Gain-of-Function Mutations in the Caenorhabditis elegans lin-1 ETS Gene Identify a C-Terminal Regulatory Domain Phosphorylated by ERK MAP Kinase

Dave Jacobs,* Greg J. Beitel,¹,² Scott G. Clark,¹,² H. Robert Horvitz¹ and Kerry Kornfeld*

*Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110 and ¹Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

ABSTRACT

Genetic analysis of lin-1 loss-of-function mutations suggests that lin-1 controls multiple cell-fate decisions during Caenorhabditis elegans development and is negatively regulated by a conserved receptor tyrosine kinase-Ras-ERK mitogen-activated protein (MAP) kinase signal transduction pathway. LIN-1 protein contains an ETS domain and presumably regulates transcription. We identified and characterized six gain-of-function mutations that define a new class of lin-1 allele. These lin-1 alleles appeared to be constitutively active and unresponsive to negative regulation. Each allele has a single-base change that affects the predicted C terminus of LIN-1, suggesting this region is required for negative regulation. The C terminus of LIN-1 was a high-affinity substrate for Erk2 in vitro, suggesting that LIN-1 is directly regulated by ERK MAP kinase. Because mpk-1 ERK MAP kinase controls at least one cell-fate decision that does not require lin-1, our results suggest that MPK-1 contributes to the specificity of this receptor tyrosine kinase-Ras-MAP kinase signal transduction pathway by phosphorylating different proteins in different developmental contexts. These lin-1 mutations all affect a four-amino-acid motif, FQFP, that is conserved in vertebrate and Drosophila ETS proteins that are also phosphorylated by ERK MAP kinase. This sequence may be a substrate recognition motif for the ERK subfamily of MAP kinases.

INTERCELLULAR signaling is one of the primary mechanisms used to establish patterns of cell fates during development. We are analyzing intercellular signaling during the development of the vulva of the nematode Caenorhabditis elegans, because signaling events between easily visualized cells have been well defined and mutations that disrupt vulval development can be readily identified and characterized (reviewed by H. Robert Horvitz and Sternberg 1991; Sundaram and Han 1996; Kornfeld 1997). Our goals are to identify the molecules that transduce signals and control cell fates, elucidate the interactions among these molecules, and understand how these molecules function in a developing animal.

In third larval stage hermaphrodites, six ventral epidermal blast cells called P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p (Pn.p cells) lie along the anterior-posterior axis. Each of these Pn.p cells can adopt any of three distinct fates: the primary (1°) vulval cell fate (eight descendants), the secondary (2°) vulval cell fate (seven descendants), or the nonvulval tertiary (3°) cell fate (two descendants) (reviewed by Horvitz and Sternberg 1991). The anchor cell of the somatic gonad signals P6.p to adopt the 1° fate. P6.p signals P5.p and P7.p to adopt the 2° fate by activating lin-12, which is similar to the receptor Notch; the anchor cell signal may also promote the 2° fate. P3.p, P4.p, and P8.p receive neither signal and adopt the 3° fate. The 22 descendants of P5.p, P6.p, and P7.p generate the vulva, a specialized epidermal structure used for egg laying.

The anchor cell and P6.p communicate using a highly conserved signal transduction pathway that includes the lin-3 ligand, which is similar to epidermal growth factor; the let-23 receptor tyrosine kinase (RTK); the sem-5 adaptase (reviewed by H. Robert Horvitz and Sternberg 1991); the let-60 Ras; the let-60 Ras pathway, which includes sem-5, sem-5, and the let-60 Ras pathway; and the let-60 Ras pathway, which includes sem-5, sem-5, and the let-60 Ras pathway. A mutation that reduces the activity of one of these genes causes all six Pn.p cells to adopt nonvulval 3° fate, resulting in a vulvaless (Vul) phenotype. By contrast, a constitutively active form of one of these genes causes all six Pn.p cells to adopt the 1° or 2° vulval fate, resulting in a multi-vulva (Muv) phenotype characterized by ectopic patches of vulval tissue. This signaling pathway functions at multiple times during development and is required for larval viability, hermaphrodite fertility, and other processes. RTK-Ras-MAP kinase signaling pathways have been conserved during evolution, and these pathways control a variety of cell-fate decisions during Drosophila and vertebrate development (reviewed by Dickson and Hafen 1994).
These observations raise two important questions: How do these signaling proteins cause a change in cell fate, and how does this conserved signaling pathway specify different cell fates in different developmental contexts? MAP kinases, a family of serine/threonine-specific protein kinases, may be important for both processes (reviewed by Davis 1995; Treisman 1996). Activated MAP kinase can translocate into the nucleus in vivo and phosphorylate a variety of proteins in vitro, including transcription factors. This activity may be how this signaling system affects gene expression and cell fate. Furthermore, it is possible that a given MAP kinase can phosphorylate different proteins in different developmental contexts and in this way contribute to the specificity of the response. These possibilities have not been carefully tested, because only a few proteins have been shown to be MAP kinase substrates in vivo and because the basis for MAP kinase recognition of particular substrates remains to be determined.

