***Caenorhabditis elegans* SOS-1 is necessary for multiple RAS-mediated developmental signals**

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Vulval induction in *Caenorhabditis elegans* has helped define an evolutionarily conserved signal transduction pathway from receptor tyrosine kinases (RTKs) through the adaptor protein SEM-5 to RAS. One component present in other organisms, a guanine nucleotide exchange factor for Ras, has been missing in *C.elegans*. To understand the regulation of this pathway it is crucial to have all positive-acting components in hand. Here we describe the identification, cloning and genetic characterization of *C.elegans* SOS-1, a putative guanine nucleotide exchanger for LET-60 RAS. RNA interference experiments suggest that SOS-1 participates in RAS-dependent signaling events downstream of LET-23 EGFR, EGL-15 FGFR and an unknown RTK. We demonstrate that the previously identified *let-341* gene encodes SOS-1. Analyzing vulval development in a *let-341* null mutant, we find an SOS-1-independent pathway involved in the activation of RAS signaling. This SOS-1-independent signaling is not inhibited by SLI-1/Cbl and is not mediated by PTP-2/SHP, raising the possibility that there could be another RasGEF.

Keywords: *Caenorhabditis elegans*/proto-oncogene/*ras* pathway/tyrosine kinase/vulval development

Introduction

Polypeptide hormones bind cell surface receptors containing tyrosine kinase activity to regulate cell proliferation, differentiation, migration and metabolism. All receptor tyrosine kinases exhibit a similar molecular architecture and apparently are activated by a common mechanism. Ligand binding to the extracellular domain induces receptor dimerization, which in turn leads to activation of the catalytic kinase domain (Lemmon and Schlessinger, 1994). Autophosphorylation of tyrosine residues that are located in the C-terminal region leads to the physical recruitment of cytoplasmic signaling molecules with phosphotyrosine (pTyr)-recognition modules, such as SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains (Pawson, 1995). These receptor-binding proteins include the adapter Grb2, which leads to Ras activation, Ras-GTPase activating protein, phospholipase C-(PLC)- γ , the tyrosine phosphatase SHP-2, the regulatory subunit of phosphatidylinositol-3-OH kinase (Ullrich and

Schlessinger, 1990; Heldin, 1995; Pawson, 1995), the scaffolding protein Shc (Pelicci *et al.*, 1992) and the proto-oncoprotein c-Cbl (Meisner *et al.*, 1997). The association between proteins with SH2/PTB domains and receptor pTyr serves as the initial step in the recruitment of an activated RTK signaling complex. Different SH2/PTB domains have distinct specificities for the pTyr residues dependent on the surrounding amino acid residues (Panayotou and Waterfield, 1993; Songyang *et al.*, 1993, 1994; Kavanaugh and Williams, 1994; Kavanaugh *et al.*, 1995). Association of RTKs with substrates is therefore dependent upon the amino acid context of the pTyr, and that, in part, determines which proteins join the signaling complex and what cellular responses are evoked (Schlessinger and Ullrich, 1992; Pawson and Saxton, 1999). The achievement of a precise amplitude and duration of the signaling required for the reproducible developmental consequences observed can be obtained by regulating the production of signaling molecules and their presentation to their substrates. Excessive or prolonged RTK signaling has been implicated in many cases of malignant transformation (Alroy and Yarden, 1997; Cheng *et al.*, 1998). Therefore, proper mechanisms must be employed to strongly control the signaling from RTKs to prevent spontaneous, ligand-independent activation and downregulate signaling after it has been activated.

EGF RTK signaling is used multiple times during the normal development of *C.elegans*, mediating vulval induction, viability, male spicule development, hermaphrodite ovulation, and differentiation of the ventral uterus and posterior ectoderm (Ferguson and Horvitz, 1985; Aroian and Sternberg, 1991; Chamberlin and Sternberg, 1994; Clandinin *et al.*, 1998; Jiang and Sternberg, 1998; Chang *et al.*, 1999). The high resolution of the vulval induction assay makes it very sensitive, allowing one to identify mutants and to reflect the relative strength of RAS-dependent signaling (Han and Sternberg, 1991). All the other developmental events are also RAS-dependent, with the exception of hermaphrodite ovulation, which is instead mediated through an inositol trisphosphate signaling pathway (Clandinin *et al.*, 1998).

The wild-type vulva is derived from three of six multipotential vulval precursor cells (VPCs), in response to an inductive signal from the anchor cell (AC) in the somatic gonad (Horvitz and Sternberg, 1991). The induced VPCs undergo three rounds of division and subsequent morphogenesis to form the vulva. VPCs that do not receive adequate signal from the anchor cell divide only once and become part of the worm's large syncytial epidermis. The inductive signal, LIN-3, is an EGF-like growth factor produced by the anchor cell. LIN-3 activates LET-23, a *C.elegans* homolog of EGF RTK, in the VPCs (Aroian *et al.*, 1990; Hill and Sternberg, 1992). LET-23 activation initiates a signaling cascade that involves downstream

effectors such as SEM-5, LET-60, LIN-45, MEK-2 and SUR-1/MPK-1, which are the nematode counterparts of the mammalian Grb2 adaptor, Ras GTP-binding protein, Raf serine/threonine kinase, MAPK kinase and MAPK, respectively (Beitel *et al.*, 1990; Han and Sternberg, 1990; Clark *et al.*, 1992a; Han *et al.*, 1993; Lackner *et al.*, 1994; Wu and Han, 1994; Kornfeld *et al.*, 1995; Wu *et al.*, 1995). Loss-of-function (*lf*) mutations in these signaling proteins result in less than three VPCs undergoing vulval differentiation, whereas gain-of-function (*gf*) mutations result in more than three VPCs undergoing vulval differentiation.

