

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

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Submitted December 11, 1996; Accepted February 3, 1997
Monitoring Editor: Judith Kimble

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 Ca²⁺ 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.

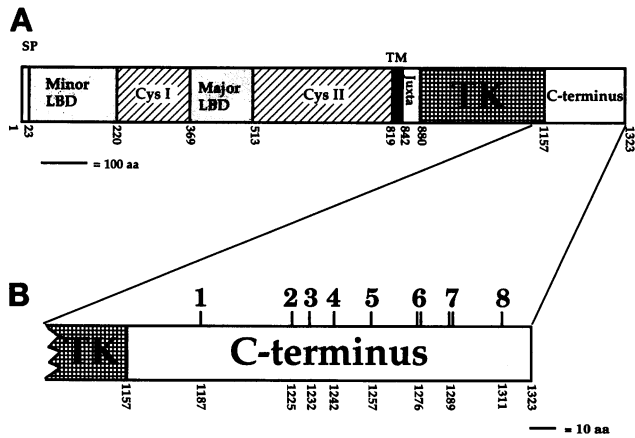


Figure 1. Structure of the LET-23 protein. All the LET-23 protein (1323 amino acids) represented with the domains characteristic of the EGFR family is shown in A. SP, signal peptide; Minor LBD, minor ligand-binding domain; Cys I, cysteine-rich domain I; Major LBD, major ligand-binding domain; Cys II, 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).

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

| 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.

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/ μ l *let-23* DNA, 15 ng/ μ l pMH86 [*dpy-20(+)*] DNA (Han and Sternberg, 1990), and 110 ng/ μ l pBluescript II carrier DNA was injected into animals from PS1484 (P0). pMH86 rescues the *Dpy* phenotype. We picked F₁ non-*Dpy* non-*Unc* hermaphrodites, corresponding to animals expressing the injected DNA. Every F₁ 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/ μ l along with 50 ng/ μ l pRF4 [a plasmid containing a dominant mutant gene of *rol-6* (Mello *et al.*, 1991)] and 100 ng/ μ l 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 \times objective, Nomarski differential interference-contrast optics for their extent of vulval induction. Vulval differentiation = number