Genetic experiments revealed that lin-1 is an important regulator of multiple cell-fate decisions in C. elegans and suggested that lin-1 functions downstream of mpk-1 ERK MAP kinase. A lin-1 loss-of-function (L) mutation causes all six Pn.p cells to adopt vulval cell fates, even in the absence of the activity of the RTK-Ras-MAP kinase signaling pathway (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985; Ferguson et al. 1987; Lackner et al. 1994; Beitel et al. 1995). A lin-1(L) mutation bypasses the requirement for the activity of the RTK-Ras-MAP kinase pathway to promote larval viability (Han et al. 1990; Kornfeld et al. 1995a,b; Sundaram and Han 1995). These observations suggest that the major function of the RTK-Ras-MAP kinase signaling pathway in at least two developmental processes is to regulate lin-1 negatively.

lin-1 is predicted to encode a 441-amino-acid protein that contains an ETS domain (Beitel et al. 1995). The ETS domain appears to be required for lin-1 function, because mutations that alter conserved residues in the ETS domain greatly reduce or eliminate lin-1 activity. The ETS domains of related proteins bind DNA, and many ETS proteins are transcription factors (reviewed by Wasylko et al. 1993). These observations suggest but do not prove that LIN-1 binds DNA and regulates transcription. ETS proteins are involved in a variety of processes, and several appear to be regulated by RTK-Ras-MAP kinase pathways, such as vertebrate Elk-1 and Drosophila Aop (anterior open; previously known as yan or pok) (reviewed by Treisman 1996).

To investigate the interaction between LIN-1 and ERK MAP kinase, we used biochemical and genetic experiments to show that the C terminus of LIN-1 is a substrate for Erk2 and is important for negative regulation of LIN-1 by the RTK-Ras-ERK MAP kinase pathway. Furthermore, we identified a C-terminal region of LIN-1 that is similar to the C box, a conserved domain present in the C-terminal regions of ETS proteins in the Elk subfamily (Treisman 1994). Our analysis suggests that the conserved elements of the C box are multiple S/TP motifs that are potential MAP kinase phosphorylation sites and an FQFP motif that we postulate to be a recognition sequence for ERK MAP kinase.

MATERIALS AND METHODS

General methods and strains: C. elegans strains were cultured as described by Brenner (1974) and were grown at 20°C unless otherwise noted. The wild-type strain and parent of all mutant strains was N2. Unless otherwise noted, mutations used in this study are described by Riddle et al. (1997) and are as follows. LGI: unc-54(r293); smg-1(r681). LGII: lin-8(n1111); lin-31(n1053). LGV: lin-3(l1761); n1790; n1835; n2515; n2525; ky54 (this study); lin-1(s1254); el777; dpy-9(e12); dpy-13(e84); dpy-20(e1282); el2(e1752); unc-7(e13); unc-24(e38); unc-35(e204); let-60(n1046gf); nT1n754; nDp1 (Rogalski and Riddle 1990). LGV: dpy-3(e1624); unc-64(e2913). LGX: lin-1(n765ts). We used standard techniques to separate the lin-l(gf) mutations from the lin-15(lf) mutation and to generate double mutants (Brenner 1974). We used DNA sequencing to confirm that n1855, n2515, and n2525 were present on recombinant chromosomes that contained let-60(+), and the linked marker dpy-13.