In addition to the main RAS-dependent signaling pathway, several accessory proteins have been identified. *ptp-2*, which encodes an SH2 domain-containing protein similar to the mammalian SHPs and *Drosophila* Corkscrew, appears to affect vulval induction only in a sensitized genetic background and behaves as a positive effector of the LET-23 pathway (Gutch *et al.*, 1998). Loss of function in *ptp-2* suppresses the multiple vulva (Muv) phenotype induced by a loss-of-function *lin-15* mutation, and an activated *let-23* or *let-60* mutation. *lin-15* encodes two novel proteins that somehow inhibit RAS-dependent vulval signaling, and *lin-15* mutation formally acts as if LET-23 was activated in a ligand-independent manner (Clark *et al.*, 1994; Huang *et al.*, 1994).

FGFR is another RTK whose developmental roles are dependent upon RAS. Genes involved in the FGFR pathway were defined by mutations affecting sex myoblast migration, including EGL-17 FGF, EGL-15, SEM-5 and several RAS-MAP kinase cascade components (Stern and Horvitz, 1991; Clark *et al.*, 1992a; Sundaram *et al.*, 1996; Chen *et al.*, 1997). Others, including *soc-1* and *soc-2*, were defined by their ability to suppress a clear (Clr) phenotype, which appears to be caused by excess EGL-15 signaling (Selfors *et al.*, 1998).

Mutations in *let-60/mek-2/mpk-1* cause a delay in pachytene exit during meiosis resulting in sterility (Church *et al.*, 1995). The inductive signal, cognate receptor, and their signal transducers used for *let-60/mek-2/mpk-1*-mediated germline meiotic cell cycle progression are still unknown (Sternberg and Han, 1998).

Ras-dependent RTK signaling pathways are well conserved at the level of their mechanistic aspects throughout metazoan evolution. Ras is activated following the recruitment of Sos to the plasma membrane, which stimulates guanine nucleotide exchange. Recruitment of Sos follows receptor activation and is dependent upon the adaptor protein Grb2. Sos contains two structural domains, CDC25 and DH, which act as exchange factors specifically upon the closely related Ras and Rho family GTPases, respectively (Chardin *et al.*, 1993; Nimnual *et al.*, 1998). An EGF RTK substrate, Eps8, and its associated protein, E3b1, have been shown recently to regulate the transduction of signals from Ras to Rac through Sos (Scita *et al.*, 1999). Despite our understanding of Sos function, obtained primarily from work on flies and mammals, the identification of the exchange factor for RAS in *C.elegans* has remained elusive. For our goal of understanding how receptor tyrosine kinase signaling is regulated, it is necessary to identify all the positive- and negative-acting components. To this end we cloned the previously unidentified *C.elegans* Sos homolog, *SOS-1*, and show that it is encoded by the *let-341* gene. We demonstrate

SOS-1 activity is required for normal RAS activation. In addition to a requirement for SOS-1, we show that there is a SOS-1 independent pathway operating in vulval induction. This SOS-1 independent pathway does not utilize PTP-2. This raises the possibility that there remain other activators of RAS signaling that are yet to be identified.

Results

Isolation of a Sos homolog in *C.elegans*

The *C.elegans* Sequencing Consortium (1998) identified an incomplete genomic sequence, which GeneFinder predicts to encode a *C.elegans* homolog of Sos. By using reverse transcription-PCR (RT-PCR) and 5' and 3' rapid amplification of cDNA ends (RACE), we have been able to obtain a nearly complete cDNA of this gene and confirm that it encodes a Sos homolog (Figure 1). We will refer to this gene as *sos-1* (for son of sevenless homolog). A SL1 trans-spliced leader sequence, which is immediately followed by the putative initiation methionine, has been identified in the 5' end of the cDNA. However, the cDNA is lacking the extreme 3' sequence as the termination codon is still missing. This is due to a highly adenylated sequence in the 3' terminus, which presumably prevents the recovery of the 3' end by use of a poly(dT) primer (Figure 1A). The longest cDNA fragment was sequenced and this 4.2 kb cDNA contains a single long open reading frame (ORF) (DDBJ/EMBL/GenBank accession No. AF251308). We obtained two shorter splice variants predicted to encode proteins that end prematurely immediately after exon 12 and 13 (see Materials and methods). Sequence analysis of the corresponding genomic region encompassing this 4.2 kb cDNA revealed that the coding region of this gene spans a region >30.6 kb and includes a long 13.6 kb intron. Conceptual translation of the 4.2 kb ORF predicted a protein of 1413 amino acids with signature motifs that match the guanine nucleotide exchange factor for Ras. The predicted structure of SOS-1 contains one Dbl homology (DH) domain followed by one pleckstrin homology (PH) domain at the N-terminus and a C-terminal CDC25 catalytic domain followed by two PxxP motifs (Figure 1B). CDC25 domain is the most conserved region in RAS-binding exchange factors among species (Figure 1C).

Phenotypic effects displayed by the progeny of *SOS-1 dsRNA-injected hermaphrodites*

To understand the biological functions of SOS-1, we used double-stranded RNA interference (RNAi) to transiently knock out gene expression during animal development. RNAi has been shown to robustly and efficiently interfere with, if not remove, gene expression in many model systems including *C.elegans*, *Drosophila*, *Trypanosoma brucei* and plants (Fire *et al.*, 1998; Sharp, 1999). A 1.6 kb SOS-1 cDNA, which represents the sequences encoding the C-terminal portion of the DH and the entire PH domains, was subcloned into the pCRII-TOPO vector. The nucleotide sequence of this 1.6 kb cDNA is not similar to any other gene identified by the *C.elegans* Sequencing Consortium (as of April 2000). Sense and antisense RNA were synthesized from T7 and Sp6 promoters, respectively, and sense/antisense annealing was carried out *in vitro* prior to microinjection.