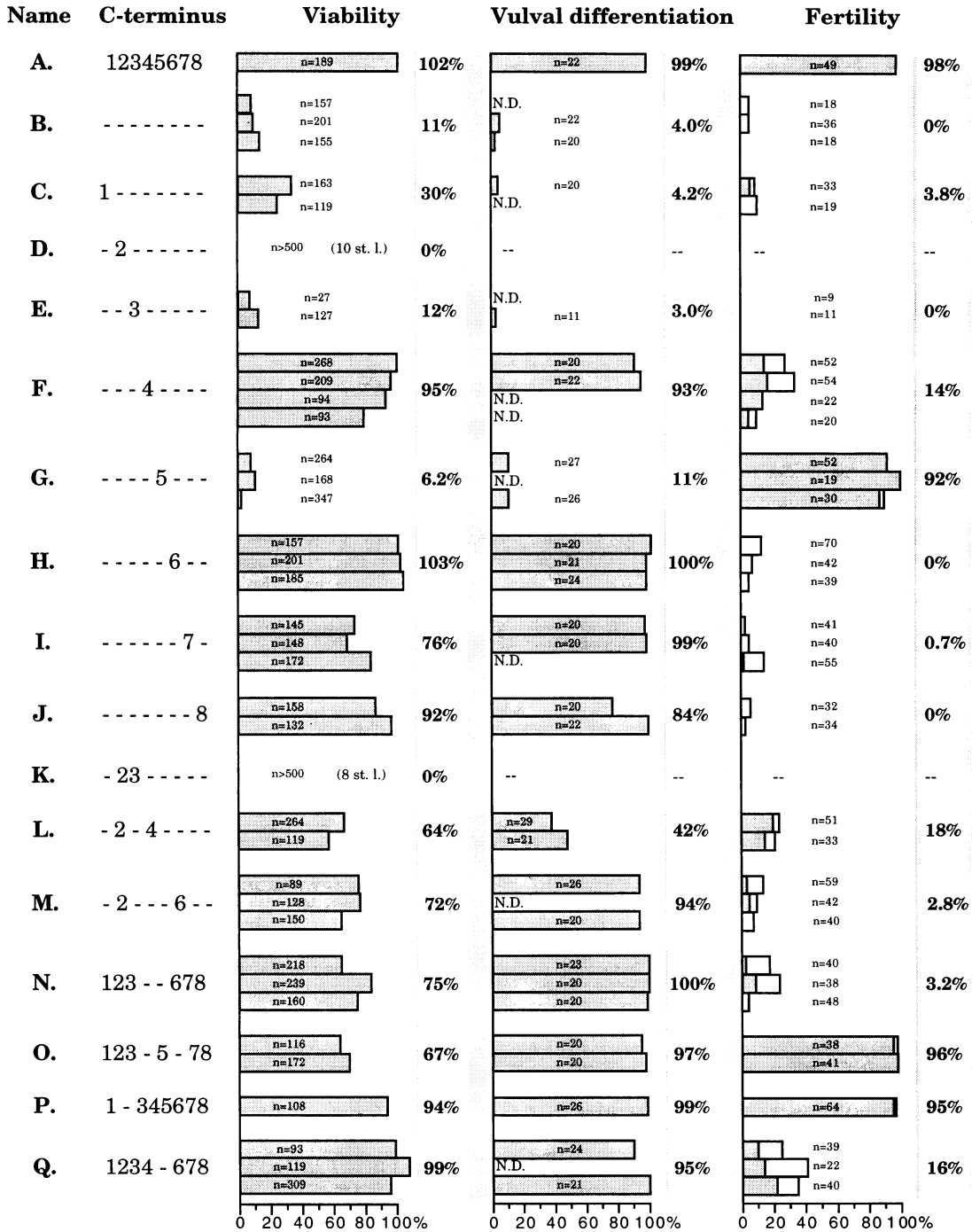


Figure 3. Ability of LET-23 carboxyl-terminal tyrosines to mediate viability, vulval differentiation, and fertility. The *let-23(null)* allele used to test all of the constructs in this figure is *let-23(syl17)*. Carboxyl-terminal sites 1, 2, 3, etc., indicate that tyrosine(s) at that putative SH2-binding 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), semi-fertile (2–10), or fertile (>10).

Site-directed Mutagenesis

Site-directed mutagenesis was carried out in *pk7-5.5*, a *HindIII* 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'-ACCGCGTG-GCTAGCGCTCTAGAAC-3') that changes the *NotI* restriction site in pBluescript II to an *NheI* site. Eighteen mutagenic primers were synthesized. One primer, pS97, (5'-TGATGGGGATAGCTT-TGAAAATTTATTAT-3'), introduces a G to A mutation at the end of intron 17. Another primer, pSTX, (5'-CGCCATTTGATG-GCTATTACTCTGAAAATTTATTA-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'-CTTCCTTGATCAAAGAA-CATCTGTGGATCGA-3'), DY-2 (site 2, 5'-AAAGGCTCCGTTTT-GAACCTAGATGAGTTT-3'), DY-3 (site 3, 5'-GTGCGGTTGAC-CCAACATAAATGCAAAAATAATT-3'), YLIP-1 (site 4, 5'-TTTGAATAAG- AAATGAATTATCTTCC-3'), YTAV-1 (site 5, 5'-GTAACAGCTGTAAACAAAACCTGCTG-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'-AAAGGCTCCGTTTTGTACCTAGATGAGTT-3'), DY-W3 (site 3, 5'-GTGCGGTTGACCCATCTAAAATG-CAAAAATAATT-3'), LIP-W (site 4, 5'-TTTGAATAAAGATAT-GAATTATCTTCC-3'), TAV-W (site 5, 5'-GTAACAGCTGTATA-CAAAACCTGCTG-3'), NQ-W (site 6, 5'-TTTGGTTGGTTGATG-TAATCGCCATTTG-3'), NE-W (site 7, 5'-TGTGGTTCATTGTA-ATATCCAGAGGAAG-3'), and ENE-W (site 8, 5'-TCTTCATTT-TCATATTGAACTGCTTC-3').

