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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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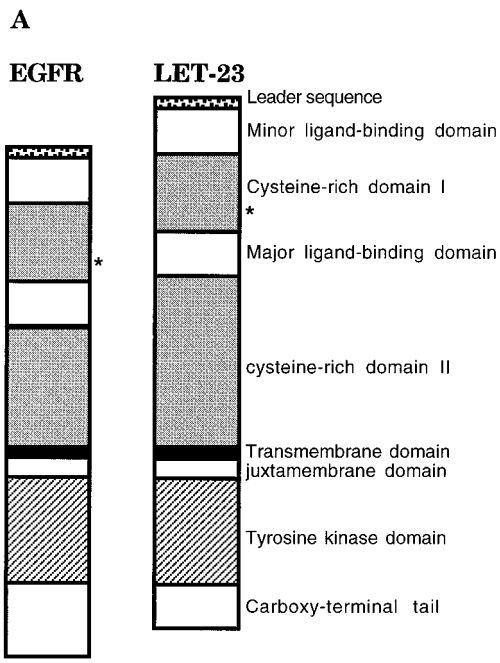

B

		305	313	
EGFR	300	RK**C**KK**C**EGP-**C**RKV**C**NGIG		317
HER2	329	QR**C**EK**C**SKP-**C**ARV**C**YGLG		346
HER3	299	KM**C**EP**C**GGL-**C**PKA**C**EGTG		316
HER4	321	KM**C**KP**C**TDI-**C**PKA**C**DGIG		338
DER	341	GE**C**VP**C**NGP-**C**PKT**C**PGVT		358
LET-23	354	RE**C**EK**C**RSSS**C**PKI**C**TVDG		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).

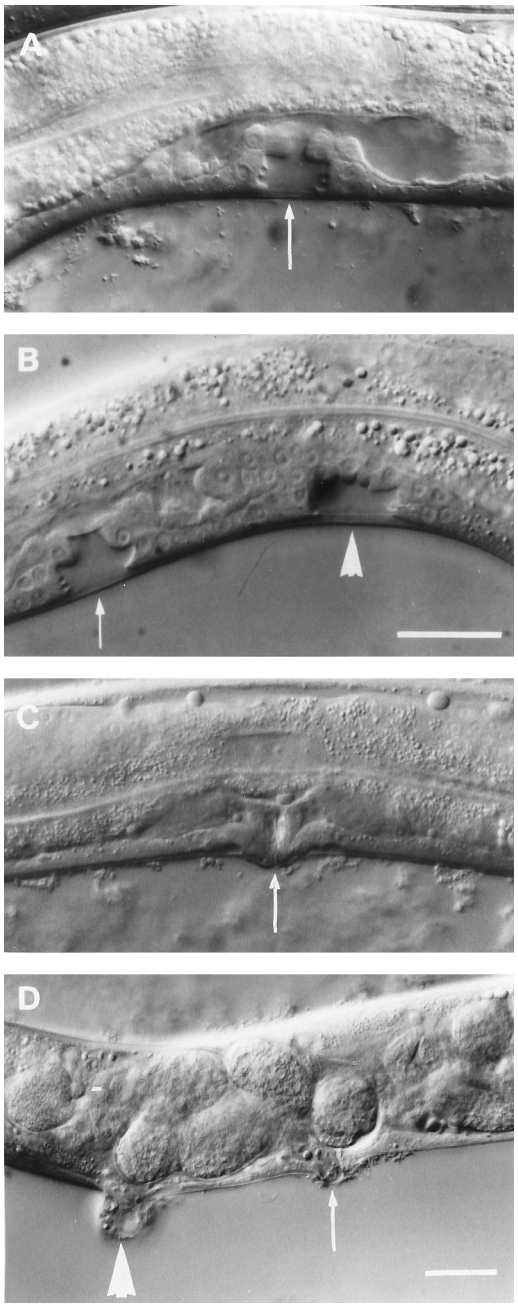

FIG. 2. Muv phenotype of *sa62*. Shown are Nomarski photomicrographs of L4 hermaphrodites (A and B) and adult hermaphrodites (C and D). (A) Wild type; (B) *sa62*; (C) *let-23*(*sy17 null*) with wild-type transgene; (D) *let-23*(*sy17 null*) with *sa62* transgene. Arrows, vulvae; arrowheads, pseudovulvae.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

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.

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).