RNAi reveals that SOS-1 participates in those functions mediated by LET-60 RAS. Progeny of hermaphrodites injected with *sos-1* dsRNA showed a low penetrance of L1-L2 lethality (8%; $n = 66$). Animals escaping this early larval lethality generally displayed slow growth and a scrawny body morphology, resembling the phenotype of hypomorphic *egl-15* mutants, an FGFR homolog (61/61 animals examined) (Figure 4B). When examined under Nomarski optics, uterine cells are generally mis-positioned or mis-specified (data not shown). During oogenesis, a significant retardation of pachytene exit is observed (11/11 animals examined) (Figure 3D). By young adult stage, the proximal germline nuclei, which normally undergo spermatogenesis in wild-type animals, remain arrested in the pachytene stage of meiosis I. The more distal nuclei, which presumably undergo oogenesis, also remain arrested in the pachytene phase. Clumped pachytene nuclei can be easily found in the distal arm of the gonad, which might be the result of the diminished size of the gonad.

In addition, adult *sos-1* [RNAi] males generally exhibit short and crumpled spicules (20/20 animals examined) (Figure 3B). *let-60(n1046gf)*, but neither a *let-23(sa62gf)* nor a *lin-15(n765ts)* mutation, suppresses the lethality and scrawny phenotype conferred by the *sos-1* RNAi, implying that SOS-1 functions downstream of LET-23 and upstream of LET-60. The hyperinduced vulval phenotypes observed in *let-60(n1046gf)*, *let-23(sa62gf)* and *lin-15(n765ts)* mutants are suppressed by the *sos-1* RNAi. However, the suppression of *let-60(n1046gf)* is weaker than that of *let-23(sa62gf)* and *lin-15(n765ts)* based on the distributions of vulval induction (Figure 2; Table I). Since all these phenotypes are also exhibited by mutants in RAS signaling pathway, we conclude that SOS-1 acts on RAS during larval development.

SOS-1 is encoded by *let-341*

sos-1 maps to the left arm of chromosome V in a region that is close to the estimated position of *let-341* on the genetic map. *let-341* alleles were originally isolated in a screen for essential genes on the left arm of V (Johnsen and Baillie, 1991). In addition, *let-341* alleles were also isolated as extragenic suppressors of the Multivulval phenotype of *lin-15(lf)* mutants (Clark *et al.*, 1992b) and in a screen for suppressors of the Clr (*soc*) phenotype of *clr-1(lf)* mutants (M.Stern, personal communication). Genetic studies suggest that *let-341* has genetic properties consistent with it encoding a RAS exchange factor, namely that it acts downstream of *lin-15* and upstream of *let-60* (Clark *et al.*, 1992b). We therefore tested whether *let-341* mutants have lesions in *sos-1*. Molecular lesions associated with three *let-341* alleles were found to affect the *sos-1* ORF, indicating that *SOS-1* transcript corresponds to *let-341* gene (Figure 1A). *let-341(s1031)* mutants display a 100% embryonic or early larval lethality, which can be suppressed by a *let-60(gf)* mutation. Sequence analysis reveals that *s1031* contains an AG→TT mutation at the splice donor of intron 13 in *sos-1*. The *n2183* mutation, provided by M.Stern, changes a glutamic acid to lysine at codon 331 within the DH domain (Figure 1). The *ku231* allele, provided by Z.Chen and M.Han, results in the substitution of proline for a conserved histidine at codon 881 right before the start of the CDC25 catalytic domain.

s1031* represents a null allele for *sos-1

As the molecular lesion associated with *s1031* is predicted to alter splicing, we looked at the *sos-1* mRNA produced in this mutant. We could not detect wild-type *sos-1* mRNA in *s1031* mutants by RT-PCR (Figure 5). The subsequent sequence analysis revealed that in *s1031* animals, upstream cryptic splice donors are used and consequently various deletions in the regions encoding the C-terminus of the CDC25 catalytic domain occur (Figures 1C and 5). The truncated transcripts are predicted to encode either a dominant negative or a loss-of-function SOS-1 protein, suggesting that *s1031* completely eliminates SOS-1 activity. Consistent with *s1031* being a null allele, a *sem-5* null or severe reduction-of-function mutation does not further reduce the vulval induction in *let-60(n1046)*; *let-341(s1031)* mutant animals (Table II). To strengthen this conclusion further, we use *sos-1* RNAi to remove any residual *sos-1* activity in *let-60(n1046)*; *let-341(s1031)* mutants. The average vulval induction displayed by the progeny of *sos-1* dsRNA-injected hermaphrodites is 2.6 ($n = 23$), which is not statistically different from that displayed by animals without *sos-1* dsRNA injection [2.7 ($n = 54$)] (Table I). Together, these three lines of observation provide compelling evidence that *s1031* is a null allele.

Since *s1031* is a null allele, we tested whether this mutation alters vulval induction. We used a *let-60(gf)* mutation to rescue the lethality of *s1031* and found that loss of *let-341 sos-1* function reduces vulval induction. Specifically, 41% of *let-60(n1046)*; *let-341(s1031)* mutant animals exhibit less than wild-type vulval induction, further supporting a role of SOS-1 during vulval induction.

Ligand-dependent and SOS-1-independent activation of RAS signaling in the vulva

To test whether *lin-3*-induced vulval signaling is solely mediated by LET-341 SOS-1, we compared the vulval induction in a mutant lacking *lin-3* to a mutant lacking *let-341* SOS-1. Since LIN-3 produced by the gonadal AC is the only inductive source for vulval differentiation, ablation of gonad eliminates the ligand for LET-23-mediated vulval signaling. The extent of vulval differentiation in *let-60(gf)*; *let-341(null)* mutants is significantly higher than that in gonad-ablated *let-60(gf)* mutants (Table II). This observation suggests that not all of the inductive signaling is mediated through SOS-1. This SOS-1-independent pathway could also be SEM-5 independent, since *let-60(gf)*; *sem-5(null)* and *let-60(gf)*; *let-341(null)*; *sem-5(null)* mutants also produce significantly more vulval differentiation than do gonad-ablated *let-60(gf)* mutants. A transgenic line that bears multiple copies of *let-60(n1046gf)* DNA displays completely ligand-independent signaling (Sundaram and Han, 1995). Importantly, this transgenic array does not confer 100% induction in all six VPCs. It has been shown that a weak *lin-45* mutation suppresses the vulval induction conferred by this transgenic line while a weak *sem-5* mutation or the gonad ablation does not. This indicates that gonad ablation does not affect signaling parallel to LET-60 RAS (Sundaram and Han, 1995). Thus, the ligand-dependent and SOS-1-independent pathway are most likely to act at the level of LET-60 RAS.

