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Requirements of Multiple Domains of SLI-1, a *Caenorhabditis elegans* Homologue of c-Cbl, and an Inhibitory Tyrosine in LET-23 in Regulating Vulval Differentiation

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SLI-1, a *Caenorhabditis elegans* homologue of the proto-oncogene product c-Cbl, is a negative regulator of LET-23-mediated vulval differentiation. Lack of SLI-1 activity can compensate for decreased function of the LET-23 epidermal growth factor receptor, the SEM-5 adaptor, but not the LET-60 RAS, suggesting that SLI-1 acts before RAS activation. SLI-1 and c-Cbl comprise an N-terminal region (termed SLI-1:N/Cbl-N, containing a four-helix bundle, an EF hand calcium-binding domain, and a divergent SH2 domain) followed by a RING finger domain and a proline-rich C-terminus. In a transgenic functional assay, the proline-rich C-terminal domain is not essential for *sli-1*(+) function. A protein lacking the SH2 and RING finger domains has no activity, but a chimeric protein with the SH2 and RING finger domains of SLI-1 replaced by the equivalent domains of c-Cbl has activity. The RING finger domain of c-Cbl has been shown recently to enhance ubiquitination of active RTKs by acting as an E3 ubiquitin–protein ligase. We find that the RING finger domain of SLI-1 is partially dispensable. Further, we identify an inhibitory tyrosine of LET-23 requiring *sli-1*(+) for its effects: removal of this tyrosine closely mimics the loss of *sli-1* but not of another negative regulator, *ark-1*. Thus, we suggest that this inhibitory tyrosine mediates its effects through SLI-1, which in turn inhibits signaling upstream of LET-60 RAS in a manner not wholly dependent on the ubiquitin–ligase domain.

INTRODUCTION

Receptor tyrosine kinase (RTK)/Ras/MAPK signaling pathways are functionally conserved among metazoans in various aspects of cell growth and differentiation (Schlessinger and Ullrich, 1992; Fantl *et al.*, 1993). Because unregulated RTK signaling can promote abnormal cell growth or differentiation, precise control of both the duration and the level of RTK activation is important (Cantley *et al.*, 1991; Rodrigues and Park, 1994), and thus it is crucial to understand the negative regulation of RTK signaling.

RTK-mediated signal transduction is initiated by ligand binding, dimerization, and subsequent autophosphorylation of cytoplasmic tyrosines within the C-terminal of the receptor (Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993; Claesson-Welsh, 1994). Phosphotyrosine (pTyr) sites in activated RTKs recruit the Grb2/SEM-5/Drk adaptor protein (Clark *et al.*, 1992a; Lowenstein *et al.*, 1992; Oliver *et al.*, 1993; Simon *et al.*, 1993). This adaptor protein is associated with the Sos guanine nucleotide exchange factor (Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993). Once recruited to the membrane, Sos activates the membrane-bound Ras protein by enhancing the exchange of GTP for GDP (Simon *et al.*, 1991; Gale *et al.*, 1993; Li *et al.*, 1993; Rozakis-adcock *et al.*, 1993; Aronheim *et al.*, 1994; Quilliam *et al.*, 1994). Activated Ras recruits the Raf serine/threonine kinase to the membrane, where Raf is activated by unknown mechanisms (Campbell *et al.*, 1998; Rommel and Hafen, 1998). Raf phosphorylates and activates MAPK kinase (MEK), which in turn phosphorylates and activates MAPK (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Tsuda *et al.*, 1993), leading to diverse cellular responses. The development of the *Caenorhabditis elegans* vulva provides a readily accessible genetic system for the

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study of RTK signaling and regulation in vivo (Sundaram and Han, 1996; Kornfeld, 1997; Sternberg and Han, 1998; Chang and Sternberg, 1999). The wild-type vulva is derived from precisely three of six multipotential vulval precursor cells (VPCs) that generate vulval tissue when induced by the gonadal anchor cell (Horvitz and Sternberg, 1991). The induced VPCs undergo three rounds of division and a characteristic morphogenesis to form the vulva. VPCs that do not receive adequate signal from the anchor cell divide only once and become part of the hyp7 syncytial epidermis. The LIN-3 protein has a single epidermal growth factor (EGF) domain and is produced by the anchor cell (Hill and Sternberg, 1992). LET-23, a homologue of the EGF receptor (EGFR) and likely receptor for LIN-3, is necessary not only for vulval differentiation but also for other aspects of development (Aroian *et al.*, 1990; Aroian and Sternberg, 1991; Clandinin *et al.*, 1998; Jiang and Sternberg, 1998; Chang *et al.*, 1999). LET-23 activation initiates a signaling cascade that involves SEM-5 (Grb2), LET-341 SOS-1 (Sos), LET-60 (Ras), LIN-45 (Raf), MEK-2 (MAPK/ERK kinase), and MPK-1 (MAP kinase) (Chang *et al.*, 2000; Clark *et al.*, 1992b; Han *et al.*, 1990; Han *et al.*, 1993; Kornfeld *et al.*, 1995; Lackner *et al.*, 1994; Wu and Han, 1994; Wu *et al.*, 1995). Reduction-of-function (rf) mutations in any of the signaling proteins in the LET-23 RTK pathway result in less than three VPCs undergoing vulval differentiation. In the most severe cases, the disruption of the LET-23-mediated signaling results in the complete failure to generate vulval tissue (vulvaless, or Vul, phenotype). In contrast, an abnormal increase in signaling due to activating mutations in the pathway or the removal of two or more negative regulators of the pathway causes the opposite effect in which greater than wild-type numbers of VPCs differentiate into vulval tissue (multivulva, or Muv, phenotype).

The *sli-1* locus was identified in a screen for suppressors of the Vul phenotype associated with *let-23(rf)* mutations (Jongeward *et al.*, 1995). *sli-1* encodes proteins of 582 and 540 amino acids that are similar to the mammalian proto-oncogene products c-Cbl, Cbl-b, and Cbl-3 (Langdon *et al.*, 1989; Keane *et al.*, 1995; Keane *et al.*, 1999). SLI-1 and c-Cbl proteins are composed of an N-terminal region, followed by a RING finger domain and a proline-rich C-terminus (see Figure 3). The N-terminal region (termed SLI-1:N/Cbl-N) contains the following three interacting domains: a four-helix bundle; an EF hand; and a divergent SH2 domain (Meng *et al.*, 1999). Genetic analysis has revealed that *sli-1* is a negative regulator of those *let-23* signaling functions that are mediated through RAS activation (Jongeward *et al.*, 1995). Mice lacking c-Cbl have inappropriate ZAP-70 activity in T cells, indicating that c-Cbl plays a negative role in signaling (Murphy *et al.*, 1998). Experiments performed in vitro suggest that c-Cbl directly controls down-regulation of RTKs. This is dependent on the SH2 domain, which mediates binding to activated receptor followed by phosphorylation of c-Cbl at a site adjacent to the RING finger domain. The RING finger domain then is essential for the last step of catalyzing receptor ubquitination (Levkowitz *et al.*, 1998; Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999).

Here we show that c-Cbl and SLI-1 are functionally equivalent. Genetic analysis reveals that SLI-1 acts to negatively regulate EGFR signaling upstream of LET-60 RAS, either at the receptor or Grb2/SEM-5 step. We compare *sli-1* function to that of other negative regulators of this pathway, *gap-1* (Hajnal *et al.*, 1997) and *ark-1* (Hopper *et al.*, 2000). We provide evidence that SLI-1 inhibition is partly mediated through an inhibitory tyrosine in the carboxy terminus of the LET-23 EGFR, and, in contrast to the ubiquitin ligase activity, this inhibition is not wholly dependent on the RING finger of SLI-1.