Identification of lin-1(gf) mutations: We previously described a screen for suppressors of the let-60(gf) Muv phenotype (Lackner et al. 1994; Kornfeld et al. 1995a,b). In brief, we mutagenized let-60(n1046gf) hermaphrodites with ethyl methanesulfonate (EMS), placed 2794 F1 self-progeny on separate Petri dishes at 25°C, and scored for the Muv phenotype. However, these mutations partially complemented several other suppressor mutations located on chromosome IV (data not shown) and thus appeared to define one complementation group. We used a three-factor cross and a four-factor cross to map the suppression of the let-60(gf) Muv phenotype caused by n1855 more precisely. Of uncoordinated (Unc) non-Dpy progeny from n1855 let-60(gf)/ unc17 dpy-13, 0/13

Genetic mapping and complementation tests: The suppression of the let-60(gf) Muv phenotype caused by n1855, n2515, and n2525 displayed linkage to let-60 and dpy-20 on chromosome IV (data not shown). To investigate complementation between these mutations, we scored the penetrance of the Muv phenotype in self-progeny from hermaphrodites with the following genotypes: n1855 let-60(n1046)/n2515 let-60(n1046) (0%, n = 120); n1855 let-60(n1046)/n2525 let-60(n1046) (2%, n = 123). The suppression of the let-60(gf) Muv phenotype caused by these three mutations was semidominant; for example, +/let-60(n1046)/n2525 let-60(n1046) animals were 15% Muv. However, these mutations partially complemented several other suppressor mutations located on chromosome IV (data not shown) and thus appeared to define one complementation group. We used a three-factor cross and a four-factor cross to map the suppression of the let-60(gf) Muv phenotype caused by n1855 more precisely. Of uncoordinated (Unc) non-Dpy progeny from n1855 let-60(gf)/ unc17 dpy-13, 0/13...
segregated n1855. From n1855 let-60(gf)/dry-9 cad-2 unc-33 hermaphrodites, we isolated Dry non-Unc progeny, identified hermaphrodites homozygous for the recombinant chromosome, and scored the CED phenotype. Of Dry non-Unc recombinants, 3/19 were cad-2(+) n1855, 2/19 were cad-2(−) n1855, and 14/19 were cad-2(−) lin-1(+). These data position n1855 left of unc-17 and between cad-2 and unc-33, an approximately 14-map-unit interval that contains the lin-1 gene.

Genetic mapping and complementation experiments were used to analyze the recessive abnormal vulva phenotype caused by n1790, n1761, and ky54. n1761 failed to complement n1790 and ky54 for this phenotype, suggesting these three mutations represent one complementation group (data not shown). Three-factor crosses were used to position n1790 and n1761 left of unc-17 on chromosome IV, an interval that contains lin-1. From n1790/unc-17 dry-13 hermaphrodites, 0/9 Unc non-Dpy progeny segregated n1790 and 8/8 Dpy non-Unc progeny segregated n1790. From n1761/unc-17 dry-13 hermaphrodites, 0/15 Unc non-Dpy segregated n1761 and 10/10 Dpy non-Unc progeny segregated n1761. To directly test whether these mutations mapped to the lin-1 locus, we placed n1761 in trans to the loss-of-function allele lin-1(e1777), and screened for recombination between the two mutations. We found zero wild-type recombinants among 1170 self-progeny of lin-1(e1777)/n1761 dry-13 hermaphrodites. These data suggest that n1761 is separated from lin-1 by less than 0.04 map units and support the hypothesis that n1761 is an allele of lin-1.**