To address the question of whether LET-23 is required for the SOS-1-independent activation of LET-60 RAS, we assayed the extent of vulval induction in *let-23(sy17 null)*; *let-60(n1046)* double mutants. The average vulval induction of *sy17; n1046* strain shows no difference from that of *n1046* single mutants (Table III). Our interpretation of this observation is that there is a LET-23-dependent negative regulation for vulval signaling, which can be lifted by the presence of the gonadal signal. This model is supported by our other experimental results. Because LET-23 has both positive and negative effects on vulval signaling, we cannot rule out a LET-23-independent response to the gonadal signal.

To test whether this putative SOS-1-independent pathway is under regulation by SLI-1, we crossed a *sli-1* mutation to a genetic background where the SOS-1-dependent pathway has been removed. SLI-1, similar to mammalian proto-oncoprotein c-Cbl, inhibits LET-23 mediated signaling during vulval development (Jongeward *et al.*, 1995; Yoon *et al.*, 1995). We found that a *sli-1(lf)* mutation fails to enhance the vulval induction in *let-60(gf); let-341(null)* mutants, arguing that SLI-1 does not negatively regulate this other pathway parallel to SOS-1 (Table III). This result also supports our

interpretation that *let-341(s1031)* represents a loss-of-function rather than a severe reduction-of-function mutation, because we expect that fluctuation in the *ras*-dependent signaling caused by a *sli-1* mutation would result in a dramatic change in vulval induction under the condition where a low level activity of LET-23/SEM-5/SOS-1 is present (C.Yoon, C.Chang and P.Sternberg, unpublished).

Gonad-ablated *let-60(gf); let-341(null)* animals have less vulval differentiation than do *let-60(gf); let-341(null)* or gonad-ablated *let-60(gf)* animals. Therefore, gonad ablation not only removes a ligand-dependent and SOS-1-independent positive signaling pathway, but also reveals a ligand-independent activation of RAS by SOS-1. Although this ligand-independent activation of RAS by SOS-1 does not contribute significantly the vulval induction in gonad-ablated *let-60(gf)* animals, it could explain the difference of vulval induction between *let-60(gf); let-341(null)* and *let-60(gf); sem-5(null)* mutants (Table II).

PTP-2 is not involved in SOS-1-independent vulval signaling

Based on the observation that the *lf* mutation in *ptp-2* reduces the vulval induction conferred by a *let-60(gf)*

Fig. 1. (A) Genomic structure of the *sos-1* locus. The solid bars represent exons, which were inferred by comparing the nucleotide sequence of a *sos-1* cDNA with that of the genomic DNA; the 5' untranslated region is depicted as an open box. Sizes of the 16 introns are noted by the numbers above each intron. The start and tentative end of the *let-341 sos-1* coding sequences are indicated by the corresponding nucleotide numbers from YAC Y61A9LA. Diagonal line labeled SL1 is the *trans*-spliced leader attachment site. The positions of *let-341* mutations and corresponding amino acid or splice site changes are shown. Exonic sequence is shown in uppercase lettering, intronic sequence in lowercase. The approximate locations of primers up8 and dn18 used to analyze *s1031* transcripts are indicated. **(B)** Comparative analyses of signature motifs in SOS-1 homologs. The protein predicted to be encoded by the *sos-1* gene is depicted schematically. The numbers listed below represent the percent identity between DH domain, PH domain and CDC25 catalytic domain of SOS-1 and those domains of human *Sos1* and *Drosophila sos*. Motifs were identified using the ProfileScan program. **(C)** Sequence alignment of CDC25 domains of RAS-binding exchange factors from *C.elegans* (Ce), *Drosophila melanogaster* (Dm) and *Homo sapiens* (Hs), with residue numbers of *C.elegans* (Ce) SOS-1 indicated. Residue identity between species is highlighted in black, and similarity is highlighted in gray. The arrows indicate the start of deletions in the *s1031* transcripts.

mutation, *ptp-2* was previously interpreted to act in the vulval signaling pathway as a positive effector downstream of, or in parallel to, *let-60 ras* (Gutch *et al.*, 1998). To determine whether the response of PTP-2 to receptor activation is parallel to that of SOS-1, we generated triple mutants with activated RAS but lacking PTP-2 and SOS-1 [*ptp-2(null)*; *let-60(gf)*; *let-341(null)*], and assessed their vulval differentiation. The *ptp-2* mutation did not further reduce the vulval induction observed in *let-60(gf)*; *let-341(null)* mutants, suggesting that PTP-2 does not mediate SOS-1-independent vulval signaling (Table III).

Lifting inhibition by GAP-1 does not explain the SOS-1-independent signaling activity

Besides a SOS-1-independent activation of RAS, an alternative explanation for the elevated signaling activity in the *let-60(gf)*; *let-341(null)* mutant animals is that activation of LET-23 by the ligand may relieve the inhibition of RAS by GAP-1, GTPase activating protein (Hajnal *et al.*, 1997). In this model, LET-23 would be able to relieve GAP-1-mediated inhibition of RAS upon activation by the ligand in *let-60(gf)*; *let-341(null)* double mutants, while *let-60(gf)* single mutants with an ablated

Fig. 2. Suppression of excessive vulval signaling by *sos-1* RNAi. Nomarski photomicrographs of the vulval induction in the L4 stage hermaphrodites. (A) *lin-15(n765)* mutant, which displays a total of six VPCs adopting vulval fates and forms three vulval invaginations. (B) *sos-1* RNAi-affected *lin-15(n765)* mutant, which displays a one cell induction that forms one vulval invagination. (C) *let-23(sa62)* mutant, which displays a total of four VPCs undergoing vulval differentiation and forms two vulval invaginations. (D) *sos-1* RNAi-affected *let-23(sa62)* mutant, which displays only two induced vulval cells that form one vulval invagination. (E) *let-60(n1046)* mutant, which displays a total of five VPCs adopting vulval fates and forms three vulval invaginations. (F) *sos-1* RNAi-affected *let-60(n1046)* mutant, which displays a wild-type vulval induction and invagination. Dorsal is up and anterior to the right in all panels. The scale bar is 20 μ m.

