphatidylinositol and inositol phosphatases, alteration of Ca^{++} fluxes, membrane ruffling, cytoskeletal rearrangements, migration, proliferation, and angiogenesis (Claesson-Welsh, 1994). Ligand binding to the PDGFR leads to tyrosine phosphorylation of six SH2 binding sites that associate with Src, PI3K, Nck, GAP, SHP-2, and PLC-γ, and other proteins (Pawson, 1995). Several studies have assigned specific functions to some SH2 binding sites. For example, the site for PI3K is essential for DNA synthesis. In contrast, the site for RasGAP is dispensable (Fantl *et al.*, 1992). By looking at Ras activation and DNA synthesis in the hepatoma cell line HepG2, Valius and Kazlauskas (1993) found that PI3K and PLC-γ are independent effectors of the PDGF-mediated mitogenic signal. In addition, they found that activation of Ras is not sufficient to confer a complete transformant phenotype. These findings suggested that there could be Ras-independent pathways leading to mitogenesis. Other evidence supporting this idea comes from studies by Roche *et al.* (1996). To inhibit the biological activity of PLC-γ, SHP-2, rasGAP, Nck, and Shc, they microinjected in NIH3T3 cells short peptides containing SH2 binding sites or antibodies. Then they looked at S-phase entry and at cFos expression (a Ras-dependent

function). They found that Shc, Nck, SHP-2, PI3K and PLC-γ, but not rasGAP, are required for PDGF-induced mitogenesis. In addition, they found that only PLC-γ, SHP-2, and Shc did stimulate cFos expression. Therefore, Nck and PI3K must stimulate S-phase entry through a Ras-independent pathway. The molecular mechanisms used by PDGF receptor do seem to depend on the cell context. Satoh *et al.* (1993) report that in fibroblast CHO cells the PI3K site is essential for Ras activation. Conversely, in BaF3 pro-B cells removal of the PI3K site does not eliminate Ras activation. These findings suggest that there must be cell-specific regulation of RTK activity.

The Fibroblast Growth Factor Receptor (FGFR) contains 3 autophosphorylation sites in its cytoplasmic portion and one of them has been shown to bind PLC-γ (Fantl *et al.*, 1992). By using receptors mutated at this site, it has been demonstrated that PLC-γ mediates phosphatidylinositol hydrolysis and Ca^{++} flux, but not mitogenesis (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). Why does PLC-γ mediate mitogenesis when activated by PDGFR but does not when activated by FGFR? Again, the simplest explanation is that the effects observed depend on the cell type. Analysis of FRFR mutants has been, in fact, carried out in a different cell type (L6 myoblasts). In support to this hypothesis, it has been shown that FGFR signals differentiation in PC12 neuronal cells, but in fibroblasts stimulates proliferation (Marshall, 1995).

One of the few examples in which the role of SH2 binding sites has been analyzed in an intact organism comes from studies of Met, the receptor for the Hepatocyte Growth Factor/Scatter Factor (HGF/SF). Met carries 2 SH2 binding sites in its C-terminus. Grb2 binds a single site,

while PLC-γ, PI3K, and pp60$^{c\text{-}src}$ bind either of the 2 sites (Ponzetto *et al.*, 1994). Activated Met induces a variety of biological responses in cultured cells (Fantl *et al.*, 1993) and *in vivo* is involved in organ regeneration, angiogenesis, tumor invasiveness, and myogenesis (Maina *et al.*, 1996). By generating transgenic mice expressing Met receptors mutated at one or both SH2 binding sites, Maina *et al.* (1996) could demonstrate that these two sites mediate all the HGF/SF-induced activity and that the site binding Grb2 is not required for early development.

Another study analyzing the role of SH2 binding sites *in vivo* has been carried out for the *Drosophila* Torso RTK (Cleghon *et al.*, 1996). Torso has two major autophosphorylation sites in its tail: ^{630}Tyr, which binds to the tyrosine phosphatase Csw, and ^{918}Tyr. It is not known which protein(s) associate with ^{918}Tyr *in vivo*, but this site binds mammalian rasGAP and PLC-γ *in vitro*. By generating transgenic flies expressing mutagenized (Tyr-to-Phe) Torso proteins, Cleghon *et al.* (1996) showed that removing ^{630}Tyr decreases Torso activity. Conversely, removing ^{918}Tyr leads to an increase in Torso signaling. Removing both sites restores wild-type Torso activity. Thus, ^{630}Tyr and ^{918}Tyr would mediate regulation of the Torso pathway activity.

While in many RTKs it has been shown that SH2 binding sites mediate specific functions, the role of EGFR SH2 binding sites is unclear. It has been demonstrated that the C-terminal tyrosines of EGFR bind Grb2, rasGAP, PLC-γ, and Shc. But even if all the five known SH2 binding sites are removed, EGFR is still able to induce mitogenesis and transformation. These experiments suggest that EGFR might phosphorylate cytoplasmic proteins like Shc, thus generating docking sites which can be used by

molecules that normally associate with EGFR phosphotyrosines (Decker, 1993; Li *et al.*, 1994; Soler *et al.*, 1994). If this is the case, then it is very difficult to understand the normal role of SH2 docking sites in EGFR. One reason why the analysis of EGFR SH2 binding sites has failed might be that all these studies use cells in culture and often the cell lines do not express endogenous receptor (G. Pauletti, personal communication). Moreover, the receptor is largely overexpressed and the basal EGFR activity could be so increased that the receptor is able to phosphorylate and activate downstream effectors even without associating with them (Soler *et al.*, 1993; Li *et al.*, 1994). In addition, there could be interaction between EGFR and other RTKs (the members of the EGFR family appear to be capable of heterodimerization (Lemmon and Schlessinger, 1994). To circumvent these problems, I decided to carry out an analysis of the LET-23 C-terminal phosphotyrosines using an intact animal, where the RTK is in its true physiological background. This analysis is described in Chapter 3.

Multiple pathway activation by RTKs

Ras

The most characterized pathway activated by RTKs is the Ras pathway. Activation of several mammalian RTKs including EGFR, PDGFR, FGFR, Met, TrkA, Insulin-like growth factor, and colony-stimulating factor (Denhardt, 1996) leads to Ras activation. What are the proteins binding RTKs that mediate Ras activation? The adapter proteins Grb-2 and Shc have been both implicated in this function. Genetic evidence in *C. elegans* (Clark *et al.*, 1992a) and *Drosophila* (Simon *et al.*, 1993), in addition to biochemical studies in mammalian cells (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*,

1992; Egan *et al.*, 1993; Skolnik *et al.*, 1993) have shown that Grb-2 is required for Ras activation. Shc binds to Grb2 and its overexpression in PC12 cells induces neurite extension, a phenotype normally dependent on Ras activation. This induction is blocked by a dominant negative Ras (Rozakis-Adcock *et al.*, 1992) suggesting that, under these conditions, Shc mediates its functions through the Ras pathway. Since dominant negative Ras does not suppress all alterations induced by overexpression of Shc or Grb2 (Bonfini *et al.*, 1996), the two adapters could also activate Ras-independent pathways. Until now there is no convincing evidence for this function.

The guanine-nucleotide exchange factor Sos has been shown to bind Grb2 and to activate Ras by exchanging GDP for GTP. Sos has never been implicated in activation of pathways other than Ras-dependent ones. However, since Sos has a Pleckstrin Homology (PH) domain, it could potentially interact with inositols or with G-proteins (Harlan *et al.*, 1994; Touhara *et al.*, 1994) leading to activation of these pathways.

RasGAP increases the intrinsic GTPase activity of Ras thus deactivating it. However, depletion of GAP by microinjection of antibodies did not have any effect on mitogenesis (Roche *et al.*, 1996). Interestingly, at least two mammalian GAPs specifically bind to inositol 1,3,4,5-tetrakisphosphate (Cullen *et al.*, 1995; Fukuda and Mikoshiba, 1996). Thus, the possibility exists that GAP interacts with the inositol pathway and vice versa.

Another adapter protein, Crk, has been shown to bind several RTKs via its SH2 domain. Crk consists of an SH2 domain and two SH3 domains. Several lines of evidence suggest that Crk exerts its functions by activating

Ras [reviewed by Matsuda and Kurata, (1996) and Birge *et al.* (1996)]. On the other hand, Crk co-immunoprecipitates with Sos, the exchange factor for Ras, and with C3G, the exchange factor for the Ras like GTPase Rap1. Rap1, in some systems, can act antagonistically to Ras (Gotoh *et al.*, 1995). It is therefore possible that Crk has two actions, one promoting Ras activation, the other inhibiting it. These two actions could be promoted by different RTKs.

Phosphatidylinositols

Phosphatidylinositol 3-OH kinase

Many RTKs interact with PI3K and PLC-γ. Both enzymes contain two SH2 domains and are involved in the generation of inositol lipids. PI3K consists of two subunits, one regulatory, p85, and one calalytic, p110. Association of p85 with RTKs is required for PI3K activation. PI3K converts PI4P and $PI4,5P_2$ to $PI3,4P_2$ and $PI3,4,5P_3$, respectively (Fry and Waterfield, 1993). Activation of PI3K has been implicated in a wide variety of cellular responses, including mitogenesis (Whitman *et al.*, 1985; Fantl *et al.*, 1992), chemotaxis (Kundra *et al.*, 1994), membrane ruffling (Wennstrom *et al.*, 1994), receptor down-regulation (Joly *et al.*, 1994), and intracellular trafficking (Herman and Emr, 1990). One of the products of PI3K, $PI3,4,5P_3$, is able to bind the SH2 domains of PI3K and of Src. By doing so, $PI3,4,5P_3$ limits the amount of PI3K associated with the RTK (Rameh *et al.*, 1995). This mechanism might downregulate PI3K activation. Alternatively, it could be a general mechanism through which $PI3,4,5P_3$ (and, indirectly, PI3K) interacts with several SH2-containing proteins. $PI3,4,P_2$ and $PI3,4,5P_3$ might also act as second messengers. The idea comes from

experiments showing that treatment of cultured cells with growth factors or hormones causes an increase of these phosphatidylinositols (Auger *et al.*, 1989). The effectors of such second messengers are yet to be discovered.

It has recently been demonstrated that PI3K is an effector of Ras. RasGTP, but not RasGDP, binds to PI3K and activates it (Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1997). Both, an active Ras and an active PI3K are necessary for an efficient transformation of NIH 3T3 cells, suggesting that Ras and PI3K activate two distinct pathways leading to cell transformation. In addition, PI3K activity is sufficient to cause membrane ruffling through Rac, a member of the Rho subfamily of Ras-like GTPases, suggesting that Rac is a downstream mediator in the PI3K pathway, but not in the Ras pathway (Rodriguez-Viciana *et al.*, 1997). Some possible members of the Rac pathway are the serine/threonine kinase Akt/PKB, or adaptin AP2 (Toker and Cantley, 1997). What could be the significance of PI3K activation by Ras? PI3K and Ras appear to activate distinct but synergistic pathways leading to cell transformation. It is conceivable that these activations, to be effective, must take place at the same time. If this is the case, the requirement of activated Ras for PI3K activation may only be a timing mechanism to ensure that both pathways signal at the same time.

Phospholipase C-γ

Phospholipases C are enzymes responsible for hydrolizing $PI4,5P_2$ to diacylglycerol (DAG) and inositol 3,4,5 trisphosphate (IP_3). DAG, in turn, leads to activation of Protein kinases C (PKC) that are able to phosphorylate many substrates. IP_3 interacts with its receptor leading to release of Ca^{++}

from intracellular stores (Lee and Rhee, 1995). PLC-γ phospholipases contain SH2 and PH domains, are cytosolic and are activated by RTKs. At least four distinct RTKs activate PLC-γ: EGFR, PDGFR, FGFR, and TrkA. Upon receptor autophosphorylation, PLC-γ binds the RTK through its SH2 domain(s) and becomes phosphorylated itself. There is controversy whether phosphorylation increases PLC-γ enzymatic activity (Cockcroft and Thomas, 1992). Phosphorylation appears to promote PLC-γ association with actin components of the cytoskeleton and translocation to the cell membrane (McBride *et al.*, 1991; Bar-Sagi *et al.*, 1993; Yang *et al.*, 1994).

One of the effectors of PLC-γ is the second messenger IP_3. IP_3 stimulates release of Ca^{++} from intracellular compartments. Since Ca^{++} modulates many physiological processes (Tsien and Tsien, 1990), PLC-γ could regulate at least some of them via production of IP_3. The physiological role of IP_3 is poorly understood. By studying the IP_3 receptor, recent studies in *Drosophila* and *C. elegans* have shed light on the functional role of IP_3. Acharya *et al.* (1997) have shown that IP_3 receptor is essential for *Drosophila* survival, but not for phototransduction. In addition, Venkatesh and Hasan (1997) reported that lethality is due to lowered levels of ecdysone, a steroid hormone requierd for moulting. In our lab, Tom Clandinin (1997) has found that the IP_3 receptor is specifically involved in one of the five LET-23 functions, fertility. This finding is very important because it establishes, for the first time, a functional connection between RTKs and Ca^{++} regulation *in vivo*.

Activation of PLC-γ decreases the amount of $PI4,5P_2$. It has been postulated that $PI4,5P_2$ has at least three roles in the cell (Lee and Rhee, 1995): a) It is involved in actin polymerization and depolymerization. By

interacting with actin-binding proteins, it promotes actin polymerization; by interacting with Ca^{++} it promotes depolymerization. b) It localizes proteins at the cell membrane by interacting with PH domains. c) It activates phospholipase D, the enzyme that hydrolizes phosphatidylcholine to choline and phosphatidic acid. Thus, activation of PLC-γ could also affect all these processes.

Studies to evaluate the role of PLC-γ in mammalian cells for EGFR and PDGFR-mediated signaling have lead to contrasting results. For example, Roche *et al.* (1996) found that activation of PLC-γ is required for PDGF-mediated mitogenesis and Ras activation. Conversely, others found that PLC-γ downregulates EGFR-mediated mitogenesis even if MAP kinase activation increases (Obermeier *et al.*, 1996). Chen *et al.* (1996) showed that downregulation of EGFR is mediated via phosphorylation of the receptor's threonine 654 by PKC. Interestingly, PLC-γ does not downregulate cell motility, the other EGF-induced, mutually exclusive cell response.

Why are the effects exerted by PLC-γ different depending on the RTK? Stimulation of distinct RTKs could activate different modulators that lead to different net outcomes. Alternatively, since the studies above have been carried out in different cell lines, the cell context could play an important role in determining the response to RTK activation. Tinhofer *et al.* (1996) have shown that stimulation of EGFR (which has a low-affinity binding site for PLC-γ) but not of TrkA (which has a high-affinity binding site for PLC-γ) leads to a Ras-dependent increase in Ca^{++} concentration. Stimulation of TrkA increases intracellular Ca^{++} independently from Ras. Thus, while TrkA might mediate the effects on Ca^{++} using a Ras-independent mechanism by activating PLC-γ, EGFR might use a different, Ras-

dependent mechanism. The Ras pathway activates phospholipase A_2, a cytoplasmic enzyme involved in the release of arachidonic acid (AA) from the plasma membrane (Lin *et al.*, 1993). AA is then metabolized, among others, to leukotrienes which are responsible for EGFR-induced Ca^{++} influx (Peppelenbosch *et al.*, 1992). Therefore, since it does not have a high-affinity binding site for PLC-γ, EGFR might use the AA pathway for its Ca^{++}-dependent effects.

Stats

Signal Transducers and Activators of Transcription (Stats) are transcription factors originally identified in interferon (IFN) signaling (Schindler and Darnell, 1995). When IFN binds its receptor, cytoplasmic kinases referred to as Janus Kinases or Jaks, associate with the receptor and phosphorylate it at specific tyrosines that serve as docking sites for Stats. Stats bind the receptor through their SH2 domains. IFN induces the formation of the complex IFN-stimulated gene factor 3 (ISGF3), which is translocated to the nucleus, binds interferon-stimulated response elements, and activates transcription (Ihle and Kerr, 1995). ISGF3 consists of three proteins: a DNA-binding component and two other dimerized proteins, p91 and p113, that must be phosphorylated in order for the complex to migrate to the nucleus. p91 and p113 are highly related and are termed Stat1 and Stat2, respectively. At least six different Stat proteins have been identified so far. They can homodimerize and heterodimerize, giving rise to a wide variety of complexes, with different DNA-binding affinities, which could account for the many responses to cytokines (Ihle and Kerr, 1995). Some, but not all, RTKs have been shown to activate Stats. For example, EGFR

induces tyrosine phosphorylation and nuclear translocation of Stat1, Stat3, and Stat5 (Fu and Zhang, 1993; Silvennoinen *et al.*, 1993; Darnell *et al.*, 1994; Ruff-Jamison *et al.*, 1995). PDGFR activates Stat1 and Stat5 (Schindler and Darnell, 1995) while there is no evidence that FGFR, HER2, HER3, and HER4 activate Stats.

In cytokine signaling, Stats are phosphorylated by Jaks. Do Jaks mediate also the RTK activation of Stats? RTKs are able to phosphorylate Jaks. For example, EGFR does phosphorylate Jak1. Using cell lines lacking individual Jaks, it has been found that none of the known Jaks is required for Stat activation by EGFR (Leaman *et al.*, 1996). Thus, EGFR appears to activate Stats directly leading to activation of transcription of early genes like c-fos (Fu and Zhang, 1993). The functional significance of Stat activation by RTKs is not yet understood.

Corkscrew/SHP-2 and Dos

Corkscrew (*csw*) encodes a non-receptor tyrosine phosphatase whose human homolog is SHP-2, also called SHPTP-2, SHPTP-3, syp, PTP2C, and PTP1D (Adachi *et al.*, 1996). *csw* was originally identified as a gene acting positively in the *torso* pathway (Perkins *et al.*, 1992; Duffy and Perrimon, 1994). Epistasis experiments suggested that *csw* acts downstream or in parallel to *torso*. Moreover, double mutants carrying loss-of-function (lf) mutations of *raf* and *csw* showed a more severe phenotype than either single mutant. This observation suggested that Csw and Raf might act in parallel to regulate the expression of several terminal genes (Perkins *et al.*, 1992). Double mutants with lf and gain-of-function mutations of *csw*, *sevenless*, *ras-1*, and *raf* were analyzed by Allard *et al.* (1996). They found

that activated Csw bypassed *sevenless* lf mutations and that *csw* was suppressed by dominant negative mutations of *ras*. In addition, activated Ras and activated Raf were both suppressed by *csw(lf)* mutations. The most likely conclusion from these epistasis analyses is that *csw* activity is required either downstream of *raf* or in a parallel pathway.

More recently, two important papers described the cloning and biochemical characterization of a possible Csw effector, *daughter of sevenless* or *dos* (Herbst *et al.*, 1996; Raabe *et al.*, 1996). *dos* was identified in a genetic screen demanding suppressors of *sev(gf)* and, in a separate screen, because it enhances the phenotype conferred by a catalytically inactive *csw* transgene. *dos* encodes a 115 kD protein with a PH domain, a putative SH3 binding site, and ten potential tyrosine phosphorylation sites (Raabe *et al.*, 1996). Genetic analyses indicated that *dos* acts downstream of *sev* and upstream or in parallel to *ras*. In addition, Csw bound Dos via its SH2 domain and could dephosphorylate it. Similarly to *csw* (Perkins *et al.*, 1996), *dos* was required for activation of the *sev*, *DER*, and *torso* pathways (Raabe *et al.*, 1996). All these findings suggest a model in which Dos functions as an adapter protein in several RTK signal transduction pathways. Since Csw acts positively in RTK signaling and it dephosphorylates Dos, Dos would be able to stimulate downstream signaling only when de-phosphorylated. At least two models explaining Dos function are possible (Herbst *et al.*, 1996): a) *dos* is required for Ras activation or b) Dos acts in a parallel pathway whose activation is required along with the activation of the Ras pathway for proper signaling. The first model is supported by studies of SHP-2 in mammalian cells indicating that Ras activation can be modulated by this phopshotyrosine phosphatase

(Milarski and Saltiel, 1994; Tang *et al.*, 1995; Zhao *et al.*, 1995). The second model is consistent with the genetic data from Allard *et al.* (1996). In conclusion, it is not clear whether Csw acts in the Ras pathway or in one parallel to it. The simplest interpretation of the epistasis data by Allard *et al.* (1996) combined with the data by Herbst *et al.* (1996) and Raabe *et al.* (1996) is that Csw has two functions, one required upstream of Ras and the other required in parallel or downstream to Raf. A possibility is that Dos might be activated by Csw and required for proper Ras activation. In addition, Csw might also activate other downstream effectors independently of Dos. A possible target of Csw action is Ksr-1, a kinase normally required for Ras signaling (Kornfeld *et al.*, 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995).

The Ras pathway in *C. elegans*

Activation of the Ras pathway in *C. elegans* is required for several processes, including development of the hermaphrodite vulva. The vulva is formed by 22 nuclei that derive from 3 of 6 equipotential cells located on the ventral side of the hermaphrodite and referred to as Vulval Precursor Cells or VPCs (Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986). Vulva formation requires the anchor cell (AC), which is located in the gonad. Removal of the gonad during the larval L2 stage prevents the VPCs from dividing and differentiating (Kimble, 1981). These experiments suggested that the AC releases the inductive signal for VPC proliferation.

Many mutations were isolated that disrupt vulva formation (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987; Sternberg, 1993;

Kornfeld, 1997). Some of them result in a decrease of the Ras pathway activity and lead to no or little VPCs induction. As a result, the animals are Vulvaless or Vul. Some other mutations increase Ras pathway activity and lead to more than 3 VPCs to be induced. In this case the animals have extra vulval tissue and are referred to as Multivulva or Muv. Genetic and molecular analyses of vulva mutations revealed that the inductive signal is LIN-3, a growth factor of the EGF family (Hill and Sternberg, 1992). LIN-3 acts through the EGFR homolog LET-23 (Aroian *et al.*, 1990) which acts upstream of *sem-5* (Clark *et al.*, 1992a), which encodes an homolog of the adapter protein Grb-2 (Lowenstein *et al.*, 1992). SEM-5 is required for activation of LET-60ras (Beitel *et al.*, 1990; Han and Sternberg, 1990). Downstream of *let-60* are *lin-45* (a Raf homolog) (Han *et al.*, 1993), *mek-2* (a MEK homolog) (Kornfeld *et al.*, 1995; Wu *et al.*, 1995), and *mpk-1/sur-1* (a MAPK homolog) (Lackner *et al.*, 1994; Wu and Han, 1994), which leads to stimulation of differentiation of VPCs (Fig. 2). In addition to reduction of function mutations it has been possible to isolate also gain of function mutations for some vulva genes. For example, *let-23(sa62)* is a semidominant mutation that results in Muv animals. The molecular and genetic characterization of this mutation is described in Chapter 2.

In addition to other genes mediating positive functions in this pathway and acting at the level of LET-23 (Kim and Horvitz, 1990; Hoskins *et al.*, 1996; Simske *et al.*, 1996), several negative regulators have been identified that act close to the LET-23 EGFR, some of them with mammallian counterparts (Sternberg, 1993; Huang *et al.*, 1994; Lee *et al.*, 1994; Sternberg *et al.*, 1994; Jongeward *et al.*, 1995; Yoon *et al.*, 1995;

reviewed by Yoon, (1997). The mechanism of action of all negative regulators identified so far in *C. elegans* is still unclear.

let-23

let-23 encodes a tyrosine kinase of the EGFR subfamily (Aroian *et al.*, 1990). All members of this family carry, in the extracellular portion, two ligand-binding domains alternated with two Cys-rich domains (Fig. 2). The intracellular portion carries the juxtamembrane domain, the tyrosine kinase domain, and the C-terminal tail. The role of the Cys-rich domains is unclear. They are probably used as a scaffold so that the ligand-binding domains can properly interact with their ligand (Ullrich and Schlessinger, 1990). Interestingly, the spacing among most of the Cys is conserved in all members of the family. The C-terminus is thought to have regulatory functions and seems to be implicated in interaction with the RTK effectors (Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992).

A functional LET-23 is required for at least five functions in the worm: vulva development, viability, fertility, correct specification of the P12 neuroectoblast, and formation of specialized structures in the male tail (Fixsen *et al.*, 1985; Aroian and Sternberg, 1991; Clark *et al.*, 1992b; L. Jiang and P. Sternberg, in preparation). Loss of *let-23* activity results in animals that die at the L1 stage. Mosaic analysis indicated that for the viability function LET-23 is probably required in the excretory cell, a cell thought to control osmotic pressure (Koga and Ohshima, 1995). Mosaic analysis also indicated that *let-23* is required cell-autonomously in the VPCs in order for them to differentiate and form vulval tissue (Koga and Ohshima, 1995; Simske and Kim, 1995). Worms carrying *let-23(lf)* mutations are sterile.

The defect seems to involve entry of the oocyte into the spermatheca. For a more detailed desription of the sterility defect produced by *let-23(lf)* mutations, see Chapter 4. Genetic evidence suggests that activation of the Ras pathway in *C. elegans* is also required in order for males to develop functional spicules, male specific copulatory structures (Chamberlin and Sternberg, 1994).

LET-23 has ten tyrosines in its C-terminus that define eight putative SH2 binding sites (two sites have two adjacent tyrosines). We had some indication that SH2 binding sites might mediate LET-23 activities. First, the cell specific mutation *let-23(sy97)* affects all *let-23* functions except fertility (see Chapter 2). *sy97* is predicted to remove the last 56 amino acids of LET-23, which include the three putative SEM-5 binding sites. Therefore, the fertility function lies upstream of the *sy97* mutation. Second, a transgene which deletes most of the C-terminal tail of the LET-23 protein (111 out of 168 amino acids, including 7 SH2 binding sites), but that has an extra putative SEM-5 binding site, results in animals viable and with vulva, but sterile. One possible explanation of these results is that the LET-23 putative SEM-5 binding sites are responsible for viability and vulva formation, but not fertility. Thus, I sought to determine whether the putative SEM-5 binding sites are specific for viability and vulva development and if they activate the Ras pathway. In addition, I addressed wether the putative SH2 binding sites located upstream of the *sy97* truncation are able to mediate fertility in a tissue specific manner. This analysis is described in Chapter III.

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Figure 1. Examples of distinct subfamilies of Receptor Tyrosine Kinases. EGFR, Epidermal Growth Factor receptor; PDGFR, Platelet Growth factor receptor; FGFR, Fibroblast Growth Factor Receptor. Open box, ligand binding domain; striped box, Cysteine-rich domain; solid box, tyrosine kinase domain; semi-circles, immunoglobulin-like domain.

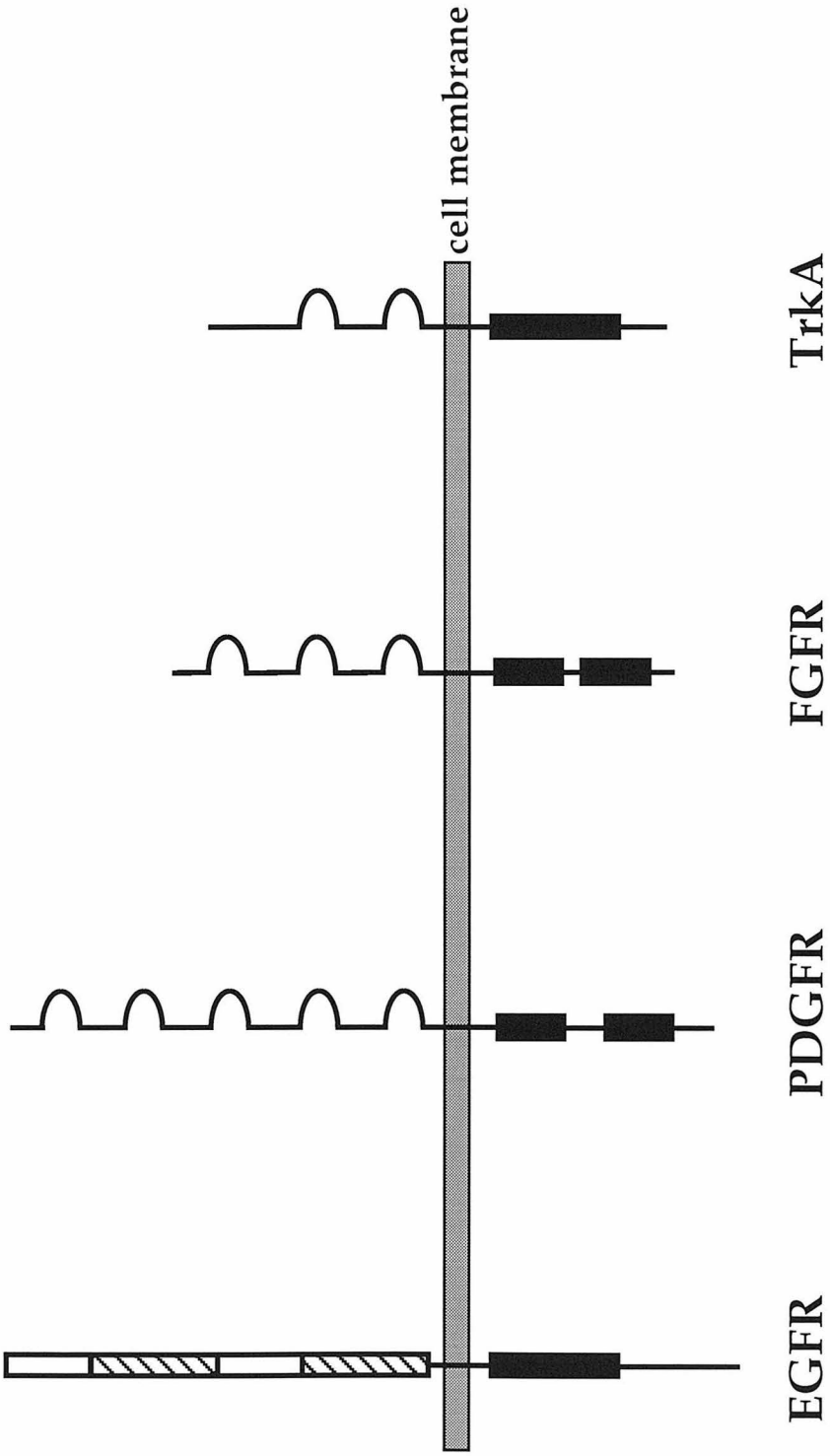
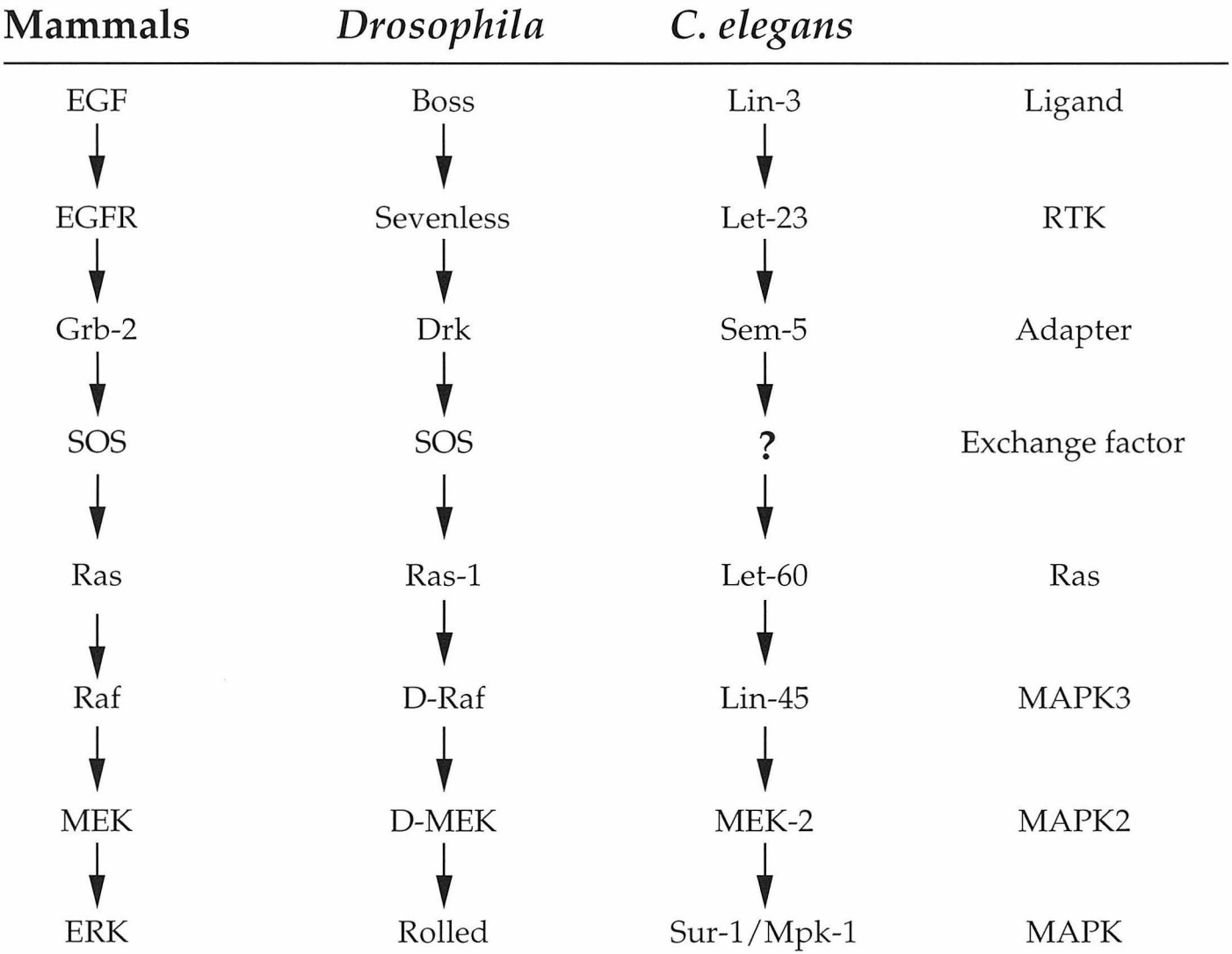

cell membrane
TrkA
FGFR
PDGFR
EGFR

Figure 2. Example of simplified Ras signaling pathways in mammalian cells, *Drosophila* and *C. elegans*. ?, not identified; RTK, Receptor Tyrosine Kinase; MAPK3, MAP kinase kinase kinase; MAPK2, MAP kinase kinase; MAPK, MAP kinase.