MATERIALS AND METHODS

Strain Construction and Maintenance

Strains were maintained at 20°C and handled according to the method of Brenner (1974). The following alleles were used for strain construction: for LGI, *unc-38(x20)*; LGII, *dpy-10(e128), let-23(sy1, sy97), unc-4(e120), clr-1(e1745)*; for LGIV, *unc-24(e138), dpy-20(e1282), ark-1(sy247), let-60(sy127, n1876, n2021, n2022, n2034, n2035)* (Beitel *et al.* 1990; Han *et al.* 1990); and for LGX, *sli-1(sy143), sem-5(n1619, ay73), gap-1(n1691), unc-2(e55)* (Clark *et al.* 1992a; M. Stern, personal communication). *szT1* is a reciprocal *I;X* translocation that is used as a balancer for *SEM-5* (Fodor and Deak, 1985). *DnT1* is a reciprocal *IV;V* translocation that is used as a balancer for *let-60* (Ferguson and Horvitz, 1985). *sDf8* is a small deficiency in LGIV that uncovers *let-60* (Rogalski *et al.*, 1982).

sli-1 sem-5 double mutants were constructed in the following manner: for *sli-1 sem-5(n1619)*, N2 males were mated into *unc-4; sli-1* hermaphrodites. Non-Unc male progeny were selected and mated into *clr-1(e1745); sem-5(n1619)* hermaphrodites. Non-Egl (egg-laying positive) healthy progeny were picked singly onto individual plates and checked for non-Clr Unc Egl progeny. These non-Clr Unc Egl progeny were again picked singly on to individual plates. If *sli-1* suppressed *SEM-5*, then only those recombinants of the genotype *unc-4; sli-1 sem-5(n1619)/+ sem-5(n1619)* would survive to give F2 progeny, because *sem-5(n1619)* homozygotes do not propagate beyond F1 from heterozygous mothers. Otherwise, no Unc Egl progeny would survive (except the small number of cases in which there was a *clr-1 unc-4* recombinant chromosome; these were selected against by checking for the lack of a Clr phenotype). *unc-4* was removed from *sli-1 sem-5(n1619)* by mating N2 males and selecting non-Unc F1 progeny. Non-Unc, sickly, slow-growing progeny were individually isolated in F2 and again in F3 and were checked for non-Unc F4 progeny. For the construction of *sli-1 sem-5(ay73)*, N2 males were mated into *sli-1* hermaphrodites. The resulting male progeny were mated into *unc-38(x20)/szT1; sem-5(ay73)/szT1* hermaphrodites. Many healthy progeny were picked singly on to individual plates from this cross. F2 notch-heads (an indication of the presence of *sli-1* then were picked singly from only those F1 plates that did not segregate phenotypically long males (*szT1* segregates *lon-2* males). The notch-head F2's could be categorized into three classes depending on their F3 progeny. The first class yielded only healthy progeny (F2 homozygous for *sli-1*). The second class yielded healthy progeny, no dead larvae, no dead eggs, and many Egl animals with dead larvae or dead eggs inside (*sli-1* almost completely suppresses the F1 lethality caused by *sem-5(ay73)* mutation). These F2s were likely to be *sli-1 sem-5(ay73)/sli-1+* recombinants. The third class produced no progeny and were Egl by themselves. This final class of F2s were *sli-1 sem-5(ay73)*. Since the *sli-1 SEM-5(ay73)* strain cannot be maintained as homozygotes, *szT1* was used to balance *sli-1 sem-5(ay73)*. The presence of *sli-1* mutations in Egl animals was further confirmed by sequencing.

To construct *sli-1 gap-1* double mutants, *sli-1* males were mated into *gap-1 unc-2* hermaphrodites. Non-Unc cross-progeny were individually isolated and checked for Unc progeny. These Unc progeny then were picked singly. Only those recombinants of the genotype *sli-1 gap-1 unc-2/+ gap-1 unc-2* can segregate Muv progeny if *sli-1 and gap-1* synergize to confer the excessive vulval differentiation. On rare plates, Muv progeny were observed and picked singly in the next generation. In their progeny, the low penetrance of

notch-head phenotype was used to confirm the presence of *sli-1* mutation.

The *let-60; sli-1* strains were constructed as follows: N2 males were mated into *unc-24(e138) let-60(rf or null)/DnT1; +/DnT1* strains. *let-60(rf)* alleles used were mentioned above. Non-Unc cross-progeny males were selected and mated into *dpy-20; sli-1* hermaphrodites. Non-Dpy cross-progeny males then were picked and mated into *dpy-20; sli-1*. Non-Dpy hermaphrodites were then picked singly. From those F1 plates that yielded Unc progeny, Unc non-Dpy and non-Unc non-Dpy progeny were individually isolated. Since *sli-1* did not suppress *let-60* lethality, nonrecombinant Unc non-Dpy progeny did not survive past F1. The strains were maintained as non-Unc non-Dpy heterozygotes of the following genotype: *unc-24(e138) let-60(rf or null) +/+ + dpy-20; sli-1*. F1 Unc non-Dpy progeny then were picked singly and scored for vulval differentiation. Each scored worm was recovered from the slide and allowed to propagate to check for F2 progeny. Vulval differentiation scores from those that gave viable F2 progeny yielding a proportion of Dpy animals were discarded as they represented recombinants that were not homozygous for *let-60*.

To construct the heteroallelic series, the strain *dpy-10(e128); unc-24(e138) let-60(n2021)/DnT1; +/DnT1* was made by standard methods. *let-60(n2021)* is a weak rf allele. To generate the various heteroallelic worms, N2 males were mated into the *unc-24(e138) let-60(rf or null)/DnT1; +/DnT1* strains carrying the different severe *let-60* mutations. Non-Unc cross-progeny males were selected and mated into the *dpy-10(e128); unc-24(e138)let-60(n2021)/DnT1; +/DnT1* strain. Unc-24, non-Dpy, non-DnT1 F1 hermaphrodites from these crosses were scored for vulval differentiation. Scored worms were recovered, and their progeny were checked to ensure that *let-60(rf or null)* remained homozygous.

Other strains were constructed following standard methods.

An In Vivo Transgenic Assay for the Activity of sli-1 Minigenes

To establish a system in which to study the relationship between SLI-1 structure and its function, we constructed *sli-1* cDNA minigene constructs driven by the heat shock promoter *hsp16–41* (Stringham *et al.*, 1992; Mello and Fire, 1995). To facilitate expression, an artificial intron is present between the promoter and the cDNA insert. Heat shock promoters were used in these experiments because the initial tests using genomic sequences 5′ to the start codon of *sli-1* fused to the full-length *sli-1* cDNA failed to confer significant wild-type SLI-1(+) activity in the vulva (our unpublished results), presumably because sequences present within the introns of *sli-1* are necessary for expression.

Germline transformation was performed according to the methods of Mello *et al.* (1991). Heat-shock constructs were injected at 50 ng/μl along with 150 ng/μl SK+ plasmid and 15 ng/μl pMH86 (*dpy-20*(+) marker) into the germline of *let-23(sy1); dpy-20; sli-1* animals. Independent non-Dpy transformant lines were maintained and selected for transgene stability. F3 stable transgenic progeny were selected for egg-lay cohorts and heat shock analysis. For the egg-lay cohorts, we selected only those worms with a egg-laying rate similar to that of wild-type hermaphrodites (~5–10 eggs laid per worm per hour).

Thirty egg-laying young hermaphrodites were moved onto fresh agar plates, 10 worms per plate. These then were allowed to lay eggs on given plates for 1 h before being moved to fresh plates. The hourly transfer of worms was continued for 6 consecutive hours, and the egg-laying hermaphrodites then were removed from the plates. The plates were maintained at 20°C during and after the establishment of the egg cohorts. 36 h after the hermaphrodites' removal from the final cohort of eggs, the plates were heat shocked for 30 min at 33°C. Thus, we generated worms synchronized in age into hourly cohorts that had been heat shocked between 36 and 42 h after egg laying. This interval encompasses the early L3 stage during which vulval induction occurs in the transgenic animals. The heat-shocked plates were returned to 20°C for 18–24 h after heat shock.