Table I. SOS-1 mediates the ectopic vulval induction during excessive *let-23* signaling

Genotype	<i>SOS-1</i> RNAi	% <3	% = 3	% >3	Numbers of animals	Average vulval induction
<i>let-60(n1046)</i>	–	0	0	100	20	5.0
<i>let-60(n1046)</i>	RNAi	0	62	38	21	3.3
<i>let-23(sa62)</i>	–	0	7	93	15	4.2
<i>let-23(sa62)</i>	RNAi	14	72	14	14	2.9
<i>lin-15(n765)</i>	–	0	0	100	20	5.3
<i>lin-15(n765)</i>	RNAi	33	67	0	12	2.5
<i>let-60(n1046); let-341(s1031)</i>	–	41	52	7	54	2.7
<i>let-60(n1046); let-341(s1031)</i>	RNAi	39	52	9	23	2.6

Vulval induction was scored at 20°C using Nomarski optics during the early-mid L4 stage. The complete genotypes for strains listed are: *let-60(n1046)unc-31(e169)* [for *let-60(n1046)*]; *let-23(sa62); him-5(e1490)* [for *let-23(sa62)*]; *lin-15(n765)* [for *lin-15(n765)*]; *let-60(n1046); let-341(s1031)unc-46(e177)* [for *let-60(n1046)*; *let-341(s1031)*]. Average vulval induction represents average number of VPCs undergoing vulval differentiation per animal; 3.0 is wild-type level.

gonad display constitutively active GAP-1 and cause a further decrease in the vulval induction. To test this model, we constructed the triple mutant *let-60(gf); let-341(null); gap-1(null)* and compared the average vulval induction in the conditions with and without the gonad. We find that gonad ablation significantly reduces the vulval induction of *let-60(gf); let-341(null)*;

gap-1(null) animals [from 3.9 ($n = 20$) to 2.4 ($n = 13$); $P = 0.0048$] (Table II), indicating that the anchor cell signal is not working solely through *let-341* and *gap-1*, which are absent already in *let-60(gf); let-341(null); gap-1(null)* animals. This result rules out the possibility that the gonad-dependent SOS-1-independent signaling is mediated solely through *gap-1*.

Fig. 3. *sos-1* RNAi affects male spicule development and hermaphrodite oocyte maturation. (A) Nomarski photomicrograph of a *him-5(e1490)* male shows a long and straight spicule, denoting by an arrow head. (B) A male progeny of *him-5(e1490)* hermaphrodite injected with *sos-1* dsRNA shows a short and crumpled spicule, denoting by an arrow head. (C and D) Fluorescence micrograph of wild-type (bottom left) and *sos-1* RNAi-affected (bottom right) hermaphrodites stained with DAPI. Regions of pachytene nuclei are indicated by a white bar and nuclei in diakinesis by a semi-gray bar. Arrow indicates the first diakinesis nucleus seen in the *sos-1* RNAi-affected hermaphrodite. All animals shown are in their young adult stage. Dorsal is up and anterior to the right in all panels. The scale bar is 20 μ m.

Fig. 4. *sos-1* RNAi-affected animals display the phenotypes associated with reduction-of-function FGFR pathway mutants. All the animals were photographed at the same magnification. (A) Wild type (N2). (B) *sos-1* RNAi-affected N2 animal, which confers a scrawny body morphology. (C) *clr-1(e1745ts)* mutants, which confer a temperature-sensitive Clr phenotype. The biological basis of the Clr phenotype is not understood but the abnormality appears to result from clear fluid accumulating in the pseudocoelom (Kokel *et al.*, 1998). (D) *sos-1* RNAi-affected *clr-1(e1745ts)* mutant, which confers a non-Clr phenotype. Animals shown in (A) and (B) are young adults and were raised at 20°C. L4 larvae shown in (C) and (D) were raised at 20°C and then switched to the nonpermissive temperature of 25°C for 10 h before being photographed.

Fig. 5. *let-341(s1031)* is a putative null allele of *sos-1*. **(A)** The sequence of the *let-341(s1031)* mutation. The upper panel shows the genomic sequence around the boundaries of exon 13–intron 13 and intron 13–exon 14. Exons are shown in uppercase lettering, introns in lowercase. The large downward-pointing arrow indicates the 5′ location of the wild-type splice. The upward-pointing arrow indicates the 3′ splice junction used. The lower panel shows the same information in the presence of the *s1031* mutation. The mutation is indicated in bold and by carets. The 5′ splice donors have shifted upstream. **(B)** RT–PCR analysis of wild-type and *s1031* RNA. PCR amplification for 35 rounds of wild-type and *s1031* reverse-transcribed RNA was performed with primers up8 and dn18 (see Figure 1A). Lanes: 1, 1 kb ladder; 2, amplification of *s1031* cDNA; 3, amplification of wild-type cDNA. Correctly spliced mRNA yields a product of 678 bp (correct joining of exons 12–16). In *s1031* animals, a 647 bp product results from usage of a upstream cryptic splice donor at nucleotide 119 792 of YAC Y61A9LA and removal of 31 bp from exon 13. Exon 13 skipping in *s1031* animals results in a 543 bp product. A putative heteroduplex (het), expected to form from two fast migrating species, is indicated.