Mammals
Drosophila
C. elegans
EGF
Boss
Lin-3
Ligand
EGFR
Sevenless
Let-23
RTK
Grb-2
Drk
Sem-5
Adapter
SOS
SOS
?
Exchange factor
Ras
Ras-1
Let-60
Ras
Raf
D-Raf
Lin-45
MAPK3
MEK
D-MEK
MEK-2
MAPK2
ERK
Rolled
Sur-1/Mpk-1
MAPK

Chapter II

Structure-to-function analysis of *let-23*

Chapter II A contains a paper published in EMBO Journal in which the tissue specific and loss-of-function mutations of *let-23* were analyzed. I discovered the molecular lesion of the vulva-specific allele *sy1* and re-sequenced all the other mutations, previously sequenced by Dr. Aroian. The main conclusion from this work is that the C-terminus of LET-23 can be divided in at least three domains, each mediating a subset of LET-23 functions.

Chapter II B contains a paper published in Molecular Biology of the Cell which describes the analysis of a gain-of-function allele of *let-23*, *sa62*. I have found that the molecular lesion of *sa62* results in a Cysteine to a Tyrosine substitution that maps in the extracellular domain of LET-23. By generating transgenic worms expressing an *in vitro* mutagenized *let-23* carrying the *sa62* mutation, I have demonstrated that the Cysteine-to-Tyrosine change is sufficient to cause the *sa62* phenotype. These results suggest that the region containing the *sa62* mutation is important for activation of LET-23 signaling. I have also carried out epistasis tests to order *lin-2*, *lin-7*, *lin-10*, and *sem-5* in the pathway. I was not first author in this paper because Dr. Katz discovered that *sa62* is a *let-23* allele and characterized it genetically.

Chapter II A

Mutations in the *Caenorhabditis elegans let-23* EGF Receptor-like gene define elements important for tissue-specificity and function

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Mutations in the *Caenorhabditis elegans let-23* EGFR-like gene define elements important for cell-type specificity and function

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The *Caenorhabditis elegans let-23* gene is a genetically characterized member of the epidermal growth factor receptor (EGFR) tyrosine kinase family. Mutations in *let-23* can produce five phenotypes in the nematode. Alleles of *let-23* include null alleles, reduction-of-function alleles and alleles that disrupt function in some cell types and not others. We have sequenced some of these mutations to identify sequences and regions important for overall *let-23* function and for *let-23* function in specific cell types. Our data indicate that *in vivo*, the receptor's C-terminus can be partitioned into at least three domains that each contribute to receptor function in different cell types. In particular, we find distinct domains that mediate hermaphrodite fertility and vulval induction. Our data also demonstrate for the first time that a single, conserved residue in the ligand binding domain is critical for function *in vivo* and that mutations in the extracellular cysteines characteristic of the EGFR family can lead to a partial or a complete reduction of receptor function.

Key words: Caenorhabditis elegans/cell-type specificity/development/epidermal growth factor receptor/signal transduction

Introduction

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases plays a diverse and important role in determining cellular states in many different cell types (Carpenter and Wahl, 1990). The human EGFR (HER) is capable of phosphorylating several substrates, including phospholipase C-γ1, GTPase-activating protein and phosphatidyl inositol-3 kinase (Hernandez-Sotomayor and Carpenter, 1992). Presumably, interactions with different substrates and with other proteins that modify its activity manifest the different effects of EGFR family members in different cell types.

Studies of EGFR family members in mammalian tissue culture cells have identified several key, functional domains (Figure 1): major and minor ligand binding domains, a transmembrane domain, a tyrosine kinase domain and a C-terminal domain that contains sites important for autophosphorylation, for interaction with substrates and for receptor internalization (Ullrich and Schlessinger, 1990; Hernandez-Sotomayor and Carpenter, 1992). There are also two extracellular cysteine-rich domains that flank the ligand binding domain. The amino acid spacing between the 40+ cysteines is notably conserved among the family members. In addition to defining the function of large domains, the functions of a limited number of individual amino acids have also been investigated, e.g. Thr654, Val664, Lys721 and the tyrosine autophosphorylation sites (Weiner *et al.*, 1989; Carpenter and Wahl, 1990).

The *let-23* gene of the nematode *Caenorhabditis elegans* encodes a member of the EGFR family (Aroian *et al.*, 1990). The LET-23 protein contains a putative tyrosine kinase domain (which shares 44% identity with the HER kinase domain), two characteristic extracellular cysteine-rich domains, a putative ligand binding domain that shares 28.7% identity with the HER ligand binding domain and an appropriately positioned transmembrane domain.

Genetic analyses indicate that complete loss of *let-23* function results in at least five unrelated phenotypes: lethality in the first larval stage, failure of vulval differentiation (i.e. a vulvaless phenotype), hermaphrodite sterility, improper differentiation of the male tail and improper development of the posterior ectoderm (Aroian and Sternberg, 1991). The cellular bases of the vulval, male tail and posterior ectoderm phenotypes have been determined and involve different cell groups (Aroian and Sternberg, 1991; H.Chamberlin and P.Sternberg, in preparation). The cells involved in larval lethality and hermaphrodite sterility, although not yet determined, are most probably distinct from one another and from the other phenotypes (Aroian and Sternberg, 1991).

Alleles of *let-23* have been isolated in genetic screens based on their ability either to cause larval lethality or to inhibit vulval development (Herman, 1978; Sigurdson *et al.*, 1984; Ferguson and Horvitz, 1985; Aroian and Sternberg, 1991). Most *let-23* alleles eliminate *let-23* function in all known cell types where *let-23* acts, i.e. they are null alleles, and lead to a completely penetrant phenotype (except for posterior ectoderm; see Aroian and Sternberg, 1991). [We measure the severity of a phenotype by its penetrance (the percent of animals displaying the mutant phenotype) and for vulval induction, the relative number of precursor cells undergoing vulval differentiation.] Other alleles reduce, but do not eliminate, *let-23* function in all its cell types. Still other alleles reduce *let-23* function in some cell types and not others. Here we present the sequences of 10 alleles that span most of the *let-23* gene. These data, combined with those of previous genetic studies, suggest that receptor function in different cell types can correlate with different regions of the receptor molecule. The data also expand our knowledge of functionally important residues, many of which are conserved among EGFR family members.

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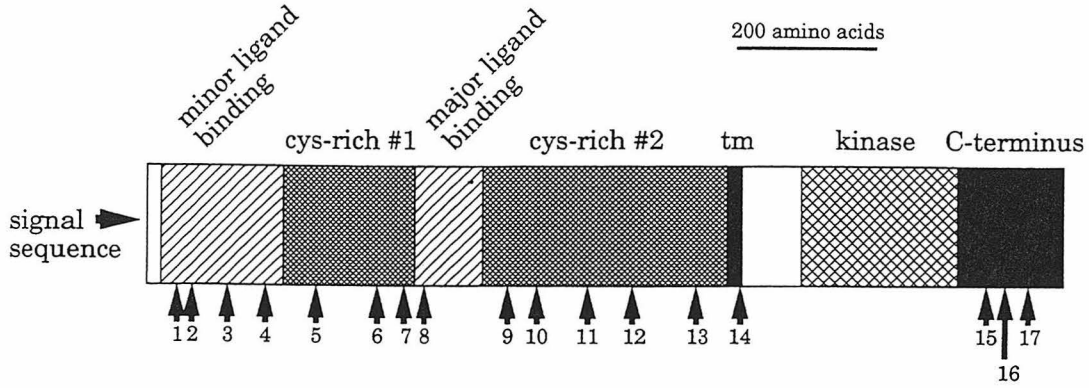

Fig. 1. The structure of the 1323 amino acid LET-23 EGFR protein represented using the domains characteristic of the EGFR family. Numbered arrows below the line indicate the number and position of the 17 introns. Domains from N-terminus to C-terminus are: the signal sequence, a minor ligand binding domain, a cysteine-rich domain with conserved inter-cysteine spacing (see Figure 3B), a major ligand binding domain, a second cysteine-rich domain, a transmembrane domain (tm), a tyrosine kinase domain and a C-terminal domain important for regulation and substrate binding. The same structure and shading are used to represent the LET-23 EGFR protein in other figures.

Results

Two cell-type specific mutations truncate the C-terminus at different points

The recessive, non-null alleles *let-23(sy1)* and *let-23(sy97)* are unusual in that they alter *let-23* function in a cell-type specific manner (Aroian and Sternberg, 1991) and therefore can provide information on how receptor tyrosine kinases operate in different cell types. Some of the genetic data that demonstrate the cell-type specificity of *sy1* and *sy97* are summarized in Table I and below.

The allele *sy1* displays only one of the five *let-23* phenotypes listed above, the vulvaless phenotype (Table I). We imagine two possible explanations for this vulval-specific defect: (i) *sy1* results in a weak, overall reduction of *let-23* function and the vulval precursor cells are the most sensitive to this reduction; or (ii) *sy1* results in a receptor that is defective specifically in the vulval precursor cells.

Genetic data strongly support the latter interpretation (Aroian and Sternberg, 1991). First, animals that harbor a single copy of *sy1 in trans* to a deletion display a completely penetrant vulvaless phenotype (i.e. 0% vulval induction), as is associated with the *let-23* null phenotype. However, in all other respects, *sy1/Deficiency* animals are virtually wild-type. On the other hand, animals homozygous for null alleles of *let-23* display, in addition to 0% vulval induction, 0% survival, 0% wild-type spicule formation and 0% hermaphrodite fertility [Table I; the complete penetrance of the *sy1/Deficiency* vulval defect in the absence of other *let-23* defects has been used to isolate suppressors of the *let-23* vulvaless phenotype (G.Jongeward and P.Sternberg, manuscript in preparation)]. Second, in comparison with all other *let-23* non-null alleles as either homozygotes (e.g. *n1045/n1045*, *sy97/sy97*, *sy10/sy10*), hemizygotes (e.g. *n1045/Deficiency*) or *trans*-heterozygotes (e.g. *n1045/sy10*), the severity of the vulval defects associated with *sy1/sy1* and *sy1/Deficiency* (Table I) should be accompanied by noticeable, if not severe, defects in other cell types as well. However, it is not. Third, *sy1* is able to rescue *in trans* all non-vulval defects associated with any null or non-null *let-23* allele virtually as well as a wild-type chromosome. On the other hand, the vulval defect, except for *sy1/sy97* as detailed below, is as severe as expected (e.g. *sy1/sy10* in Table I). These data all indicate that *let-23(sy1)* is not a simple overall reduction of *let-23* function allele but rather an allele that specifically impairs *let-23* function in the vulval precursor cells.

We have sequenced the entire *let-23* coding sequence of *sy1/sy1* homozygotes and found that the vulva-specific mutation *let-23(sy1)* harbors a single C-to-T alteration in coding sequence such that Q_{1318}(CAA) is mutated to $STOP_{1318}$(TAA) (Figure 2A). As a result, six amino acids are predicted to be truncated from the full-length 1323 amino acid protein. None of the truncated six amino acids are tyrosines (potential autophosphorylation sites).

Whereas the allele *sy1* impairs function specifically in one tissue, the allele *sy97* retains functions specifically in one tissue. Namely, *sy97* animals are essentially wild-type for *let-23* fertility but severely defective for all other phenotypes (Table I). There are two possible explanations for this retention of fertility function: (i) *sy97* is a strong, overall reduction of *let-23* function allele, and fertility is the *let-23* function least sensitive to a reduction of *let-23* function; or (ii) *sy97* specifically retains the ability to promote fertility while compromising *let-23* functions in other cell types. Genetic data strongly support the latter, cell-type specific interpretation. First, *sy97* homozygotes display severe *let-23* phenotypes. The levels of vulval induction and normal spicule development seen in *sy97* homozygotes are close to those associated with the null phenotype (Table I). However, unlike the null phenotype, the *sy97* phenotype is essentially wild-type with regard to fertility (the null phenotype is 0% fertility). Also, in comparison with other non-null *let-23* alleles, given the severity of the defects in viability, vulval induction and production of wild-type spicules associated with *sy97* homozygotes, one would expect to see severe fertility defects in *sy97* animals. For example, *sy97* homozygotes are at least as severe as *n1045/Deficiency* and *sy10/sy10* animals in their lack of viability, vulval induction and production of wild-type spicules (Table I). However, *sy97* homozygotes show only a slight defect in fertility, whereas all *n1045/Deficiency* and *sy10/sy10* animals are sterile. Second, *sy97* can rescue *in trans* the fertility defect associated with *n1045* or *sy10* to the same extent as a wild-

Table I. Penetrance of *let-23* phenotypes for different allele combinations

Allele[a]	Viability	Vulva	Spicules	Fertility	P12
+/+	100	100	100	100	100
n1045/n1045[b]	42	44	24	100	
n1045/null[c]	25	4.4	0	0	
sy10/sy10	14	1.6	4.8	0	
sy10/null[d]	0	ND	ND	0	
null/null[d,e]	0	0	0	0	
sy1/sy1	100	14	100	100	100
sy1/sy10	94	8.7	95	100	
sy1/null[d]	100	0	95	100	
sy97/sy97	11	0	0	95	40
sy97/sy10	21	0	5.6	100	
sy97/null[d]	0.4	0	0	100	

See Aroian and Sternberg (1991) for data and details. 'Viability' is the percent survival for a given allele combination [in two cases (*sy1/sy1* and *sy1/null*), viability was >100% due to statistical variation.] 'Vulva' is the percent vulval differentiation (100% is normal, 0% is completely vulvaless). 'Spicules' is the percent of males with wild-type spicules. 'Fertility' is the percent of fertile hermaphrodites. Although not present with any of these allele combinations, intermediate levels of fertility are possible. 'P12' is the percent of animals with wild-type posterior ectoderms. It was not measured for all allele combinations due to maternal effects. The data for *sy1/sy1* and *sy97/sy97* are taken from homozygous mothers and are included to indicate that *sy1* is wild-type and *sy97* is defective. The 40% penetrance associated with *sy97/sy97* is as severe as any measured to date for this phenotype.

[a] This is a partial listing of allele combinations made. Other combinations support the trends shown here (Aroian and Sternberg, 1991). The allele combinations shown are grouped in three sets. The first set includes combinations with effects on all *let-23* functions (ordered from least severe to most severe); the second and third sets illustrate how *sy1* and *sy97* respectively affect *let-23* functions in a cell-type specific manner.

[b] Both here and in the text, data for the temperature sensitive allele *n1045* are given at 20°C.

[c] Null allele is *mnDf68*, a deficiency that removes *let-23* and genes to either side of it.

[d] Null allele is *sy15*, a genetically defined null *let-23* allele. Except for viability, the sample sizes used to generate the data for *sy10/sy15* and *sy97/sy15* are very small due to the severe lethality of these combinations. ND = not determined.

[e] Since *let-23* null alleles are inviable, the data for vulva, spicules and fertility were inferred genetically.

type chromosome, although these *sy97/n1045* and *sy97/sy10* animals are severely defective in viability, vulval induction and production of wild-type spicules (*sy10/sy97* data are given in Table I). Third, although the *sy97/null* heterozygote is essentially inviable, we have managed to isolate one surviving hermaphrodite of this genotype. That one hermaphrodite was fertile, although all its progeny died (see Table I). Thus, even in the *sy97/null* genotype, when LET-23 function is severely compromised for most cell types, the fertility function of LET-23 still functions. These data suggest that *sy97* is not a simple reduction-of-function allele, but rather an allele in which the ability of the LET-23 receptor to promote fertility is intact while all other functions are compromised.

The entire *let-23(sy97)* coding sequence was scanned for point mutations using hydroxylamine mismatch detection (see Materials and methods). A single base change was detected. Sequencing revealed a G-to-A transition in the ultimate nucleotide of intron 17 (Figure 2B). The effect of this point mutation on splicing was determined by sequencing *sy97* cDNAs that were generated by PCR amplification on reverse transcribed RNA isolated from *sy97* animals (see Materials and methods). We found that splicing in *let-23(sy97)* occurs exclusively at the adjacent exonic AG (Figure 2B). Protein translated from this mutant mRNA would be wild-type up to and including T_{1267}. Due to a shift in reading frame, the 56 amino acids that follow in wild-type are replaced with 23 novel amino acids. These 23 mutant amino acids are AIPIKWRLLQPTKHSFIFLWILQ.

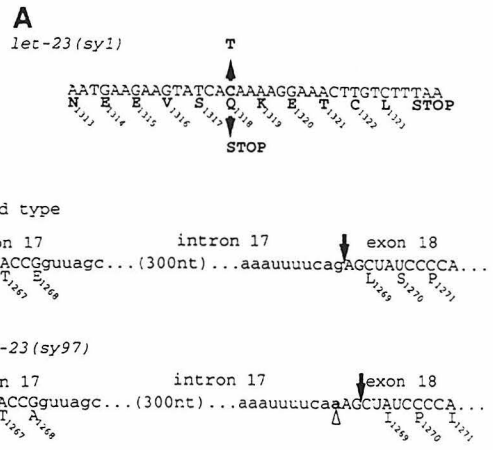

Fig. 2. Analyses of the cell-type specific mutations *sy1* and *sy97*. (**A**) The sequence of the *let-23(sy1)* mutation. The wild-type coding sequence (upper line) and protein sequence for the final 11 amino acids (lower line) are shown. The *sy1* alteration is shown in coding sequence and protein sequence. Numbers refer to amino acid residue number. (**B**) The sequence of the *let-23(sy97)* mutation. The upper panel shows the genomic sequence around the exon 17–intron17–exon18 boundaries. Exon 18 is the last *let-23* exon. Exons are shown in uppercase lettering, introns in lowercase. The amino acid reading frame is shown below the sequences. Numbers refer to residue number. The large arrow indicates the 3′ location of the wild-type splice. The lower panel shows the same information in the presence of the *sy97* mutation. The mutation is indicated in bold and by an open triangle. The 3′ splice site has shifted over two nucleotides (large arrow), resulting in a new reading frame.

Sequences of let-23 null and reduction-of-function alleles reveal new, functionally important residues

In contrast to *sy1* and *sy97*, most *let-23* alleles eliminate all LET-23 function, i.e. they are null alleles. A few others are overall reduction-of-function alleles (Aroian and Sternberg, 1991). We sequenced a number of these alleles to provide insight into residues and domains important for overall LET-23 function. All of these alleles are recessive. As with *sy97*, we identified the location of the mutations in these alleles using hydroxylamine mismatch detection.

Three null alleles affect residues in the tyrosine kinase domain (Figure 3A). The allele *let-23(sy5)* has been previously reported to alter W_{1078}(TGG) to $STOP_{1078}$(TGA) in kinase subdomain IX (Aroian *et al.*, 1990). [The catalytic domain of protein kinases can be subdivided into 12 subdomains based on regions of high conservation (Hanks and Quinn, 1991).] The alleles *let-23(sy7)* and *let-23(sy16)* also both mutate residues in kinase subdomain IX: G_{1074}(GGA) to E_{1074}(GAA) and T_{1065}(ACC) to I_{1065}(ATC) respectively. To our knowledge, these residues in the kinase domain of the EGFR subfamily have not been previously shown to be critical for activity.

Two other null alleles, *let-23(sy17)* and *let-23(sy14)*, mutate splice donor sites, altering the first base of introns

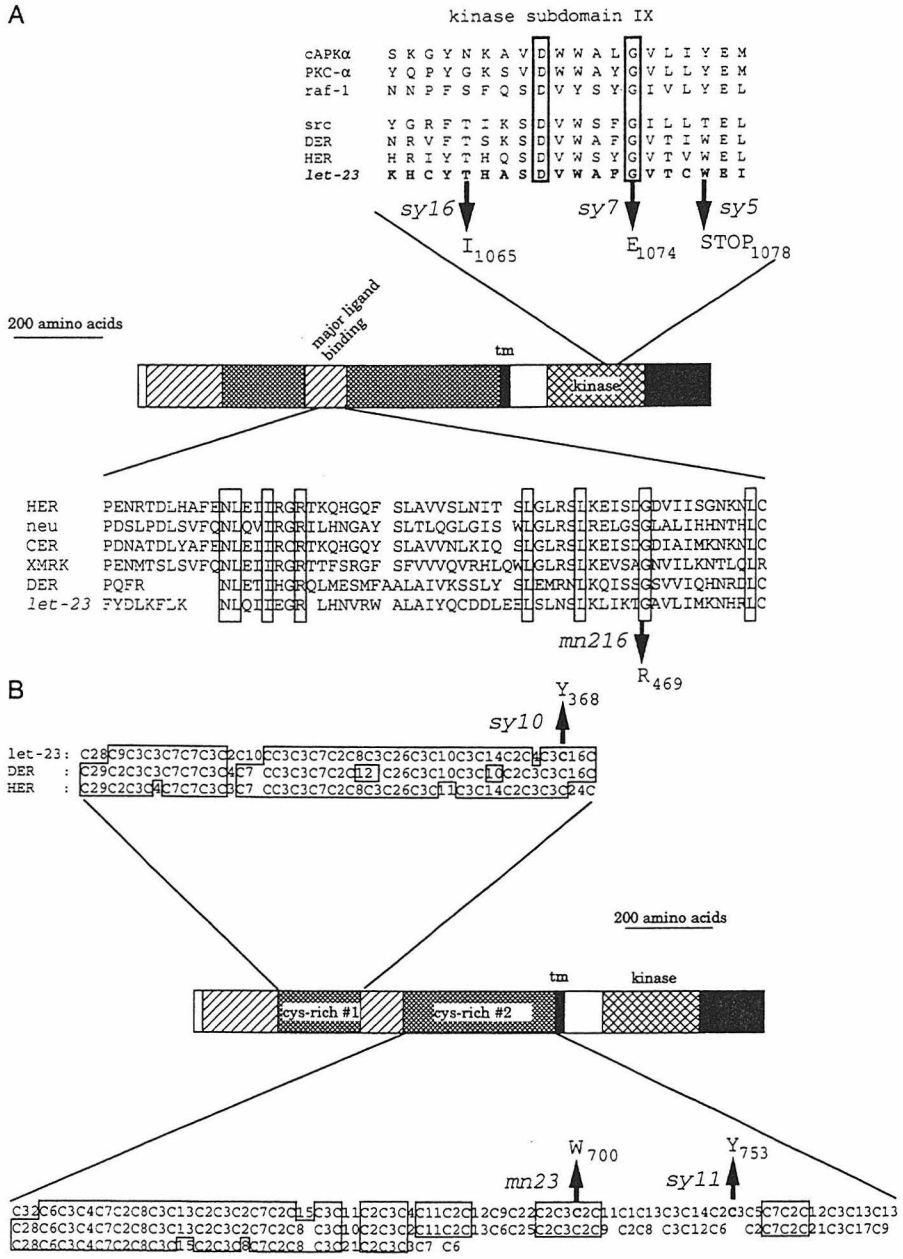

Fig. 3. Sequence of *let-23* null alleles. Numbers associated with mutated residues indicate residue number. The LET-23 protein is schematically depicted as in Figure 1. (**A**) Above the diagram of the protein, the sequence of LET-23 in kinase subdomain IX is shown. The location and amino acid sequences of the kinase domain mutations *sy5*, *sy7* and *sy16* in kinase subdomain IX are indicated (the sequence of *sy5* has been previously reported; Aroian *et al.*, 1990). For comparison, the sequences of six other kinases (Hanks *et al.*, 1988) are shown and conserved residues boxed (serine/threonine kinases: cAPKα, cAMP-dependent protein kinase catalytic subunit, α form from bovine cardiac muscle; PKC-α, protein kinase C, α form from bovine brain; raf-1, cellular homolog from human fetal liver of oncogene products from 3611 murine sarcoma virus and Mill Hill 2 avian acute leukemia virus; tyrosine kinases: src, cellular homolog from human fetal liver of oncogene product from Rous avian sarcoma virus; DER, *Drosophila* EGFR; HER, human EGFR). Below the diagram of the protein, the sequence of about two-thirds of the LET-23 major ligand binding domain is shown. The location and amino acid sequence of the ligand binding domain mutant *mn216* is indicated. For comparison, the ligand binding domain sequences of six other members of the EGFR family are shown (neu = human cellular homolog of the *neu* oncogene, also known as erb-b2 and HER2; CER = chicken EGFR; XMRK = EGFR from *Xiphophorus*). Absolutely conserved residues are boxed. (**B**) Location and amino acid sequences of the cysteine mutations *sy10*, *mn23* and *sy11* in the first and second extracellular cysteine-rich domains (respectively, above and below the diagram). The sequence of LET-23 in these domains is represented by listing the cysteines and the number of non-cysteine residues between them (e.g. C2C indicates a cysteine followed by two non-cysteine amino acids followed by a cysteine). For comparison, the same is shown for DER and HER. Regions with conserved inter-cysteine spacing are boxed. Note the extension of cysteine-rich domain #2 for LET-23 and DER.

4 and 11 respectively from G to A (not shown; see Figure 1 for location of these introns). The first base of virtually all eukaryotic introns is a G (Shapiro and Senapathy, 1987) and this nucleotide is essential for proper splicing (Aebi *et al.*, 1986; Sakuraba *et al.*, 1992). Thus, the *sy17* and *sy14* mutations are expected to lead to aberrant splicing in the extracellular domain of LET-23, consistent with the null phenotype of these alleles.

Four other *let-23* alleles, *let-23(mn23)*, *let-23(mn216)*, *let-23(sy10)* and *let-23(sy11)*, were found to harbor missense mutations in the extracellular part of the molecule. The extracellular region of the EGFR family, which includes the ligand binding domain and the cysteine-rich regions, has not been studied at the level of individual amino acids. The null allele *let-23(mn216)* alters a residue in the putative major ligand binding domain conserved among all members of the EGFR family: G_{469}(GGA) to R_{469}(AGA) (Figure 3A). Three other mutations affect extracellular cysteines (Figure 3B). The null allele *let-23(mn23)* mutates C_{700}(TGC) to W_{700}(TGG), the null allele *let-23(sy11)* mutates C_{753}(TGC) to Y_{753}(TAC) and the strong pleiotropic reduction-of-function allele *let-23(sy10)* mutates C_{368}(TGT) to Y_{368}(TAT).

Possible upstream extent of the let-23 fertility domain

We took advantage of an existing construct to test our hypothesis that a domain of LET-23 N-terminal to the *sy97* truncation is required for fertility.

A strain carrying a null allele of *let-23* was transformed with two *let-23* genomic constructs: one construct contains the entire *let-23* gene and serves as a positive control; the other truncates the C-terminus of LET-23 after the position of amino acid D_{1212}. We found that the former construct was able to rescue fully the sterility of the *let-23* null mutation in four stably transformed lines, whereas the truncated construct could not rescue the sterility at all in four stably transformed lines. Although there are caveats to our interpretation of this experiment (see Materials and methods), this result suggests that sequences required for providing fertility reside upstream of the *sy97* truncation and downstream of residue 1212.

Discussion

We have investigated the molecular defects associated with alleles of the *C. elegans let-23* gene, a member of the EGFR family. These molecular data have been combined with genetic studies to reveal insights into the functioning of this important family of receptor tyrosine kinases.