**Determination of DNA sequences of lin-1 alleles: For each of the six lin-1 alleles, genomic DNA was derived from homozygous mutant adult hermaphrodites and amplified by polymerase chain reaction (PCR), according to Williams et al. (1992). lin-1 contains six exons (Beitel et al. 1995). The oligonucleotide primers GBO25f (5’-CCACGTGCTACCTTAGTC) and GBO26r (5’-GAGCAGATTTGCTTTTAGTAGC) were used to amplify a 322-base pair (bp) DNA fragment containing 64 bp upstream of exon 1, intron 1, exon 2, and 63 bp of intron 2. GBO7f (5’-CCCTATACAGGCTTCGATCTC) and GBO14r (5’-CGTTCATATCAACCTACTAC) amplified a 436-bp fragment containing 77 bp of intron 2, exon 3, and 69 bp of intron 3. GBO16f (5’-TACCCGAATATGACG) and GBO17r (5’-CCGTCCTCTTTCAAAATTTAG) amplified a 490-bp fragment containing 72 bp of intron 3, exon 4 and 137 bp of intron 4. GBO28f (5’-CTTCTGCAAAAAGAGTGCC) and GBO29r (5’-GGTCTGCAGCAGAAAGGCGG) amplified a 583-bp fragment containing 119 bp of intron 4, exon 5 and 110 bp of intron 5. GBO21f (5’-GAATTTTCAACATTCTTCCA) and GBO27r (5’-GAAGACTATGCAACCCATGG) amplified a 410-bp fragment containing 106 bp of intron 5 and 305 bp of exon 6 extending 116 bp beyond the stop codon. We purified PCR-amplified DNA fragments and determined the complete sequences of both strands using an automated ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

**Protein analysis:** To produce LIN-1 protein fused to glutathione-S-transferase (GST) or maltose-binding protein (MAL), we cloned fragments of a lin-1 cDNA (Beitel et al. 1995) into pGEX-2T (Pharmacia LKB Biotechnologies, Piscataway, NJ) or pMALP2 (New England Biolabs, Beverly, MA), respectively, using standard techniques (Sambrook et al. 1989). The plasmid pDG29 encodes GST:LIN-1(1-441), pDG14 encodes GST:LIN-1(1-379), pAT4 encodes GST:LIN-1(1-278), pAT2 encodes GST:LIN-1(1-281-441), pGB35 encodes GST:LIN-1(1-154-294), and pGB34 encodes MAL:LIN-1(1-154-294). We used standard in vitro mutagenesis techniques to generate pDG29, which encodes GST:LIN-1(1-441P384L). We produced and partially purified fusion proteins using glutathione-Sepharose (Pharmacia), essentially according to the manufacturer’s instructions. In brief, we transfected plasmids into Escherichia coli strain BL21, induced expression with isopropyl thiogalactoside, and made protein extracts using sonication to disrupt cells. Proteins were either soluble in phosphate-buffered saline (PBS), or they were solubilized in 7 M urea and then dialyzed against PBS. GST fusion proteins were bound to glutathione-sepharose, eluted using glutathione, glycine, or triethylamine, and dialyzed against MAP kinase assay buffer. To estimate the amount of intact fusion protein in a partially purified sample, we fractionated protein samples by SDS-PAGE, stained the proteins with Coomassie blue, and compared the intensity of the intact fusion protein band to the intensity of known amounts of bovine serum albumin in adjacent lanes. Purified, recombinant, murine Erk2 (New England Biolabs) was assayed as described by Alessi et al. (1995). A standard 50 µl reaction was incubated 15 min at 30°C and contained 100 µmol [32P]ATP (0.15 Ci/mmol) and 0.05 pmol Erk2. To quantify phosphorylation, we measured radioactive protein bound to a P81 filter using a scintillation counter. Purified myelin basic protein was from GIBCO BRL (Gathersburg, MD).

Anti-LIN-1 antisera were generated by immunizing rabbits with 1 mg of MAL:LIN-1(1-154-294) protein in Freund’s complete adjuvant (Sigma, St. Louis). After 4, 8, and 12 wk, rabbits were immunized with 1 mg of GST:LIN-1(1-154-294) in Freund’s incomplete adjuvant (Sigma). Sera were collected 2 weeks after each immunization. Western blots were generated as described by Traub et al. (1993) and visualized using chemiluminescence.

**RESULTS**

**Isolation and molecular characterization of six novel lin-1 mutations:** We isolated six lin-1 alleles that are unlike previously described lin-1(If) alleles by performing genetic screens for mutations that prevented Pn.p cells from adopting vulval cell fates (see materials and methods). The alleles n1855, n2515, and n2525 were isolated in screens for mutations that suppressed the Muv phenotype caused by a gain-of-function (gf) mutation that constitutively activates the let-60 ras gene. The alleles n1761 and n1790 were isolated in a screen for mutations that suppressed the Muv phenotype caused by a lin-15(If) mutation. lin-15 is a negative regulator of vulval cell fates and appears to act upstream of or parallel to let-60 ras (reviewed by Korner and 1997). The allele ky54 was recovered as a mutant with a defective vulva in a genetic screen for mutants with an unrelated phenotype.