Vulval differentiation was scored in non-Dpy progeny at early to mid L4 stages. A minimum of two independent lines was scored per heat shock construct. Fifteen to 30 worms were scored per 1-h time interval per stable line after heat shock. The distributions generated from each heat shock construct (as seen in the histograms of Figure 4B) were compared in pairs using the Mann-Whitney test to generate two-tailed p values. There is approximately a two-cell range in the average induction levels between the no-insert control and the full-length *sli-1* cDNA construct. This range allows comparisons of the various induction levels in different minigene constructs. In the structure–function experiments, SLI-1 activity is inferred in those minigene constructs that lower average vulval differentiation to significantly <2.5 vulval cells per worm.

sli-1 Minigenes and Mutagenized let-23 Constructs

Site-directed in vitro mutagenesis was carried out in double-stranded DNA according to the method prescribed by Deng and Nickoloff (1992) and with reagents and specific protocols from Clonetech (Palo Alto, CA). The mutagenesis was carried out in the plasmid pCY-D6, a pSK+ vector containing the full-length *sli-1* cDNA inserted into the *Eco*RI site. A selection primer that changed the novel *Not*I site in the vector to an *Nhe*I site was used in conjuction with mutagenic primers that mutated or removed sequences of varying length within the *sli-1* coding region. Mutagenized *sli-1* cDNA then was digested with *Spe*I/*Eco*RV and was inserted into the *Nhe*I/*Eco*RV sites of the pPD49.83 and pPD49.78 nematode heat shock minigene vectors. Mutagenesis was confirmed by restriction mapping and by sequencing. The deletion constructs SLI-1.ΔNΔRINGΔC, SLI-1.ΔC, SLI-1.ΔNΔRING, SLI-1.ΔPro2, and SLI-1.ΔPro3 replace the deleted sequences with an *Nhe*I site that codes for alanine and serine in frame with the remainder of the *sli-1* construct. Primers used for in vitro mutagenesis of the *sli-1* cDNA are as follows: SKNotNhe; Del-NC(Nhe); Del-C(Nhe); Del-N(Nhe); Altspli; Del-PRO2(Nhe); Del-PRO3(Nhe); Del-RING.

For the deletion of the putative myristylation site, we utilized a selection primer which changed a unique *Xho*I site into a *Cla*I site in the various mutagenized *sli-1* cDNAs. Primers used were SKXhoCla and Del-MYR1. The cloning into heat shock vectors followed identical procedures as above.

The conserved N-terminal domain of human c-Cbl was PCR amplified from the cDNA in a pUC vector (provided by W. Langdon) using the primers hCbl-N1A(Nhe) and hCbl-N2R(Nhe). The ends of the amplification primers contained in-frame *Nhe*I sites. The amplified conserved N-terminal fragment of c-Cbl then was purified, digested, and ligated into the *Nhe*I site of the SLI-1.ΔN construct in pPD49.83. The directionality of the insert was checked by restriction digests, and the sequence of the amplified c-Cbl fragment was verified by DNA sequencing. DNA sequencing was carried out on automated sequencers (Applied Biosystems, Foster City, CA) by the California Insitute of Technology DNA Sequencing Facility.

All the systematically mutagenized *let-23* constructs were made as described by Lesa and Sternberg (1997). Engineered *let-23* constructs were injected into the germline of *let-23(sy17)unc-4/mnc1; dpy-20; sli-1* or *let-23(sy17)unc-4/mnc1; dpy-20 ark-1* mothers; the Unc-4, non-Dpy stable transformed progeny (F2 or later generation) were tested for effects in vulval differentiation.

Primer Designations

Primer names and their corresponding sequences used during in vitro mutagenesis are as follows:

SKNotNhe:5′- ACCGCGGTGGCTAGCGCTCTAGAAC

Del-NC(Nhe):5′- GCCCGGTTTCTGCAGTGAAGAGGCTAGCT-AGACTTGTGTAAATGTTCATCTTACC

Del-C(Nhe):5′- GTGTGATTATTGACAGGTTCAAGCCCGCTA-GCTAGACTTGTGTAAATGTTCATCTTACCG

Del-N(Nhe):5′- GATGCCCGGTTTCTGCAGTGAAGAGGCTAGCACTCCGGTAGAAATTGAAAAAGCG
Altspli:5′- CCCGACGTGCCTCCCAGAACGTCGTCACAAACATCCTCTTCATACG
Del-PRO2(Nhe):5′- CTCAATTCCGTCGGTCGACGAGGCTAGCGCATTGGGTACCCTGGACAC
Del-PRO3(Nhe):5′- GGCACAAGTGGTAAACCGGCAACGGGCTAGCTCAGCGAGCGAGCACCAACCACACC
DEL-RING:5′- TTGTGAGATGGGCACAACATTCGAGTACGAAATCAAAGGAACAAATCGTGT
SKXhoCla:5′- ACCGTCGACATCGATGGGGGGCCCG
Del-MYR1:5′- GTTTCACCGGGAATGGCTAGCATAAACACAATTTTTC
hCbl-N2R(Nhe):5′- GCCACTGCTAGCAGGATCAAACGGATCTACCAC
hCbl-N1A(Nhe):5′- CCGCCGGGGGCTAGCGACAAGAAGATGGTGGAG

Microscopy

The extent of vulval differentiation was measured by examining vulval anatomy in early-to-mid-L4-stage animals (Han *et al.*, 1990). Hermaphrodites were placed on 5% Noble Agar pads and were scored with a Plan 100x objective, Nomarski differential interference-contrast optics. Vulval fates of 1° and 2° were scored as vulval cells. Wild-type was equal to three VPCs undergoing vulval differentiation per worm, which equaled 100% vulval differentiation.

RESULTS

sli-1(lf) Suppresses a Severe rf Allele but Not a Null Allele of SEM-5

Mutations in *SEM-5* result in a partial or complete lack of a vulva. *SEM-5* encodes an SH3-SH2-SH3 domain containing the Grb2 homologue, which is required to transduce the signal from LET-23 to LET-60 Ras (Clark *et al.*, 1992a, 1992b; Stern *et al.*, 1993; Katz *et al.*, 1996; Lesa and Sternberg, 1997). *SEM-5(ay73)* is an early missense mutation, Q10amber (Q at codon 10 mutated into an amber stop), that removes all of the functional domains of the SEM-5 protein and is, therefore, a putative null allele (M. Stern, personal communication). Animals homozygous for *ay73* are inviable in the F2 generation, with F1 escapers (due to maternal rescue) being completely vulvaless (Table 1). The *sem-5(n1619)* allele generates a P49L substitution, which most likely results in a nonfunctional N-terminal SH3 domain (Clark *et al.*, 1992a). Animals homozygous for *n1619* are also inviable in the F2, and viable F1 animals have, on average, only 0.4 vulval cells induced per animal. In wild-type animals, three cells are invariably induced to form the vulva.