Non-specific mutations

We have explored the basis of mutations in alleles that compromise LET-23 functions uniformly. We found that null alleles *let-23(sy14)* and *let-23(sy17)* result in splice donor mutations in the portion of the gene encoding the extracellular part of the protein. These mutations affirm a correlation between genetic and molecular data—their molecular defects are consistent with their genetic, loss-of-function behavior.

The other severe *let-23* alleles examined are mutated at residues characteristic of either the EGFR family or tyrosine kinases in general, showing for the first time that these residues are important for receptor function *in vivo*. Two mutations associated with null alleles reside in the kinase domain (Figure 3A). The glycine mutated in *let-23(sy7)* is found in virtually all kinases and the threonine altered in *let-23(sy16)* is conserved as either a threonine or a serine in all tyrosine kinases (Hanks *et al.*, 1988). That these null mutations affect residues found in almost all tyrosine kinases suggests that these residues are important for tyrosine kinase activity. Alternatively, these mutations could eliminate function less specifically, e.g. by grossly affecting protein folding or stability. Interestingly, the residues mutated, T_{1065} and G_{1074}, flank the nearly invariant D_{1069}. This residue is thought to stabilize the catalytic loop of cyclic adenosine monophosphate-dependent protein kinase (Knighton *et al.*, 1991). The two residues mutated in *sy7* and *sy16* might contribute to the positioning of this critical residue.

Two null alleles (*mn23* and *sy11*) and one strong reduction-of-function allele (*sy10*) result from mutations in extracellular cysteines characteristic of the EGFR family (Figure 3B). That the hypomorphic allele *sy10* alters the same type of residue as is altered in two null alleles is consistent with the genetic observation that *sy10* behaves like an overall reduction-of-function allele, unlike *sy1* and *sy97*. Our results with *mn23*, *sy10* and *sy11* further suggest that individual extracellular cysteines play an important role in the function of this family. It has been hypothesized that the many extracellular cysteines form a network of bridges that provide scaffolding for a conformation that mediates receptor–receptor interaction and/or for the transduction of the ligand binding signal across the plasma membrane (Yarden *et al.*, 1986). If so, then these mutations might disrupt function by perturbing one or more of the bridges in that network. We note that in the two invertebrate members of this family, LET-23 and the *Drosophila* EGFR (DER), the second cysteine-rich region is noticeably extended and that both the *mn23* and *sy11* mutations reside in this extension. This suggests that the invertebrate-specific extension is functionally significant. The cysteine mutated in *sy10*, on the other hand, is in the first cysteine-rich region and is conserved in both DER and HER.

Finally, the null allele *let-23(mn216)* alters one of the few conserved residues in the putative ligand binding domain (Figure 3A). Amino acid conservation among family members in this domain is limited, and few residues are conserved among all family members. For example, DER and LET-23 show only 26.6% identity in the ligand binding domain (Aroian *et al.*, 1990). Nonetheless, our result demonstrates for the first time that at least one of the conserved residues in this important domain is functionally critical.

Cell-specific mutations

The *let-23(sy1)* and *let-23(sy97)* mutations in the *C. elegans let-23* gene suggest that the C-terminus of this receptor tyrosine kinase can be partitioned into at least three domains with different cell-type specific functions: (i) a vulva-specific domain at the very C-terminus, (ii) a domain upstream of this that is important for viability, spicule development and posterior ectoderm development and (iii) a domain important for fertility (Figure 4). The *sy1* allele specifically impairs the functioning of the LET-23 protein in one cell type, the vulval precursor cells, whereas the *sy97* allele specifically retains the function of LET-23 that controls fertility. The *sy1* truncation (Figure 2A) demonstrates that the last six amino acids of LET-23 are critical for function specifically in the vulva. The *sy97* truncation (Figure 2B) indicates that the wild-type 56 amino acids at the end of LET-23 (from T_{1267} onwards) are dispensable for providing fertility but are important for all other known LET-23 EGFR activities.

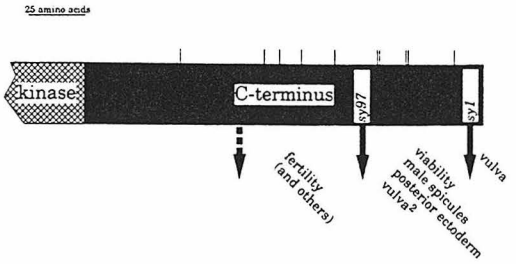

Fig. 4. A model for cell-type specific C-terminal domains. The C-terminal domain and part of the kinase domain are depicted as in Figure 1. The locations of the *sy1* and *sy97* mutations are indicated. Based on genetics and molecular sequence, the C-terminus (i.e. the region downstream of the kinase domain) of LET-23 EGFR can be split into three domains (delineated by down arrows). Based on the sequence of the *sy1* mutation, the very C-terminus is a domain specific to functioning in the vulva. Based on the sequence of the *sy97* mutation, just upstream of this vulva-specific domain is a domain required for all LET-23 functions but fertility. Genetic data suggest that the vulval function in this domain (vulva^2) is somewhat separable from the vulval function at the very C-terminus (see text). Upstream of this second domain is a domain essential for fertility and probably other functions as well. As inferred from germline transformation results (see text), this third domain exists downstream of D_{1212} (represented with a dashed arrow), but its position has not been definitively determined (D_{1212} is located 71 amino acids downstream of the kinase domain). The locations of tyrosines are indicated by vertical lines above the boxes.

The *sy97*, but not the *sy1*, truncation lacks the probable binding sites for the SH2 domain-containing protein, SEM-5 (Songyang *et al.*, 1993). Like *let-23*, the *sem-5* gene is required for vulval formation (Clark *et al.*, 1992). The sy97 protein might therefore be defective in the vulva because the mutant LET-23 is unable to interact with SEM-5. SEM-5 is apparently not necessary for fertility (Clark *et al.*, 1992) and thus a SEM-5-independent pathway might mediate fertility. We have begun to delineate the domain required for fertility further. We have found that elements critical for *let-23*-controlled fertility may reside just upstream of the *sy97* truncation (at T_{1267}) since high-copy germline transformation of a *let-23* construct that truncates LET-23 at D_{1212} is not able to rescue the sterility of a null *let-23* allele. The sy1 mutant protein alters function differently than does sy97, perhaps by making vulval substrate binding sites inaccessible or by destabilizing the protein in the vulva.

sy1 and *sy97* display intragenic complementation in the vulva—i.e. the vulval defects in *sy1/sy97* animals are noticeably less severe than the defects in either *sy1/sy1* or *sy97/sy97* animals (Aroian and Sternberg, 1991). *sy1/sy1* hermaphrodites have 14% vulval induction, *sy97/sy97* hermaphrodites have 0% vulval induction, but *sy1/sy97* hermaphrodites have 36% vulval induction. This difference is even more notable when looking at egg-laying percentages: only 8% of *sy1/sy1* and 0% of *sy97/sy97* hermaphrodites lay eggs due to deficient vulval development, but 50% of *sy1/sy97* hermaphrodites lay eggs (100% of wild-type hermaphrodites lay eggs). This intragenic complementation suggests that LET-23 receptors function as multimers, consistent with what is known about HER (Kashles *et al.*, 1991). In addition, sy1 and sy97 are likely to be defective in two different functions required for vulval development. For example, the heterodimer sy97−sy1 might be more active in the vulva than either mutant alone because of an enhanced ability both to bind substrate and to expose substrate binding sites or remain stable.

Our finding that the *in vivo* functions of LET-23 required in different cell types reside on different parts of the receptor molecule is consistent with several observations from vertebrate systems. First, C-terminal truncations in HER can affect the receptor's ability to transform one cell type and not another (Khazaie *et al.*, 1988). Second, the binding of different substrates to receptor tyrosines kinases can occur at different sites along the receptor tyrosine kinase (Kashishian *et al.*, 1992; Fantl *et al.*, 1992; Songyang *et al.*, 1993). An intriguing extension of all these results is that it might be possible to alter and/or target the functioning of receptor tyrosine kinases other than LET-23 in a cell-type specific fashion. The cell-type specific behavior of the *sy1* and *sy97* mutations may also have a parallel in some mutations of the *Drosophila* EGFR-like gene. Several mutations in that gene are able to affect differentially various developmental phenotypes (Clifford and Schupbach, 1989). Cell-specific action may be a general feature of this class of receptor tyrosine kinases.

Materials and methods

Sequencing of the let-23(sy1) point mutation

The sequence of *sy1* was determined by sequencing the entire *let-23* coding region and exon−intron boundaries from gel-excised, PCR-amplified genomic DNA (Kretz *et al.*, 1989; primers used for PCR are listed below). Only this one change was found. It has been verified with multiple independent PCRs and with multiple genomic DNA preparations. Although this mutation generates a mismatched C, it was not detected with the hydroxylamine scheme described below, perhaps due to problems of context.

Localization and sequencing of other point mutations

Due to the large size of the *let-23* gene, the *sy97* mutation and all other mutations but *sy1* were localized using hydroxylamine mismatch detection, which detects mismatched C residues. The entire *let-23* coding sequence and exon−intron boundaries (spanning ~9 kb) were partitioned into eight sets of slightly overlapping, 1 kb PCR fragments. Primer sets from the 5′ to the 3′ end of the gene are as follows. Set 1, 5′-TTGGGTATCACATG-TATAAGAGG and 5′-GCAAAAAATTAGTTTTGCCTGGG; set 2, 5′-CCGGATCGACGAAGTAACCATCC and 5′-GAAACTTTTGATCG-TTCTGTGTC; set 3, 5′-GACACAGAACGATCAAAAGTTTC and 5′-GCTTTGAAGTACTTCTAGACACACAC; set 4, 5′-GACTTAAAA-GTGCATCTTTAGAGAGC and 5′-CCTTAGACGGTAGCTAAAATC-ATTCG; set 5, 5′-CGTTCCATCTGCAAAGAAGGC and 5′-CCATTC-AAGTGAATGCATACCC; set 6, 5′-GAAGGAGACAATCTGGCTTC-ACGC and 5′-CCAGGACGAGACTTTGGATCCGCC; set 7, 5′-TGGC-TTGCGATCGAAATCTTCTCC and 5′-CCAGATCTTCATTTGTAAC-AGCTG; set 8, 5′-CTCATCTAGGTACAAAACGGAGCC and 5′-CCA-AGCTTGTATGAGATGAATGGCAACGG.

Genomic DNA from heterozygous *let-23(mutant)/mnC1[let-23(+)]* hermaphrodites was amplified by PCR using these primers. For each allele, all heterozygous, amplified fragments were tested for a mismatched C as described previously (Cotton *et al.*, 1988). Given a random point mutation, a mismatched C should appear 83% of the time and since all of our alleles were generated with the mutagen EMS (which preferentially produces G-to-A transitions), it is very likely that mutations generated by EMS will be detectable by this scheme (all but one mutation, *mn23*, does in fact originate from a G-to-A transition). It is possible that point mutations exist in each of the alleles in addition to the one described here but we think this unlikely: (i) hydroxylamine detection detected only one mismatch in all the coding sequence for all alleles; (ii) mutageneses to generate these alleles were carried out under conditions that favor single point mutations; and (iii) no point mutations were found by sequencing within 100 bases of either side of each mutation. Mutations for each allele were sequenced from populations of PCR-amplified genomic DNA as for *sy1*. Mutant sequences were verified with multiple, independent PCRs using multiple genomic DNA preparations. In all cases, the location of the mutation as indicated by hydroxylamine detection and by sequencing coincided.

Analysis of sy97 splicing products

To determine the pattern of splicing in *let-23(sy97)*, 30 μg of *sy97* total RNA was reverse transcribed with random hexamers. 1/50th of the reaction

was then PCR-amplified with *let-23*-specific primers from exon 16 and exon 18 (primer set # 8). The PCR products were subjected to gel electrophoresis, revealing two bands: a major band at the size expected for a wild-type splice and a minor band at the size expected for unspliced intron 17 message. The major band was excised from the gel and cloned into pBluescript. Sixteen random subclones were sequenced in the region around the splice site mutation. The minor band, if translated, would result in a protein that truncates one amino acid after the end of exon 16 (residue number 1269).

Analysis of D_{1212} truncation

50 μg/ml of either NGROS213-13.3 or pK7-13.8 were injected into *let-23(mn23) unc-4(e120)/mnC1* hermaphrodites using a transgenic protocol we previously described (Aroian *et al.*, 1990). NGROS213-13.3 is a 12 kb genomic subclone that contains the *let-23* promoter and coding sequence up to D_{1212}. pK7-13.8 is a 15 kb genomic subclone that contains the *let-23* promoter, the entire gene and 2 kb of 3′ untranslated sequence. The *let-23(mn23)* allele eliminates LET-23 function and thus has no LET-23 fertility function. We established four independent lines stably transformed with NGROS213-13.3 that were able to rescue *let-23* lethality. Thus, at high copy number, NGROS213-13.3 is capable of providing some *let-23* activity. However, none of four lines rescued *let-23* sterility, indicating they were incapable of providing fertility function. We also established four independent lines stably transformed with pK7-13.8 that were able to rescue *let-23* lethality. All four of these lines showed rescue of *let-23* sterility. F_1 transient rescue experiments with both subclones agree with the results using stably transformed lines (data not shown). We note two caveats to our interpretation of these results: (i) it is conceivable that the 3′ untranslated region is important for fertility function since it is missing from NGROS213-13.3; (ii) the predicted translated product from NGROS213-13.3 encodes an additional 48 amino acids after D_{1212} from in-frame vector sequences. These mutant amino acids are PGNSISSLSIPSTSRGGPVPNS-PYSESYYNSLAVVLQRVTGKTLAYPT. It is possible that these additional amino acids might function to allow viability because of the potential SEM-5 binding site YXN (YYNS) (Songyang *et al.*, 1993) in this extension. Another possibility is that at high copy the D_{1212}-truncated LET-23 can provide the essential function of *let-23*.

Acknowledgements

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Chapter II B

A point mutation in the extracellular domain activates LET-23, the *C. elegans* EGF receptor homolog

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A Point Mutation in the Extracellular Domain Activates LET-23, the *Caenorhabditis elegans* Epidermal Growth Factor Receptor Homolog

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The *let-23* gene encodes a *Caenorhabditis elegans* homolog of the epidermal growth factor receptor (EGFR) necessary for vulval development. We have characterized a mutation of *let-23* that activates the receptor and downstream signal transduction, leading to excess vulval differentiation. This mutation alters a conserved cysteine residue in the extracellular domain and is the first such point mutation in the EGFR subfamily of tyrosine kinases. Mutation of a different cysteine in the same subdomain causes a strong loss-of-function phenotype, suggesting that cysteines in this region are important for function and nonequivalent. Vulval precursor cells can generate either of two subsets of vulval cells (distinct fates) in response to *sa62* activity. The fates produced depended on the copy number of the mutation, suggesting that quantitative differences in receptor activity influence the decision between these two fates.

Cells differentiate in response to a variety of extracellular signals; receptor tyrosine kinases (RTKs) receive some of these signals and transduce the information to other components within the cell. Signal transduction pathways involving RTKs are conserved in mammals, insects, and nematodes (reviewed in references 16 and 56). RTKs normally are activated by ligand binding, followed by receptor dimerization and covalent modification of the receptor by autophosphorylation on tyrosine. This activation transduces the signal into the cytoplasm (16, 56, 71). Mutations may cause inappropriate RTK activity by interfering with regulation or by conferring hypersensitivity to ligand or ligand-independent activity via a change in conformation. Because many RTK pathways control cell proliferation, such mutations can be oncogenic (42, 71, 78).

The *let-23* gene of the nematode *Caenorhabditis elegans* encodes a member of the epidermal growth factor (EGF) receptor (EGFR) family (1) (Fig. 1A). *let-23* is required for larval viability, vulval differentiation, formation of male mating structures, fertility, and specification of the P11 and P12 neurectoblasts (3, 17, 23). Complete loss of *let-23* function causes death in the first stage of larval development. Reduction-of-function mutations in *let-23* are pleiotropic; some alleles cause only a subset of defects. For example, the allele *let-23(sy10)* causes partial reduction of survival, vulval differentiation, male spicule differentiation, and complete loss of fertility (3). LET-23 acts in a genetically defined signal transduction pathway that parallels the biochemically defined EGFR pathway of mammalian cells (reviewed in reference 63). Downstream effectors include SEM-5, a GRB2 homolog that has SH2 and SH3 domains (10, 61), LET-60 and LIN-45, homologs of *ras* and *raf*, respectively (20, 21), a Mek homolog (39, 77), and a mitogen-activated protein kinase homolog (43, 76).

LET-23 is essential for vulval differentiation. The vulva of *C. elegans* is made from the progeny of three of six multipotent vulval precursor cells (VPCs). In response to the LIN-3 inductive signal, produced by the anchor cell in the adjacent gonad (24), these three cells adopt vulval fates and differentiate to form vulval tissue. The vulval fates are of two types, designated 1° and 2°. The other three VPCs normally adopt a nonvulval epidermal fate, designated 3° (reviewed in reference 30).

Reduction-of-function mutations in *let-23* or other genes in the pathway cause fewer than three VPCs to adopt vulval fates. In extreme cases, all six VPCs adopt epidermal fates and no vulva is made. This phenotype is called vulvaless (Vul) (reviewed in reference 63). Excess pathway activity, due to *lin-3* overexpression or *let-60 ras* gain-of-function mutations, causes more than three VPCs to adopt vulval fates. This leads to formation of ectopic pseudovulvae, a phenotype called multivulva (Muv) (6, 19, 34).

We have characterized *sa62*, the first known activating mutation of *let-23*. This mutation causes a semidominant phenotype, excess differentiation of the VPCs, that occurs even in the absence of inductive signal from the gonad. The ligand-independent activity can induce either the 2° or 1° vulval fate, depending on the copy number of the mutant gene. We compared the gain-of-function phenotypes to loss-of-function phenotypes caused by a similar codon change in the same domain and tested the effects of analogous mutations in the human EGFR.

MATERIALS AND METHODS

General methods. Methods for culturing, handling, and genetic manipulation of *C. elegans* were performed as described previously (8). Unless otherwise noted, strains were maintained at 20°C. We use the standard *C. elegans* cellular and genetic nomenclature (29, 66). Mutations used are as described in reference 8 or as noted. The following mutations were used: LG I, *dpy-5(e61)*, *lin-10(e1439)* (17), *unc-13(e51)*; LG II, *let-23(mn23)*, (*sy16*), (*sy17*), (*sy1*) (3, 23), *dpy10(e128)*.

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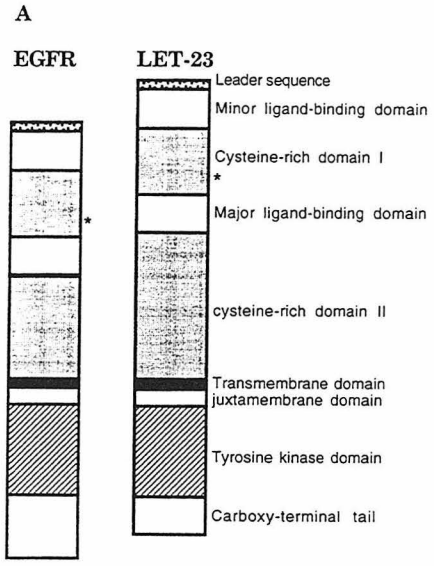

B

		305	313	
EGFR	300	RKCKKCEGP-CRKVCNGIG		317
HER2	329	QRCEKCSKP-CARVCYGLG		346
HER3	299	KMCEPCGGL-CPKACEGTG		316
HER4	321	KMCKPCTDI-CPKACDGIG		338
DER	341	GECVPCNGP-CPKTCPGVT		358
LET-23	354	RECEKCRSSSCPKICTVDG		372
sa62		.....Y.....		
sy10		.....Y....		
		359	368	

FIG. 1. Comparison of LET-23 and EGFR. (A) Schematic representations of LET-23 and EGFR structures. Asterisks mark approximate locations of mutated cysteines. (B) Sequence comparison of EGFR family members. The numbers flanking each sequence indicate the positions of the first and last amino acids shown (1, 11, 44, 49, 50, 70, 72).

unc-4(e120), *rol-6(e187)*, *unc-52(e444)*, *rol-1(e91)*, *mnC1[dpy-10(e128) unc-52(e444)]* (23), *lin-7(e1413)* (17, 31); LG III, *unc-32(e189)*; LG IV, *dpy-20(e1282)* (25, 66), *lin-3(n378)*, *(n1059)* (17), *unc-22(e66)*, *unc-24(e138)*, *let-60(sy100)* (19), *nT1[unc(n754dm) let]* (*IV*; *V*) (17), *nT1[let(m435)]* (52); LG V, *dpy-11(e224)*, *him-5(e1490)* (26); and LG X, *lon-2(e678)*, *sem-5(n2019)* (10), *lin-2(e1309)* (17, 28).

Genetic analysis. (i) Backcrossing. *sa62*, isolated from a screen of ethyl methanesulfonate-mutagenized animals, was generously provided by J. H. Thomas. We backcrossed the *sa62* strain twice, first to a *lon-2(e678)* strain and then to the wild-type strain N2. In the course of the second backcross, we isolated a line of animals that had lost a linked recessive lethal mutation, which we designated *sy220*.

Further backcrossing revealed the presence of an interacting mutation which we designated *sy322*. This unlinked mutation increased the penetrance of the *sa62* Muv phenotype. In *sa62 e120*; *sy322* homozygotes, 4 to 6 (an average of 5.3) VPCs adopted vulval fates (100% of animals were Muv, $n = 54$) at 20°C. In comparison, in *sa62 e120* homozygotes, 3 to 5.5 (an average of 4.2) VPCs adopted vulval fates (90% were Muv) at 20°C (Table 1). In *sa62 e120/mnC1*; *sy322* animals, 3 to 5 (an average of 3.6) VPCs adopted vulval fates (70% of animals were Muv, $n = 93$), while in *sa62 e120/mnC1* animals, 3 to 4 (an average of 3.04) VPCs adopted vulval fates (6% were Muv). *sy322* does not suppress the Vul phenotype of *let-23(sy1)* or *let-23(sy97)* (data not shown).

(ii) Mapping to chromosome II. We mated *let-23(sa62)/+*; *lon-2* or *let-23*

TABLE 1. Vulval differentiation in *let-23(sa62)* animals

Genotype[a]	No. of VPCs differentiated[b]	% Muv animals	No. of animals examined
sa62/+[c]	3.04	6	162
sa62/sa62[d]	4.2	89	147

[a] *sa62* is linked to *unc-4(e120)* as a marker, and *let-23(+)* is on the *mnC1* balancer chromosome.

[b] Average number of VPCs per animal that differentiated to vulval fates. The wild-type value is 3.00.

[c] *sa62e120/mnC1* heterozygote descended from *sa62 e120/mnC1* heterozygotes or from mated *sa62 e120/sa62 e120* homozygotes.

[d] *sa62 e120* homozygotes descended from *sa62 e120/mnC1* heterozygotes or from *sa62 e120/sa62 e120* homozygotes.

(sa62) let(sy220)/+−; *lon-2* animals with males heterozygous for one of the chromosomal markers. From Muv (*sa62/+*; *marker/+* or *sa62+/−+*; *marker/+*) non-Lon F_1 progeny from each cross, we isolated F_2 progeny that were homozygous for the marker. F_3 progeny were scored for presence of the Muv phenotype (and the *sy220* Let phenotype if applicable). If the marker is linked to *sa62* and *sy220*, most animals homozygous for the marker should give no Muv or Let progeny. If the marker is unlinked, two-thirds of animals homozygous for the marker should produce Muv and Let progeny. *let-23(sa62)* and *let(sy220)* showed linkage to *dpy-10* and assorted independently from *dpy-5*, *unc-32*, *dpy-20*, *dpy-11*, and *lon-2*.

Three-factor mapping located *let(sy220)* close to or to the right of *rol-1* [15 of 16 Dpy non-Let recombinants from *rol-1(e91)/dpy-10(e128) sy220* animals produced Rol progeny].

(iii) Three-factor mapping of *sa62*. To balance the right arm of chromosome II, we used *mnC1[dpy-10(e128) unc-52(e444)]* (23). *sa62/mnC1*; *him-5(e1490)* males were mated to *let-23(mn23) unc-4(e120)/mnC1* or *rol-6(e187) let-23(sy16)/mnC1* hermaphrodites. Both *mn23* and *sy16* are lethal alleles of *let-23* (3, 23). Progeny of the *let-23(sa62)/let-23(mn23) unc-4* or *let-23(sa62)/rol-6 let-23(sy16)* heterozygotes were screened for viable Unc or Rol progeny. These recombinants were isolated, and their progeny were examined for the presence of Muv animals. All 36 Unc non-Let animals and all 24 Rol non-Let animals produced Muvs, indicating that they carried *sa62*. These values placed *sa62* within 0.1 map unit of *let-23* with 95% confidence.

(iv) Mutagenesis and *cis-trans* test. Since animals carrying *sa62* in *trans* to lethal *let-23* alleles were healthy with a partially penetrant Muv phenotype (Table 2), if *sa62* were caused by a mutation in a linked but different gene, it should also produce the Muv phenotype in *cis* to a *let-23* loss-of-function (lethal) allele. In contrast, if *sa62* were a *let-23* mutation, we would expect a lethal allele of *let-23* to be a *cis*-dominant suppressor of the *sa62* Muv phenotype.

We isolated lethal alleles of *let-23* in *cis* to *sa62* by mutagenesis. Animals were mutagenized with ethyl methanesulfonate (8), and 10 to 20 mutagenized *let-23 (sa62) unc-4(e120)* hermaphrodites per plate were mated with eight *let-23(sy1)*; *him-5(e1490)* males. *let-23(lethal)/let-23(sy1)* animals are viable but defective in egg laying (Egl phenotype) because they are Vul (3). We picked Egl F_1 progeny from independent plates and isolated descendants homozygous for *him-5*.

From 4,800 F_1 progeny we isolated three Egl mutants that produced dead larvae of the *let-23(lethal)* type. The three alleles were designated *sy264*, *sy265*, and *sy266*. We outcrossed two of these (*sy264* and *sy265*) and showed that they failed to complement the lethality of *let-23(sy16)* and the Egl defect of *sy1*. The third isolate (*sy266*) could not be outcrossed, as males had severely crumpled spicules, and hermaphrodites were Vul.

We constructed *let-23(sa62 sy264) unc-4/mnC1* and *let-23(sa62 sy265) unc-4/mnC1* strains and examined them with Nomarski optics to score vulval differen-

TABLE 2. Vulval differentiation in transgenic animals

Genotype (chromosome, transgene)[a]	% Muv	No. of animals examined
mn23/+, Tyr-359	28[b]	157
mn23/mn23, Tyr-359	31[b]	155
mn23/+, Cys-359	≤0.1[c]	>500
mn23/mn23, Cys-359	≤0.1[c]	>500

[a] The lethal allele *let-23(mn23)* is linked to *unc-4(e120)* as a marker and placed in *trans* to *mnC1* in heterozygotes. Transgenic strains also carry the unlinked marker *dpy-20(e1282)*. The transgene is present on an extrachromosomal array consisting of pk7s62 (Tyr-359) or pk7-13.8 (Cys-359) together with pMH86 (a plasmid that rescues the Dpy-20 defect) as a marker.

[b] Data from three stable lines.

[c] Data from 10 stable lines.

tiation and P11/P12 fate. All 109 *sa62 sy264*/++ and 51 *sa62 sy265*/+− animals examined were wild type. Therefore, *sy264* and *sy265* are dominant suppressors of the Muv phenotype when in *cis* to *sa62*. For analysis of the *trans* phenotype, we used *rol-6(e187) let-23(sy17)*/*sa62 unc-4(e120)*. *sy17* mutates the 5′ splice donor of intron 4 and causes a larval lethal phenotype; thus, it is likely to cause complete loss of LET-23 function (2). Of these 60 *trans* heterozygotes, 7 were Muv. Since *sy264* and *sy265* are *cis*-dominant suppressors of *sa62*, we concluded that *sa62* is a *let-23* allele.

(v) Epistasis tests. We performed epistasis tests with alleles of *lin-3*, *sem-5*, *let-60*, *lin-2*, *lin-7*, and *lin-10*. We constructed double-mutant strains heterozygous for *sa62* and one of the Vul mutations and examined the vulval phenotypes of their progeny. If *sa62* is epistatic to the Vul mutation, then Muv progeny will produce only Muv progeny, but Vul progeny may segregate Vul (genotype *sa62*/+; *vul*) or Muv (genotype *sa62*; *vul*) progeny. If the Vul mutation is epistatic to *sa62*, then Muv progeny may segregate Muv (genotype *sa62*; *vul*/+) or Vul (genotype *sa62*; *vul*) progeny, while Vul progeny will produce only Vul progeny. When homozygous double mutants were identified, we quantitated vulval differentiation by examination with Nomarski optics. In wild-type animals, exactly three of the six VPCs adopt vulval fates. Vulval differentiation of more than 3.0 VPCs indicates a Muv phenotype; vulval differentiation of fewer than 3.0 VPCs indicates a Vul phenotype.

For tests with *let-23(sa62)* and *lin-3*, we used two *lin-3* alleles: a genetically null allele, *n1059*, and an allele which retains some activity, *n378* (17). *n1059*/*n378* is the *lin-3* genotype that has the most severe Vul phenotype and yet is viable. *let-23(sa62) unc-4(e120)*/*mnC1* males were mated to *lin-3(n378) unc-22(e66)* hermaphrodites. L4 (fourth larval stage) males from the cross were mated to *unc-24(e138) lin-3(n1059) dpy-20(e1282)*/*DnT1* hermaphrodites. As *DnT1* dominantly confers paralysis, nonparalyzed cross progeny carry *lin-3(n1059)* rather than *DnT1*. A strain of genotype *sa62 unc-4*/+; + *lin-3(n378) unc-22* +/*unc-24 lin-3(n1059)* + *dpy-20* was isolated from these cross progeny.

For tests with *let-23(sa62)* and *sem-5(n2019)*, *let-23(sa62) unc-4(e120)*/*mnC1* males were mated to *sem-5(n2019)* hermaphrodites (10), and F_1 hermaphrodites of genotype *sa62 unc-4*/++; *sem-5*/− were isolated.

For tests with *let-23(sa62)* and *let-60(sy100dn)*, we used *let(m435)nT1*[*unc (n754dm let)*], referred to as *let nT1*, to balance the *lin-3* region. *let-23(sa62) unc-4(e120)*/*mnC1* males were mated to *let nT1*/*let-60(sy100dn) dpy-20(e1282)* hermaphrodites. As *let nT1* confers embryonic lethality and *let-60(sy100dn)* is a recessive larval lethal mutation (19), F_1 hermaphrodites that segregated dead larvae but no dead embryos and no *mnC1* progeny had the desired genotype [*sa62 unc-4*/++; *let-60(sy100dn) dpy-20*/++].