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.

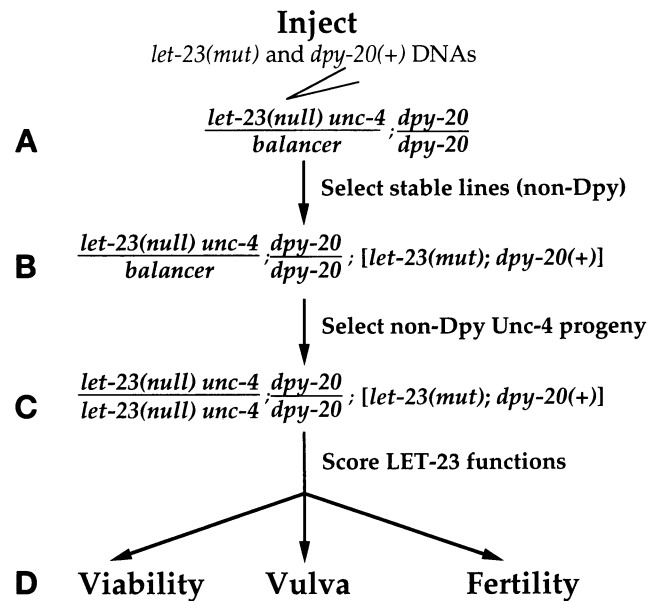


Figure 4. Strategy used to analyze the effects of in vitro-modified *let-23* genes on viability, vulval induction, and fertility. Worms with a *let-23(null)* and *dpy-20(-)* background (A) were injected with a wild-type [*let-23(+)*] (control) or altered [*let-23(mut)*] *let-23* clone along with a marker 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 *Sall* and *HindIII* and ligated with an ~12-kb *EcoRI-Sall* genomic *let-23* fragment and with an ~3-kb *EcoRI-Sall* 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