We constructed *sli-1 sem-5* double mutants using the *sli-1* reference allele, *sy143*. The molecular lesion associated with *sy143* is Q152amber, which removes the carboxyl-terminal 75% of the SLI-1 protein. In addition, it is the most severe allele and likely generates a nonfunctional product (Jongeward *et al.*, 1995; Yoon *et al.*, 1995). *sli-1* strongly suppresses the vulval defect in *sem-5(n1619)* mutants: *sli-1 SEM-5(n1619)* have near wild-type levels of vulval differentiation (average, 3.0 vulval cells per worm; not all animals have wild-type level of vulval induction; Table 1, Figure 2D). The lethality of the *sem-5(n1619)* allele is also partially suppressed: double mutants can be maintained as homozygotes. In contrast, *sli-1* does not suppress the *sem-5(ay73)* null allele: *sli-1 sem-5(ay73)* double mutants are Vul when scored as F1 homozygotes and cannot be maintained as homozygotes beyond F2 from heterozygous mothers (Table 1, Figure 2F). However, there is increased survival of *sli-1 sem-5(ay73)* F1 homozygotes from heterozygous mothers in comparison with *sem-*

Table 1. *sli-1* suppression of mutations in *let-23, sem-5*, and *let-60*[a]

	Average no. of VPCs undergoing vulval differentiation	
Genotype	*sli-1(+)*	*sli-1(sy143)*
+	3.0 (many animals)	3.0 (n = 20)[b]
let-23(sy1)	0.8 (n = 29)[b]	4.3 (n = 30)
let-23(sy97)	0.0 (n = 20)[b]	2.9 (n = 131)
sem-5(n2019)	0.5 (n = 20)[b]	2.6 (n = 23)[b]
sem-5(n1619)	0.4 (n = 40)	3.0 (n = 84)
sem-5(ay73)	0.0 (n = 20)	0.02 (n = 23)
gap-1(n1691)	3.0 (n = 21)	3.2 (n = 24)
let-60(n1876)	0.0 (n = 62)	0.08 (n = 80)
let-60(n2034)	0.02 (n = 62)	0.03 (n = 87)
let-60(n2035)	0.05 (n = 66)	0.09 (n = 81)

[a] Extent of vulval differentiation in *let-23, sem-5, gap-1*, and *let-60* mutants in *sli-1(+)* and *sli-1(sy143)* backgrounds. The number values represent the average number of VPCs undergoing vulval differentiation per worm (3.0 is wild-type level).
[b] Data from Jongeward *et al.* (1995).

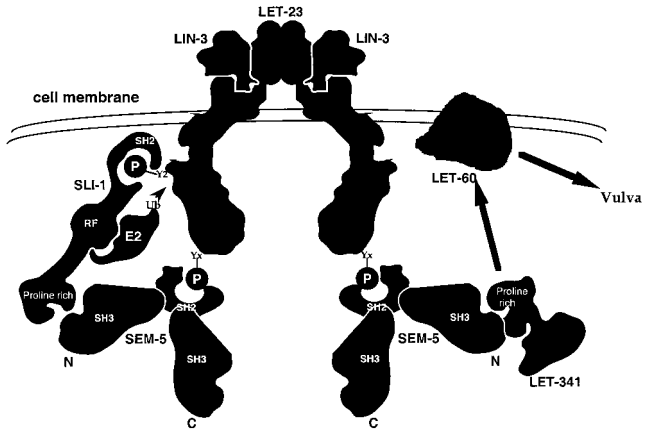


Figure 1. Models for negative regulation of LET-23 EGFR signaling by SLI-1. The interpretation is based on our molecular genetic analyses presented here or on biochemical assays of others presented in other model systems. LIN-3 binding to the extracellular domain induces LET-23 dimerization and autophosphorylation of tyrosine residues that are located in its C-terminal region (C), including Y2. The latter targets for the SH2 domain of SLI-1. Localization of SLI-1 to the active receptor presumably enables RING finger (RF) domain to recognize the substrates and promote their ligation to ubiquitin via E2 molecule, marking active receptor for degradation. The recruitment of SLI-1 to the active receptor might be helped by the binding of the SEM-5 to the receptor Yx sites. The interaction between SLI-1 and SEM-5, which is mediated by the proline-rich domain of SLI-1 and the SH3 domain of SEM-5, might interfere with the localization of LET-341 to the cell membrane by SEM-5. Yx represents the docking sites in LET-23 for SEM-5.

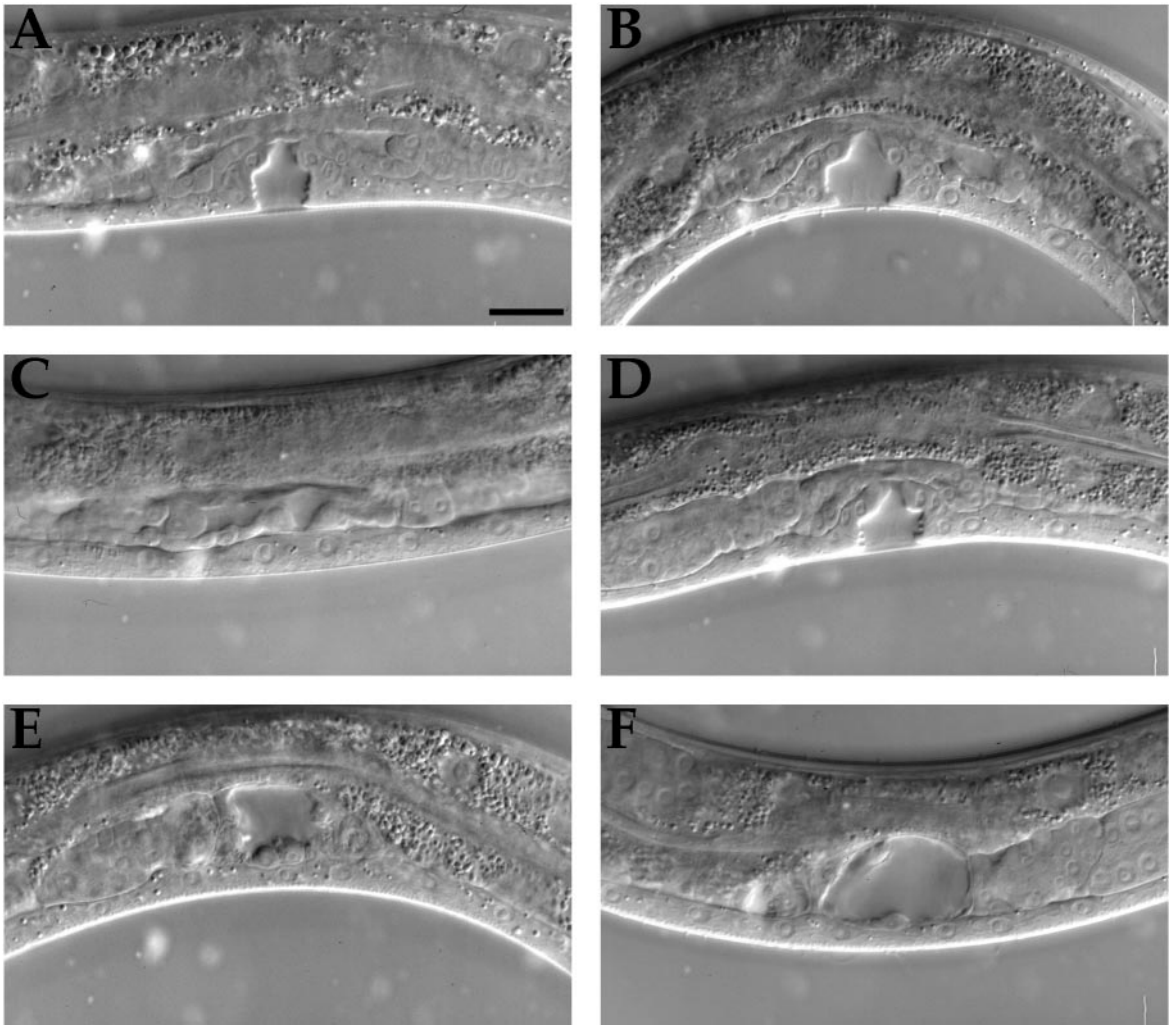


Figure 2. Nomarski micrographs of vulval induction in various genetic backgrounds: (A) wild-type; (B) *sli-1(sy143)*; (C) *sem-5(n1619)*; (D) *sli-1(sy143) sem-5(n1619)*; (E) *sem-5(ay73)*; and (F) *sli-1(sy143) sem-5(ay73)*. A, B, and D have phenotypically wild-type vulvae. C, E, and F are phenotypically Vul, where all VPCs have generated epidermal progeny (note the lack of vulval openings in these panels). In all panels, anterior is to the left and dorsal is toward the top. The scale bar is 20 μm.