For tests with *let-23(sa62)* and *lin-2(e1309)*, *let-23(sa62)*/*mnC1*; *him-5(e1490)* males were mated to *rol-6(e187)*; *lin-2(e1309)* hermaphrodites (17, 31), and F_1 progeny of genotype *sa62*/*rol-6*; *lin-2*/+ were isolated.

For tests with *let-23(sa62)* and *lin-7(e1413)*, *let-23(sa62)*/*mnC1*; *him-5(e1490)* males were mated to *unc-4(e120) lin-7(e1413)* (17, 31) hermaphrodites. From *sa62*/*unc-4 lin-7* progeny, we isolated Vul non-Unc recombinants (genotype *sa62* + *lin-7*/ + *unc-4 lin-7*). To confirm the wild-type genotype, N2 [*let-23(sa62)*] males were mated to *unc-4(e120) lin-7(e1413)* hermaphrodites. Their male progeny (genotype *unc-4 lin-7*/−) were mated to putative *let-23(sa62) lin-7* (Muv) homozygotes. Vul animals [genotype + *unc-4 lin-7*/*let-23(sa62)* + *lin-7*] were isolated. Their progeny segregated both Muv and Vul progeny, confirming that both *let-23(sa62)* and *lin-7(e1413)* were present.

For tests with *let-23(sa62)* and *lin-10(e1439)*, *let-23(sa62)*/*mnC1*; *him-5(e1490)* males were mated to *unc-13(e51) lin-10(e1439)* hermaphrodites (17). To confirm the genotype of the double homozygote, putative *let-23(sa62) unc-13 lin-10* hermaphrodites were mated to N2 males. Their progeny segregated Muvs and Vuls, confirming that *let-23(sa62)* and *lin-10* were present.

Molecular analysis. Unless otherwise noted, subcloning and DNA manipulations were performed according to standard methods (4, 54).

(i) Sequence analysis. Eight sets of primers that span the entire coding sequence and the intron-exon boundaries of *let-23* (~8.5 kb) were designed (1, 2). By using these primers, DNA from nematodes homozygous for *let-23(sa62)* and from wild-type nematodes were amplified by PCR under the following conditions: 94°C for 3 min, then 30 cycles of 94°C for 1 min, 55°C for 0.5 min, and 72°C for 1.5 min, followed by 72°C for 7 min. Each amplified fragment was gel purified by using Geneclean II (Bio 101 Inc., La Jolla, Calif.) and sequenced by the dideoxy double-strand method, using a Sequenase kit (U.S. Biochemical, Cleveland, Ohio) (40). The mutation was sequenced twice, using DNA from two independent animals.

(ii) Molecular reconstruction of the Cys-359→Tyr (C359Y) mutation in *let-23*. pk7-13.8 is a 15-kb subclone that contains the *let-23* promoter, the entire gene, and ~2 kb of 3′ untranslated region (1). A 5.9-kb *Bam*HI-*Sal*I fragment from pk7-13.8 was subcloned into pBluescript KS(+) (Stratagene, La Jolla, Calif.), generating the clone pk7-6.1. Site-directed mutagenesis was carried out in pk7-6.1 by the method of Deng and Nickoloff (14), which permits direct mutagenesis of double-stranded circular DNA (Clontech Laboratories Inc., Palo Alto, Calif.). Two mutagenic primers were synthesized: SKNot (5′-ACCGCGGTGGCTA GCGCTCTAGAAC-3′), which changes the *Not*I restriction site in pBluescript to an *Nhe*I site, and SA62 (5′-GAGAGTGTGAAAAATACAGAAGTTCCAG CTG-3′), which introduces the *let-23(sa62)* mutation at position 5673 of *let-23*. The mutated *Bam*HI-*Sal*I fragment was placed back into pk7-13.8, generating pk7s62, which is different from pk7-13.8 only in that it carries the G-to-A mutation at the same position in which it was detected in *let-23(sa62)* animals.

We constructed transgenic *C. elegans* by high-copy-number germ line transformation (47). A mix of 20 ng of pk7s62 per μl, 10 ng of pMH86 (a plasmid which rescues the Dpy-20 phenotype) per μl, and 170 ng of pBluescript carrier DNA per μl was injected into *let-23(mn23) unc-4(e120)*/*mnC1*; *dpy-20(e1282)* worms, and non-Dpy animals were picked. Some of these non-Dpy F_1 transgenic animals produced non-Dpy transgenic F_2 progeny; these stable lines were analyzed to give the data in Table 2. *mn23* is a null allele of *let-23* (2, 3, 23).

Construction of C305Y and C313Y mutations in HER14. The C305Y and C313Y mutants were constructed by using an Altered Sites in vitro mutagenesis system from Promega. The mutant clones were excised from the pAlter vector with *Kpn*I and *Xba*I restriction digestion and inserted into the pRK5 vector by blunt-end cloning into the blunt-ended *Xba*I site of the vector. The transcription in these cells is driven by the cytomegalovirus promoter.

Alternatively, these two mutants as well as the wild-type and K721A clones were cloned into the pLXSHD retroviral vector (48), using the *Xho*I site in the vector polylinker.

NIH 3T3 2.2 cells, which lack endogenous EGFR (45), were transfected with C305Y or C313Y clones together with a neomycin-resistant plasmid by the calcium phosphate precipitation method (74). Two days after transfection, the cells were split, seeded at a density of 100,000 cells per 10-cm-diameter dish, and put under neomycin resistance selection by addition of 0.8 mg of Geneticin G418 (GIBCO) per ml to the medium. Resistant clones were picked after 3 weeks and screened for EGFR by Western blotting (immunoblotting) with antibody RK-2 (41).

Alternatively, BOSC cells were transfected with the retroviral vector and viral supernatants were used to infect NIH 3T3 cells as described previously (48). Resistance to histidinol was selected by addition of 0.8 mg of histidinol (Sigma) per ml. Resistant clones were picked and screened as described above.

Scoring vulval differentiation. Twenty young adult hermaphrodites were placed on a plate, allowed to lay eggs for 2 h, and then removed from the plate. All larvae on a plate were examined by Nomarski optics when they were at the late L3 stage or early L4 stage. Vulval differentiation was scored as described elsewhere (3). The values reported are the averages of three independent experiments.

Cell ablation experiments and VPC fate assignment. Cell ablations were performed as described previously (5, 68), using 3.5 mM sodium azide in the mounting agar. We ablated all cells in the gonad primordium of *let-23(sa62) unc-4(e120)*/*mnC1* or *let-23(sa62) unc-4(e120)*/*let-23(sa62) unc-4(e120)* larvae before the first division of the somatic gonad precursors Z1 and Z4. Ablations were confirmed at or before the L2 lethargus. Animals were maintained at 20°C during the ablation procedure and all of development. VPC fates were assigned as described elsewhere (34, 64, 66).

Scoring brood size. We picked *let-23(sa62) unc-4(e120)* animals or *unc-4* control animals at the L4 stage, placing a single animal on each plate, and observed them approximately every 12 h, counting the number of eggs or larvae produced. (Some animals, at the end of their reproductive life, apparently became egg laying defective, as larvae appeared while no eggs were observed. This typically occurred in the last two to four 12-h intervals.) At each time point, we transferred the parents to new plates and incubated the eggs left on the plates to score larval viability or counted and removed the eggs, leaving the parent on the plate. The brood was judged complete when no eggs or larvae were produced for three consecutive 12-h intervals.

Mating test. We picked animals for the mating test at the L4 stage. Males of genotype *let-23(sa62)*/*let-23(sa62) unc-4(e120)*; *him-5(e1490)*/+ were placed singly on plates with four *unc-52(e444)* hermaphrodites each. After the animals matured and produced progeny, we counted the non-Unc progeny that arose from mating. The control cross used males of genotype *unc-4*/+; *him-5*/+.

Immunoprecipitation. Dishes of confluent cells were treated with 100 ng of EGF per ml for 5 min and then washed three times with phosphate-buffered saline, drained well, and scraped into 0.5 ml of lysis buffer (27). After 5 min of incubation on ice, the lysate was centrifuged for 5 min in an Eppendorf centrifuge at 4°C, and the supernatant was either used immediately or frozen at −70°C. EGFR was immunoprecipitated with antibody RK-2, a rabbit antiserum directed against a synthetic peptide from the cytoplasmic domain, or with 108, a monoclonal antibody specific for human EGFR (7). Lysates were incubated with protein A-Sepharose-antibody complex for 90 min at 4°C and then washed three times with lysis buffer.

Autophosphorylation. Immunoprecipitates were incubated on ice in lysis buffer containing 5 mM $MnCl_2$, 200 μM sodium orthovanadate, 15 μM unlabeled ATP, and 1 μCi of [γ-^{32}P]ATP for 15 min. The reaction was stopped by addition of sample buffer. The proteins were separated by electrophoresis on a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel.

^{125}I-EGF binding experiments and Scatchard analysis. For all ^{125}I-EGF binding assays, cells were plated at a density of 100,000 cells per well in 24-well dishes coated with 10 mg of human plasma fibronectin (Meloy Laboratory) and grown for 48 h to confluency in Dulbecco modified Eagle medium containing 10% calf serum. Human recombinant EGF (Intergen) was iodinated by using the chloramine T method to a specific activity of 1.5×10^8 cpm/mg.

Binding experiments and Scatchard analysis were performed as described previously (27).

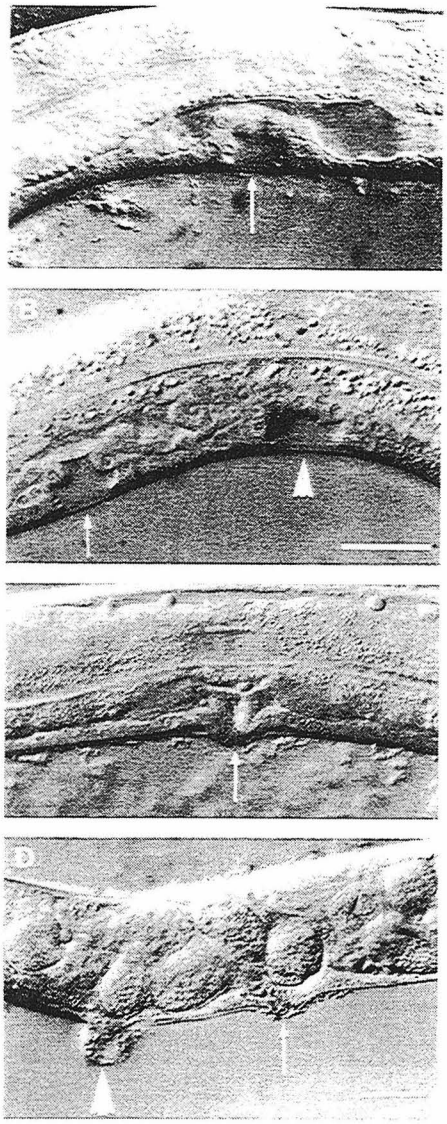

FIG. 2. Muv phenotype of *sa62*. Shown are Nomarski photomicrographs of L4 hermaphrodites (A and B) and adult hermaphrodites (C and D). (A) Wild type; (B) *sa62*; (C) *let-23(sy17 null)* with wild-type transgene; (D) *let-23(sy17 null)* with *sa62* transgene. Arrows, vulvae; arrowheads, pseudovulvae. Scale bars = 20 μm.

TABLE 3. Vulval differentiation after gonad ablation

Genotype[a]	No. of animals examined	No. of VPCs adopting indicated fate				
		3°[b]	Half-vulval	2°	Intermediate	1°
−/+	Many	All	0	0	0	0
sa62/+	67	330	13	25	0	0
sa62/*sa62*	14	20	6	35	12	5

[a] *sa62* is linked to *unc-4(e120)* as a marker; heterozygotes are balanced in *trans* to *mnC1*.

[b] P3.p fused with the epidermal syncytium hyp7 without dividing in 34 heterozygotes and 6 homozygotes. This occurs in approximately 50% of intact wild-type animals (33a, 64).

RESULTS

***sa62* confers a semidominant Muv phenotype.** A strain carrying the *sa62* mutation was isolated in the laboratory of J. H. Thomas. We isolated *sa62* from a complex genetic background and examined vulval differentiation. In wild-type animals, 3.0 VPCs differentiate to vulval fates. The Muv phenotype was observed in *sa62* animals when more than three VPCs adopted vulval fates, giving rise to ectopic pseudovulvae (Fig. 2B). In animals homozygous for *sa62*, 3 to 5.5 (4.2 on average) VPCs adopted vulval fates at 20°C; 89% of homozygotes displayed excess vulval differentiation (Table 1). In animals heterozygous for *sa62*, three to four VPCs adopted vulval fates at 20°C; approximately 6% of heterozygotes displayed excess vulval differentiation. The effect of the *sa62* mutation is exerted zygotically; maternal genotype did not affect the phenotype of the progeny.

***sa62* is caused by a point mutation in the extracellular domain of *let-23*.** We mapped *sa62* genetically to within 0.1 map unit of *let-23*, a region corresponding to approximately 100 kb on the physical map. As other tyrosine kinases are present in this interval, we performed a *cis-trans* test to demonstrate that *sa62* is an allele of *let-23* (see Materials and Methods).

We then sequenced the coding region of *let-23* in *sa62* animals and found a single point mutation (G to A) in cysteine-rich domain I, close to the major ligand-binding domain (Fig. 1B). The mutation converts cysteine 359 to tyrosine. We reconstructed the C359Y by site-directed mutagenesis. This reconstructed mutant allele, when expressed as a transgene in *let-23(lethal)* animals, was able to rescue the lethal phenotype. Transgenic animals bearing this construct displayed excess vulval differentiation (Table 2; Fig. 2D). While we have not rigorously ruled out the possibility that an additional mutation in noncoding sequence leads to overexpression of *sa62* gene product, extra copies of wild-type *let-23* do not cause excess vulval differentiation in transgenic animals (Table 2; Fig. 2C). Therefore, the change at codon 359 is necessary and likely sufficient to cause the semidominant Muv phenotype of *sa62*.

***sa62* activity induces both vulval fates in a ligand-independent, dose-dependent manner.** In wild-type animals, a signal from the anchor cell of the gonad is required to induce vulval differentiation. When a laser microbeam is used to ablate the gonad primordium of wild-type animals, no VPCs differentiate to vulval fates (37, 68). When we ablated the gonad primordium in *sa62* animals, both *sa62*/+ heterozygotes and *sa62* homozygotes displayed vulval differentiation (Table 3). Twenty-two of 67 heterozygotes and all 14 homozygotes tested displayed vulval differentiation after gonad ablation. This result demonstrates that *let-23(sa62)* can act in a ligand-independent manner.

We observed a correlation between the copy number of *sa62*, the number of VPCs induced to vulval fates, and the types of vulval fates observed in gonad-ablated animals (Table 3; Fig. 3). In *sa62* homozygotes, 58 of 78 VPCs adopted vulval fates. Five of these 58 adopted the 1° fate, while 12 adopted a fate that has properties of both 1° and 2° fates, designated intermediate (34). In *sa62* heterozygotes, only 38 of 368 VPCs adopted vulval fates, and the 38 VPCs induced to vulval fates adopted either the 2° fate or a fate having properties of both 3° and 2° fates (half-vulval). Strikingly, in these animals no VPCs adopted the 1° or intermediate fate. This result indicates that

Genotype	Lineage generated by each VPC					
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
sa62/+	S S	S S	S S	S S	S S	S S
	S	S S	S ss	LL S	S S	S S
	S S	S S	LLTN	S S	S S	S S
	S	S S	LLTN	S S	LLOT	S S
sa62/*sa62*	S	ss S	LLLN	ss LO	LLLN	S S
	LLON	DDTT	LLLN	LDTT	NLLL	S S
	LLLT	LDNT	LLLN	LTTT	LLLN	S S
	S LL	LLLL	LLLN	TTTT	NTLL	S S

FIG. 3. Representative vulval lineages observed after gonad ablation. Lineages were determined and classified as previously described (34). 3° lineages are indicated as S S or S ss. In some animals, P3.p does not divide and is indicated as S. Hybrid fates include S LL and ss LO. 2° fates include LLTN, LLLN, LLON, LDTT, DDTT, and LLLN. Dotted boxes indicate intermediate fates; the solid box indicates a 1° fate.

receptor activity influences VPC fate specification in a dose-dependent manner. The observation that a twofold difference in *sa62* copy number causes a large difference in the fraction of VPCs that adopt vulval fates suggests that a threshold level of receptor activity may be required for a VPC to adopt a vulval fate.

Phenotypes affected by other *let-23* mutations. *let-23* acts at multiple points in development. Reduction-of-function mutations in *let-23* cause four defects in addition to the vulval defect: deformed male spicules (structures necessary for mating), infertility, larval lethality, and misspecification of the P11 and P12 neurectoblast fates. Since *sa62* causes a vulval defect that is the opposite of the *let-23* reduction-of-function defect, we investigated its effect on other *let-23* functions.

We tested mating efficiency and examined male spicule structure in *sa62* homozygotes. *sa62* males were able to sire non-Unc cross progeny when mated with *unc-52* hermaphrodites but at reduced frequency (6 of 22 *sa62* males were able to sire cross progeny, while 17 of 20 control males were able to do so). Hyperactivating the pathway by overexpressing LIN-3 in transgenic animals gives rise to deformed spicules due to misspecification of cell fates (9). Inspection of *sa62* male mating structures revealed a defect in the bursa of the male tail but not the spicule defect expected for the gain-of-function lineage alteration. The bursa of the *sa62* male tail appeared constricted, and some of the rays were abnormally short or curved (Fig. 4). This novel defect may identify another role for *let-23* in development or may reflect aberrant activity of the mutant receptor.

The average brood size of unmated *sa62* hermaphrodites was 173 at 20°C, with a range of 45 to 276 (n = 24). Control animals assayed in parallel had an average brood size of 283, with a range of 204 to 360 (n = 15). *sa62 e120* animals laid eggs at a slower rate (average peak rate of 24 eggs per 12 h) than *e120* controls (average peak rate of 73 eggs per 12 h) but produced eggs over a slightly longer period. *sa62* homozygotes are scrawny and slow growing; the low brood size that we observed in comparison with that of wild-type control strains could indicate a partial defect in fertility or could merely reflect the sickliness of *sa62* homozygotes. As described above, transgenic *sa62* rescued the lethality of *let-23* null alleles. It also rescued the sterile phenotype of *let-23* null alleles: 28 of 30 transgenic animals were fertile.

No significant embryonic or larval lethality was observed in *sa62* strains. P11/P12 fate specification was normal in 79 *sa62* animals examined (31a).

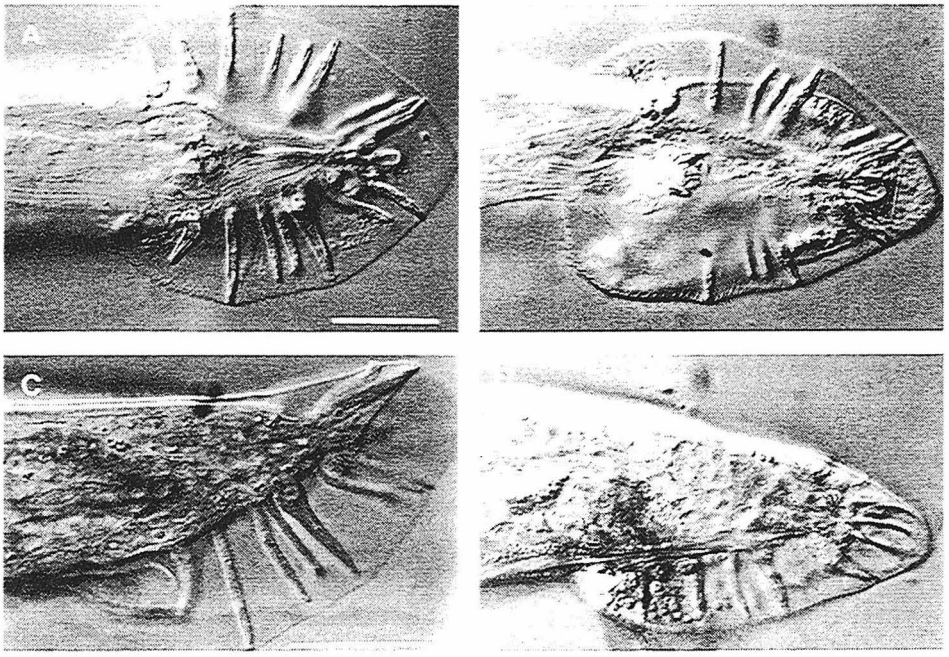

FIG. 4. Morphologies of mating structures in *sa62* and wild-type males. (A) Wild type, ventral view; (B) *sa62*, ventral view; (C) wild type, lateral view; (D) *sa62*, lateral view. Scale bars = 20 μm.

TABLE 4. Vulval differentiation in *sa62*; *vul* double mutants

Expt[a]	Genotype	*let-23*(+)		*sa62*	
		No. of VPCs induced[b]	No. of animals examined	No. of VPCs induced[c]	No. of animals examined
1	+	3.0	Many	4.2[d]	58
2	*lin-3(weak)*	0.8	22	4.4	21
3	*lin-3(strong)*	0.09	20	4.4	20
4	*sem-5*	0.5	20	0.4	21
5	*let-60(dn)*[e]	0[f]	10	0.8	22
6	*lin-2*	0.5	20	3.5	23
7	*lin-7*	1.0	20	4.3	22
8	*lin-10*	0.5	17	3.9	18

[a] Genotypes used in experiments 1 to 8 were as follows: 1, *N2*; 2, *lin-3(n378) unc-22(e66)*; 3, *lin-3(n378)unc-22(e66)/unc-24(e138) lin-3(n1059) dpy-20(e1282)*; 4, *sem-5(n2019)*; 5, *let-60(sy100) dpy-20(e1282)*; 6, *lin-2(e1309)*; 7, *lin-7(e1413)*; 8, *unc-13(e51) lin-10(e1439)*.

[b] Average number of VPCs per animal that differentiated to vulval fates in *vul*; *let-23*(+) animals. Data from reference 32.

[c] Average number of VPCs per animal that differentiated to vulval fates in *vul*; *sa62* animals. *vul* genotypes are the same as in footnote *a*. *sa62* is linked to *unc-4(e120)* in experiments 1 to 5.

[d] From Table 1.

[e] *dn*, a dominant negative allele.

[f] From reference 19.

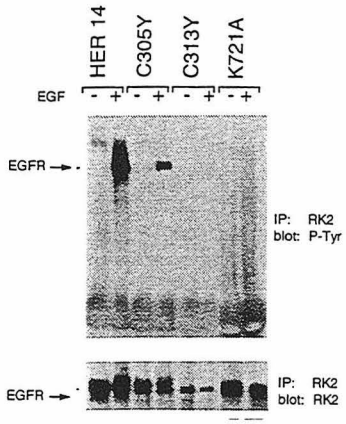

FIG. 5. EGF-dependent Tyr phosphorylation of EGFR and mutant receptors. Cells expressing either wild-type or mutant receptors were exposed to 100 ng of EGF per ml for 5 min and then subjected to cell solubilization, immunoprecipitation (IP) with anti-EGFR antibody RK-2, separation by SDS-polyacrylamide gel electrophoresis, blotting, and analysis with an antiphosphotyrosine (P-Tyr) antibody (top). The nitrocellulose filter was stripped and reblotted with anti-EGFR antibody RK-2 (bottom).

Genetic epistasis tests. The Muv phenotype of *sa62* permits genetic epistasis tests with genes in the signal transduction pathway that have Vul mutant phenotypes. By analogy with the activities of their homologs in other systems, the LIN-3 growth factor is expected to act before LET-23, and the SEM-5 adaptor and LET-60 RAS to act after LET-23 in the signal transduction pathway.

We constructed double mutant strains and compared their vulval differentiation with that of single mutants (Table 4). *sa62* did not rescue the lethal phenotype of severe *lin-3* alleles. Therefore, we analyzed the strongest viable reduction of function *lin-3* genotype. This genotype confers almost complete lack of vulval differentiation. Animals homozygous both for *sa62* and severe *lin-3* showed the Muv phenotype of *sa62*. Thus, *sa62* is epistatic to the *lin-3* vulval defect, supporting the hypothesis that LET-23 acts after LIN-3 in vulval differentiation.

Animals with either a *sem-5* mutation or a dominant negative *let-60* mutation display little or no vulval differentiation (6, 10, 19). Animals doubly mutant for *sa62* and either *sem-5* or *let-60(dn)* also displayed very little vulval differentiation. Thus, *sem-5* and *let-60* vulvaless mutations are epistatic to *sa62*, consistent with activity after LET-23 in vulval differentiation.

lin-2, *lin-7*, and *lin-10* mutations cause reduced vulval differentiation (17, 18, 31, 36, 65, 67). The functions of their gene products are not known; *lin-10* encodes a novel protein (36). Therefore, it is of interest to constrain their point of action with respect to the signal transduction pathway. We found that animals doubly mutant for *sa62* and *lin-2*, *lin-7*, or *lin-10* had excess vulval differentiation, while *lin-2*, *lin-7*, or *lin-10* alone caused incomplete vulval differentiation. Thus, *sa62* is epistatic to these mutations, consistent with *let-2*, *let-7*, and *let-10* acting to help *let-23* function effectively.

Activity of an analogous mutation in human EGFR. To analyze further the activity of the gain-of-function mutation, we constructed the analogous mutation in the human EGFR (70) by in vitro mutagenesis, converting cysteine 305 to tyrosine. We also constructed a loss-of-function mutation analogous to *let-23(sy10)* by converting cysteine 313 to tyrosine (Fig. 1B). We transfected NIH 3T3 cells that lack endogenous EGFR, isolated stable cell lines containing each mutation, and compared them in parallel experiments with well-characterized cell lines carrying either wild-type HER14 or the kinase-inactive mutant K721A (27). Confluent cells were incubated for 5 min with EGF and then lysed. The receptor was immunoprecipitated from the cell lysates with antibody RK-2, raised against a synthetic peptide from the cytoplasmic domain (41).

To assay tyrosine phosphorylation, immunoprecipitated proteins were analyzed by Western blotting with an antiphosphotyrosine antibody (33). The C305Y mutant receptor showed EGF-dependent tyrosine phosphorylation, as did wild-type receptor (Fig. 5). However, neither the C313Y mutant receptor nor the loss-of-function mutant K721A showed tyrosine phosphorylation, consistent with loss of function caused by the C313Y mutation. Since equal amounts of protein were loaded in all lanes, the low level of C313Y in the lower panel of Fig. 5 may be due to instability of the mutant gene product.

To exclude the unlikely possibility that the extracellular cysteine mutation C313Y might inactivate the intracellular kinase domain, we performed an in vitro kinase assay. Both C305Y and C313Y showed kinase activity in vitro, confirming that the mutations do not exert their effects directly on receptor kinase activity (Fig. 6).

To test whether the ligand-binding site was altered in the mutant receptors, we performed immunoprecipitation with

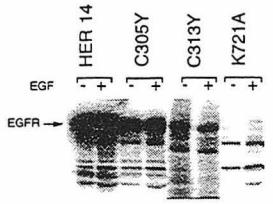

FIG. 6. In vitro kinase assay of EGFR and mutant receptors. EGFR and mutant receptors were immunoprecipitated with antibody RK-2. The immunoprecipitates were phosphorylated with [γ-^{32}P]ATP and separated by SDS-polyacrylamide gel electrophoresis.

monoclonal antibody 108, raised against the major ligand-binding domain of EGFR (7). The antibody immunoprecipitated C305Y but not C313Y (data not shown). This result indicates that the ligand-binding site is not significantly altered in the C305Y receptor but is altered in the C313Y receptor. The loss of antibody binding suggests a conformational change affecting the ligand-binding domain of the C313Y mutant receptor.

Assays of ^{125}I-EGF binding to EGFR on living cells revealed two distinct affinity states (38, 57). We performed Scatchard plot analysis of ^{125}I-EGF binding to cells expressing wild-type receptor (275,000 receptors per cell) or C305Y mutant receptor (50,000 receptors per cell). Binding curves at room temperature are consistent with the existence of two classes of binding sites in both cell lines. In the case of HER14, 2 to 3% of the sites were in the high-affinity state ($K_d = 0.3 \times 10^{-10}$ to 0.7×10^{-10} M), and 97 to 98% were in the low-affinity state ($K_d = 5 \times 10^{-9}$ to 6×10^{-9} M). In the case of C305Y, 3 to 4% of the sites were in the high-affinity state with a slightly higher K_d (0.15×10^{-9} to 0.2×10^{-9} M), and 96 to 97% were in the low-affinity state ($K_d = 4 \times 10^{-9}$ to 5×10^{-9} M). Cell lines expressing the wild-type and mutant receptors, made by infection with a retroviral vector, gave similar K_d values. Infected cell lines carrying wild-type, C305Y, or K721A receptor all expressed similar numbers of receptors (approximately 300,000 per cell). Thus, the C305Y mutation does not alter the steady-state number of receptors at the cell surface. The amount of C313Y receptor per cell could not be estimated, since the mutant receptor does not show any EGF binding.

DISCUSSION

We have shown that a single amino acid substitution in a cysteine-rich extracellular domain of the LET-23 RTK causes a gain-of-function phenotype. This is the first example in the EGFR subfamily of activation caused by a single extracellular amino acid substitution. In contrast, *sy10*, another point mutation nine residues away in the same domain, which also substitutes tyrosine for cysteine, reduces LET-23 function. While *sy10* is pleiotropic, *sa62* visibly affects only one of several developmental functions previously shown to depend on LET-23. Genetic epistasis and cell ablation experiments suggest that the *sa62* phenotype may arise from ligand-independent activity of the mutant gene product or from hypersensitivity to very low levels of ligand. The copy number of *sa62* influenced the vulval fates produced, suggesting that levels of receptor activity affect the choice of VPC fate.

Activation of RTKs by mutations in the extracellular domain. RTKs are grouped into classes based on molecular structure. Receptors of the EGFR class include EGFR, HER2/neu, HER3/c-erbB-3, and HER4/p180erb4 of vertebrates, DER of *Drosophila melanogaster*, and LET-23 of *C. elegans* (1, 49, 51, 71). These receptors consist of an extracellular domain, a single transmembrane domain, and an intracellular domain (Fig. 1A). The extracellular domain contains four subdomains: two that are thought to contribute most to ligand binding, alternating with two cysteine-rich domains (56, 71).

Ligand binding appears to promote receptor dimerization (reviewed in reference 22). Receptor dimerization leads to intermolecular cross-phosphorylation of receptor, which results in signal transduction (22, 55, 56, 59, 71).