***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).

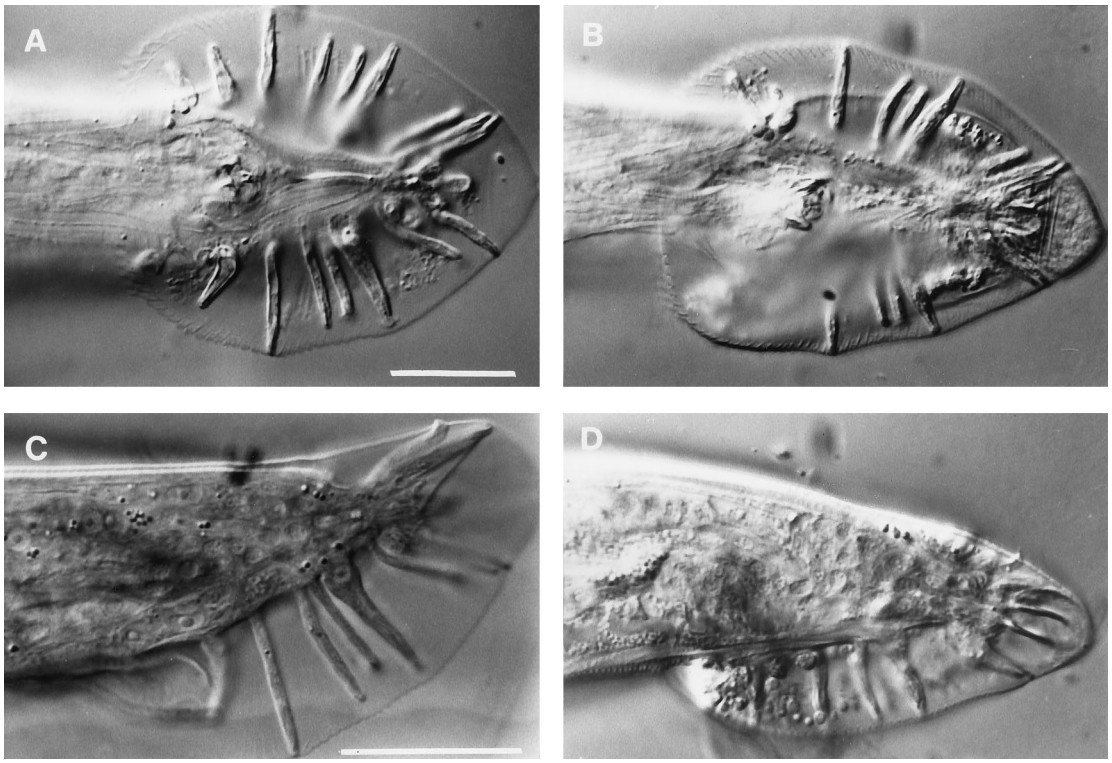

FIG. 4. Morphologies of mating structures in *sa62* and wild-type males. (A) Wild type, ventral view; (B) *sa62*, ventral view; (C) wild type, lateral view; (D) *sa62*, lateral view. Scale bars = 20 μm.

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.

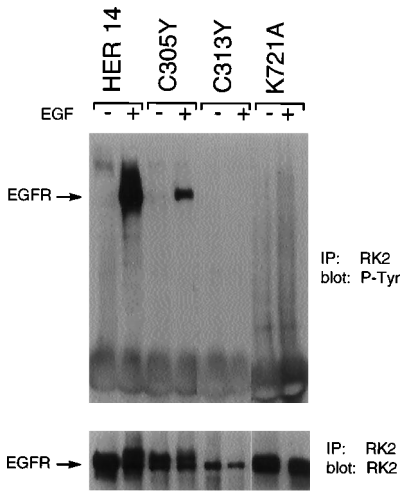

FIG. 5. EGF-dependent Tyr phosphorylation of EGFR and mutant receptors. Cells expressing either wild-type or mutant receptors were exposed to 100 ng of EGF per ml for 5 min and then subjected to cell solubilization, immunoprecipitation (IP) with anti-EGFR antibody RK-2, separation by SDS-polyacrylamide 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

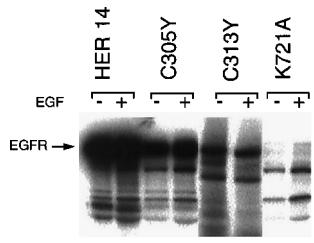

FIG. 6. In vitro kinase assay of EGFR and mutant receptors. EGFR and mutant receptors were immunoprecipitated with antibody RK-2. The immunoprecipitates were phosphorylated with [γ-^{32}P]ATP and separated by SDS-polyacrylamide gel electrophoresis.

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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