5(ay73) single-mutant F1 homozygotes. Thus, mutations in *sli-1* can enhance maternal rescue of lethality associated with *sem-5*, which is consistent with the absence of *sli-1* compensating for a reduction in *sem-5* activity.

sli-1(lf) Does Not Suppress Severe rf Alleles of let-60

We tested three *let-60* alleles for suppression by *sli-1*. These *let-60* alleles, *n1876* (Ala66Thr), *n2034* (Ala66Thr), and *n2035* (Ala66Val), are described by Beitel *et al.* (1990). Corresponding mutations have been made in Ha-Ras and have been shown to interfere with exchange factor interaction (Howe and Marshall, 1993; Boriack-Sjodin *et al.*, 1998). We find that *sli-1* fails to suppress the vulvaless phenotype of these alleles (Table 1). In addition, *sli-1* also fails to suppress the lethality associated with these three *let-60* alleles. However, as in the case with *sem-5(ay73)*, there is an increase in F1 homozygote survival from *let-60(rf)/+* heterozygous parents in a *sli-1* background, indicating enhanced maternal rescue of lethality (our unpublished results).

sli-1 fails to suppress null alleles of *let-23* and *sem-5*, while it strongly suppresses severe rf alleles of *let-23* and *sem-5* (Jongeward *et al.*, 1995; this study). It was therefore crucial to determine whether the tested *let-60* alleles, *n1876*, *n2034* and *n2035*, were non-null alleles. To distinguish between severe non-null alleles and null *let-60* alleles, we measured the extent of vulval differentiation in a series of F1 trans-heteroallelic animals using a weak rf *let-60* allele (*n2021*), *in trans* to the three *let-60* alleles (*n1876*, *n2034*, and *n2035*), as well as a putative *let-60* null allele (*sy127*) and *sDf8*, a deficiency that uncovers the *let-60* locus as controls. *let-60(n2021)* is also an exchange factor defective mutant (G75S) (Howe and Marshall, 1993). If *n1876*, *n2034*, and *n2035* are non-null mutations then *in trans* to *let-60(n2021)* should be less severe

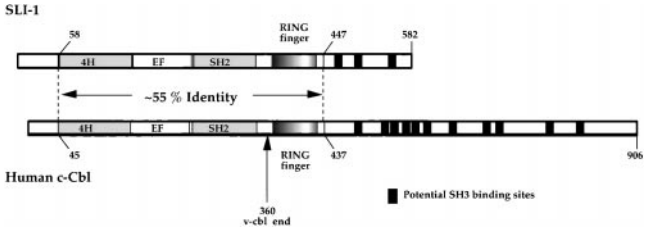


Figure 3. Schematic alignment of SLI-1 and human c-Cbl. Conserved domains between the two proteins have been marked in the same manner. Residues 58–447 of SLI-1 share 55% amino acid sequence identity to residues 45–437 of human c-Cbl. The four-helix bundle (4H), EF hand, SH2 domain, the RING finger, and the putative SH3-binding polyproline domains are indicated. An arrow marks the C-terminal truncation point of v-Cbl. The figure is a modified version from Yoon *et al.* 1995 and Meng *et al.* 1999. The keys for this figure are retained in the structure–function analysis presentation in Figure 4.

than *let-60(null)/let-60(n2021)* heterozygote controls in a vulval induction assay. In these experiments, *n2035* and *n1876* had significantly more activity than the *sy127* null allele and the *sDf8* deficiency in the vulva (1.6 and 1.4 cells versus 0.8 and 0.6 cells being induced on average [$n > 22$]). Therefore, *n1876* and *n2035* are likely to be rf alleles. Thus, *sli-1(rf)* fails to suppress non-null *let-60(rf)* mutations in the vulva. Since a lack of *sli-1* activity can compensate for the decreased function of *let-23* and *SEM-5*, but not *let-60,* we conclude that SLI-1 acts upstream of RAS activation.

Mutations in sli-1 Give a Muv Phenotype in a gap-1 Mutant Background

GAP-1 is a negative regulator of vulval signaling that likely acts at the level of LET-60 Ras (Hajnal *et al.*, 1997). A *gap-1(lf)* reference allele suppresses the Vul phenotype of *let-60(n1876)* while only weakly suppressing the *sem-5(n2019)* mutation (Hajnal *et al.*, 1997). As is the case with *sli-1* single mutants, *gap-1* mutations by themselves are silent.

We constructed *sli-1 gap-1* double mutants. This strain has a partially penetrant Muv phenotype, with 7 of 24 observed double mutants exhibiting an excessive vulval induction (Table 1). *gap-1* corresponds to a Q149ochre nonsense mutation, which results in early truncation and presumably defines a null allele (Hajnal *et al.*, 1997). The simplest interpretation of this result is that SLI-1 and GAP-1 have, at least partially, distinct activities.

The Conserved N-terminal Region and RING Finger Domain Rather Than the Proline-rich C-terminal Region Is Sufficient for the Inhibitory Function of SLI-1

We established an in vivo transgenic assay for the activity of *sli-1* minigenes being expressed from the *hsp16–41* heat-shock promoter in the genetic background of *let-23(sy1); sli-1* (see MATERIALS AND METHODS). The vulvaless phenotype of *let-23(sy1)* animals is strongly suppressed by *sli-1* (see Table 1). In control transgenic animals of genotype *let-23(sy1); dpy-20; sli-1; syEx*[dpy-20(+); hsp16–41] that were heat shocked at the time of vulval induction, the average vulval induction is 2.5 cells per worm (Figure 4). Under the same experimental conditions and genetic background, the full-length *sli-1* cDNA driven by the *hsp16–41* promoter confers full SLI-1(+) activity with animals having, on average, 0.5 VPCs executing a vulval fate per worm (henceforth referred to as vulval cells per worm; Figure 4). This is a full two cells lower than the vulval induction seen in the no-insert control and is comparable to the levels of vulval differentiation seen in *let-23(sy1)* single mutants in a non-Dpy-20 germline-transformed background (0.3 vulval cells per worm) (Yoon *et al.*, 1995).

In this transgenic assay, SLI-1 proteins lacking either the second or third proline-rich domain retained full activity (average induction, 0.7 and 0.3 vulval cells per animal, respectively; $p = 0.14$ and 0.39, respectively). Likewise, the expression of the alternatively spliced *sli-1* cDNA, which deletes the 10th exon of the genomic sequence, has activity similar to that of full-length SLI-1 (average, 0.9 vulval cells per worm; $p = 0.05$; Figure 4A). Moreover, we found that expression of a SLI-1 protein truncated after residue 447, and thus lacking the entire proline-rich C-terminal domains, also retains significant SLI-1(+) activity (average, 1.0 vulval cells per worm; $p < 0.0001$ versus no-insert control; Figure 4, A and B). However, this level of activity is also significantly weaker than that of the full-length *sli-1* cDNA ($p = 0.0002$). To test whether proline-rich C-terminal domains are partially sufficient for SLI-1 function, a *sli-1* construct lacking the N-terminal region and RING finger domain (deleting residues 58 to 447) was tested; it does not have SLI-1(+) activity under identical assay conditions (vulval induction levels similar to the no-insert control; $p = 0.8$; Figure 4A, B).