Changes in the structure of the extracellular domain can activate RTKs. The leukemogenic insertionally activated version of c-erbB deletes the extracellular binding domain (42); an N-terminal truncation of human EGFR allows constitutive self-renewal of erythroblasts (35). In-frame deletion of 7 to 12 extracellular residues activates Neu/c-erbB-2 (58). Insertion of a cysteine residue in the extracellular juxtamembrane region of EGFR increases affinity for ligand and kinase activity of the receptor (59).

Point mutations in the exoplasmic domain of RTKs of other subclasses can cause ligand-independent activation of RTKs. Three *torso* gain-of-function alleles are caused by different point mutations in the extracellular domain (60). Both the feline and human *c-fms* proto-oncogenes are activated by a point mutation at identical positions in the extracellular domain (53, 75). The *neu* oncogene contains a Val-to-Glu change in the transmembrane domain. This change is postulated to enhance oligomerization, leading to increased activity (62, 73). An Arg-to-Cys change in the exoplasmic domain of the erythropoietin receptor causes factor-independent growth and accumulation in the endoplasmic reticulum and prevents the rapid degradation characteristic of the wild-type receptor. The new cysteine makes sulfide linkages postulated to mimic the dimerization of the ligand-bound receptor (78).

The Cys-to-Tyr change in *sa62* lies in cysteine-rich region II of the extracellular domain. The cysteine-rich subdomains are conserved, flank the major ligand-binding domain, and are in a position to interact with adjacent receptors (56, 71). Thus, this region of the extracellular domain may be involved in conformational change upon ligand binding. The fact that the nearby *sy10* Cys-to-Tyr mutation causes the phenotype opposite that of *sa62* suggests that cysteines in this domain are important for function but not all are equivalent in function.

The hypothesis that the *sa62* mutation activates the RTK by promoting ligand-independent dimerization and activation is not supported by in vitro analysis of the analogous mutation in human EGFR. This may reflect a unique interaction between LET-23 and factors specific to vulval differentiation, or vulval differentiation may be more sensitive to receptor activity than other inductive events mediated by the same pathway.

Role of LET-23 in *C. elegans* vulval pattern formation. Recent work has demonstrated that the EGF-like domain of LIN-3, the vulval inductive signal, can influence vulval cell fate in a dose-dependent manner (34). Here we demonstrate that the *sa62* mutation can act in a similar manner. Specifically, in gonad-ablated animals, we observed a correlation between the number of copies of the *sa62* mutation, the extent of vulval differentiation, and the presence or absence of the 1° fate. The dependence of VPC fate on *sa62* copy number is consistent with a model in which graded levels of inductive signal induce different vulval fates by stimulating distinct levels of LET-23 activity in VPCs receiving different levels of inductive signal. Given the importance of these quantitative differences, we propose that modulation of receptor activity in different VPCs could be important in the specification of VPC fate.

It will be of interest to learn how quantitative differences in signal or receptor activity give rise to qualitatively different responses. One possibility is that quantitative information is transduced via the Ras pathway. Investigations using cultured mammalian cells have shown that quantitative differences in EGFR activity are transduced via mitogen-activated protein kinase and may affect the duration of phosphorylation of downstream effectors (15, 69). Alternatively, different levels of signal or receptor activity may stimulate distinct signal transduction pathways. Investigations of other mammalian cell types, as well as genetic studies of *C. elegans*, offer precedent for this model (13, 32). These models can be tested by molecular genetic investigation of the activities of downstream effectors in the vulval induction pathway and by genetic analysis of interacting genes.

LIN-12, a transmembrane protein structurally similar to the *Drosophila* Notch protein, appears to function downstream of

LET-23 in a lateral signaling pathway that normally passes between vulval precursor cells and is thought to be required for specification of the 2° fate (reviewed in reference 63). Our observation of 2° fates in gonad-ablated *sa62* heterozygotes suggests that intermediate levels of LET-23 activity can promote the 2° fate. While our data do not rule out a LIN-12-independent mode of 2° fate specification, the LIN-12 signaling pathway might be activated in response to intermediate LET-23 activation.

Role of LET-23 in *C. elegans* signal transduction. We have used *let-23(sa62)* to confirm the order of action of LET-23 in the signal transduction pathway for vulval differentiation. Taken together with previous observations that LET-23 is necessary for vulval differentiation in response to overexpressed LIN-3 (24), our epistasis and cell ablation experiments indicate that LET-23 acts after LIN-3. By contrast, SEM-5 is necessary for vulval differentiation stimulated by the *sa62* gene product, consistent with SEM-5 acting to transduce signal from LET-23 to LET-60. These results support the likelihood of interactions proposed on the basis of the biochemical activity of mammalian homologs. In particular, since human GRB2 can replace SEM-5 functions in transgenic nematodes (61), we expect that SEM-5 associates with activated LET-23 via its SH2 domain, as its homolog GRB2 does with activated EGFR (46).

Three other genes, *lin-2*, *lin-7*, and *lin-10*, are only partly required for vulval differentiation (17, 18, 31, 36, 65, 67). This partial requirement is consistent with their helping the efficacy of signaling, or acting in one of two parallel signal transduction pathways. *sa62* bypasses the requirement for *lin-2*, *lin-7*, and *lin-10*. If these are indeed null alleles, our results suggest that *lin-2*, *lin-7*, and *lin-10* help in receptor synthesis or activation, not as components of a second pathway.

Although we observed a novel defect in the male tail, we did not observe the predicted defect in the male spicules, nor did we find abnormalities in several other nonvulval phenotypes that are affected by *let-23* loss-of-function mutations or pathway hyperactivity. Moreover, *sa62* does not suppress the lethal phenotype caused by a loss of function mutation of *lin-3*. These differences suggest that the VPCs may be more sensitive than other cells to increases in pathway activity. In support of this possibility, the reduction-of-function mutation *sy10* displays more activity in vivo than the analogous mutation C313Y displays in vitro. Similarly, *sa62* displays elevated activity in the VPCs, while the analogous mutation C305Y has no significant effect on the growth of 3T3 cells or on receptor activity in vitro. Alternatively, interacting factors unique to the VPCs might mediate the vulval differentiation phenotype of *sa62*. For example, *sa62* might cause a conformational change in the receptor that affects an interacting protein expressed only in the VPCs. In either case, these observations support the possibility that vulval differentiation provides a sensitive assay for subtle variations in pathway activity. Analysis of vulval development in *C. elegans* holds promise to elucidate novel aspects of receptor tyrosine kinase regulation and signal transduction.

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Chapter III

Positive and negative tissue-specific signaling by a nematode EGF receptor

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Positive and Negative Tissue-specific Signaling by a Nematode Epidermal Growth Factor Receptor

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The major determinants of receptor tyrosine kinase (RTK) signaling specificity have been proposed to be Src homology 2 (SH2) binding sites, phosphotyrosine-containing oligopeptides in the cytoplasmic domain of the receptor. The *Caenorhabditis elegans* epidermal growth factor receptor homologue LET-23 has multiple functions during development and has eight potential SH2-binding sites in a region carboxyl terminal to its kinase domain. By analyzing transgenic nematodes for three distinct LET-23 functions, we show that six of eight potential sites function in vivo and that they are required for most, but not all, of LET-23 activity. A single site is necessary and sufficient to promote wild-type fertility. Three other sites activate the RAS pathway and are involved only in viability and vulval differentiation. A fifth site is promiscuous and can mediate all three LET-23 functions. An additional site mediates tissue-specific negative regulation. Putative SH2 binding sites are thus key effectors of both cell-specific and negative regulation in an intact organism. We suggest two distinct mechanisms for tissue-specific RTK-mediated signaling. A positive mechanism would promote RTK function through effectors present only in certain cell types. A negative mechanism would inhibit RTK function through tissue-specific negative regulators.

INTRODUCTION

Growth factor receptor tyrosine kinases (RTKs)[1] play a critical role in intercellular communication in both vertebrates and invertebrates, providing a link between extracellular signals and intracellular effectors. Their activation influences a wide variety of cellular responses including growth, metabolic homeostasis, and survival (Carpenter and Wahl, 1990; Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993; Dickson and Hafen, 1994). Activation of RTKs upon ligand binding results in oligomerization followed by autophosphorylation or transphosphorylation on specific tyrosine residues. This phosphorylation generates short sites that have been shown to bind specific proteins via Src homology 2 (SH2) domains. These proteins include the adapter GRB-2, which leads to RAS activation, phospholipase C-γ (PLC-γ), the tyrosine phosphatase SHP-2 (Adachi *et al.*, 1996), Ras GTPase-activating protein (GAP), and the regulatory subunit of phosphatidylinositol-3OH-kinase (Ullrich and Schlessinger, 1990; Koch *et al.*, 1991; Hernandez-Sotomayor and Carpenter, 1992; Cohen *et al.*, 1995; Heldin, 1995; Pawson, 1995). Interaction of RTKs with different substrates is thought to result in activation of distinct signaling pathways, thus producing different cellular responses (Schlessinger and Ullrich, 1992).

Some growth factor RTKs have indeed been shown to exert different activities through different SH2-binding sites. For example, the sites for PLC-γ and phosphatidylinositol-3OH-kinase in the platelet-derived growth factor receptor-β (PDGFR-β) promote chemotaxis, whereas the site for GAP mediates suppression of migration (Kundra *et al.*, 1994). In the fibroblast growth factor receptor, the site binding PLC-γ is required for phosphatidylinositol turnover and Ca^{2-} flux but not for mitogenesis (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). These studies suggest that the exact

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[1] Abbreviations used: EGFR, epidermal growth factor receptor; GAP, GTPase-activating protein; PDGFR, platelet-derived growth factor receptor; PLC-γ, phospholipase C-γ; RTK, receptor tyrosine kinase; SH2, src homology 2; VPC, vulval precursor cell.

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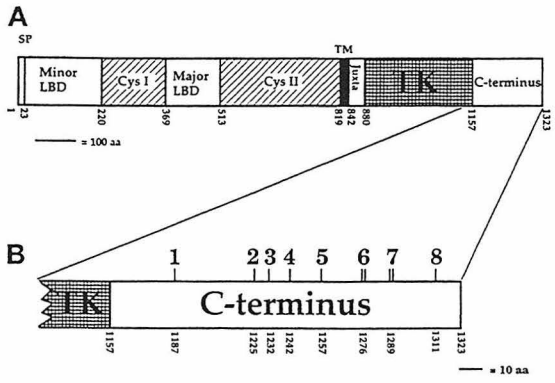

Figure 1. Structure of the LET-23 protein. All the LET-23 protein (1323 amino acids) represented with the domains characteristic of the EGFR family is shown in A. SP, signal peptide; Minor LBD, minor ligand-binding domain; Cys I, cysteine-rich domain I; Major LBD, major ligand-binding domain; Cys II, cysteine-rich domain II; TM, transmembrane domain; Juxta, juxtamembrane domain; TK, tyrosine kinase domain; C terminus, carboxyl-terminal tail. Part of the TK domain and all of the carboxyl terminus are shown in B. Vertical bars represent tyrosines. There are 10 tyrosines in the carboxyl-terminal tail that define eight putative SH2- binding sites (sites 1–8).

number and type of SH2-binding sites are the major determinant of RTK signaling specificity. Nonetheless, few biological assays on SH2-binding sites have been carried out in intact organisms (for example, see Maina *et al.*, 1996) and little is known about the physiological significance of SH2-binding sites in RTK signaling (Cohen *et al.*, 1995). Moreover, previous studies in cell culture have not been able to assign functions to the SH2 sites in epidermal growth factor receptor (EGFR). For example, although specific EGFR sites have been shown to bind specific substrates (Rotin *et al.*, 1992; Batzer *et al.*, 1994; Batzer *et al.*, 1995), elimination of one or some of these sites has never been shown to result in specific functional alterations. In contrast, even when all known SH2-binding sites are eliminated by site directed mutagenesis and no association with known SH2 domain–containing proteins can be detected, EGFR can still induce mitogenesis and transformation (Decker, 1993; Li *et al.*, 1994; Soler *et al.*, 1994).

EGFR	DER	LET-23
YRAL	YTSQ	YFDQ
YLIP	YLQP	YKTE
YSSD	YCKD	YGST
YINQ	YLMP	YLIP
YHNQ	YMDL	YTAV
YQDP	YLLN	YYNQP
YLNT	YYNDT	YYNEP
YQQD	YAFA	YENE
YLRV		

Figure 2. Putative carboxyl-terminal SH2- binding sites in human EGFR, *Drosophila* EGFR (DER), and LET-23.

LET-23 is a *Caenorhabditis elegans* member of the EGFR family (Aroian *et al.*, 1990): its extracellular portion contains two ligand-binding domains alternated with two cysteine-rich domains (Figure 1). The cytoplasmic region contains a tyrosine kinase domain and a carboxyl-terminal tail. The tail contains tyrosines that define eight putative SH2-binding sites (Figures 1 and 2) and is implicated in multiple functions (Aroian and Sternberg, 1991): the portion containing sites 6, 7, and 8 is important for viability and vulval differentiation; the portion containing sites 2, 3, 4, and 5 mediates fertility (Aroian *et al.*, 1994). Mutations that eliminate LET-23 activity result in complete loss of these functions (Aroian and Sternberg, 1991). Complementation analysis indicated that a general decrease of *let-23* activity parallels with a decrease of all *let-23*-mediated functions. In addition, it indicated that *let-23* has qualitatively distinct functions (Aroian and Sternberg, 1991; Aroian *et al.*, 1994). These functions act to control development in distinct tissues. Mosaic analysis indicated that *let-23* acts cell-autonomously in the vulval precursor cells (VPCs) to promote vulval differentiation (Koga and Ohshima, 1995; Simske and Kim, 1995) and suggested that the essential *let-23* function is in cells other than the VPCs, possibly the excretory cell, a cell thought to control osmotic pressure (Koga and Ohshima, 1995). Sterility produced by a *let-23* mutation arises from a defect in ovulation (McCarter and Schedl, personal communication) and likely has a focus in the gonad.

LET-23 is likely to stimulate viability and vulval differentiation via SEM-5 and LET-60. The vulvaless (no vulva) and lethal phenotypes caused by *sem-5* and *let-60* mutations are similar to those of *let-23* mutations. Also, genetic epistasis tests with gain-of-function alleles of *let-23* and *let-60* revealed that the three genes act in a common pathway (Beitel *et al.*, 1990; Han *et al.*, 1990; Clark *et al.*, 1992; Katz *et al.*, 1996).

To better understand how EGFR family tyrosine kinases transduce their signals in vivo, we have tested the significance of the LET-23 putative SH2-docking sites (Figure 2) in such a manner that the intrinsic regulatory complexity of the signal transduction pathway is intact. We have altered the potential SH2-binding sites of LET-23 in vitro and expressed the resulting constructs in nematodes lacking LET-23 activity. We have then used functional assays to detect LET-23 activity in these transgenic nematodes and

show that six of the eight LET-23 putative sites function in vivo. These sites are required to mediate most but not all of LET-23 activity and they are not equivalent. Some activate specific functions, one mediates tissue-specific negative regulation, and one is promiscuous, promoting all LET-23 functions analyzed. To a first approximation, viability and vulval induction are stimulated by the same sites. Our data demonstrate the physiological importance of SH2-binding sites in an intact organism and suggest that at least two independent mechanisms are used for RTK-mediated tissue specificity.

MATERIALS AND METHODS

Strains and General Methods

The following mutations were used: LG II, *let-23(nulls), mn23, sy15, sy17,* (Herman, 1978; Aroian and Sternberg, 1991; Aroian *et al.*, 1994), *unc-4(e120)* (Brenner, 1974), *mnC1[dpy-10(e128) unc-52(e444)]* (Herman, 1978); LG IV, *dpy-20(e1282)* (Hosono *et al.*, 1982); and LG X, *sem-5(n2019)* (Clark *et al.*, 1992). *let-23(null) unc-4(e120)/mnC1; dpy-20(e1282)* and *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282); sem-5(n2019)* were constructed by following standard procedures. Strains of the form *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282); sem-5(n2019); Ex* were constructed by mating males from a *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282); Ex* stable line with *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282); sem-5(n2019)* hermaphrodites and then following standard procedures. Statistical tests of significance were performed with Fisher's exact test with the InStat 2.00 program (GraphPad Software, San Diego, CA).

Assay for Modified let-23 Genes In Vitro

An intact *let-23* genomic clone, pk7–13.8, rescues the defects associated with loss-of-function alleles of *let-23* (Aroian *et al.*, 1990; Aroian *et al.*, 1994; Simske and Kim, 1995; Katz *et al.*, 1996; Simske *et al.*, 1996; Figure 3A). One or more carboxyl-terminal tyrosines encoded by this rescuing clone were changed to phenylalanines by site-directed mutagenesis to alter LET-23 putative SH2-binding sites (Figure 2). Each construct was expressed in a strain of genotype *let-23(null) unc-4(e120)/mnC1 [dpy-10(e128) unc-52(e444)]; dpy-20(e1282)* [in most cases strain PS1484, which has the *sy17* allele of *let-23*; for the *let-23(null)* used in each experiment, see text and legends]. *unc-4* is a recessive mutation tightly linked to *let-23* that renders the worms uncoordinated: Unc-4 animals are also homozygous for *let-23(null). mnC1[dpy-10(e128) unc-52(e444)]* is a balancer that provides a wild-type allele of *let-23* and *unc-4* and inhibits recombination in the region (Herman, 1978). It is used because *let-23(null)/let-23(null)* animals die as young larvae (therefore, PS1484 does not segregate Unc-4 animals). *mnC1/mnC1* worms are immobile, semisterile, and easily recognizable under the dissecting microscope.

High copy number germline transformation was carried out according to Mello *et al.* (1991). Young adult hermaphrodites were placed live on pads of 5% Noble agar under an inverted differential contrast-interference (Nomarski) microscope (Carl Zeiss, Oberkochen, Germany) and DNA was injected into the gonad with an Eppendorf microinjector model 5242 (Eppendorf Gerätebau Netheler, Hamburg, Germany). Except where noted, a mixture of 50 ng/μl *let-23* DNA, 15 ng/μl pMH86 *[dpy-20(+)]* DNA (Han and Sternberg, 1990), and 110 ng/μl pBluescript II carrier DNA was injected into animals from PS1484 (P0). pMH86 rescues the Dpy phenotype. We picked F_1 non-Dpy non-Unc hermaphrodites, corresponding to animals expressing the injected DNA. Every F_1 worm able to propagate the transgene to the following generations defines a stable line. We maintained balanced stable lines and from those we analyzed non-Dpy Unc-4 worms for the ability of the mutagenized *let-23* gene to rescue three phenotypes in the hermaphrodite: vulval induction, viability, and fertility (the transformation scheme is outlined in Figure 4). As a control we injected a construct that provides a wild-type copy of *let-23* and, therefore, is able to completely rescue all three phenotypes considered (Figure 3A). NGros213–13.3 was injected at 50 ng/μl along with 50 ng/μl pRF4 [a plasmid containing a dominant mutant gene of *rol-6* (Mello *et al.*, 1991)] and 100 ng/μl pBluescript II as carrier DNA.

Scoring Viability

When we inject *let-23(+)* DNA (Figure 3A), which completely rescues defects associated with the *let-23(null)* alleles, including lethality (Figure 5), along with pMH86 *[dpy-20(+)]* into a *let-23(null) unc-4(e120)/mnC1; dpy-20(e1282)* mother (see Figure 4), all progeny expressing the transgene are non-Dpy; the number of non-Dpy Unc animals [nDU, genotype *let-23(null) unc-4(e120); dpy-20(e1282); Ex-(construct A, pMH86)*] should be half the number of non-Dpy non-Unc animals [nDnU, genotype *let-23(null) unc-4(e120)/mnC1; dpy-20(e1282); Ex(construct A, pMH86)*] (Figure 4; *Ex* indicates an extrachromosomal array). If a construct only partially rescues lethality, then the number of nDU will be less than one-half of the number of nDnU. In general, the percentage of viability = 2x·100/y, where x is the number of nDU and y is the number of nDnU.

To test whether *let-23* constructs can overcome the reduced viability due to decreased SEM-5 activity, we constructed strains of the form *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282); sem-5(n2019); Ex. Ex* contains the extrachromosomal transgene of interest (A, F, or H) plus *dpy-20(+)* DNA. We also determined the viability of an isogenic strain with no extrachromosomal transgene (62.7%, n = 523). Since transgenic animals have copies of *dpy-20(+)* DNA, they are non-Dpy whereas nontransgenic animals are Dpy. Dpy non-Unc animals (DnU) and nDnU are heterozygous for *let-23*. Animals homozygous for *let-23(sy17)* and carrying the transgene are nDU. We determined the transmission frequency (TF) of the transgene in the strains *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282); sem-5(+); Ex*. Since TF = nDnU/(nDnU + DnU), a viability increase in transgenic nDnU animals results in higher TF. By comparing the nDnU observed with the nDnU expected in case of complete overcome of *sem-5(n2019)* inviability or no overcome at all of *sem-5(n2019)* inviability, we can infer whether the transgene activity is SEM-5 dependent in a *let-23(sy17)/let-23(+); sem-5(n2019)* background. To determine whether viability increases from a *let-23(sy17)/let-23(+); sem-5(n2019)* background to a *let-23(sy17); sem-5(n2019)* background, we counted nDnU and nDU. If viability does not differ, then nDU/nDnU = 1/2. If viability does differ, nDU/nDnU > 1/2. By comparing the number of nDU observed with the expected number in case of a bypass of *sem-5(n2019)* inviability or no bypass of *sem-5(n2019)* inviability, we infer whether the transgene requires SEM-5 for its activity.

Scoring Vulval Induction

In *C. elegans* six cells, the VPCs, have the potential to divide three times and generate vulval cells (i.e., adopt vulval fates; Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986). In wild-type hermaphrodites, only three VPCs adopt vulval fates (Figure 5C), and the other three VPCs divide once and fuse with the epidermis.

Vulval induction was scored under Nomarski optics at the L3 molt when the VPCs' nuclei that will form the vulva are at the four-cell stage and easily visible. Vulval induction is 100% when three VPCs are induced. Non-Dpy Unc-4 hermaphrodites were placed live on pads of 5% Noble agar (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1981) and observed with a Plan 100× objective, Nomarski differential interference-contrast optics for their extent of vulval induction. Vulval differentiation = number

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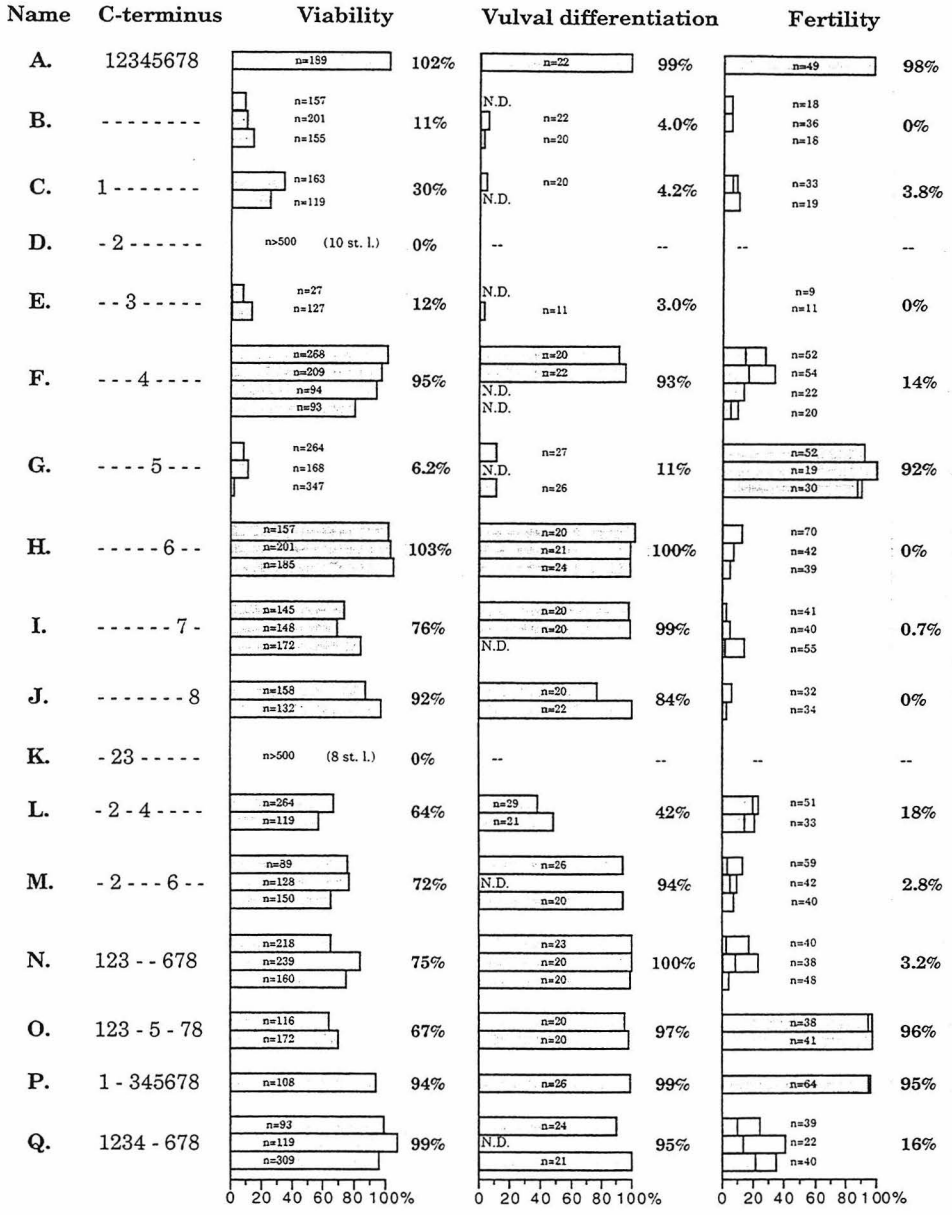

Figure 3. Ability of LET-23 carboxyl-terminal tyrosines to mediate viability, vulval differentiation, and fertility. The *let-23(null)* allele used to test all of the constructs in this figure is *let-23(sy17)*. Carboxyl-terminal sites 1, 2, 3, etc., indicate that tyrosine(s) at that putative SH2-binding site has not been changed; a dash indicates that each tyrosine in the corresponding site has been substituted with phenylalanine. Unshaded bars in the fertility column represent semifertile animals (see below). The percent on the right of each column represents the weighted average of all stable lines analyzed per construct. The tyrosine is essential, when phosphorylated, for binding a given SH2 domain and its elimination prevents binding (Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990; Fantl *et al.*, 1992). n indicates the number of animals scored. Analysis of phenotypes and relative calculations are fully explained in MATERIALS AND METHODS and in Figure 5. Viability is the proportion of surviving animals. Vulval differentiation is the number of VPCs generating vulval progeny compared with wild-type (always 3) and scored with Nomarski optics. To score fertility, brood size per hermaphrodite was counted: 0–1 progeny, sterile; 2–10, semifertile; >10 fertile. Typically, the number of progeny observed (including dead larvae) was 40–70 for egg-laying–defective worms and >150–200 for

of VPCs induced/3 (100% vulval differentiation = three VPCs induced).

Scoring Fertility

let-23 sterility is different from that observed in animals defective in *let-60(ras)* (Beitel *et al.*, 1990; Han and Sternberg, 1990), *mpk-1/sur-1* (Lackner *et al.*, 1994; Wu and Han, 1994), and *mek-2* (Wu *et al.*, 1995), where sterility is caused by alterations in meiotic cell cycle progression within the germline (Church *et al.*, 1995). L4 or young adult hermaphrodites were placed one per plate and checked every day. Each plate was followed for 3 to 5 d for the presence of larvae or vulvaless worms (see Figure 5). All progeny were counted, including dead larvae. Animals were classified as follows, depending on the number of progeny (shown in parentheses): sterile (0–1), semifertile (2–10), or fertile (>10).

Site-directed Mutagenesis

Site-directed mutagenesis was carried out in pk7–5.5, a *Hin*dIII clone of *let-23* that contains the last 3 kb of a 3′ coding sequence plus ~2 kb of 3′ untranslated sequence. We used the method of Deng and Nickoloff (1992), which permits direct mutagenesis of double-stranded circular DNA (Clontech, Palo Alto, CA). A selection primer was synthesized, SKNot (5′-ACCGCGGTGGCTAGCGCTCTAGAAC-3′) that changes the *Not*I restriction site in pBluescript II to an *Nhe*I site. Eighteen mutagenic primers were synthesized. One primer, pS97, (5′-TGATGGGGATAGCTTTGAAAATTTATTAT-3′), introduces a G to A mutation at the end of intron 17. Another primer, pSTX, (5′-CGCCATTTGATGGCTATTACTCTGAAAATTTATTA-3′), introduces two stop-codons so that the resulting LET-23 protein is predicted to be truncated at amino acid 1268. Eight primers alter the putative SH2-binding sites: DY-1 (site 1, 5′-CTTCCTTGATCAAAGAACATCTGTGGATCGA-3′), DY-2 (site 2, 5′-AAAGGCTCCGTTTTGAACCTAGATGAGTTT-3′), DY-3 (site 3, 5′-GTGCGGTTGACCCAACTAAAATGCAAAATAATT-3′), YLIP-1 (site 4, 5′-TTTGGAATAAG- AAATGAATTATCTTCC-3′), YTAV-1 (site 5, 5′-GTAACAGCTGTAAACAAAACTGCTG-3′), YYNQ-2 (site 6, 5′-TTTGGTTGGTTGAAGAAATCGCCATTTG-3′), YYNE-2 (site 7, 5′-TGTGGTTCATTGAAAAATCCAGAGGAAG-3′), and YENE-1 (site 8, 5′-TCTTCATTTTCAAATTGAACTGCTTC-3′).

Eight primers restore the putative SH2-binding sites: DY-W1 (site 1, 5′-CTTCCTTGATCAAAGTACATCTGTGGATCGA-3′), DY-W2 (site 2, 5′-AAAGGCTCCGTTTTGTACCTAGATGAGTTT-3′), DY-W3 (site 3, 5′-GTGCGGTTGACCCATCTAAAATGCAAAATAATT-3′), LIP-W (site 4, 5′-TTTGGAATAAGATATGAATTATCTTCC-3′), TAV-W (site 5, 5′-GTAACAGCTGTATACAAAACTGCTG-3′), NQ-W (site 6, 5′-TTTGGTTGGTTGTAGTAATCGCCATTTG-3′), NE-W (site 7, 5′-TGTGGTTCATTGTAATATCCAGAGGAAG-3′), and ENE-W (site 8, 5′-TCTTCATTTTCATATTGAACTGCTTC-3′).