Complementation tests between these alleles, which caused recessive or semidominant phenotypes, and genetic mapping together suggested that these mutations define a single locus located within an interval on the left arm of chromosome IV that contains lin-1 (see materials and methods). Complementation tests with a lin-1(If) mutation could not be used to test the hypothesis that these mutations affect the lin-1 gene, because the new mutations did not cause the same abnormalities as a lin-1(If) mutation. Therefore, we investigated whether these were lin-1 alleles by determining the DNA sequence of the entire lin-1 coding region and the regions of introns close to the splice sites; we discovered a base
genes involved in vulval induction: to that caused by loss-of-function mutations in many of rigid, rod-like larval lethality appeared to be identical invariant, and wild-type hermaphrodites have 22 descen-

60 ras

1. Most affected animals died during the first or second examine the cells that form the vulva in fourth larval

n2525 alleles contained the same base change, a mis-
n1855 and intron 5, shown underlined and lower case. Each base change was a GC-to-AT transition, the character-
istic mutation caused by ethyl methane-sulfonate, the mutagen used to generate all of these mutations (Coulondre and Miller 1977). The predicted LIN-1 pro-
teins are drawn to scale, and the ETS domain is stippled. n1761 mRNA is likely to be spliced abnormally; if intron 5 is not removed, then 45 amino acids encoded by intron 5 will follow residue 379 (hatching). Alternatively, a GT sequence located five bases upstream or five bases downstream of the mutation might function as a cryptic splice site resulting in 64 amino acids

Figure 1.—Base changes in lin-1(gf) alleles and predicted mutant proteins. The wild-type amino acid, codon num-
ber, and DNA sequence are followed by the corresponding information for the mutant alleles. Opal is a termination co-
don. An arrowhead indicates the boundary between exon 4, which ends with codon 379, and intron 5, shown underlined and lower case. Each base change was a GC-to-AT transition, the character-
istic mutation caused by ethyl methane-sulfonate, the mutagen used to generate all of these mutations (Coulondre and Miller 1977). The predicted LIN-1 pro-

close. Western blot analysis of extracts prepared from a wild-type and these mutants showed that LIN-1 protein is predicted to lack the C-terminal 90 amino acids. The n1761 allele contained a mutation in the splice site at the 5' end of intron 5, following codon 379. Intron 5 is likely to be retained or removed by the use of a cryptic splice site; in either case about 62 amino acids at the C terminus would be replaced by about 50 new amino acids (see Figure 1 legend). The n1855 and n2525 alleles contained the same base change, a mis-
sense mutation that changes amino acid 384 from pro-
line to serine. The n2515 allele also contained a base change in codon 384; this mutation changes proline to leucine (Figure 1). Thus, these six independently derived mutations represent four different molecular changes. All four affect the predicted C terminus of LIN-1.

The novel lin-1 mutations caused larval lethality: To investigate the abnormalities caused by these lin-1 muta-
tions, we observed the development of mutant animals using a dissecting microscope. These mutations caused larval lethality that varied in penetrance from 73% for n1761 to 5% or less for n2515, n2525, and n1855 (Table 1). Most affected animals died during the first or second larval stage and were thin and straight. This phenotype of rigid, rod-like larval lethality appeared to be identical to that caused by loss-of-function mutations in many genes involved in vulval induction: lin-3, let-23, sm-5, let-
60 ras, lin-45 raf, ksr-1, mek-2, and mpk-1 (Ferguson et al. 1997). By contrast, n1790 and n1761 hermaphrodites had an average of 20 descendants of these cells and sometimes had as few as 17 descendants (Table 1). Furthermore, these mutants often had grossly abnormal vulval invaginations and large Pn.p descendants that appeared to have undergone too few cell divisions. We never observed these abnormalities in wild-type animals. These abnormalities are similar to the defects in vulval formation caused by partial loss-of-function mutations