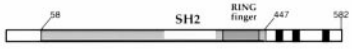
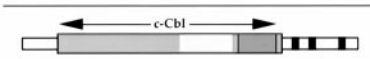
Consensus Myristylation Site Is Not Required for SLI-1(+) Activity

In the conceptual translation of SLI-1, the initiator methionine is followed in sequence by Gly-Ser-Ile-Asn-Thr, a myristylation site (reviewed by Resh 1994). Myristylation may play a role in localizing proteins to the cell membrane, usually in combination with other lipid modifications or nearby basic residues (Resh, 1994). Using site-directed mutagenesis, we substituted the second codon glycine with alanine (G2A substitution); alanine should maintain the charge-neutral hydrophobic character of glycine but should prevent the covalent addition of myristate at the second codon. Constructs expressing the full-length and truncated SLI-1 proteins with G2A substitutions do not have less activity than control constructs (Figure 4A; $p > 0.2$). In addition, the SLI-1 construct that lacks the N-terminal conserved SH2 and RING finger domains has no activity in the vulva after the G2A substitution (Figure 4A). As mentioned previously, the first ~50 amino acid residues of c-Cbl are highly divergent in sequence from those of SLI-1, and the consensus myristylation sequence is not conserved in c-Cbl, Cbl-b or Cbl-3.

The Conserved N-terminal Region of c-Cbl Containing the SH2 and RING Finger Domains Can Functionally Replace that of SLI-1

The human c-Cbl protein is 906 amino acid residues in length. The ~400 amino acid stretch of high sequence similarity (~55% identity) between c-Cbl and SLI-1 begins after

A

Injected sli-1 heatshock minigene constructs (schematic)	Construct designation	Average number of VPCs undergoing vulval differentiation (individual lines)	Average number of VPCs undergoing vulval differentiation (combined)
No cDNA insert	Control	2.54 (n=40) 2.42 (n=18)	2.5 (n=58)
	SLI-1	0.36 (n=40) 0.56 (n=40)	0.5 (n=80)
	SLI-1.ΔPro3	0.30 (n=33) 0.37 (n=30)	0.3 (n=63)
	SLI-1.ΔPro2	0.47 (n=31) 1.00 (n=24)	0.7 (n=55)
	SLI-1.ΔExon10	0.82 (n=26) 0.89 (n=30)	0.9 (n=56)
	SLI-1.ΔC	0.86 (n=40) 1.14 (n=35)	1.0 (n=75)
	SLI-1.ΔNΔRING	2.66 (n=40) 2.16 (n=40)	2.4 (n=80)
	SLI-1.Δmyr	0.48 (n=35) 0.55 (n=32)	0.5 (n=67)
	SLI-1.ΔCΔmyr	0.89 (n=53) 0.83 (n=45)	0.9 (n=98)
	SLI-1.ΔNΔRING Δmyr	2.46 (n=40) 2.90 (n=20)	2.6 (n=60)
	SLI-1.ΔNΔRING ΔC	2.56 (n=40) 2.40 (n=40)	2.5 (n=80)
	SLI-1.ΔNΔRING +CBL.N-RING	1.38 (n=60) 1.75 (n=40)	1.5 (n=100)
	SLI-1.ΔRING	1.19 (n=31) 1.69 (n=35)	1.5 (n=66)

B

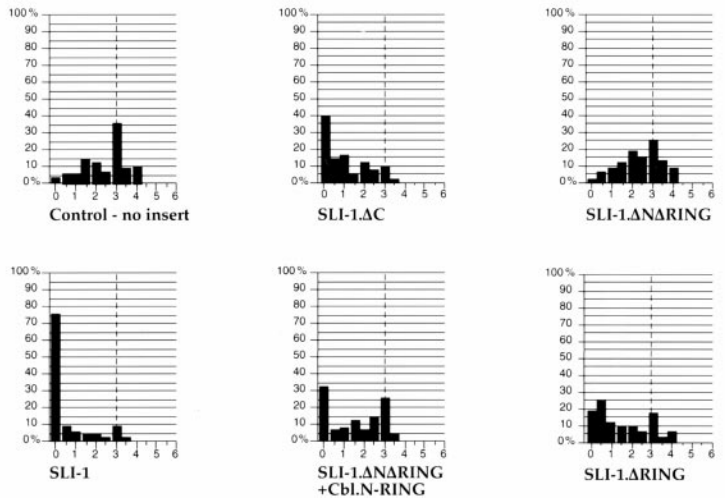


Figure 4. (A) Vulval differentiation in germline-transformed progeny carrying various minigene constructs, as indicated. Vulval differentiation is scored in L4 non-Dpy progeny after heat shock in early L3 (genotype *let-23(sy1); dpy-20; sli-1; syEx[heat shock construct + pMH86(dpy-20+)]*). The first column contains schematic representations of the mutant constructs inserted into the heat-shock minigene pPD49.83. The second column lists construct designations. Average number of VPCs that undergo vulval differentiation are calculated in the third column for each independent stable line. The results are averaged from the combined data of column 3 in column 4. (B) Histograms. The distribution of vulval differentiation levels of the combined data (column 4, panel A) obtained using the various heat-shock minigene constructs described in panel A. The horizontal scale (1–6) represents the number of VPCs that became vulval tissue per worm, scored in half-cell increments. Vertical scale (0–100%) represents the percentage of total worms scored with a given level of vulval differentiation. The constructs are listed directly below each histogram, and the names correspond to the construct designations of panel A.

a stretch of ~50 N-terminal residues in the two proteins (Figure 3). This region contains the SH2 and RING finger domains strongly implicated in Cbl function (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999). The N-terminal 45 and 58 residues of c-Cbl and SLI-1, respectively, are highly divergent in sequence. Furthermore, although both proteins have several putative SH3-binding polyproline motifs in their respective C-terminal domains, the amino acid sequence after residue 447 in SLI-1 and residue 437 in c-Cbl are also largely divergent.

To test for the functional similarity between the conserved domains of SLI-1 and c-Cbl, we constructed a chimeric sli-1/c-cbl cDNA by replacing the coding sequence of SLI-1's N-terminal region and RING finger domain (residues 58–447) with that of c-Cbl (residues 45–437). We find that this c-Cbl/SLI-1 chimeric construct driven by the *hsp16–41* promoter exhibits partial SLI-1(+) activity (1.5 vulval cells per worm; $p < 0.0001$ versus both the no-insert control and the full-length *sli-1* cDNA; Figure 4, A and B). As a control, a *sli-1* construct lacking the N-terminal region and RING finger domain (deleting residues 58–447) was tested. It was shown not to have SLI-1(+) activity under identical assay conditions (i.e., vulval induction levels similar to the no-insert control; $p = 0.8$; Figure 4, A and B). However, the full-length c-Cbl cDNA driven by the *hsp16–41* promoter does not have SLI-1(+) activity in the vulva (our unpublished results).

A SLI-1 Protein Lacking the RING Finger Domain Retains Some Activity

Both *sli-1* and *c-Cbl* function as negative regulators of signaling. c-Cbl recently has been shown to have a ubiquitin ligase activity that is proposed to explain the negative regulation of RTKs signaling observed (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999). Thus, the SH2 domain presumably mediates binding to the activated receptor, and the RING finger domain then catalyzes receptor ubiquitination. The SH2 and RING finger domains of c-Cbl share 55% identity with those of SLI-1 and can functionally replace them in our transgenic assay. We sought to test whether the RING finger domain of SLI-1 was essential for activity. We found that significant SLI-1(+) activity is observed in constructs lacking the RING finger (average, 1.5 vulval cells per worm; $p < 0.0001$ versus no-insert control; Figure 4, A and B). However, in comparison with the full-length cDNA, the RING deletion significantly reduces the SLI-1(+) activity ($p < 0.0001$). Thus, although the RING finger domain is important for SLI-1 activity, a ubiquitin ligase function for SLI-1 is not the sole inhibitory function of *sli-1*.

pYKTEP Defines an Inhibitory Site in the C-terminal Tail of LET-23, Mediating the Negative Effect of SLI-1