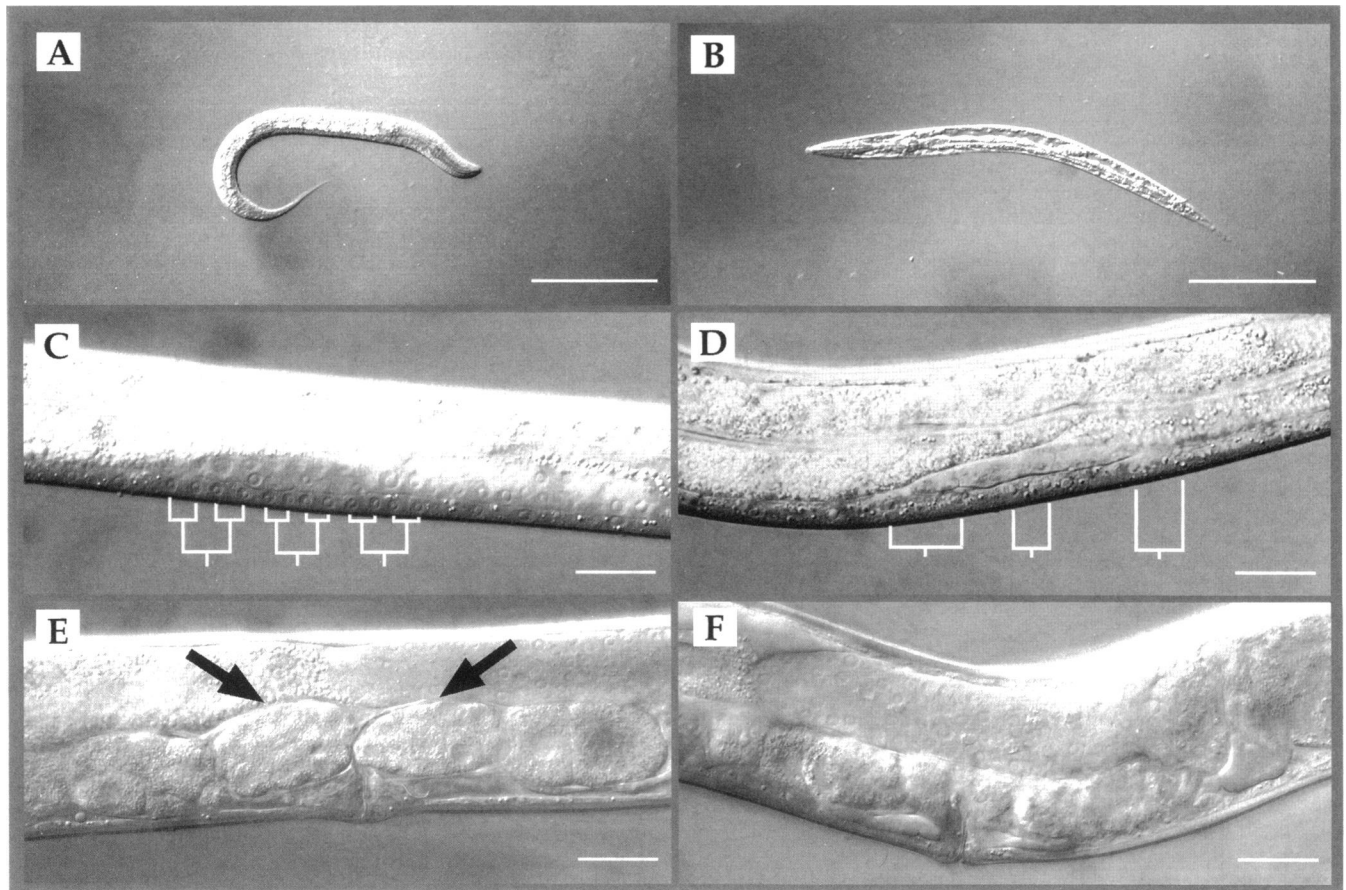


Figure 5. Nomarski photomicrographs of three distinct phenotypes associated with loss of LET-23 function. Lethality (A and B); vulval differentiation (C and D); fertility (E and F). Wild-type (A, C, and E). *let-23(loss-of-function)* phenotypes (B, D, and F). Wild-type L1 larva (A). Dead L1 larva (B). 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 C⁷⁰⁰ to W⁷⁰⁰) and analyzed their progeny. Injection mixes containing 5 ng/ μl (F₁ analysis, viability = 24%, n = 67) or 20 ng/ μl (F₂ 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 Ohs-

hima, 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/ μ l and pSTX at 20, 50, or 185 ng/ μ l 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/ μ l 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

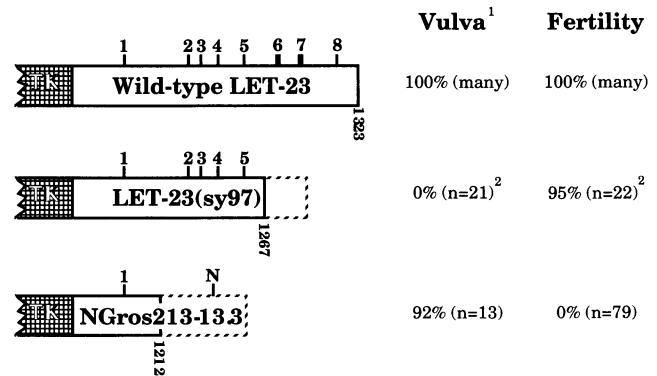


Figure 6. Vulval differentiation and fertility functions conferred by wild-type LET-23, LET-23(sy97), and NGros213-13.3. On the left the carboxyl-terminal end of LET-23 is shown. *let-23*(sy97) is predicted to truncate LET-23 at amino acid 1267. NGros213-13.3 is predicted to truncate LET-23 at amino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

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

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

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

pS97 and pSTX at 20 ng/ μ l and at 50 ng/ μ l were injected into *let-23*(*mn23*) *unc-4*(*e120*)/*mnC1*; *dpy-20*(*e1282*) animals. pSTX at 185 ng/ μ l 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).

^aAnimals that laid eggs.

