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

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

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

Introduction

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

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

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

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

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

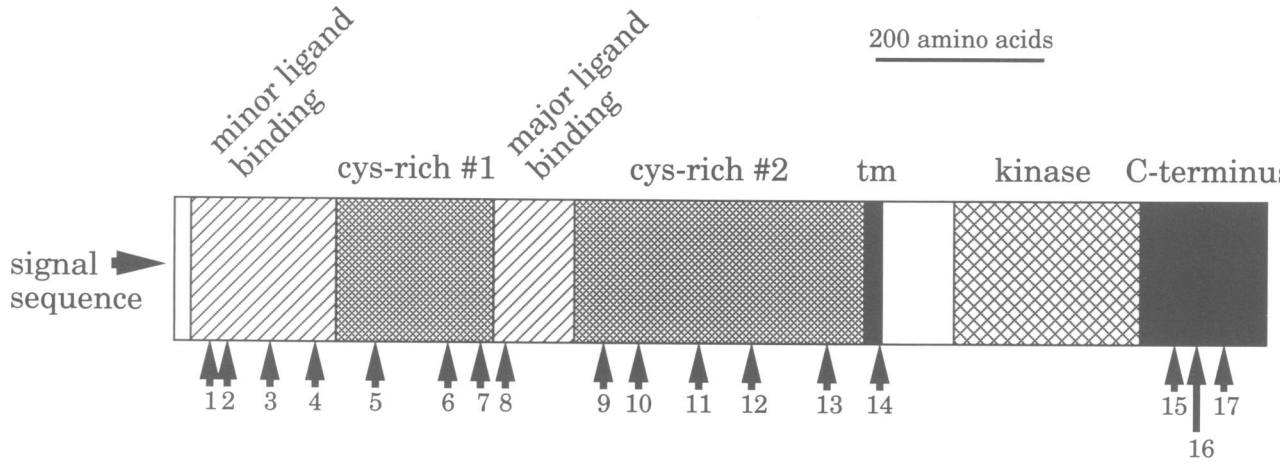

Fig. 1. The structure of the 1323 amino acid LET-23 EGFR protein represented using the domains characteristic of the EGFR family. Numbered arrows below the line indicate the number and position of the 17 introns. Domains from N-terminus to C-terminus are: the signal sequence, a minor ligand binding domain,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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

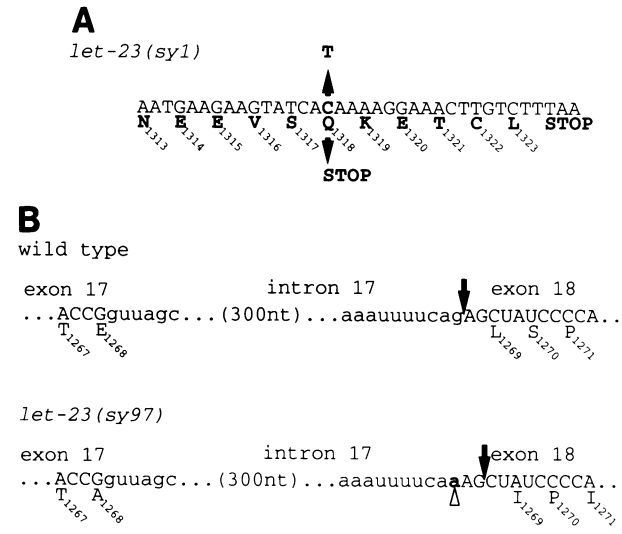

Fig. 2. Analyses of the cell-type specific mutations *sy1* and *sy97*. (**A**) The sequence of the *let-23(sy1)* mutation. The wild-type coding sequence (upper line) and protein sequence for the final 11 amino acids (lower line) are shown. The *sy1* alteration is shown in coding sequence and protein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