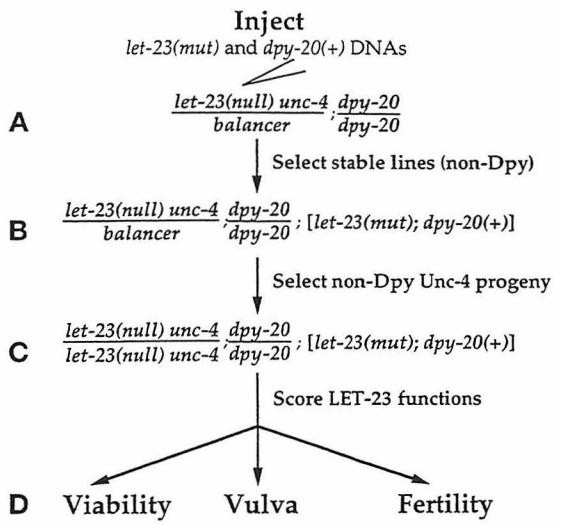

Figure 4. Strategy used to analyze the effects of in vitro-modified *let-23* genes on viability, vulval induction, and fertility. Worms with a *let-23(null)* and *dpy-20(−)* background (A) were injected with a wild-type [*let-23(+)*] (control) or altered [*let-23(mut)*] *let-23* clone along with a marker DNA [*dpy-20(+)*]. Stable lines were selected based on segregation of non-Dpy progeny (B). Progeny (C) were used for analysis. Since *let-23* is required for viability, all viable non-Dpy Unc-4 progeny will carry the transgene (C). For every *let-23* clone, we analyzed the extent of rescue of each LET-23 function in multiple independent transgenic lines (D).

The mutated fragments were then excised with *Sal*I and *Hin*dIII and ligated with an ~12-kb *Eco*RI-*Sal*I genomic *let-23* fragment and with an ~3-kb *Eco*RI-*Sal*I pBluescript II fragment generating the plasmids in Figure 3. The correctness of the constructs was verified by sequencing the mutated region plus the surrounding 200–300 bp.

RESULTS

An In Vivo Genetic System for RTK Function

Studies in cultured cells have demonstrated that, after ligand binding, RTKs become phosphorylated at specific tyrosines (Schlessinger and Ullrich, 1992). In the same experimental conditions, when these tyrosines are removed, alternative sites normally not used could become phosphorylated (Walton *et al.*, 1990; Hernandez-Sotomayor and Carpenter, 1992). In addition, very little is known about the actual phosphorylation sites of RTKs in intact organisms. Herein, we analyze LET-23 function in different tissues and, therefore, would need to know the precise phosphorylation state of LET-23 in all of these tissues. Because at present this is technically impossible, we used a different approach and studied the functional significance of all LET-23 potential SH2-binding sites.

Before starting a systematic analysis of carboxyl-terminal tyrosines, we carried out a series of control

Figure 3 (cont). egg-laying–competent worms. Some dead larvae could be missed because they degrade and are difficult to see. Wild-type brood size is ~300 but egg-laying–defective animals have a maximum of 70 progeny. For a given construct and a given function, data from multiple stable lines were not statistically different from each other ($p > 0.05$), with two exceptions: one of three lines of construct G showed lower viability; one of two lines of construct J showed less vulval induction. These differences could arise from mosaicism and differential stability of the transgene (Mello *et al.*, 1991). The data presented are consistent with data from transient (F_1)-transformed animals and with observation of additional transformed lines. Analysis of F_1-transformation tests a much larger number of independent transformation events.

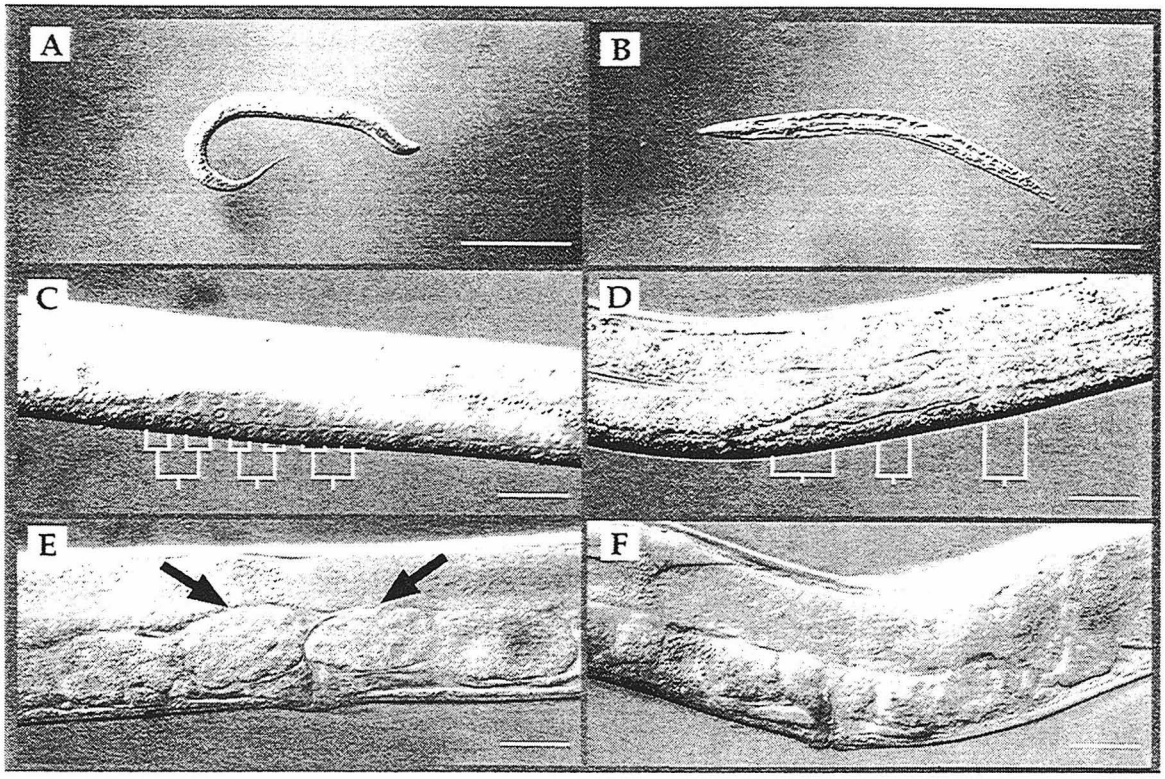

Figure 5. Nomarski photomicrographs of three distinct phenotypes associated with loss of LET-23 function. Lethality (A and B); vulval differentiation (C and D); fertility (E and F). Wild-type (A, C, and E). *let-23(loss-of-function)* phenotypes (B, D, and F). Wild-type L1 larva (A). Dead L1 larva (B). Lineage of the three VPCs at the four-cell stage that will divide again and form the vulva in a wild-type L3 larva (C) is shown. The vulva is necessary for copulation and egg laying. In *C. elegans* three of six VPCs divide three times and form a vulva; the other three divide once and fuse with a syncytial epidermis (hyp7; Horvitz and Sternberg, 1991). The same VPCs shown in C have divided only once in a vulvaless L4 larva (D). When all VPCs divide only once and fuse with hyp7, the vulva does not form (vulvaless animal). An adult wild-type hermaphrodite is shown in E (arrows, eggs). An adult sterile hermaphrodite (no eggs) is shown in F. Bars, 100 μm (A and B) and 20 μm (C–F).

experiments. We injected a wild-type *let-23* clone in *let-23(mn23) unc-4/mnC1; dpy-20* animals (*mn23* is a *let-23* genetic null allele that changes C^{700} to W^{700}) and analyzed their progeny. Injection mixes containing 5 ng/μl (F_1 analysis, viability = 24%, n = 67) or 20 ng/μl (F_2 analysis, viability = 76%, n = 177) only partially rescued the lethality associated with *mn23/mn23*. We found that, in this functional assay, a wild-type *let-23* clone at 50 ng/μl mimics wild-type chromosomal gene activity and rescues the defects associated with *let-23(sy17)*, a genetic null allele (Figure 3A). *sy17* is a splice donor mutation predicted to truncate LET-23 in the extracellular domain (Aroian *et al.*, 1994). We and others had previously shown that the same genomic clone does indeed rescue defects associated with other *let-23* null or reduction-of-function alleles (Aroian *et al.*, 1990, 1994; Koga and Ohshima, 1995; Simske and Kim, 1995; Katz *et al.*, 1996; Simske *et al.*, 1996).

We next tested the extent to which a multicopy transgene would reproduce a chromosomal mutation, choosing a mutation that would be the most sensitive. *sy97* is a *let-23* genomic mutation in a splice acceptor site at the ultimate exon, predicted to truncate LET-23 at amino acid 1267 and to add 23 new amino acids (Aroian *et al.*, 1993, 1994). As a result *sy97* eliminates putative SH2 binding sites 6, 7, and 8. No animals homozygous for *sy97* develop a vulva, only 11% are viable, but 95% are fertile. (Figure 6; Aroian and Sternberg, 1991). We created mutant forms of *let-23* that would mimic the *sy97* mutation. First we generated pS97, a *let-23* construct carrying the same G to A splice acceptor mutation as *sy97*. Although mRNA analysis has not shown any wild-type transcripts in *sy97* animals (Aroian *et al.*, 1993), the

possibility exists that splicing nonetheless occurs at low frequency at the mutated 3′ splice site (AA) as it does at other *let-23* introns (Aroian *et al.*, 1993). For this reason, we generated pSTX, a mutant *let-23* clone predicted to eliminate the last 55 amino acids of LET-23 by inserting two consecutive stop codons. We injected pS97 at 20 and 50 ng/μl and pSTX at 20, 50, or 185 ng/μl and found that rescue does not increase when the dose of the transgene is varied ninefold (Table 1). These experiments show that, in the range of doses used, the pS97 and pSTX transgenes confer viability and fertility comparable to the ones observed in the *sy97* genomic mutation. There is an increase in the extent of vulval differentiation, consistent with previous observations that *sy97*, although conferring very little vulval induction on its own, can confer vulval induction in the absence of negative regulators (Huang *et al.*, 1994; Jongeward *et al.*, 1995). Moreover, these observations demonstrate that increasing the amount of *let-23* transgene does not increase the extent of rescue, rendering unlikely the hypothesis that the differential rescue observed is solely due to different stability of the LET-23 protein in different tissues. The decrease of LET-23 function observed when we injected 185 ng/μl pSTX (Table 1), may depend on the different *let-23(null)* background used. Since *mn23* is a point mutation predicted to generate a protein with an amino acid substitution in the extracellular domain of LET-23, it could allow some signaling to occur. We therefore used the *sy17* null allele of *let-23* in the recipient strain for our analysis.

NGros213–13.3 is a genomic clone generated during the molecular cloning of *let-23* (Aroian *et al.*, 1990) and is predicted to be translated into a chimera containing the LET-23 protein up to amino acid 1212 (putative SH2-binding sites 2–8 are eliminated) plus 48 new amino acids translated from in-frame vector sequences (Aroian *et al.*, 1994; Figure 6). The 48 new amino acids contain a putative SH2-binding site, YYNS (Songyang *et al.*, 1993; van der Geer *et al.*, 1996) similar to the LET-23 putative binding sites 6, 7, and 8 (Figures 2 and 6). NGros213–13.3 expressed at high copy number does not rescue sterility but does rescue lethality and vulvaless phenotypes associated with the *let-23(null)* allele *mn23* (Figure 6; see also Aroian *et al.*, 1994). If the phenotypes observed depend on LET-23 stability and if fertility is the function most sensitive to LET-23 activity, then since NGros213–13.3 confers viability and vulval differentiation, it should be able to confer fertility as well. These findings, along with the *sy97* data, suggested that the fertility function resides upstream of the *sy97* mutation (Aroian *et al.*, 1994) and

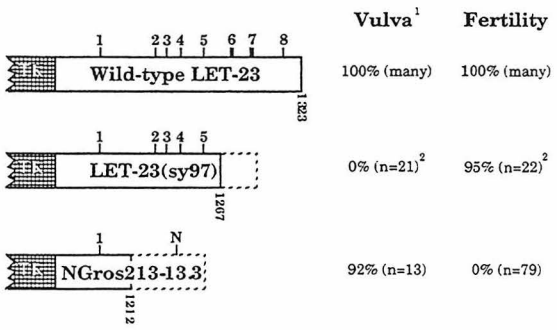

Figure 6. Vulval differentiation and fertility functions conferred by wild-type LET-23, LET-23(sy97), and NGros213–13.3. On the left the carboxyl-terminal end of LET-23 is shown. *let-23(sy97)* is predicted to truncate LET-23 at amino acid 1267. NGros213–13.3 is predicted to truncate LET-23 at amino acid 1212. The area surrounded by a dotted line represents new amino acids (see text). N represents a new putative SH2-binding site (YYNS). [1]The presence of a vulva was determined under Nomarski optics. [2]Data from Aroian and Sternberg (1991).

Table 1. Ability of pS97 and pSTX transgenes to mediate viability, vulval differentiation, and fertility at different doses

let-23 background	Transgene	Dose injected (ng/μl)	Viability (%)	Vulva[a] (%)	Fertility (%)	No. stable lines
mn23 or *sy15*	—	—	0	(0)[b]	(0)[b]	NA
mn23	pS97	20	14 (n = 169)	33 (n = 21)	100 (n = 21)	3
mn23	pS97	50	12 (n = 217)	51 (n = 45)	100 (n = 45)	2
mn23	pSTX	20	20 (n = 475)	32 (n = 91)	100 (n = 91)	7
mn23	pSTX	50	29 (n = 259)	30 (n = 71)	100 (n = 71)	4
sy15	pSTX	185	17 (n = 47)	10 (n = 65)	95 (n = 65)	2
sy97	—	NA	11 (n = 389)	0 (n = 21)	95 (n = 22)	NA

pS97 and pSTX at 20 ng/μl and at 50 ng/μl were injected into *let-23(mn23) unc-4(e120)/mnC1; dpy-20(e1282)* animals. pSTX at 185 ng/μl was injected into *let-23(sy15) unc-4(e120)/mnC1; dpy-20(e1282)* animals. *let-23(sy15)* is a genetic null mutation whose defect has been localized in the extracellular domain of LET-23 (Aroian, 1992).

[a]Animals that laid eggs.

[b]If they survived, based on complementation tests (Aroian and Sternberg, 1991).

NA, not applicable.

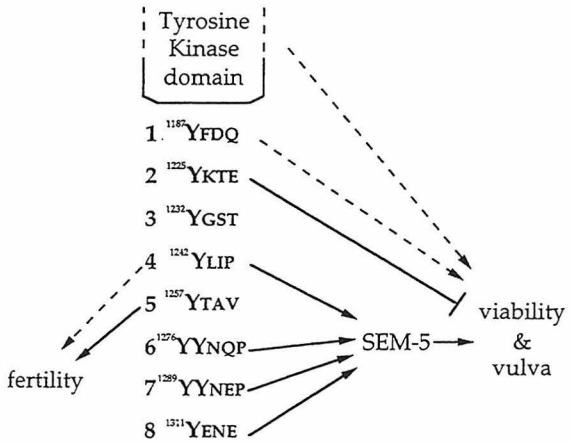

Figure 7. Model of in vivo LET-23-mediated activity for viability, vulval differentiation, and fertility. The eight putative SH2-binding sites in the carboxyl terminus of LET-23 are shown. Sites 1 and 3 have a minor role. Site 4 promotes all three functions. Dotted lines indicate incomplete activation. Site 5 is necessary and sufficient to activate wild-type fertility. Sites 6, 7, and 8 are specific for viability and vulval differentiation. Site 2 has a negative effect on viability and vulval development but not on fertility. Single-letter amino acid code is used. Numbers to the left of the Ys indicate the amino acid number (Aroian *et al.*, 1990). All sites are conserved in *Caenorhabditis vulgaris*, except site 7 (YYNQP) and site 8 (YQNE; Sakai *et al.*, 1996).

that it is not the most sensitive to LET-23 activity. Moreover, they raised the possibility that the putative SH2-binding site YYNS might be sufficient to confer full viability and vulval activity. Therefore, by a systematic functional analysis of all of the carboxyl-terminal LET-23 putative SH2-binding sites, we investigated whether they play a role in mediating LET-23 functions.

For the experiments described below, we set up an assay that makes use of the intact *C. elegans* (Figure 4). We mutated copies of a genomic wild-type *let-23* clone (Figure 3A) and then expressed them in animals lacking LET-23 activity. We then analyzed three phenotypes associated with *let-23* function (Figure 5). Since the phosphotyrosines are essential for SH2 binding, we systematically mutagenized in vitro codons specifying all LET-23 carboxyl-terminal tyrosine residues (Figures 2 and 7), substituting them with phenylalanine codons. The tyrosine to phenylalanine change is expected to prevent SH2 binding (Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990; Fantl *et al.*, 1992). As described by Mello *et al.* (1991), we introduced the engineered *let-23* constructs into nematodes with a *let-23(null)* background and assayed their activity by scoring transgenic animals for viability, vulval differentiation, and fertility (for more details, see Figure 4 and MATERIALS AND METHODS). Each construct was injected at 50 ng/μl, except where noted.

The Carboxyl-Terminal Tyrosines Mediate Most but Not All of LET-23 Activity

To clarify the functional relationship between the activity of LET-23 and the carboxyl-terminal tyrosines, we analyzed a LET-23 protein in which all carboxyl-terminal tyrosines were substituted with phenylalanine residues (Figure 3B). Association between this mutated LET-23 protein and the effectors that normally bind LET-23 via carboxyl-terminal tyrosines should be prevented.

By analyzing transgenic worms expressing this construct, we found that most of LET-23 activity depends on its putative SH2-binding sites (Figure 3B): viability, vulval induction, and sterility are all severely impaired but not completely abolished. This result indicates that these sites are required in vivo for most, but not all, of LET-23-mediated function. Similar results were obtained with a construct-bearing site 3 alone (Figure 3E), suggesting that this site has little or no role in LET-23-mediated activity. Addition of site 1 increases viability slightly but has no other effect (Figure 3C), implying that this site can play only a minor role in mediating LET-23 function. We expect that site 3 and probably site 1 are not phosphorylated.

A Single Site Is Necessary and Sufficient to Confer Wild-Type Fertility

Experiments with *let-23(sy97)* and NGros213–13.3 suggested that the fertility function resides in the carboxyl-terminal portion of LET-23 containing sites 2, 3, 4, and 5 (Figure 6; Aroian and Sternberg, 1991; Aroian *et al.*, 1994). Consistent with these findings, we observed that none of the sites deleted by the *let-23(sy97)* mutation is able to confer fertility (Figure 3, H–J). Under these conditions only two LET-23 carboxyl-terminal sites can mediate fertility: sites 4 and 5 (Figure 3, F and G). A *let-23* construct bearing only site 5 confers full fertility with no apparent increase in viability and vulval differentiation (Figure 3G). Removal of site 5 from an otherwise wild-type *let-23* construct decreases fertility to 15% (Figure 3Q). Site 4 alone is able to confer 14% fertility (Figure 3F). Although removal of site 4 has no significant effect on fertility (Figure 3O), removal of both sites 4 and 5 decreases fertility to approximately 0% (Figure 3N). Thus, site 5 appears to be the major mediator of fertility in the wild-type LET-23 protein.

Three Sites Are Specific for Viability and Vulval Differentiation

We tested the physiological significance of sites 6, 7, and 8 and found that each individually is able to mediate viability and vulval differentiation but not fertility (Figure 3, H–J). For example, site 7 increases viability to 76%, and vulval differentiation to 99% of wild type. Sites 6, 7, and 8 match consensus binding

sites for SEM-5 (YXNX; Songyang *et al.*, 1993; Figures 2 and 7). SEM-5, which is required for viability and vulval differentiation (Clark *et al.*, 1992), is homologous to the adapter protein GRB-2 (Lowenstein *et al.*, 1992) and associates with activated human EGFR (Stern *et al.*, 1993). Interestingly, an interaction between the YYN motif (present in both sites 6 and 7) and GRB-2 has been recently documented in vivo (van der Geer *et al.*, 1996). All of these findings, along with the proposed role of SEM-5 as an adapter protein acting in the RAS-dependent vulva and viability pathways in *C. elegans* (Clark *et al.*, 1992; Katz *et al.*, 1996), suggest that SEM-5 might interact with LET-23 through one or all of sites 6, 7, and 8 and lead to RAS activation.

A Single Site Mediates Negative Regulation

The presence of site 2 in the absence of other carboxyl-terminal sites or in the presence of site 3 results in complete inviability (Figure 3, D and K). This observation suggests that site 2 acts negatively on viability in vivo because the presence of only site 2 leads to 0% viability compared with 11% viability in the absence of all carboxyl-terminal sites.

To clarify the negative effect of site 2, we generated transgenic worms expressing either a construct bearing only site 2 and site 4 or a construct bearing only site 2 and site 6 (Figure 3, L and M). The presence of site 2 significantly decreases both viability and vulval differentiation conferred by site 4 and site 6 but does not affect fertility (Figure 3, compare L and M with F and H; for vulval differentiation, the proportion of animals with less than three VPCs induced versus animals with three VPCs induced was found to be statistically significant, $p < 0.016$ in all cases). Therefore, site 2 acts in a subset of tissues requiring LET-23 and inhibits viability and vulval differentiation, the functions mediated by SEM-5 (Clark *et al.*, 1992) and RAS (Beitel *et al.*, 1990; Han and Sternberg, 1990; Han *et al.*, 1990). Site 4 confers less viability and vulval activity than site 6 (Figure 3, F and H) and it may be more strongly down-regulated by site 2 (Figure 3, L and M). Elimination of site 2 from an otherwise wild-type receptor does not cause any detectable phenotype (Figure 3P).

By analogy to the human EGFR, LET-23 is likely to act as a multimer. This hypothesis is supported by the fact that certain *let-23* mutations display intragenic complementation (Aroian and Sternberg, 1991). Thus, a site might work only in cis or might affect the signaling ability of another polypeptide in the complex. To test whether the negative effect of site 2 is maintained in trans, we constructed transgenic animals that express both a construct bearing only site 2 (100 ng/μl) and a construct bearing only site 4 (20 ng/μl). As a control we constructed transgenic animals that express a construct with no carboxyl-terminal tyrosines (100 ng/μl) along with a construct bearing only site 4 (20 ng/μl). The construct bearing only site 4 in combination with the construct without carboxyl-terminal tyrosines promoted 71% viability (Table 2), but in combination with a construct bearing only site 2 promoted only 31% viability ($p = 0.0051$). We interpret this significant decrease in viability as reflecting the action of site 2 in trans to site 4. Similarly, site 2 in cis to site 4 injected at 50 ng/μl decreased viability from 95% (Figure 3F) to 64% (Figure 3L; $p = 0.0012$).

One Site Is Promiscuous and Essential for Complete Viability

Sites 5, 6, 7, and 8 specifically activate a subset of LET-23 functions. By contrast, site 4 contributes, to different extents, to all functions. In worms expressing a construct bearing only site 4, viability and vulval differentiation are virtually wild-type but fertility is 14% of wild-type (Figure 3F).

Site 4 is not required for fertility but is required for wild-type levels of viability, in the presence of site 2. Site 4 alone, site 6 alone, sites 7 and 8, or sites 6, 7, and 8 together are not able to overcome the negative effect of site 2 and confer complete viability (Figure 3, L–O). Removal of site 4 in the presence site 2 and of all other sites mediating viability and vulval differentiation, decreases survival from 99% to 75% (Figure 3, N and Q).

let-23 Constructs Do Not Overcome the Reduced Activity of SEM-5

LET-23 is very likely to stimulate viability and vulval formation via SEM-5. Loss-of-function mutations of *let-23* and *let-60* are lethal (Herman, 1978; Beitel *et al.*, 1990; Han and Sternberg, 1990; Han *et al.*, 1990; Aroian and Sternberg, 1991). Reduction-of-function mutations of *sem-5* are partially lethal (Clark *et al.*, 1992). Once we found that site 6 and site 4 could stimulate viability and vulval formation, we sought to test whether they do so via SEM-5. To do this we tested the alternative hypothesis by asking whether constructs bearing only either site 6 or site 4 can bypass the requirement for SEM-5. For this test we used the strong reduction-of-function *sem-5(n2019)* allele (Clark *et al.*, 1992). If these clones did overcome *sem-5(n2019)* inviability, we would conclude that multiple copies of *let-23* constructs allow wild-type signaling independently of SEM-5 and thus our results are not physiological. If they did not overcome *sem-5(n2019)* inviability, we would conclude that an overexpressed *let-23* construct still requires SEM-5 to confer its activities.

Briefly, we calculated expected values of viability based on transgene transmission (TF) and penetrance of *sem-5(n2019)* for two hypotheses: that viability stimulated by a *let-23* transgene does or does not bypass a

Table 2. Viability conferred by a construct bearing only site 4 coinjected with a construct bearing only site 2 or a construct bearing no putative SH2-binding sites

Constructs coinjected	Carboxyl-terminal sites	Viability (%)	p	No. stable lines
B	- - - - - - - -			
and	and	71 (n = 135)		2
F	- - - 4 - - - -			
			0.0051	
D	- 2 - - - - - -			
and	and	31 (n = 121)		2
F	- - - 4 - - - -			

Either constructs B and F or constructs D and F along with pMH86 and pBluescriptII were coinjected into *let-23(null) unc-4(e120)/mnC1; dpy-20(e1282)* hermaphrodites and viability was evaluated as described in MATERIALS AND METHODS. Amount of DNA injected: constructs B and D, 100 ng/μl; construct F, 20 ng/μl, pMH86, 15 ng/μl; pBluescriptII, 40 ng/μl.

sem-5 mutant. For example, in the presence of wild-type chromosomal *let-23* (*sy17/+*), with a wild-type *let-23* transgene (Table 3A, construct A) we observed 286 Dpy non-Unc (DnU) animals (since they are Dpy, they do not carry the transgene). From this value we calculated the expected number of non-Dpy non-Unc (nDnU) animals (since they are non-Dpy, they have the transgene) if there is no bypass of *sem-5(n2019)* or nDnU = DnU·(TF/1 − TF) = 286·(38/62) = 175. If there is bypass we would expect nDnU = DnU·(TF/1 −TF)/viability of *sem-5(n2019)* = 175/0.627 = 279. To make the same test in the absence of chromosomal *let-23* (*sy17/sy17*), we counted the non-Dpy Unc (nDU) segregants from the same parents. The *unc-4* mutation is tightly linked to *let-23* and, therefore, essentially all Unc animals are also homozygous for *let-23(sy17)*. Since the nDU will be 1/2 the nDnU, we normalized by a factor of 2. We observed 173 nDnU and, therefore, expected 173/2 = 86 nDU animals. Testing the difference between the observed and the expected number in both experiments, we conclude that the *let-23* transgenes cannot overcome *sem-5(n2019)* inviability in the presence of wild-type chromosomal *let-23* [*let-23(sy17)/let-23(+)*] (Table 3A) or in the absence of wild-type chromosomal *let-23* [*let-23(sy17)/let-23(sy17)*] (Table 3B).

DISCUSSION

In this study, we have used an intact organism to analyze the functional significance of eight putative SH2-docking sites in a *C. elegans* EGFR tyrosine kinase homologue, LET-23. Since we do not yet know the in vivo autophosphorylation sites of LET-23 in the specific cells of interest, we have analyzed transgenic constructs altered in all of their putative SH2-binding sites. We have found that six of eight sites have a physiological role. We have also demonstrated that these putative SH2-binding sites are not equivalent in vivo and can mediate either positive or negative tissue-specific regulation. Our results suggest that RTK tissue specificity in vivo is regulated by at least two independent mechanisms. We propose that tissue-specific effectors and tissue-specific negative regulators act together to allow RTK activity in some cell types and not in others.

Since the transgenes are present in multiple copies, one concern is that their overexpression leads to results that are difficult to interpret. Overexpression of *let-23* does not cause hyperactivation of the vulval induction pathway (Aroian *et al.*, 1994; Simske and Kim, 1995; Katz *et al.*, 1996; Simske *et al.*, 1996). By contrast, overexpression of LIN-3, the ligand for the LET-23 receptor (Katz *et al.*, 1995), or overexpression of *let-60(ras)* (Han and Sternberg, 1990) does produce hyper activation of the vulva pathway. Moreover, our experiments with different doses of pS97 and pSTX, transgenes that mimic the predicted effect of the chromosomal tissue-specific *let-23* mutation *sy97*, show that both *let-23* transgenes have a similar pattern of activities as *let-23(sy97)* (Table 1). *let-23(sy97)* results in no vulval differentiation but has vulval activity in the absence of negative regulation (Huang *et al.*, 1994; Jongeward *et al.*, 1995). The increased vulva activity of the pS97 and pSTX transgenes compared with the *sy97* chromosomal mutation suggests that the vulva is to some extent sensitive to an increase of *let-23* copy number. Because of our control experiments and the internal consistency of our results, we believe that our transgene experiments reflect to a first approximation the physiological roles of the carboxyl-terminal tyrosines of LET-23 and thus provide significant insights on how a member of the EGFR family of RTKs signals in an intact animal. We cannot rule out that some of the effects observed in transgenic lines would not appear in the corresponding gene-replacement experiments.

Table 3. *let-23* transgenes do not overcome reduced SEM-5 activity

A. *let-23(sy17)/let-23*(+) background[a]

Construct	Carboxyl-terminal sites	TF[b] (%)	No. of DnU observed	No. of nDnU expected with hyp1[c]	No. of nDnU expected with hyp2[d]	No. of nDnU observed	P_{hyp1}[e]	P_{hyp2}[f]
A	1 2 3 4 5 6 7 8	38.0 (n = 166)	286	175	279	173	0.9459	0.0002
F	- - - 4 - - - -	49.0 (n = 104)	135	130	207	100	0.1513	<0.0001
H	- - - - - 6 - -	44.8 (n = 116)	356	289	461	285	0.9108	<0.0001

B. *let-23(sy17)/let-23(sy17)* background[g]

Construct	Carboxyl-terminal sites	No. of nDnU observed	No. of nDu expected with hyp3[h]	No. of nDu expected with hyp4[i]	No. of nDU observed	P_{hyp3}[j]	P_{hyp4}[k]
A	1 2 3 4 5 6 7 8	173	87	138	86	1.0000	0.0076
F	- - - 4 - - - -	70	35	56	14	0.0119	<0.0001
H	- - - - - 6 - -	297	149	237	162	0.5757	0.0043

Viability conferred in a *sem-5* reduction-of-function background [*sem-5(n2019)*] by *let-23* constructs bearing all carboxyl-terminal sites or site 4 only or site 6 only. (*A*) Viability in a *let-23(sy17)/let-23*(÷); *sem-5(n2019)* background. (*B*) Viability in a *let-23(sy17)/let-23(sy17); sem-5(n2019)* background. We first determined both the lethality caused by *sem-5(n2019)* as well as the TF of each array. If a given transgene does not overcome the reduced activity of *sem-5(n2019)* to mediate its effect on viability, the TF of the transgene should be the same in both *sem-5*(+) and *sem-5(n2019)* backgrounds. Conversely, if a transgene at least partially overcomes *sem-5(n2019)* inviability, transgenic animals in a *sem-5(n2019)* background should be more viable than nontransgenic siblings. Thus, the TF of such a transgene should be higher. We performed similar analysis to test whether three *let-23* transgenes could overcome the reduced SEM-5 activity in a *let-23(sy17)/let-23*(+) background (*A*). Then we carried out the same analysis in a *let-23(sy17)* background (Unc animals, *B*). Statistical analysis was used to compare the number of animals observed with the number of animals expected in case of complete SEM-5 dependence of the transgene or in case of complete SEM-5 independence of the transgene. The fact that with construct F we observed significantly less than expected viability in a *let-23(sy17)* background (*B*) is consistent with our observation (Fig. 3F) that this construct has less activity than wild-type LET-23.