We next explored the possibility that SLI-1 exerts its inhibitory effect by direct or indirect binding to specific pTyr sites in LET-23. The *let-23(sy97)* mutation deletes the last 56 amino acids of the receptor, which include the only three tyrosines (pTyr sites 6, 7, and 8) in the receptor carboxy-terminal tail, which, if phosphorylated, would create SH2 binding sites matching the consensus binding site for the SEM-5 SH2 domain. *sli-1(lf)* strongly suppresses *let-23(sy97)*, which suggests that pTyr site 1 through pTyr site 5 could mediate, directly or indirectly, negative regulation of *let-23* signaling by *sli-1* under the above hypothesis. We introduced engineered *let-23* constructs, with certain codons specifying LET-23 carboxy-terminal tyrosine residues mutated to phenylalanine codons, into nematodes with a *let-23(null)* background with or without a *sli-1* or *ark-1* mutation and assayed their activity by scoring transgenic animals for vulval differentiation. Our results suggest that SLI-1 function is dependent on LET-23 carboxy-terminal tyrosine residues. Particularly, we observed that the inhibition of signaling by the presence of pTyr2 could be overcome by mutations in *sli-1* (Table 2). We therefore sought to determine whether the absence of this site would synergize with mutations in genes known to negatively regulate *let-23* signaling. We did not observe any synergy between *let-23*(*null*); *syEx*[LET-23 ($Y2^-$)] and *sli-1*. However, we observed that *let-23*(*null*); *syEx*[LET-23 ($Y2^-$)] did synergize with *ark-1*, resulting in 34% of animals having greater than wild-type levels of vulval induction (Table 2). This synergy was not observed in *ark-1* animals overexpressing an intact LET-23 construct (Table 2). *ark-1* was isolated as an enhancer of *sli-1* and encodes a non-RTK related to Ack that inhibits LET-23 signaling (Hopper *et al.*, 2000). Forty percent of *ark-1*; *sli-1* double-mutant animals have a hyperinduced vulval phenotype, with the extra induction usually being in P4.p (Table 3). This phenotype is distinct from *ark-1*; *gap-1* double-mutant animals, which have a higher penetrance of extra induction, usually as a consequence of P8.p induction (Table 3). It is striking that the hyperinduced vulval phenotype of

Table 2. pTyr site 2 in the carboxyl-terminal tail of LET-23 mediates negative regulation of LET-23 signaling by SLI-1[a]

	Average vulval induction (%)[b]		
C-terminus	*ark-1(+); sli-1(+)*[c]	*sli-1(−)*	*ark-1(−)*
1 2 3 4 5 6 7 8	99 (n = 22)		91 (n = 63)[d]
– – – – – – – –	4 (n = 42)		
– 2 – – – – – –	. . .[e]	. . .[e]	
– – 3 – – – – –	3 (n = 11)	8 (n = 28)	
– – – 4 – – – –	93 (n = 42)	94 (n = 8)	
– – – – 5 – – –	11 (n = 53)	21 (n = 22)	
– – – – – 6 – –	100 (n = 65)	89 (n = 67)	
– 2 – 4 – – – –	42 (n = 85)	86 (n = 47)	48 (n = 76)
1 – 3 4 5 6 7 8	99 (n = 26)	82 (n = 41)	107 (n = 32)[f]

[a] Carboxyl-terminal sites 1, 2, 3, etc., indicate that tyrosine(s) at that putative SH2-binding sites have not been changed; a dash indicates that each tyrosine in the corresponding site has been substituted with phenylalanine. At least two stable lines were analyzed per construct, except for the site 3 construct, which has only one stable line analyzed.

[b] Average percentage of vulval precursor cells adopting vulval fates per animal.

[c] Taken from Lesa and Sternberg (1997).

[d] One animal of 63 had greater than three cells induced (3.5-cell induction).

[e] The presence of site 2 in the absence of other carboxyl-terminal sites confers animals inviability in the genetic backgrounds of *let-23(lf). sli-1(lf)* weakly suppresses this lethality.

[f] Thirty-four percent of scored animals are Muv.

Table 3. Frequency of induction of the Pn.p cells in different mutants[a]

Mutants	Frequency of being induced (%)						Average induction	n
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p		
ark-1; sli-1	1.3	26.3	100	100	100	6.3	3.35	40
ark-1; gap-1	5	15	100	100	100	80	4.0	20
ark-1; Y2$^-$	3.2	25.8	100	100	100	5.6	3.35	62

Animals scored at 20°C. *ark-1; Y2*$^-$ animals actual genotype is *let-23(null) unc-4; dpy-20 ark-1; syEx[dpy-20(+); let-23(Y2*$^-$*)]*. Only those animals with greater or equal to three cells induced scored for *ark-1; Y2*$^-$. Data were pooled from three independent lines.

let-23(*null*); *ark-1*; *syEx*[LET-23 (Y2$^-$)] is nearly identical to that seen in *ark-1*; *sli-1* animals, both in penetrance and frequency of P4.p induction versus P8.p induction (Table 3).Together, this led us to conclude that pTyr 2 (pYKTEP) mediates its effects through *sli-1*. This conclusion is strengthened by a recently solved crystallographic structure of c-Cbl complexed to a tyrosine-phosphorylated inhibitory site of protein tyrosine kinase ZAP-70 (Meng *et al.*, 1999). The interaction is mediated by a divergent SH2 domain of c-Cbl, which is conserved in SLI-1, and a pTyr of ZAP-70, which is in a similar amino acid context to the one in LET-23 that we identified (pYXXEP versus pYKTEP; XX represents pY+1 and pY+2 residues, which are not in the interface of the binding complex).

DISCUSSION

The *sli-1* locus was originally defined by mutations that suppress partially defective LET-23, a *C. elegans* homologue of EGF-receptor subfamily tyrosine kinases (Jongeward *et al.*, 1995). The overall sequence of SLI-1 resembles that of the mammalian proto-oncoprotein c-Cbl and related proteins (Cbl-b and Cbl-3). In this study, we have found the following: (1) mutations in SLI-1 suppress rf mutations in SEM-5 but not LET-60 RAS, which are mutations that likely interfere with exchange factor interaction; (2) mutations in SLI-1 do not bypass the requirement for SEM-5, and a *sem-5* null allele is not suppressed by *sli-1*; (3) the SLI-1 N-terminal region and RING finger domain is sufficient to confer partial wild-type SLI-1 activity in the vulva; (4) the conserved N-terminal region and RING finger domain in c-Cbl can substitute for those of SLI-1 such that a chimeric protein can negatively regulate vulval differentiation; and (5) the ubiquitin ligase domain of SLI-1 is not absolutely required for negative regulation by SLI-1.

Our genetic analyses led us to believe that SLI-1 inhibits signaling between LET-23 and RAS for the following reasons. The reduced activity of SLI-1 can increase vulval signaling in a *sem-5(rf)* background: *sli-1*, the reference allele, strongly suppresses the vulvaless phenotype of non-null *sem-5* rf alleles (Jongeward *et al.*, 1995; this study). However, we also have shown that *sli-1* does not suppress a *sem-5* null allele. Thus, SEM-5 activity is essential for RTK-mediated vulval signaling with or without the presence of wild-type SLI-1. We conclude that SLI-1 is a negative regulator of SEM-5-dependent signaling after LET-23 activation. In contrast, severe reduction of LET-60 Ras activity is not compensated for by the absence of SLI-1. We thus infer that SLI-1 affects vulval signaling upstream of LET-60 Ras. Furthermore, the Muv phenotype of *sli-1 gap-1* double-mutant animals indicates that SLI-1 and GAP-1 define, at least partly, independent pathways. Recently, another GAP gene, *gap-2*, has been identified in *C. elegans* (Hayashizaki *et al.*, 1998); however, mutations of *gap-2* do not suppress the vulvaless phenotype of *let-23* mutations (Hayashizaki *et al.*, 1998); (C. Chang, unpublished observations). *gap-1* and *gap-2* are the only RasGAPs found in *C. elegans* by genome-wide blast search.