Table II. A SOS-1-independent activation of <i>ras</i> signaling in the vulva									
Genotype					% <3	% = 3	% >3	Numbers of animals	Average vulval induction
<i>let-60</i>	<i>let-341</i>	<i>gap-1</i>	<i>sem-5</i>	<i>gonad</i>					
1. <i>n1046</i>	+	+	+	+	0	15	85	20	4.4
2. <i>n1046</i>	+	+	+	–	89	0	11	18	1.3
3. <i>n1046</i>	<i>s1031</i>	+	+	+	41	52	7	54	2.7
4. <i>n1046</i>	+	+	<i>ay73</i>	+	0	87	13	30	3.2
5. <i>n1046</i>	<i>s1031</i>	+	<i>ay73</i>	+	50	25	25	8	2.8
6. <i>n1046</i>	<i>s1031</i>	+	+	–	95	5	0	22	0.8
7. <i>n1046</i>	<i>s1031</i>	<i>n1691</i>	+	+	0	35	65	20	3.9
8. <i>n1046</i>	<i>s1031</i>	<i>n1691</i>	+	–	54	15	31	13	2.4

Vulval induction was scored at 20°C using Nomarski optics during the early-mid L4 stage. *let-341(s1031)* was marked with *unc-46(e177)*. *sem-5(ay73)* is a putative null allele resulting in a Q10Stop (Clark *et al.*, 1992a; M.Stern, personal communication). *gap-1(n1691)* corresponds to a Q149Stop, which defines a putative null allele. The distributions generated from each genotype were entered into the InStat program (GraphPad Software, version 2.0) and compared in pairs using the Mann–Whitney test to generate two-tailed *P* values. The differences resulting from the following comparisons are considered very significant: 2 versus 3, *P* <0.0001; 2 versus 4, *P* <0.0001; 2 versus 5, *P* = 0.0021; 3 versus 6, *P* <0.0001; 3 versus 7, *P* <0.0001; 7 versus 8, *P* = 0.0048. With a *P* value of 0.7448, 3 versus 5 is considered not significant.

A role for SOS-1 in FGFR-mediated signaling during development

Hyperactive EGL-15 FGF receptor signaling, conferred by either an activated *egl-15* or a *lf clr-1* mutation, results in a Clr phenotype. Decreased EGL-15 signaling, conferred by a partial *lf egl-15* mutation, results in a scrawny body morphology phenotype (Kokel *et al.*, 1998). *clr-1*, which encodes a receptor tyrosine phosphatase, is believed to attenuate signaling from FGFR by dephosphorylation. A

scrawny phenotype was observed in the *SOS-1* RNAi-affected animals (Figure 4B), suggesting that SOS-1 normally mediates FGFR signaling. To test this possibility, we removed SOS-1 function in the temperature-sensitive *clr-1(e1745ts)* mutants by *SOS-1* RNAi and assessed their Clr phenotype. We found that the Clr phenotype of *clr-1(e1745ts)* mutants was suppressed by *SOS-1* RNAi (77/77 animals examined) (Figure 4C and D), indicating a positive role for SOS-1 in FGFR-mediated

Table III. *sli-1* regulates *let-23* signaling upstream of *ras*, in a SOS-1-dependent manner

Genotype					Average vulval induction	Proportion of animals with >3 cells induced
<i>ptp-2</i>	<i>let-23</i>	<i>let-60</i>	<i>let-341</i>	<i>sli-1</i>		
1. +	+	<i>n1046</i>	+	+	4.4 ^a	17/20
2. +	<i>sy17</i>	<i>n1046</i>	+	+	4.6	19/20
3. +	+	<i>n1046</i>	+	<i>sy143</i>	5.5 ^a	20/20
4. +	+	<i>n1046</i>	<i>s1031</i>	+	2.7	4/54
5. +	+	<i>n1046</i>	<i>s1031</i>	<i>sy143</i>	2.9	1/20
6. <i>op194</i>	+	<i>n1046</i>	<i>s1031</i>	+	3.0	2/20

Vulval induction was scored at 20°C using Nomarski optics during the early-mid L4 stage. *ptp-2(op194)* and *let-341(s1031)* were marked with *unc-4(e120)* and *unc-46(e177)*, respectively. *ptp-2(op194)* is a putative null allele resulting in a genomic deletion of 1458 nucleotides (Gutch *et al.*, 1998). *sli-1(sy143)* is also a putative null allele resulting in a Q152Stop (Yoon *et al.*, 1995).

^aThe difference of average vulval induction between *let-60(n1046)* and *let-60(n1046); sli-1(sy143)* mutants is highly significant ($P = 0.0002$) based on the Mann–Whitney test. The most significant difference occurs in the P8.p induction with 33% of P8.p induced for *let-60(n1046)* and 78% for *let-60(n1046); sli-1(sy143)*. The differences result from the following comparisons are considered not significant: 4 versus 5, $P = 0.1629$; 4 versus 6, $P = 0.1313$.

signaling. Indeed, the *let-341(ku231)* mutation also suppresses the Clr phenotype of *clr-1(e1745ts)* mutants (data not shown) and *let-341(n2183)* was isolated as a *soc* mutant, as mentioned before. Since many *sem-5* alleles were also isolated as suppressors of the Clr phenotype of *clr-1(lf)* mutant (Clark *et al.*, 1992a), it is likely that SEM-5-dependent recruitment of SOS-1 to the active FGFR (EGL-15) plays an analogous role in activating RAS as with the EGFR (LET-23).

Discussion

SOS-1 participation during vulval development

We have found the *C.elegans* homolog of Sos, and demonstrated by RNAi and by phenotypic analysis of a null allele that it is required for vulval induction. *sos-1* RNAi does not affect vulval induction in the wild-type animals at an appreciable frequency (1/50 animals examined vulvaless; data not shown). It might be that vulval induction occurs late in development or a certain property of vulval lineages renders this developmental process more resistant to *sos-1* RNAi. However, the effect of *sos-1* RNAi on vulval signaling becomes obvious in sensitized genetic backgrounds. Although SOS-1 acts downstream of *let-23* and upstream of *let-60*, *sos-1* RNAi affects excessive vulval signaling in both *let-23(gf)* and *let-60(gf)* mutants. The fact that vulval signaling was affected in a greater degree in *let-23(gf)* than *let-60(gf)* mutants is consistent with our expectation that *sos-1* RNAi abrogates both ligand-dependent and -independent activation by *let-23(gf)*, while only abrogating ligand dependent activation by *let-60(gf)*. The involvement of SOS-1 during vulval development is further confirmed by our observation that 41 percent of the *let-60(gf); let-341(null)* animals examined displayed less than a wild-type extent of vulval differentiation (Table II).