[a]The genotype is *let-23(sy17)/let-23*(+); *dpy-20(e1282); sem-5(n2019); Ex*.

[b]Transmission frequency of the transgene (TF) is the fraction of progeny that carries the transgene and equals nDnU/(nDnU + DnU), where nDnU are non-Dpy non-Unc animals and DnU are Dpy non-Unc animals. Dpy animals have lost the transgene; non-Dpy animals carry the transgene.

[c]nDnU expected if there is no SEM-5 bypass (hypothesis 1) = DnU·[TF/(1−TF)].

[d]nDnU expected if there is SEM-5 bypass (hypothesis 2) = DnU·[TF/(1−TF)]/viability of *sem-5(n2019)* = DnU·[TF/(1−TF)]/0.627.

[e]p obtained by comparing the number of nDnU expected if there is no SEM-5 bypass (hypothesis 1) with the nDnU observed.

[f]p obtained by comparing the number of nDnU expected if there is SEM-5 bypass (hypothesis 2) with the nDnU observed.

[g]The genotype is *let-23(sy17)/let-23(sy17); dpy-20(e1282); sem-5(n2019); Ex*.

[h]Number of non-Dpy Unc animals (nDU) expected if there is no SEM-5 bypass (hypothesis 3). By Mendelian segregation nDU are expected to be one-half of nDnU.

[i]Number of nDU expected if there is SEM-5 bypass (hypothesis 4) = (nDnU/2)/viability of *sem-5(n2019)* = (nDnU/2)/0.627.

[j]p obtained by comparing the number of nDU expected if there is no SEM-5 bypass (hypothesis 3) with the nDU observed.

[k]p obtained by comparing the number of nDU expected if there is SEM-5 bypass (hypothesis 4) with the nDU observed.

Most of LET-23 Activity Depends on Carboxyl-Terminal Tyrosines

We have shown that a LET-23 construct with no carboxyl-terminal tyrosines has less than 10% of wild-type LET-23 activity. Some of these tyrosines define sites that, in other experimental systems, have been shown to interact with SH2 domains (Songyang *et al.*, 1993). These findings, combined with the data presented herein, suggest that downstream targets of LET-23 interact with its putative SH2-binding sites and that the interaction is required for LET-23-mediated functions. Since we do not have direct biochemical evidence for phosphorylation or binding of proteins, other models are possible. The residual activity

observed when all sites are mutated may reflect the existence of effectors activated independently of direct SH2 association or the fact that tyrosines in other domains of the receptor can also be used for signaling (Decker, 1993; Soler *et al.*, 1993; Gotoh *et al.*, 1994; Li *et al.*, 1994).

Viability and Vulval Differentiation

LET-23 tyrosine sites 6, 7, and 8 specifically mediate viability and vulval differentiation and match consensus binding sites for SEM-5 (YXNX; Songyang *et al.*, 1993; Figures 2 and 7). Mutant animals with very low SEM-5 activity show viability and vulval defects but are not sterile (Clark *et al.*, 1992), suggesting that SEM-5 is not required for fertility. SEM-5 is structurally and functionally homologous to the RAS activator GRB-2 (Lowenstein *et al.*, 1992; Egan *et al.*, 1993) and can associate with activated human EGFR (Stern *et al.*, 1993). Moreover, it has recently been shown that the YYN motif when phosphorylated at both tyrosines has a strong affinity for GRB-2 in vivo (van der Geer *et al.*, 1996). In addition, site 6 does not overcome the inviability associated with a reduction of SEM-5 activity. Therefore, it seems likely that SEM-5 binds to LET-23 through site 6, and probably through sites 7 and 8, and leads to RAS activation.

Site 4 is different from sites 6, 7, and 8 in that it is able to confer fertility as well as viability and vulval differentiation. Site 4-mediated viability is SEM-5 dependent, suggesting that site 4 also leads to RAS activation and might bind SEM-5. We cannot rule out the possibility that site 4 can bind SEM-5 only in the absence of sites 6, 7, and 8. Moreover, site 4 does not match the consensus for SEM-5 binding and might interact with another adapter to mediate its functions.

Fertility

Only two sites appear to mediate fertility: site 5 and site 4. Since removal of site 4 has no significant effect on fertility and since removal of both sites 4 and 5 results in almost complete sterility, we conclude that site 4 is not required when site 5 is present but can partially compensate for the absence of site 5. Therefore, site 5 is necessary and sufficient to activate fertility. Site 4 could confer some fertility by interacting at low efficiency with the protein(s) that normally binds to site 5.

Since sites 6, 7, and 8 appear to activate RAS to mediate viability and vulval differentiation, our results suggest that the pathway branches at the level of the receptor and that the fertility pathway activated by LET-23 through site 5 employs a distinct set of effectors (Figure 7; Jongeward *et al.*, 1995). Indeed, genes involved in LET-23-mediated fertility but not in viability and vulval induction have been identified (Clandinin and Sternberg, unpublished observation).

Since site 5 has the most specific effect, it is possible that the fertility function is the most sensitive to a reduction of *let-23* activity. However, data from extensive complementation analysis (Aroian and Sternberg, 1991) are not consistent with this possibility. Indeed, the *let-23(sy1)* mutation is the most specific, affecting only vulva formation.

Negative Regulation

We have shown that LET-23 site 2 specifically inhibits viability and vulval differentiation, two RAS-dependent functions. There are a few other examples of tyrosine sites mediating negative regulation in RTKs. Valius *et al.* (1995) have shown that a GAP-binding site in PDGFR-β down-regulates PLC-γ-mediated activity. Cleghon *et al.* (1996) have found that a tyrosine site in the *Drosophila* PDGFR homologue Torso inhibits signaling. Weidner *et al.* (1995) have demonstrated that the Met receptor carries a tyrosine with a negative role in its juxtamembrane domain.

The fact that viability and vulval differentiation (that are approximately 100%) are decreased whereas fertility (which is 14%) is not (Figure 3, compare F and H with L and M) argues against a general destabilizing effect of site 2 on the LET-23 protein. If this were the case, we would expect a decrease in the fertility function as well. Moreover, site 2 can negatively act on site 4-mediated viability in trans as well as in cis (Table 2). Thus, these results suggest that site 2 interacts with effectors leading to activation of tissue-specific negative regulation.

Four sites (sites 4, 6, 7, and 8) are required to overcome the negative effect of site 2 and confer wild-type viability. This observation suggests that there is no redundancy for the viability function and that sites 4, 6, 7, and 8 might all be used in vivo. For vulval induction, instead, a subset of the sites 4, 6, 7, and 8 seems to be sufficient to confer full vulval induction, suggesting that they are functionally redundant. However, this difference could of course be due to the apparent sensitivity of vulval differentiation to overexpression of *let-23* constructs.

We have also found that elimination of site 2 does not result in inappropriate RAS pathway activation. Why does elimination of site 2 produce no detectable phenotype? Partially redundant negative regulators of LET-23 have been genetically characterized and inactivation of at least two of them is required to generate a visible phenotype (Ferguson and Horvitz, 1989; Lee *et al.*, 1994; Jongeward *et al.*, 1995; Yoon *et al.*, 1995). Therefore, it is likely that there is a threshold of detectability for loss of negative regulation of LET-23. Site 2 could mediate the function of one such negative regulator.

We have shown that specific sites can activate different pathways to produce distinct functions. We

have also provided evidence of negative regulation specific for two RAS-mediated functions. Our findings, therefore, suggest that RTK tissue specificity is regulated in at least two ways in vivo. First, specific SH2-binding sites could activate effectors present only in particular cell types. Second, tissue-specific negative regulators could be used to modulate RTK activity in some tissues but not in others. A combination of these mechanisms would allow fine control of tissue-specific pathway activation.

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Appendix to Chapter III

Additional *let-23* constructs

Table 1. Ability of LET-23 C-terminal sites 4 (YLIP) and 5 (YTAV) to mediate viability, vulval differentiation, and fertility in the absence or in the presence of the negatively acting site number 2 (YKTE). The transgene was injected into the strain: *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282)*. Constructs were injected at 50 ng/µl with 15 ng/µl of pMH86 (*dpy-20(+)* DNA) and 110 ng/µl of pBluescript (carrier).

Construct	C-term. sites	Stable Line(s)	Viability	Vulval ind.	Fertility	Semi-fertility
A. pk7GL22	45	5.1 6.1 12.3	94.1% (n=102) 93.3% (n=120) 88.6% (n=79)	N.D. 62.7% (n=21) 68.9% (n=22)	95.7% (n=23) 76.9% (n=39) 96.0% (n=25)	0% (n=23) 7.7% (n=39) 0% (n=25)
B. pk7GL26	2 45	14.2 14.3	14.7% (n=177) 22.4% (n=98)	80.8% (n=20) 85.6% (n=22)	93.8% (n=48) 85.0% (n=20)	0% (n=48) 5.0% (n=20)

Table 2. Ability of genes driven by the *sem-5* promoter to mediate viability, vulval differentiation, and fertility. *let-23::egl-15* is predicted to encode a chimera in which the extracellular and the transmembrane portion are derived from l*et-23* sequences and the intracellular portion is derived from *egl-15*. *egl-15* encodes a *C. elegans* Fibroblast Growth Factor receptor homolog (Devore *et al.*, 1995). The transgene was injected into the strain: *let-23(sy17) unc-4(e120)/mnC1; dpy-20(e1282)*. Constructs were injected at 50 ng/µl with 15 ng/µl of pMH86 (*dpy-20(+)* DNA) and 110 ng/µl of pBluescript (carrier).

Construct	*sem-5* driven	Stable Line(s)	Viability	Vulval ind.	Fertility	Semi-fertility
A. pk7GL34	*let-23*		38% (n=100) (F1 data)	27.8% (n=18) bagged	66.7% (n=24)	N.D.
B. NH193	*let-23::egl-15*	6.1 8.1 9.1	96.7% (n=120) 57.1% (n=21) 103% (n=85)	11.1% (n=6) N.D. N.D.	0% (n=15) 0% (n=6) 0% (n=11)	0% (n=15) 0% (n=6) 9.1% (n=11)

Table 3. Ability of LET-23 C-terminal tyrosines to mediate viability, vulval differentiation, and fertility in different genetic backgrounds. *sli-1(lf)* background is: *let-23(sy17)unc-4(e120)/mnC1; dpy-20(e1282); sli-1(sy143). sem-5(rf)* background is: *let-23(sy17)unc-4(e120)/mnC1; dpy-20(e1282); sem-5(n2019)*. Constructs were injected at 50 ng/µl with 15 ng/µl of pMH86 (*dpy-20(+)* DNA) and 110 ng/µl of pBluescript (carrier).

Construct	C-term. sites	Background	Stable Line(s)	Viability	Vulval ind.	Fertility	Semi-fertility
A. pk7RL	none	*sli-1(lf)*	6.1 11.1	3.6% (n=110) 10.8% (n=37)	N.D. N.D.	0% (n=2) 0% (n=9)	0% (n=2) 0% (n=9)
B. pk7GL17	2	*sli-1(lf)*	3.1 10.1	15.8% (n=76) 31.0% (n=84)	66.7% (n=1) N.D.	0% (n=4) 0% (n=12)	0% (n=4) 0% (n=12)
C. pk7GL15	4	*sli-1(lf)*	several		93.8% (n=8)		
D. pk7GL20	24	*sli-1(lf)*	1.1 3.1 8.2 15.1 17.1	59.5% (n=74) 92.7% (n=110) 116.7% (n=36) 41.5% (n=188) 65.0% (n=80)	N.D. N.D. N.D. N.D. N.D.	25.0% (n=4) 16.7% (n=6) N.D. N.D. 33.3% (n=6)	N.D. N.D. N.D. N.D. N.D.
E. pk7GL24	5	*sli-1(lf)*	10.2 10.3 10.4	8.3% (n=24) 13.9% (n=43) 10.5% (n=19)	58.3% (n=2) 41.7% (n=4) N.D.	91.9% (n=37) (all lines)	2.7% (n=37) (all lines)
F. pk7STX	6 7 8 deleted	*sli-1(lf)*	7.1 8.1 9.1	50.7% (n=75) (F1 data)	N.D. N.D. N.D.	95.2% (n=21) 100% (n=19) 87.5% (n=8)	N.D. N.D. N.D.
G. pk7GL23	6	*sem-5(rf)*	M5 N6	113.7% (n=95) 107.0% (n=202)	N.D. N.D.	60.0% (n=10) 90.9% (n=11)	20.0% (n=10) 9.1% (n=11)

Reference

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Chapter IV

A screen for genes involved in *let-23*-mediated fertility

Introduction

let-23 was originally identified because it is required for viability (Herman, 1978) and vulval development (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987; Aroian and Sternberg 1991). It is now clear that *let-23* activates both these functions via the Ras pathway. Indeed, double mutant animals carrying genetic null mutations of *let-23* and gain-of-function (gf) mutations of *let-60ras* or loss-of-function (lf) mutations of *lin-1* are viable and Multivulva (Muv) (Han *et al.*, 1990; Clark *et al.*, 1992; Sternberg, 1993). Reduction-of-function mutations of *let-23* result in at least three other defects: hermaphrodites are sterile; the neuroectoblast P12 is not correctly specified and appears to follow the fate of the neighboring cell P11; the male spicules do not develop correctly, become crumpled and, as a result, males can not mate with hermaphrodites. Genetic studies suggest that also P12 specification and spicule development are mediated through the Ras pathway (Aroian and Sternberg, 1991; Han *et al.*, 1993; Chamberlin and Sternberg, 1994; L. Jiang and P.W. Sternberg, in preparation).

Several lines of evidence indicate that *let-23* mediates fertility using a pathway distinct from Ras. First, *let-23(null); let-60(gf)* and *let-23(null); lin-1(lf)* double mutants are viable, Muv, and sterile (Han *et al.*, 1990). Thus, activated *let-60* rescues the viability and vulva defects associated with *let-23* null mutations, but not fertility. Second, the negative regulator *sli-1* specifically inhibits the Ras pathway (Jongeward *et al.*, 1995; Yoon *et al.*, 1995; Yoon, 1997). Third, distinct LET-23 domains are used to mediate either fertility or viability and vulval development (see Chapters II and III). Fourth, 2 loci, *lfe-1* and *lfe-2* were identified (*lfe* stands for *l*et-23 *f*ertility *e*ffector), acting selectively on the *let-23*-mediated fertility

(Clandinin, 1997). Mutations in these two loci suppress the sterility of *lin-3* and *let-23* alleles in addition to that of *let-23(null); let-60(gf)* and *let-23(null); lin-1(lf)* double mutants. Thus, at least two pathways originate from the LET-23 EGFR tyrosine kinase: one required for the Ras-mediated functions; the other required for hermaphrodite fertility (Fig. 1).

For the fertility function, *let-23* activity appears to be required at the level of the proximal hermaphrodite gonad. In wild-type animals, oocytes mature while they are moving along the gonadal arm. They are completely mature when they arrive at the proximal gonad, which is adjacent to the spermatheca. One oocyte at a time matures in each gonad arm and after nuclear envelope breakdown it is ready for ovulation. At ovulation, the mature oocyte exits the proximal gonad and enters into the spermatheca, where it is fertilized. Contractions of the proximal gonad and probably dilation of the spermathecal valve are determinant for the relocation of the oocyte. Contractions are generated by the sheath cells, six myoepithelial cells surrounding the proximal gonad. In animals carrying mutated *let-23*, exit of the oocytes from the gonad into the spermatheca is blocked. The nature of this block is unclear. It could be a defect in the contractions pushing the oocytes or a defect in the dilation of the spermathecal valve, or both (J. McCarter and T. Schedl, personal communication). As a result, in *let-23* defective worms, the mature oocyte sits in the proximal gonad and replicated its DNA endomitotically (endo mitotic oocyte or Emo phenotype, Fig. 2). Note that the sterility defect induced by *let-23* alleles is different from that observed in *let-60* mutations (Church *et al.*, 1995). *let-60* affects oocyte maturation and, in particular, exit from pachytene. Therefore, *let-60* is required at an earlier stage than *let-23*.

My molecular genetic analysis of *let-23* C-terminal mutants indicates that the LET-23 protein uses a putative SH2 binding site to mediate fertility (Chapter III) suggesting that protein(s) binding at this site are mediators required for *let-23* fertility function. We were, therefore, interested in characterizing further the *let-23* fertility pathway, hoping to find proteins that interact with the LET-23 C-terminus. To this end, Tom Clandinin and I designed a genetic screen demanding sterile mutations suppressed by *lfe-2* alleles (Fig. 3). With this screen we should identify genes acting positively in the *let-23* fertility pathway (like *lin-3* or *let-23*) and acting upstream of *lfe-2*. We do not expect to find many *lin-3* or *let-23* alleles because tissue specific mutations of these genes are rare (2 out of 100,000 gametes screened for *let-23* (Ferguson and Horvitz, 1985; Han *et al.*, 1990; J. Liu, personal communication).

Materials and methods

Methods of culturing, handling, and genetic manipulation of *C. elegans* were as described in (Brenner, 1974). Strains were grown at 20°C.

Strains

LGI: *unc-38(x20) lfe-2(sy326)* (Clandinin, 1997), *dpy-5(e61) unc-15(e73)*, *bli-4(e937) unc-13(e51)*, *dpy-14(e188ts) unc-13(e51)*, *dpy-14(e188ts) unc-29(e1072)*, *dpy-5(e61) unc-101(m1)*, *daf-8(e1393) unc-29(e1072)*, *unc-13(e1091) lin-11(n566)*, *unc-29(e1072) unc-75(e950) aex-6(sa24)*, *unc-29(e1072) lin-11(n566)*, *lin-11(n566) unc-75(e950) aex-6(sa24)*, *unc-38(x20) lfe-2(sy326) dpy-5(e61) lin-10(e1439) sy499*, *dpy-5(e61) lin-10(e1439) sy499/dpy-14(e188ts) unc-13(e51)*,

dpy-5(e61) lin-10(e1439) sy499/dpy-5(e61) unc-15(e73), bli-4(e937) lin-10(e1439) sy499, hDf9/szT1. hDf9 deletes about 0.5 map units including *let-82*.

LGIV: *lfe-1(sy290) unc-24(e138)* and *lfe-1(sy290) dpy-20(e1282)* (Clandinin, 1997).

LGI and LGIII: *unc-13(e1091) sy499/hT2(dpy-18); +/hT2(bli-4)*.

LGI and LGIV: *dpy-5(e61) lin-10(e1439) sy499; lfe-1(sy290) unc-24(e138). unc-13(e1091) sy499; lfe-1(sy290) dpy-20(e1282)*.

LGI and LGX: *dpy-5(e61) unc-13(e450) hDf9/szT1[lon-2(e678)]; +/szT1*.

Transgenic strains: *unc-38(x20) lfe-2(sy326) lin-10(e1439); syEx146* (PS2474). *unc-38(x20) lfe-2(sy326) lin-10(e1439); dpy-20(e1282); syEx223* (PS2712). *syEx146* is an extra chromosomal array containing a cosmid with *lfe-2(+)* DNA (100 ng/µl) and the plasmid pRF4 (rol-6^{D}, 50 ng/µl) (Clandinin, 1997). *syEx223* contains a cosmid with *lfe-2(+)* DNA (76 ng/µl) and the plasmid pMH86 [*dpy-20(+)* DNA, 15 ng/µl].

Mutagenesis

PS2474 was mutagenized with 50mM EMS for 4 hours, as described in Brenner (1974). F1 Unc Roller hermaphrodites were cloned and allowed to self fertilize. three-five days later, these plates were observed under the dissecting microscope. Plates containing sterile Unc Roller hermaphrodites but not sterile Unc non-Rollers were kept. From each of these plates, ~24 Unc Rollers and ~24 Unc non-Rollers were cloned. If ~1/4 of the Unc Rollers but none (or << 1/4) of the Unc non-Rollers were sterile, the plates were kept. At the same time, F2 Unc non-Roller hermaphrodites were used to map a mutation to a specific linkage group. At this time it was determined that *sy499* was linked to *unc-38*, on LGI.

Construction of *sy499* doubles with *lfe-1* or *lfe-2*

Construction of *unc-38 lfe-2 lin-10 sy499*. From *unc-38 lfe-2/bli-4 lin-10 sy499* heterozygous, we picked individually ~200 Unc-38 hermaphrodites and looked for plates that segregated Unc and egg-laying defective (bag) animals. We found three plates that segregated bags.

Construction of *dpy-5 lin-10 sy499; lfe-1 unc-24*. N2 (wild-type) males were mated into *dpy-5 lin-10 sy499/dpy-14 unc-13* hermaphrodites. Males cross progeny were mated into *lfe-1 unc-24* hermaphrodites and cross progeny was cloned. Four independent plates segregated Dpy Unc animals. These animals were individually picked and scored for fertility.

Construction of *unc13 sy499; lfe-1 dpy-20*. N2 males were mated into *unc-13 sy499/hT2; +/hT2* hermaphroodites. Males from this cross were mated into *lfe-1 dpy-20* hermaphrodites and cross progeny were individually picked. Five independent plates were found that segregated Unc Dpy animals. These animals were individually picked and scored for fertility.

Mapping

dpy-5 was linked to *sy499* as follows. *dpy-5 unc-15/ + +* males were crossed with *unc-38 lfe-2 lin-10 sy499* hermaphrodites and Dpy non Unc recombinant animals were picked individually (genotype *dpy-5 lin-10 sy499/dpy-5 unc-15*). We mapped by crossing *dpy-5 lin-10 sy499/dpy-5 unc-15* or *bli-4 lin-10 sy499/bli-4unc-13* hermaphrodites with males of the type m_1m_2/+ + (where m_1m_2 are two linked mutations on chromosome I). Strains of the type *dpy-5 lin-10 sy499/*m_1m_2 or *bli-4 lin-10 sy499/*m_1m_2 were generated. M_1 non M_2 and M_2 non M_1 animals were cloned and checked for segregation of sterile progeny. Mapping was performed with the following

m_1m_2 strains: *dpy-5 unc-15*, *bli-4 unc-13*, *dpy-14 unc-29*, *dpy-5 unc-101*, *daf-8 unc-29*, *unc-13 lin-11*, *unc-29 unc-75 aex-6*, *unc-29 lin-11*, *lin-11 unc-75 aex-6*, and with the deficiency *hDf9*.

Scoring the sterile phenotype

unc-13 sy499 or *bli-4 lin-10 sy499* hermaphrodites were placed live on a 5% Noble agar pad and observed with a 100X Plan Nomarski objective for sterility defects. Videos of oocyte ovulation and exit from the spermatheca were also recorded from N2 and *sy499* hermaphrodites, to identify the *sy499* defects. Worms were anesthetized for 45 minutes with 0.1% tricaine and 0.01% tetramisole in M9 buffer (Kirby *et al.*, 1990; Iwasaki *et al.*, 1996; Sigma Chemical Co., St. Louis, MO). Tricaine/tetramisole paralyzes the body muscles, but it allows oocyte maturation and ovulation. Hermaphrodites were viewed with a Nomarski microscope (Axioplan; Carl Zeiss, Oberkochen, West Germany) on a 5% Noble agar pad. The microscope was connected to a video camera (CCD72; Dage MTI, Michigan City, IN) and to a VHS video recorder (SLV-440; Sony Corp., Park Ridge, NJ). Hermaphrodites were followed for up to three ovulation (~ 3 hours). Experiments were carried out at 20°C.

Measuring survival

Survival of *sy499* homozygous worms coming from heterozygous mothers was measured as follows. *unc-13 sy499*/*dpy-24 unc-75* worms were cloned and all Unc, Dpy Unc, and non-Unc non-Dpy animals were counted. If viability is 100%, then the number of Unc worms (x) will be half the number of non-Unc non-Dpy worms (y). Therefore, % viability = $2x \cdot 100/y$.

Measuring vulval induction

In wild type hermaphrodites, three of six equipotential VPCs undergo vulval fates (Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986). *unc-13 sy499* hermaphrodites at L3 molt were viewed under Nomarski optics and scored as described in Chapter III.

Scoring the lethal phenotype

15-20 *unc-13 sy499* hermaphrodites were placed on a plate and allowed to lay eggs for ~2 hours. Hermaphrodites were then removed and eggs were scored ~10-12 hours later. They were suspended in M9 (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$), placed on a 5% Noble agar pad, and observed for 3-5 hours with a Plan 100X Nomarski objective.

Results

We performed a screen to isolate new genes involved in the *let-23* fertility pathway and acting upstream of *lfe-2* (Fig. 3). We screened about 8,000 gametes and found one candidate, *sy499*.

Approach

The screen shown in Fig. 3 is designed to identify recessive sterile mutations which are suppressed by *lfe-2(lf)* alleles. Therefore, we need to distinguish animals homozygous for our sterile mutation (phenotype: sterile) from animals homozygous for both, our sterile mutation and *lfe-2(lf)* (phenotype: fertile). To this end we constructed the strain PS2474: *unc-38 lfe-2 lin-10; syEx146*. *unc-38* is used as a marker to follow *lfe-2*. To

distinguish more easily between sterile and fertile animals, we used a vulvaless mutation (*lin-10*); progeny will remain inside their mother, and therefore fertile animals will form a bag of worms, while sterile animals will not. The extra chromosomal array *syEx146* contains wild-type copies of *lfe-2* DNA and a gene conferring a dominant Roller phenotype. Thus, a worm carrying the transgene will be phenotypically Roller. In addition, since transgenic worms carry *lfe-2(+)* DNA, any mutation in the *let-23* fertility pathway will not be suppressed, i.e., the worms will be sterile. For example, *unc-38 lfe-2 lin-10; let-23; syEx146* would be Roller and sterile. Conversely, the same strain without the extra chromosomal array is non-Roller and fertile. Since extra chromosomal arrays are not passed to all progeny, a Roller fertile worm will generate both Roller and non-Roller progeny (usually about 1/2 each).

Phenotypically Roller PS2474 mothers were EMS mutagenized and F1 Roller hermaphrodites were picked individually to Petri dishes. There are three possible scenarios:

1) F1 Rollers do not carry any mutation in genes affecting fertility and will produce no sterile progeny.

2) F1 Rollers are heterozygous for mutations that affect fertility differently from genes in the *let-23* fertility pathway (general sterile mutation). For example, many mutagenized worms carry mutations that do not allow proper development of the gonad and 1/4 of their progeny are sterile. These kinds of mutations are not suppressed by *lfe-2(lf)* mutations and, therefore, F1 worms carrying a general sterile mutation segregate fertile Rollers, fertile non-Rollers, sterile Rollers, and sterile non-Rollers.

3) F1 Rollers are heterozygous for a mutation in one of the genes mediating *let-23* fertility. These worms will segregate sterile Rollers, fertile Rollers and fertile non-Rollers. Therefore, we looked for plates that segregated some sterile Roller progeny, but no sterile non-Rollers.

Since we have found that the Roller phenotype conferred by the transgene is not completely penetrant, we have more recently mutagenized the strain *unc-38 lfe-2 lin-10; dpy-20; syEx223* (PS2712). *syEx223* contains *lfe-2(+)* and *dpy-20(+)* DNAs. Thus, worms expressing the transgene will be non-Dpy. Apart from using rescue of the Dpy phenotype as the transformation marker instead of rolling, the screen is performed as above.

Mapping

dpy-5 was linked to *sy499* by placing *unc-38 lfe-2 lin-10 sy499* in trans to *dpy-5 unc-15* and picking Dpy non-Unc animals (Table 1 A). Mapping was performed placing *dpy-5 lin-10 sy499* in trans to the m_1m_2 double mutant chromosomes and picking M_1 non-M_2 and M_2 non-M_1 animals (Table 1 B). The data reported on Table 1 indicate that *sy499* maps on chromosome I, close to *lin-11*.

***sy499* phenotypes**

sy499 was identified as a sterile mutation suppressed by a *lfe-2* allele. A more careful analysis has indicated that *sy499* displays two phenotypes, an incompletely penetrant sterile phenotype and an embryonic lethal phenotype. At the plate level, most of the hermaphrodites homozygous for *sy499* lay eggs which never hatch. *sy499* is not a temperature sensitive mutation, since it confers a phenotype at 15°C, at 20°C, and at 25°C.

Spermathecal phenotype. About 12% (n=16) of the *sy499* animals have Emo oocytes (Fig. 4 and Table 2). The *sy499* Emo phenotype is very similar to that displayed by *let-23(sy10)* (compare Fig. 2 C with Fig. 4 F) and by *lin-3(n1058)* [not shown; Clandinin (1997) and J. McCarter and T. Schedl personal communication]. Video analysis showed that in these animals oocytes mature normally moving towards the proximal gonad but fail to enter the spermatheca and therefore can not be fertilized. Mature oocytes undergo multiple rounds of endomitotic DNA replication and become polyploid (Emo phenotype, Fig. 4 F) In *sy499* hermaphrodites that do not display the Emo phenotype mature oocytes can relocate into the spermatheca and can be fertilized. Preliminary observations indicate that in these animals the oocyte transfer from the proximal gonad into the spermatheca takes about twice as long when compared to wild-type animals (data not shown). Moreover, portions of the oocyte yolk appear to remain trapped into the spermatheca. It is not clear yet if these defects are responsible for any functional consequence.