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

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

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

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

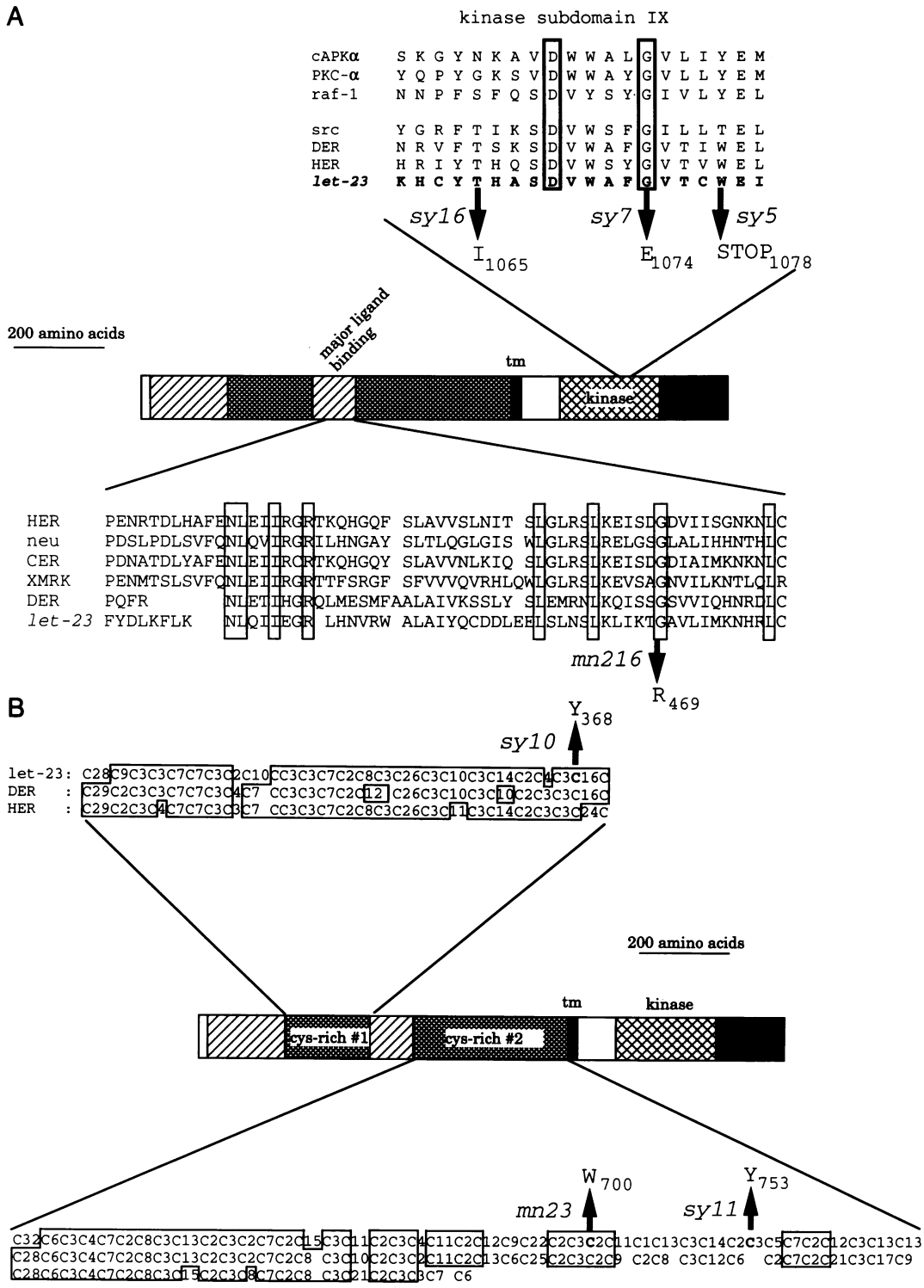

Fig. 3. Sequence of *let-23* null alleles. Numbers associated with mutated residues indicate residue number. The LET-23 protein is schematically depicted as in Figure 1. (**A**) Above the diagram of the protein, the sequence of LET-23 in kinase subdomain IX is shown. The location and amino acid sequences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

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

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

Possible upstream extent of the let-23 fertility domain

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

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

Discussion

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

Non-specific mutations

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

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

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

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

Cell-specific mutations

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

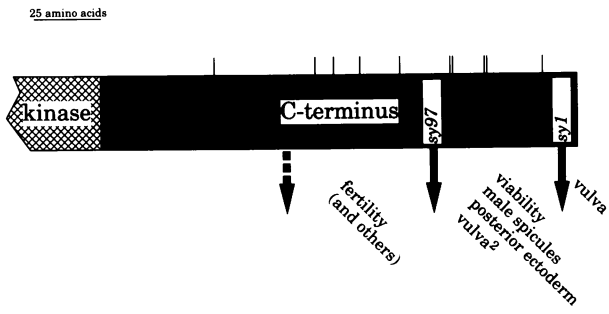

Fig. 4. A model for cell-type specific C-terminal domains. The C-terminal domain and part of the kinase domain are depicted as in Figure 1. The locations of the *syl* and *sy97* mutations are indicated. Based on genetics and molecular sequence, the C-terminus (i.e. the region downstream of the kinase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

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

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

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

Materials and methods

Sequencing of the let-23(syl) point mutation

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

Localization and sequencing of other point mutations

Due to the large size of the *let-23* gene, the *sy97* mutation and all other mutations but *syl* were localized using hydroxylamine mismatch detection, which detects mismatched C residues. The entire *let-23* coding sequence and exon−intron boundaries (spanning ~9 kb) were partitioned into eight sets of slightly overlapping, 1 kb PCR fragments. Primer sets from the 5′ to the 3′ end of the gene are as follows. Set 1, 5′-TTGGGTATCACATGTATAAGAGG and 5′-GCAAAAAATTAGTTTTGCCTGGG; set 2, 5′-CCGGATCGACGAAGTAACCATCC and 5′-GAAACTTTTGATCGTTCTGTGTC; set 3, 5′-GACACAGAACGATCAAAAGTTTC and 5′-GCTTTGAAGTACTTCTAGACACACAC; set 4, 5′-GACTTAAAAGTGCATCTTTAGAGAGC and 5′-CCTTAGACGGTAGCTAAAATCATTCG; set 5, 5′-CGTTCCATCTGCAAAGAAGGC and 5′-CCATTCAAGTGAATGCATACCC; set 6, 5′-GAAGGAGACAATCTGGCTTCACGC and 5′-CCAGGACGAGACTTTGGATCCGCC; set 7, 5′-TGGCTTGCGATCGAAATCTTCTCC and 5′-CCAGATCTTCATTTGTAACAGCTG; set 8, 5′-CTCATCTAGGTACAAAACGGAGCC and 5′-CCAAGCTTGTATGAGATGAATGGCAACGG.

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

Analysis of sy97 splicing products

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

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

Analysis of D_{1212} truncation

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

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