change in each of these six strains (Figure 1). The n1790 and ky54 alleles contained the same base change, a nonsense mutation at codon 352; the mutant LIN-1 protein is predicted to lack the C-terminal 90 amino acids. The n1761 allele contained a mutation in the splice site at the 5' end of intron 5, following codon 379. Intron 5 is likely to be retained or removed by the use of a cryptic splice site; in either case about 62 amino acids at the C terminus would be replaced by about 50 new amino acids (see Figure 1 legend). The n1855 and n2525 alleles contained the same base change, a mis-
sense mutation that changes amino acid 384 from pro-
line to serine. The n2515 allele also contained a base change in codon 384; this mutation changes proline to leucine (Figure 1). Thus, these six independently derived mutations represent four different molecular changes. All four affect the predicted C terminus of LIN-1.

The novel lin-1 mutations caused larval lethality: To investigate the abnormalities caused by these lin-1 muta-
tions, we observed the development of mutant animals using a dissecting microscope. These mutations caused larval lethality that varied in penetrance from 73% for n1761 to 5% or less for n2515, n2525, and n1855 (Table 1). Most affected animals died during the first or second larval stage and were thin and straight. This phenotype of rigid, rod-like larval lethality appeared to be identical to that caused by loss-of-function mutations in many genes involved in vulval induction: lin-3, let-23, sm-5, let-
60 ras, lin-45 raf, ksr-1, mek-2, and mpk-1 (Ferguson et al. 1997; Han et al. 1990, 1993; Clark et al. 1992; Wu and Han 1994; Kornfeld et al. 1995a,b; Sundaram and Han 1995). In let-60(lf) mutants, this lethality results from a failure to establish the excretory duct cell fate (Yochem et al. 1997).

A lin-1 loss-of-function mutation does not cause larval lethality and can suppress the larval lethality caused by a loss-of-function mutation in these signaling genes (Han et al. 1990; Kornfeld et al. 1995a,b; Sundaram and Han 1995). These observations suggest that lin-1 activity causes larval lethality and the RTK-Ras-MAP ki-

n1855 also contained a C-to-T mutation in intron 5, 21 bp upstream of exon 6; this mutation is probably probably silent.
in signaling genes, such as lin-45 raf (Han et al. 1993), and are less severe than the defects in vulval formation caused by strong loss-of-function mutations in signaling genes. For example, a null allele of mek-2 causes P5.p, P6.p, and P7.p to adopt the 3rd fate and generate a total of six descendants that form no vulval invagination (Kornfeld et al. 1995a). We interpret the defects in n1790 and n1761 mutants as a weak Vul phenotype that results from vulval lineage defects. The vulval invaginations of n2515 and n2525 animals appeared normal and had an average of 22 descendants of P5.p, P6.p, and P7.p (Table 1). Thus, we observed a correlation between the number of descendants of P5.p, P6.p, and P7.p and the severity of the abnormal vulval phenotype, suggesting that these cell-fate defects caused the gross vulval abnormalities.

To characterize further the effect of these lin-1 alleles on vulval development, we examined double mutants containing let-60(ras) or lin-15 mutations that cause a Muv phenotype. The lin-1 alleles partially suppressed the lin-15(lf) Muv phenotype and partially or completely suppressed the let-60(gf) Muv phenotype (Table 2). These alleles can be ordered on the basis of their ability to suppress these two Muv phenotypes: n2515 and n2525 were the most effective, followed by n1761 and n1790. To investigate the cellular basis for the suppression of the let-60(gf) Muv phenotype, we examined the descendants of the Pn.p cells in L4 animals. Whereas in let-60(gf) animals, 53% of the cells P3.p, P4.p, and P8.p adopted vulval fates (n = 10 animals), in lin-1(n2515) let-60(gf) animals, 0% of the cells P3.p, P4.p, and P8.p adopted vulval fates (n = 10 animals; Figure 2, A and B). Thus, the lin-1(n2515) mutation prevented P3.p, P4.p, and P8.p from adopting vulval fates in response to mutationally activated LET-60 Ras, but n2515 did not prevent P6.p from adopting a vulval fate in response to the anchor cell signal. Our interpretation of these findings is that n2515 partially suppresses Ras-mediated signaling; the anchor cell signal overcomes the n2515 suppression by strongly activating let-60 ras in P6.p, whereas the let-60(gf) mutation does not overcome the n2515 suppression since it only partially activates let-60 ras in P3.p, P4.p, and P8.p. The anchor cell signal may also activate pathways that function in parallel to let-60 ras, and factors other than the anchor cell signal may cause P6.p to be more likely to adopt a vulval fate than P3.p, P4.p, and P8.p.