Lethal phenotype. *sy499* animals lay eggs that, at an early stage, are indistinguishable from wild-type eggs. However, *sy499* eggs usually do not hatch. Those that hatch (~10%) only yield dead larvae (Fig. 4 B). The penetrance of the embryonic lethal phenotype is 100% (Table 2). It appears, therefore, that in addition to the Emo phenotype *sy499* displays a fully penetrant lethal phenotype. On the other hand, *sy499* homozygous hermaphrodites coming from heterozygous mothers do not show any lethality (Table 2). Thus, *sy499* shows maternal rescue. The lethal phenotype caused by *sy499* animals is different from the one observed in

animals homozygous for *let-23(lf)* mutations. Animals with defective *let-23* die as young L1 larvae (Aroian *et al.*, 1991).

sy499 does not have a vulval phenotype. To test if *sy499* is involved in vulval induction, we scored *unc-13 sy499* hermaphrodites for vulval induction. All 18 animals examined had wild-type vulva with 3 VPCs induced (Fig. 4 C). This result suggests that *sy499* does not affect vulval induction.

lfe-1 and _lfe-2_ mutations partially suppress _sy499_ phenotypes

Preliminary observations suggest that both *lfe-1* and *lfe-2* partially suppress *sy499* phenotypes. We have tested lethality in the *unc-38 lfe-2 dpy-5 lin-10 sy499* strain and found that these worms segregate ~19% (n=188) dead eggs in comparison to 100% dead eggs of the *sy499* homozygous animals (Table 2).

lfe-1 partially suppresses *sy499* lethality: only 13% (n=144) of *dpy-5 lin-10 sy499; lfe-1 unc-24* do not segregate any viable progeny. It has not been possible to deconstruct this strain probably because the vulvaless *lin-10* phenotype is enhanced in this genetic background. When we used *unc-13* as a marker of *sy499* instead of *dpy-5* and *lin-10*, we could not detect any *lfe-1* suppression of *sy499* lethality. *unc-13* encodes a protein with homology to the Protein Kinase C diacylglycerol binding domains C1 and C2 (Maruyama and Brenner, 1991; Ahmed *et al.*, 1992) and is likely involved in calcium signaling. Therefore, we believe that this failure to suppress is possibly due to interactions between *unc-13* and *lfe-1* or *sy499* or both.

Discussion

We have performed a genetic screen for sterile mutations suppressed by a *lfe-2* allele. From the ~8,000 gametes screened we have isolated one candidate mutation, *sy499*. *sy499* displays two phenotypes: an incompletely penetrant sterile Emo phenotype (similar to that of *lin-3* and *let-23* reduction of function mutations) and a fully penetrant maternal rescued late embryonic lethal phenotype. *sy499* is partially suppressed by mutations in both the *lfe-1* and the *lfe-2* genes. *lfe-1* and *lfe-2* have been cloned and found to encode an inositol(1,4,5)trisphosphate receptor (IP_3R) and D-myo-inositol(1,4,5)trisphosphate 3-kinase (IP3K), respectively (Clandinin, 1997). Both these proteins are involved in calcium signaling. IP3K converts inositol(1,4,5) trisphosphate (IP_3) to inositol(1,3,4,5) tetraphosphate (IP_4). The role of IP_4 is unclear (Berridge, 1993). When IP_3 binds to its receptor, it causes the release of calcium from intracellular stores (Ferris and Snyder, 1992; Berridge, 1993). Therefore, this screen should identify additional genes involved in the regulation of intracellular calcium by a receptor tyrosine kinase (RTK).

Tom Clandinin (1997) has found that *lfe-1(sy290)* suppresses the sterility associated with loss-of-function mutations of *lin-3* and *let-23*. There is genetic evidence that *sy290* is a recessive gain-of-function mutation and therefore would mimic the effect of an increase of IP_3. *lfe-2(lf)* mutations also suppress *lin-3* and *let-23* sterility presumably by increasing the amount of available IP_3 (less IP_3 is converted to IP_4) in the spermatheca (Clandinin, 1997). Based on these arguments, one would expect that lowering the concentration of IP_3 in the spermatheca would result in a sterile phenotype.

Formation of IP_3 is catalyzed by phospholipases C (PLC), which hydrolyze phosphatidyl inositol (4,5) bisphosphate to diacylglycerol and IP_3. Since RTKs activate the γ family or phospholipases (PLC-γ), it is likely that PLC-γ mediates the *let-23*-dependent production of IP_3. Thus, I expect to mutate PLC-γ in this screen. However, *sy499* is not likely to be PLC-γ because the genome project has not identified PLC-γ homologs in the area where *sy499* maps.

Calcium signaling is essential for most cell types. Removing a PLC-γ expressed in many cell types should therefore result in a lethal phenotype. Although we can not recover lethal mutations with this screen, we could recover a PLC-γ specifically expressed in the spermatheca. This may be possible, since *lfe-2* is expressed specifically in the spermatheca, in the pharynx, and in the intestine (Clandinin, 1997). Alternatively, we could recover a mutated PLC-γ which is only defective in a subset of tissues, not essential for viability.

This screen was designed to identify components upstream of *lfe-1* and *lfe-2*. Even if we do not mutate PLC-γ, we could identify other components acting between the RTK and the enzymes involved in calcium signaling. We could potentially identify components that positively regulate PLC-γ activity. It is known that PLC-γ binds to RTKs, but it is still debated if PLC-γ is activated by direct phosphorylation or by other means (Cockcroft and Thomas, 1992). If specific PLC-γ activators exist, this screen should identify them. On the other hand, if *let-23*-mediated fertility uses a PLC-γ-independent pathway, we should find its components with this screen.

In conclusion, we believe that this genetic screen will identify additional components of the *let-23*-mediated fertility pathway. This will

increase our understanding of what molecules and what mechanisms RTKs use to control calcium signaling. In addition, it will shed light on the strategies employed by RTKs to activate distinct pathways leading to tissue-specific functions.

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Table 1. Mapping of *sy499*. Mapping was done with strains of two different backgrounds: *unc-38 lfe-2 lin-10 sy499*/m_1 m_2 (A) or *dpy-5 lin-10 sy499*/m_1 m_2 (B). M_1 non-M_2, fraction of M_1 non-M_2 recombinants that picked up *sy499*. M_2 non-M_1, fraction of M_2 non-M_1 recombinants that picked up *sy499*.

Tester strain	m_1m_2	M_1 non-M_2	M_2 non-M_1
A *unc-38 lfe-2 lin-10 sy499/m_1m_2*	*dpy-5 unc-15*	1/8	--
B *dpy-5 lin-10 sy499/m_1m_2*	*bli-4 unc-13*	1/3	0/4
	dpy-14 unc-13	--	0/3
	dpy-14 unc-29	--	0/6
	daf-8 unc-29	--	0/2
	unc-13 lin-11	7/7	0/11
	unc-75 unc-101	0/6	--
	unc-75 aex-6	1/1	--
	unc-29 unc-75	--	2/4
	unc-29 lin-11	1/1	0/4
	lin-11 unc-75	0/6	2/2

Table 2. Comparison of phenotypes between *unc-13* and *unc-13 sy499* animals. n is the number of animals scored, except where noted.

[1]Viability was measured in the strain *unc-13 sy499*/*dpy-24 unc-75*.

% viability = 2·100·(Number of Unc non-Dpy)/(Number of non-Dpy non-Unc).

[2]Emo phenotype, number of animals whose oocytes endomitotycally replicate their DNA before entering the spermatheca.

[3]% Lethality = (dead eggs/total eggs laid)·100. n here represents the number of eggs scored.

	unc-13	***unc-13 sy499***
Viability	N.D.	102.9% (n= 313)[1]
Vulval induction	100% (n=18)	100% (n=18)
Emo phenotype[2]	0% (n=24)	12% (n=16)
Lethality[3]	0% (n= 91)	100% (n=559)

Figure 1. At least two pathways originate from the receptor tyrosine kinase LET-23. The Ras pathway mediates viability and vulval differentiation. A pathway involving *lfe-1* and *lfe-2* is required for *let-23*-mediated fertility.

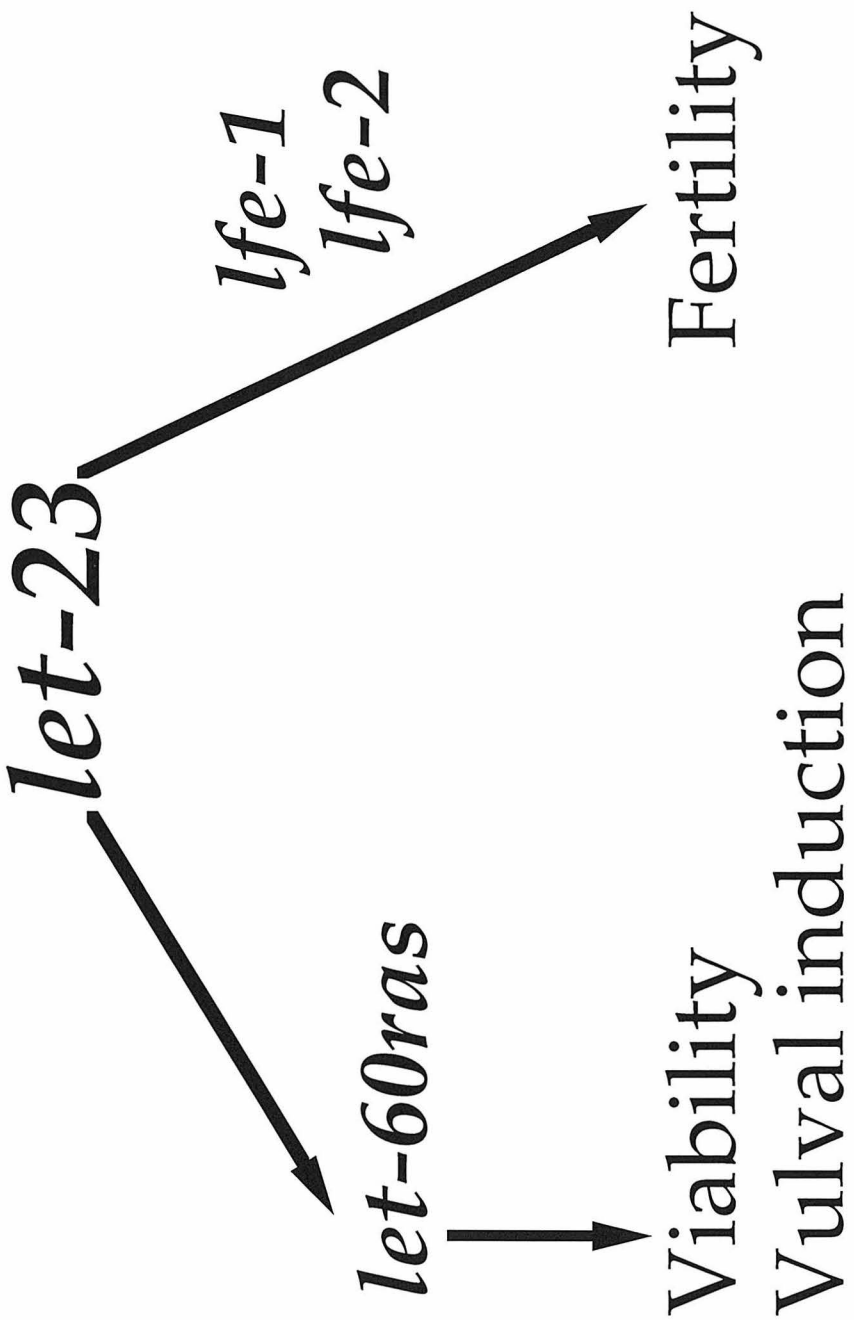

let-23
lfe-1
lfe-2
Fertility
let-60ras
Viability
Vulval induction

Figure 2. The Emo phenotype. In panel (A) a cartoon representing the hermaphrodite gonad is shown. Oocytes mature whilst passing along from the distal gonad to the proximal gonad. A mature oocyte (Oo) sits in front of the spermatheca (SP). Upon entering the spermatheca it is fertilized. After fertilization the oocyte is surrounded by an egg shell and passes into the uterus (UT). (B), wild-type mature oocyte. The big arrow indicates a normal nucleus. (C), the small arrow an Emo oocyte with two nuclei. Bar, 20 μm.

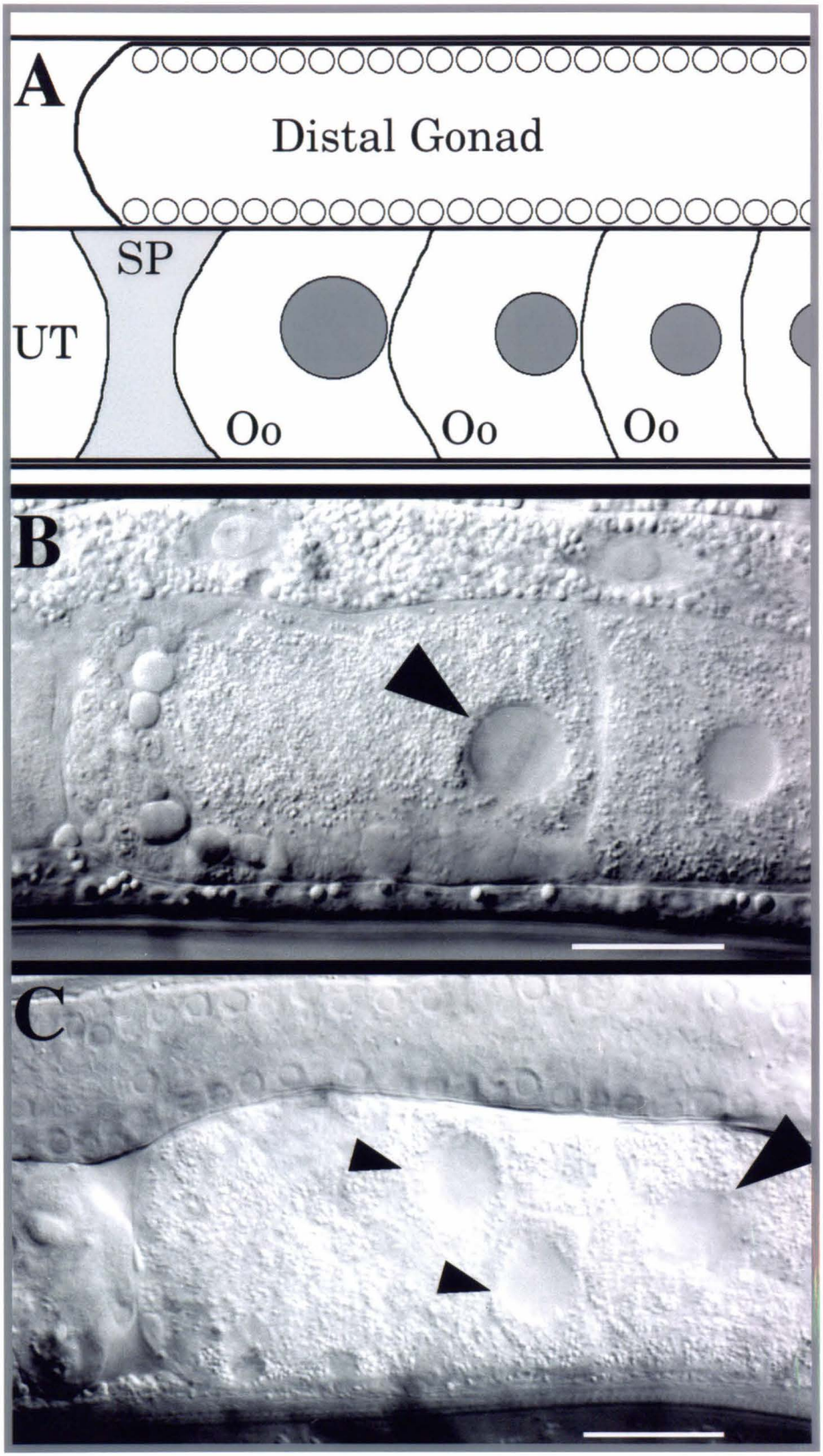

A
Distal Gonad
SP
UT
Oo
Oo
Oo
B
C

Figure 3. A genetic screen to identify more components of the *let-23*-mediated fertility pathway. A strain homozygous for a *lfe-2(lf)* mutation and carrying an extrachromosomal array with *dpy-20(+)* and *rol-6*^{D} DNAs is mutagenized with EMS. F1 Roller hermaphrodites are individually picked and their progeny analyzed. Worms carrying the mutation of interest *m** will segregate fertile Rollers and non-Rollers and sterile Rollers.

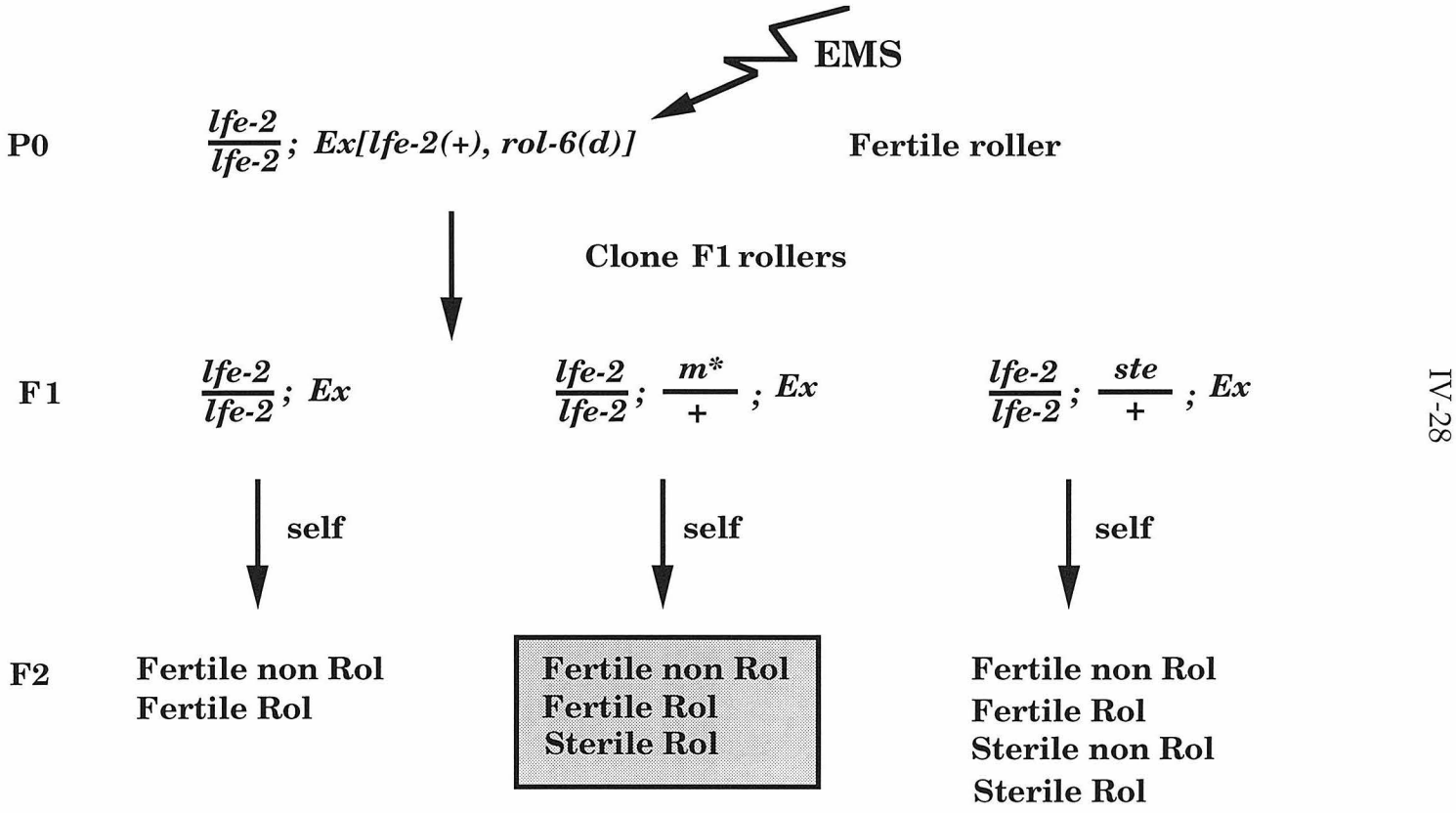
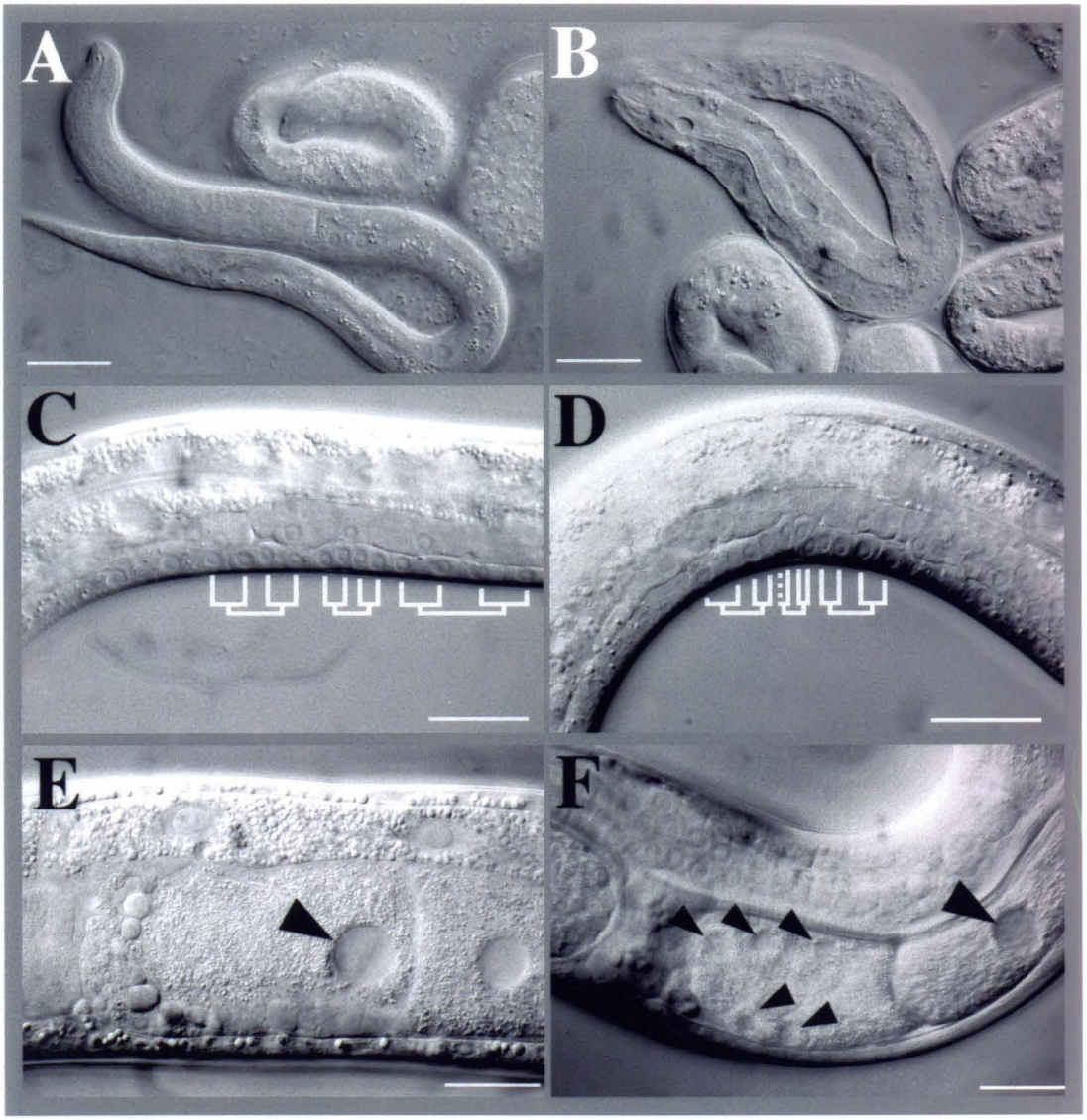

EMS
P0
lfe-2 / lfe-2 ; Ex[lfe-2(+), rol-6(d)]
Fertile roller
Clone F1 rollers
F1
lfe-2 / lfe-2 ; Ex
lfe-2 / lfe-2 ; m* / + ; Ex
lfe-2 / lfe-2 ; ste / + ; Ex
self
self
self
F2
Fertile non Rol
Fertile Rol
Fertile non Rol
Fertile Rol
Sterile Rol
Fertile non Rol
Fertile Rol
Sterile non Rol
Sterile Rol

Figure 4. Analysis of *sy499* lethality (A and B), vulval induction (C and D), and sterility (E and F). A, C, and E wild-type. B, D, and F *sy499*. (A), wild-type larva at early L1 stage and wild-type eggs. (B), *sy499* dead larva and eggs (B). Lineage of three VPCs at the four cell stage in a *unc-13* larva (C). Lineage of three VPCs in a *unc-13 sy499* larva (D). Here two inner daughters of the central VPC have divided transversally (only the two towards the viewer are visible). Wild-type mature oocyte (E). Emo oocyte from an *unc-13 sy499* hermaphrodite (F). Bar, 20 μm.


A
B
C
D
E
F

Chapter V

Summary

This thesis combines molecular and genetic techniques to analyze signal transduction mediated by LET-23, a *Caenorhabditis elegans* Epidermal growth Factor Receptor (EGFR) homolog.

When I began research towards my thesis, very little was known about signaling by tyrosine kinases of the EGFR family. *let-23* had just been cloned (Aroian *et al.*, 1990) and there were many genetically characterized *let-23* loss-of-function alleles available (Aroian and Sternberg, 1991). To understand more about how *let-23* structure relates to *let-23* functions, Raffi Aroian decided to sequence all *let-23* alleles. I joined him in this project, the results of which are reported in the first part of Chapter II (Aroian *et al.*, 1994). The main conclusion from this work is that the C-terminus of LET-23 can be divided in at least three domains, each mediating a subset of LET-23 functions.

Chapter II describes also the analysis of an activating *let-23* allele, *sa62* (Katz *et al.*, 1996). Activating mutations of Receptor Tyrosine Kinases (RTK) are important for the study of structural determinants involved in transmembrane kinase activation and regulation of signaling functions. By analyzing *sa62*, I have demonstrated that a single point mutation in the extracellular domain activates LET-23 in a signal-independent manner, and further that the Cysteines in the Cysteine-rich domain are important for signaling and are non-equivalent.

Based on the structure-to-function analysis reported in Chapter II and on previous work on mammalian members of the EGFR family, I hypothesized that tissue-specific signaling would depend on LET-23 C-terminal tyrosines. All the members of the EGFR family carry C-terminal tyrosines, some of which are phosphorylated upon ligand binding. They

have been proposed to be the major determinant of RTK signaling, but their role in the EGFR family was unclear. I therefore decided to dissect the complexity of LET-23 signaling, clarifying the role of its C-terminal tyrosines *in vivo*. This analysis is described in Chapter III (Lesa and Sternberg, 1997). Using *in vitro* mutagenesis and transgenic technology, I show that, *in vivo*, the C-terminal tyrosines are required for wild type activity and that they are differently used to mediate cell-specific, positive and negative regulation. In particular, one tyrosine is necessary and sufficient for wild-type fertility. Three other tyrosines are involved in the activation of the Ras pathway and are involved only in viability and vulval differentiation. Another tyrosine appears to mediate tissue-specific negative regulation. These data suggest that there are two mechanisms for tissue-specific RTK-mediated signaling: a positive mechanism which promotes and a negative mechanism which inhibits RTK function.

One may expect that tissue-specificity could arise by expressing some effectors only in certain cell types. For example, the effector SEM-5, which mediates only viability and vulval development, would not be expressed in the cells required for *let-23*-mediated fertility. To test this hypothesis I have built a *let-23* gene driven by the *sem-5* promoter. If *sem-5* is not expressed in the cells required for fertility, we expect transgenic animals expressing *sem-5*-driven *let-23* to be sterile. I found that 67% of these animals are fertile (Appendix to Chapter III, Table 2 A). This result suggests that *sem-5* is expressed in the cells required for fertility. Then, what is its function? One possibility is that, in these cells, *sem-5* is used to activate pathways leading to a function unrelated to fertility. An interesting finding (Appendix to Chapter III, Table 3 G) is that a LET-23 protein carrying only site 6 (a SEM-

5 putative binding site) is able to mediate fertility in a *sem-5* reduction-of-function background. One interpretation of this result is that the normal function of SEM-5 in these cells is to inhibit the fertility function, perhaps by the recruitment and activation of negative regulators. However, other explanations are possible. For example, an incompletely functional SEM-5 could bind with much less affinity to site 6 and could allow components mediating fertility to interact with site 6 and to be activated [specificity of SH2 binding sites is not absolute (Songyang *et al.*, 1993; Songyang *et al.*, 1994)].

Since Ras is activated by distinct RTKs in many different cell types, it is possible that the Ras pathway is used by different means in different cells. Thus, the highly conserved Ras signaling cassette would be used to ultimately activate different effectors depending on the cell type. This hypothesis would be consistent with the observation that the same RTK expressed in different cells leads to different outcomes. For example, the Fibroblast Growth Factor receptor stimulates differentiation in PC12 neuronal cells, but in fibroblasts stimulates proliferation (Marshall, 1995).

Another way to achieve tissue-specificity would be to make use of tissue-specific negative regulators. Some cell types would not activate certain pathways or certain effectors because inhibitors present only in those cells would prevent them to do so. Alternatively, negative regulators could be present in all cell types and inhibit a certain pathway. Since they would have an effect only where this pathway is actually used, they would appear tissue-specific. For example, the LET-23 C-terminal site 2 acts through a "tissue-specific" negative regulator. Since Ras does not seem to be used in the cells required for fertility, then the negative effect mediated by

site 2 would take place only where Ras is used, that is in the cells required for viability and in the vulval precursor cells. The action of these inhibitors could take place at the level of the receptor or more downstream. Some of these tissue-specific negative regulators have been identified. For example, in our laboratory Charles Yoon has found that the c-Cbl homolog SLI-1 acts negatively in the cells required for viability and in the vulval precursor cells (Yoon *et al.*, 1995; Yoon, 1997).

let-23 is known to activate the highly conserved Ras pathway. In addition, the experiments described in Chapter III showing that distinct *let-23* domains are required to activate fertility and the Ras-mediated functions suggest that *let-23* mediates fertility employing a set of genes different from the Ras pathway. Indeed, two suppressor loci of sterile *lin-3* and a sterile *let-23* allele have been identified that do not interfere with the *ras*-mediated functions, *lfe-1* and *lfe-2* (*lfe*=*l*et-23 fertility effector). These two genes encode proteins involved in calcium signaling (Clandinin, 1997). To find additional, positively acting components of the *let-23* fertility pathway, I screened for sterile mutations that can be suppressed by *lfe-2*. This screen is described in Chapter IV. In addition, the initial characterization of a gene acting positively in the *let-23*-mediated fertility pathway is reported.

The study reported in this thesis has contributed to the clarification of the complexity of signaling by RTKs *in vivo*. A systematic analysis of potential LET-23 SH2 binding sites has been carried out almost uniquely in intact animals, where subtle changes in activation, regulation and tissue-specific signaling can be studied and where all the components normally interacting with RTKs are present. Therefore, the findings presented in this thesis are of high physiological significance.

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