SOS-1 involvement in oocyte maturation and uterine differentiation

Consistent with the observation that *sos-1* RNAi prevents meiotic prophase progression, *sos-1* RNAi-affected hermaphrodites also display a late onset sterile phenotype in the adult stage (10/10 animals examined; data not shown).

Furthermore, this sterile phenotype can be suppressed by a *let-60(gf)* mutation (data not shown). Taken together, these results identify SOS-1 as an activator for LET-60 RAS in regulating oogenesis. It remains unknown what is required upstream of SOS-1 to permit exit from pachytene (Church *et al.*, 1995).

As judged by anatomy, we can not tell whether abnormalities in the dorsal and ventral uterine lineages are caused by a mis-determination of cell fates, defects in cell migration, or both. Most of the adult hermaphrodites affected by *sos-1* RNAi have an abnormal everted vulva that consists of vulval cells and somatic gonadal tissues that have been extruded out of the body cavity (data not shown). Uterine cells are known to be required for the proper attachment between the uterus and vulva (Newman and Sternberg, 1996), a process that has been suggested to prevent the vulva and uterus from slipping out of the body cavity during vulval eversion (Seydoux *et al.*, 1993).

SOS-1-dependent and -independent signaling

The finding that the *sli-1(null)* mutation fails to enhance the vulval induction in *let-60(gf); let-341(null)* mutants suggests that the synergy between *sli-1(null)* and *let-60(gf)* in the vulva is dependent upon SOS-1. This result is consistent with our previous data suggesting that *sli-1* acts upstream of *let-60 ras* to inhibit signaling from *let-23* (C.Yoon, C.Chang and P.Sternberg, unpublished). The finding that the extent of vulval induction in a *let-60(gf); let-341(null)* background is higher than in gonad ablated *let-60(gf)* animals reveals that there is a ligand-dependent and SOS-1-independent signaling by EGF RTK. The simplest explanation is that there is a SOS-1-independent positive signaling pathway in *let-60(gf); let-341(null)* animals. Alternatively, gonad-ablated *let-60(gf)* animals may be subject to a form of negative regulation in the vulva that is independent of SOS-1 and is lifted in a *let-60(gf); let-341(null)* background, perhaps by the presence of the ligand. A SOS-1-independent positive signaling pathway elicited by LIN-3 might involve another guanine nucleotide exchange factor for RAS or the DOS/PTP-2-cascade. It has been previously shown that there may be more than one activator of LET-60 RAS. Genetic analysis of *sur-5* revealed that a *sur-5(lf)* mutation

Fig. 6. Models for EGFR, FGFR and an unknown RTK pathways. The order of linear pathways was either determined by double mutants analyses or inferred from biochemical assays in other model systems. For example, the relationship between *sem-5* Grb2 and *let-341* Sos1 was adapted from the previous molecular characterization of mammalian counterparts. Arrows indicate a positive effect and bars indicate a negative effect. For simplicity, only events upstream of RAS are presented. The dashed pathway X is insufficient to allow signaling in the absence of *let-60* activity. The putative relationships between *ptp-2* and target genes are yet to be defined. A, B and X are hypothetical receptor tyrosine kinase, adaptor and RasGEF, respectively.

selectively suppresses one group of *let-60(dn)* alleles but not the other, supporting the hypothesis that there could be more than one activator for LET-60 RAS (T.Gu *et al.*, 1998). *let-60(dn)* alleles have been shown to affect signaling by titrating upstream activators rather than downstream effectors (Han and Sternberg, 1991). At least seven additional putative guanine nucleotide exchange factors have been predicted from the *C.elegans* genome sequence (as of April 2000), and one or more of these could be the hypothetical activator.

Regulation of RTK pathways by PTP-2

Csw and SHP-2 are PTP-2 homologs in *Drosophila* and vertebrates, respectively. Csw is normally required for Sevenless, Breathless, Torso and DER pathways, while SHP-2 has been shown to function in EGFR, PDGFR, FGFR and HGFR signaling pathways. The best characterized substrates for Csw/SHP-2 are Daughter of sevenless (Dos)-type proteins (Herbst *et al.*, 1996; Raabe *et al.*, 1996; H.H.Gu *et al.*, 1998). It is believed that dephosphorylation of Dos by Csw generates a positive downstream signal. However, it remains unclear how Dos dephosphorylation creates such a positive signal. It is conceivable that PTP-2 might dephosphorylate specific phospho-tyrosyl residue(s) on a Dos-like protein, thus controlling the composition of an assembled signaling complex. Based on our results that PTP-2 function could be dependent upon SOS-1, several models for PTP-2 action can be proposed (Figure 6). PTP-2 could modulate the SEM-5-directed recruitment of SOS-1 or the catalytic activity of SOS-1. Alternatively, PTP-2 could release a negative regulator, such as SLI-1, from the signaling complex.