^bIf they survived, based on complementation tests (Aroian and Sternberg, 1991).

NA, not applicable.

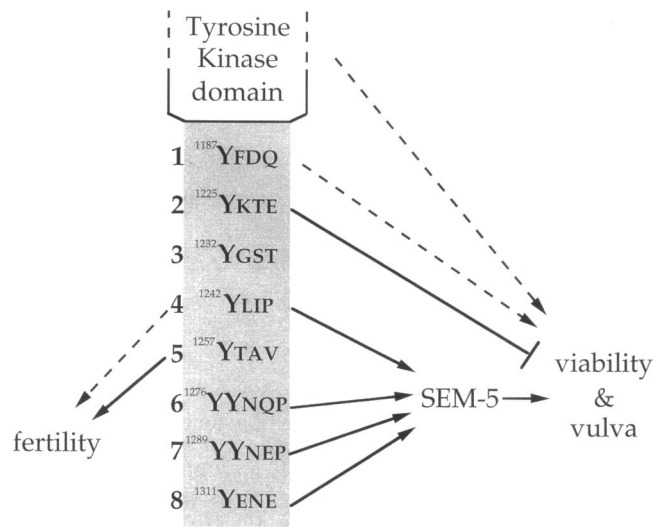


Figure 7. Model of in vivo LET-23-mediated activity for viability, vulval differentiation, and fertility. The eight putative SH2-binding sites in the carboxyl terminus of LET-23 are shown. Sites 1 and 3 have a minor role. Site 4 promotes all three functions. Dotted lines indicate incomplete activation. 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/ μ l, 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/ μ l) and a construct bearing only site 4 (20 ng/ μ l). As a control we constructed transgenic animals that ex-

press a construct with no carboxyl-terminal tyrosines (100 ng/ μ l) along with a construct bearing only site 4 (20 ng/ μ l). 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/ μ l 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 F | - - - - - and - - - 4 - - - - | 71 (n = 135) | 0.0051 | 2 |
| D and F | - 2 - - - - - and - - - 4 - - - - | 31 (n = 121) | | 2 |

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/ μ l; construct F, 20 ng/ μ l, pMH86, 15 ng/ μ l; pBluescriptII, 40 ng/ μ l.

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 (nDnU) 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 $nDnU = DnU \cdot (TF/1 - TF) = 286 \cdot (38/62) = 175$. If there is bypass we would expect $nDnU = DnU \cdot (TF/1 - TF) / \text{viability of } sem-5(n2019) = 175 / 0.627 = 279$. 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. <i>let-23(sy17)/let-23(+)</i> 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. <i>let-23(sy17)/let-23(sy17)</i> 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.

^aThe genotype is *let-23(sy17)/let-23(+)*; *dpy-20(e1282)*; *sem-5(n2019)*; *Ex*.

^bTransmission 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.

^cnDnU expected if there is no SEM-5 bypass (hypothesis 1) = DnU/[TF/(1-TF)].

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

^ep obtained by comparing the number of nDnU expected if there is no SEM-5 bypass (hypothesis 1) with the nDnU observed.

^fp obtained by comparing the number of nDnU expected if there is SEM-5 bypass (hypothesis 2) with the nDnU observed.

^gThe genotype is *let-23(sy17)/let-23(sy17)*; *dpy-20(e1282)*; *sem-5(n2019)*; *Ex*.

^hNumber 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.

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

^jp obtained by comparing the number of nDU expected if there is no SEM-5 bypass (hypothesis 3) with the nDU observed.

^kp 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 (Clanadinin 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.

ACKNOWLEDGMENTS

We thank Raffi Aroian whose contribution was invaluable to the completion of this work. We are also deeply indebted to Tom Clandinin for suggestions and discussions. We thank Raffi Aroian, Tom Clandinin, Marie-Anne Félix, Neil Hopper, Giovanna Lalli, and Robert Palmer for critical reading of the manuscript. This work was supported by United States National Institutes of Health grant HD23690 to P.W.S. P.W.S. is an investigator with the Howard Hughes Medical Institute. G.M.L. was a Merck Graduate Fellow.

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