Results from our SLI-1/c-Cbl chimeric construct indicate that the N-terminal region and RING finger domains of SLI-1 and c-Cbl are functionally conserved with respect to vulval signaling. Our structure–function studies show that the conserved N-terminal region and RING finger domain of SLI-1 are necessary and almost sufficient for negative regulatory activity in LET-23-mediated signal transduction. That the N-terminal region and RING finger domain may have a conserved regulatory function in RTK signaling is supported by two lines of evidence from elsewhere. First, identification of a *Drosophila* c-*cbl* homologue (*Drosophila-Cbl*; or D-*cbl*) revealed that the D-Cbl product shares the conserved N-terminal domain with c-Cbl and SLI-1 (Hime *et al.*, 1997; Meisner *et al.*, 1997). As in the alignment of SLI-1 and c-Cbl, the sequence similarity of D-Cbl begins after a stretch of divergent N-terminal residues that are different from both c-Cbl and SLI-1. However, unlike SLI-1 and c-Cbl, the D-Cbl sequence ends shortly C-terminal to the RING finger motif and contains no polyproline motifs. As expected from the lack of poly-proline motifs, D-Cbl does not bind Drk, the *Drosophila* homologue of SEM-5/Grb2 adaptor (Hime *et al.*, 1997; Meisner *et al.*, 1997). Furthermore, both reports show that D-Cbl associates with the *Drosophila* EGFR in an activation-dependent manner. Finally, Meisner *et al.* (1997) show that the expression of D-cbl under the *sevenless* enhancer in *Drosophila* negatively regulates R7 photoreceptor development. These results suggest that the conserved N-terminal domains of SLI-1 and D-Cbl play analogous roles and that these domains are sufficient for the negative regulation of the LET-23 and *Drosophila sevenless* pathways. Second, it was previously shown that introduction of a hypomorphic Gly-to-Glu missense mutation found in SLI-1's N-terminal domain (Yoon *et al.*, 1995) can abolish c-Cbl binding to ZAP-70 and EGFR and can ablate the transforming function of v-Cbl (Lupher *et al.*, 1996; Thien and Langdon, 1997). Thus, identical mutations in a conserved residue in the N-terminal domains disrupts function in both SLI-1 and c-Cbl, suggesting structural and functional conservation.

It is unclear why the full-length c-Cbl construct does not have SLI-1(+) function in our assays despite the fact that the conserved N-terminal domain of c-Cbl can functionally replace the corresponding domain in SLI-1 in a chimeric construct. Several explanations are possible. One reason may be that the size of the C-terminal domain of c-Cbl prevents sterically an effective association with the cytoplasmic portion of the LET-23 protein; the C-terminal domain of c-Cbl is three times the mass of that of SLI-1. Or, the nematode translational machinery does not properly recognize the initiator methionine of c-Cbl, therefore preventing effective expression. A third possibility is that the highly divergent stretch of residues N-terminal to the conserved domains could serve important species–specific functions.

The C-terminal polyproline motifs, although not essential for SLI-1 function, are necessary for the full wild-type negative regulatory activity; the SLI-1:N+RING finger protein is significantly less effective than the full-length SLI-1 (39% Vul for the SLI-1:N+RING finger protein versus 74% Vul for full-length SLI-1; Figure 4B; $p = 0.0002$). This effect may simply be caused by a reduction in protein stability due to early truncation. However, we have found that the polyproline-rich C-terminus of SLI-1 can interact with SEM-5 in a yeast 2 hybrid assay (this study; Walhout *et al.*, 2000). This raises another possibility for the function of the C-terminal polyproline motifs: SEM-5, or a similar adaptor, may bind to SLI-1 and increase the efficacy of SLI-1 localization to the RTK complex, due to the binding of the adaptor to the receptor pTyr sites. In mammalian cells, the C-terminal polyproline domains of c-Cbl have been shown to bind adaptors such as Grb2 and Nck via PPII helix-SH3 interactions (Rivero-Lezcano *et al.*, 1994; Meisner and Czech, 1995; Donovan *et al.*, 1996; Clements *et al.*, 1999). Furthermore, it has been shown that Grb2 can mediate an indirect association between c-Cbl and EGFR (Meisner and Czech, 1995). In a similar manner, the C-terminal polyproline domains of SLI-1 may bind to SH3 domains of adaptor proteins, which in turn enhance the localization of the N-terminal domain of SLI-1 to the activated LET-23 receptor. In addition, it is also possible that the polyproline domains of SLI-1 may compete with the polyproline domains of Sos in binding SEM-5, thereby enhancing the inhibition of the LET-23 RTK pathway by SLI-1.

The RING finger domain of c-Cbl has been shown recently to enhance ubiquitination of active RTKs by acting as an E3 ubiquitin–protein ligase (Levkowitz *et al.*, 1998; Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999). Thus, the effects of the loss of *sli-1* activity might be explained by the failure of the LET-23 RTK to be down-regulated by a ubiquitination-dependent degradation pathway. However, we find that the RING finger domain of SLI-1 is partially dispensable: an SLI-1 variant lacking its RING finger domain retains a significant amount of biological activity. Thus, the ubiquitin–ligase activity of SLI-1/c-Cbl is unlikely to be the sole activity of SLI-1. This conclusion is strengthened by other findings. It might be expected that if the sole function of SLI-1 was to target-activated receptor for degradation so that (1) there might be more LET-23 observable in a *sli-1(lf)* background, and (2) that over expression of LET-23 might mimic *sli-1(lf)*. Neither of these effects has been observed (Table 2).

In summary, we find that SLI-1 inhibits LET-60 RAS activation by LET-23. We suggest that SLI-1 interacts with the LET-23 signaling complex via at least two domains. One of these domains, the proline-rich C-terminal portion, is necessary for the wild-type level of activity of SLI-1 in the context of a full-length protein; its role is likely to interact with an SH3 domain-containing protein. SEM-5 is a candidate due to the interaction between SLI-1 and SEM-5 that we observed in our yeast two-hybrid assays. Since the C-terminal proline-rich domain alone is not sufficient to inhibit signaling, we infer that SLI-1 does not simply titrate SEM-5 from the RTK/Ras pathway. In addition, an SLI-1 protein lacking this proline-rich C-terminal portion retains inhibitory function on signaling. Thus, the remaining N terminal and RING finger domains must be able to interact with some of the components of the signaling complex directly by a different mechanism. In mammalian cell lines, the conserved N-terminal domain of c-Cbl associates directly with the autophosphorylated C-terminal region of the EGFR (Bowtell and Langdon, 1995; Galisteo *et al.*, 1995; Lupher *et al.*, 1997). The N-terminal domain also has been shown to associate with the non-RTK ZAP-70 in a phosphorylation-dependent manner in T cells (Lupher *et al.*, 1996). In both cases, the association requires the N-terminal divergent SH2 domain and not the C-terminal polyproline motifs. We explored the possibility that SLI-1 may exert its inhibitory effect by direct or indirect binding to specific pTyr sites in LET-23. By analyzing the systematically mutagenized *let-23* constructs containing substitutions in the carboxyl-terminal tyrosine residues, we have identified an inhibitory tyrosine residue that can overcome the negative regulation by *sli-1* when it is mutated. Our current models for *sli-1* functions propose two roles (Figure 1). The major role of *sli-1* might be to attenuate signaling after activation has occurred. On induction, SLI-1 is recruited into the receptor-signaling complex by itself or an adaptor protein, and negative regulation ensues by some other means, possibly by preventing the association and activation of downstream effectors such as the SEM-5 adaptor and/or LET-341 SOS-1, via catalyzing the ubiquitination of the receptor, thus targeting it for degradation. The minor role of *sli-1* might be to regulate the basal activity of signaling in a quiescent state by competing with LET-341 SOS-1 for the binding of SEM-5, thereby decreasing the chance that the spontaneously activated receptor recruits the SOS-1-bound SEM-5 in the absence of ligand. Other models are possible, since we cannot rule out the possible existence of uncharacterized catalytic domains in SLI-1.

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