SOS-1 acts in multiple, RAS-dependent signaling pathways

SEM-5 recruitment of SOS-1 to the cell membrane seems to be a common mechanism involved in transducing signals from the activated EGFR and FGFR to RAS (Figure 6). Although SOS-1 also participates in signaling during the maturation of germline cells, how it is directed and to what signal it responds are unclear. Regulation of signaling upstream of RAS has been intensively studied from EGFR by using vulval development as a major paradigm. Several negative regulators have been previously implicated as affecting RAS activation by LET-23, including SLI-1/Cbl, UNC-101/AP47 and GAP-1/RasGAP. Biochemical studies suggest that RasGAP inhibits the signaling by directly acting on Ras (Boguski and McCormick, 1993), while Cbl desensitizes the signaling through binding to active receptors (Meisner *et al.*, 1997). It is not clear how the UNC-101 adaptor antagonizes the RAS signaling pathway. In this report, we have provided evidence for the existence of a SOS-1-independent signaling pathway, which is dependent upon ligand, independent of PTP-2, and is not negatively regulated by SLI-1. Recently, Cbl was shown to function as a ubiquitin-protein ligase for active RTKs (Joazeiro *et al.*, 1999). The failure to see an effect of SLI-1 on the SOS-1-independent pathway argues that promoting the degradation of active EGFR might not be the major activity of SLI-1. SLI-1 and another negative regulator of LET-23 EGFR signaling, ARK-1, both have proline-rich domains that can interact with the SH3 domain of SEM-5 (C.Yoon, C.Chang, P.Sternberg, unpublished; Hopper *et al.*, 2000). Competition between SOS-1 and negative regulators for binding to the SEM-5 adaptor is a potential mechanism of direct negative regulation of LET-60 RAS activation. Alternatively, the SH3 domains of SEM-5 may, in addition to the recruitment of SOS-1, also recruit negative regulators simultaneously with SOS-1. It is less likely that *ptp-2* activates signaling by inhibiting *gap-1* function, since a *ptp-2* mutation did not further reduce the vulval induction in *let-60(gf)*; *let-341(null)* mutants, and a *let-60(gf)*; *let-341(null)* mutant is sensitive to *gap-1* regulation (Tables II and III). Regulation of Sos1 by phosphorylation has been proposed previously (Buday *et al.*, 1995; Hu and Bowtell, 1996). Enhanced phosphorylation of Sos1, while not altering its catalytic activity for Ras, weakens its binding affinity for Grb2 or uncouples the Sos1-Grb2 complex from tyrosine kinase substrates (Buday and Downward, 1993). Although Sos1 is phosphorylated mostly on serine and threonine, regulated tyrosine phosphorylation has never been ruled out for Sos1. Therefore, it is possible that protein tyrosine kinases/phosphatases might modulate RAS signaling strength by directly regulating the phosphorylation state of SOS-1.

Materials and methods

Strains

Strains were handled according to the standard protocol and maintained at 20°C (Brenner, 1974). The following alleles were used: for LGII, *let-23(sa62)*, *ptp-2(op194)* (Gutch *et al.*, 1998), *unc-4(e120)*, *clr-1(e1745)* (Hedgecock *et al.*, 1990); for LGIV, *let-60(n1046)* (Ferguson and Horvitz, 1985), *unc-31(e169)*; for LGV, *him-5(e1490)*, *let-341(s1031)* (Johnsen and Baillie, 1991), *let-341(n2183)* (M.Stern, unpublished), *let-341(ku231)* (Z.Chen and M.Han, unpublished); for

LGX, *lin-15(n765)*, *sem-5(ay73)* (M.Stern, unpublished), *gap-1(n1691)* (Hajnal *et al.*, 1997), *sli-1(sy143)*.

Construction of double and triple mutants

Double and triple mutants were constructed using standard genetic methods, and markers used are indicated in the tables. For *let-60(n1046)*; *let-341(s1031)* and *let-60(n1046)*; *sli-1(sy143)* double mutants and for *let-60(n1046)*; *let-341(s1031)*; *sem-5(ay73)*, *let-60(n1046)*; *let-341(s1031)*; *gap-1(n1691)*, *let-60(n1046)*; *let-341(s1031)*; *sli-1(sy143)* and *ptp-2(op194)*; *let-60(n1046)*; *let-341(s1031)* triple mutants, the presence of *let-341*, *sli-1*, *sem-5*, and *gap-1* mutations was confirmed by sequencing the appropriate region of genomic DNA from each strain.

sos-1 cDNA isolation and allele sequencing

Partial *sos-1* cDNA was derived from an incomplete genomic sequence, which was provided by *C.elegans* Sequencing Consortium (1998) and predicted to encode a Sos homolog using the program GeneFinder. RT-PCRs were performed to rectify the GeneFinder prediction and confirmed our further derivation based on the comparative analyses. Two splice variants were recovered in addition to the major transcript. One variant results from failure to use the normal splice donor site of intron 12. The other results from usage of an alternative splice acceptor site in intron 13 (AG/GAATTGCCGAACATATACTAATCACTCTAA). 5' and 3' RACE reactions were carried out according to the manufacturer's instructions to obtain sequences corresponding to the 5' and 3' ends of the *sos-1* transcript (5'/3' RACE kit, Boehringer Mannheim).

To identify molecular lesions, PCR was performed on both reverse-transcribed RNA and genomic DNA from mutants, the products were purified and sequenced. The consequence of splice site mutation was determined by PCR amplification of reverse-transcribed RNA from mutants and sequencing purified PCR fragments directly.

RNAi

dsRNA corresponding to *sos-1* was generated by *in vitro* transcription using 1.6 kb *sos-1* cDNA corresponding to the nucleotides 1045–2613 relative to the initiation codon inserted into pCRII-TOPO (Invitrogen). Transcripts were prepared using T7 and Sp6 RNA polymerase and annealed prior to injection (Fire *et al.*, 1998). Progeny of injected animals were assayed at 20°C unless indicated otherwise.

Vulval induction assay

The extent of vulval induction was determined by examining vulval anatomy in the early to mid-L4 stage of development under Nomarski optics using a Plan 100 objective (Sternberg and Horvitz, 1986). Wild-type animals have three VPCs generating vulval progeny; vulvaless animals have fewer than three VPCs generating vulval progeny; multivulva or hyperinduced animals have more than three VPCs generating vulval progeny. In some cases, a VPC generates one daughter that makes vulval progeny and another daughter that becomes nonvulval epidermis; such VPCs are scored as one-half VPC differentiating into vulval tissue.

Cell ablation

Ablations of the AC were performed with a laser microbeam as described by Sulston and White (1980).

DAPI staining

Animals were washed in M9 buffer before picked into a small drop of Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories) in a depression slide. After 30–60 min at room temperature, animals were dried in a vacuum for 1 h before mounted on agarose pads for observation.

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