A lin-1(lf) mutation causes a Muv phenotype that is epistatic to the Vul phenotype caused by loss-of-function mutations in genes in the RTK-Ras-MAP kinase signaling pathway (Ferguson et al. 1987). These observations suggest that lin-1 activity causes Pn.p cells to adopt the nonvulval 3rd fate and that the RTK-Ras-MAP kinase signaling pathway promotes the 1st vulval cell fate by regulating lin-1 negatively. The finding that these new lin-1 alleles caused Pn.p cells to adopt nonvulval fates sug-

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage dead larvae</th>
<th>Percentage abnormal vulva</th>
<th>n</th>
<th>P5.p–P7.p descendants</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>22 (22)</td>
<td>10</td>
</tr>
<tr>
<td>n1761</td>
<td>73</td>
<td>31</td>
<td>359</td>
<td>20 (17–22)</td>
<td>10</td>
</tr>
<tr>
<td>n1790</td>
<td>17</td>
<td>54</td>
<td>285</td>
<td>20 (17–24)</td>
<td>10</td>
</tr>
<tr>
<td>ky54</td>
<td>49</td>
<td>59</td>
<td>143</td>
<td>ND</td>
<td>10</td>
</tr>
<tr>
<td>n2515</td>
<td>5</td>
<td>3</td>
<td>167</td>
<td>22 (22)</td>
<td>9</td>
</tr>
<tr>
<td>n2525</td>
<td>1</td>
<td>3</td>
<td>184</td>
<td>22 (21–22)</td>
<td>10</td>
</tr>
<tr>
<td>n1855</td>
<td>2</td>
<td>6</td>
<td>185</td>
<td>ND</td>
<td>10</td>
</tr>
<tr>
<td>smg-1; n1790</td>
<td>43</td>
<td>81</td>
<td>168</td>
<td>15 (10–22)</td>
<td>11</td>
</tr>
</tbody>
</table>

For percent dead larvae and abnormal vulva, we placed each egg on a separate Petri dish and observed development at 20°C. Percent dead larvae, the percent of all hatched eggs that generated dead larvae; most dead larvae displayed a rigid, rod-like morphology. Percent abnormal vulva, the percent of all adult hermaphrodites with a severe egg-laying defect or a protruding vulva. P5.p–P7.p descendants, the number of nuclei that appeared to be descendants of P5.p, P6.p, and P7.p based on appearance and position in L4 hermaphrodites at the "Christmas tree" stage of vulval development. The number is an average followed by the smallest and largest values observed. Differences between the phenotypes of the n1790 strain and the ky54 strain were probably caused by genetic differences at sites other than the lin-1 locus, presumably as a result of prior mutagenesis.

\*a n, number of hatched eggs analyzed.

\*b n, number of L4 hermaphrodites examined.

\*c ND, not determined.

\*d These strains also contained dpy-13. dpy-13 mutants did not display significant larval lethality or abnormal vulval morphology (data not shown).

\*e This strain also contained unc-54(r293); the Unc phenotype is suppressed by smg-1 (Pulak and Anderson 1993).
TABLE 2
lin-1 double mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage Muv</th>
<th>n×10²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>let-60(gf)×</td>
<td>64</td>
<td>637</td>
</tr>
<tr>
<td>n2515 let-60(gf)×</td>
<td>0</td>
<td>515</td>
</tr>
<tr>
<td>n2525 let-60(gf)×</td>
<td>0</td>
<td>516</td>
</tr>
<tr>
<td>n1855 let-60(gf)×</td>
<td>1</td>
<td>547</td>
</tr>
<tr>
<td>n1761 let-60(gf)×</td>
<td>8</td>
<td>527</td>
</tr>
<tr>
<td>n1790 let-60(gf)×</td>
<td>44</td>
<td>449</td>
</tr>
<tr>
<td>lin-15(lf)×</td>
<td>100</td>
<td>624</td>
</tr>
<tr>
<td>n2515; lin-15(lf)×</td>
<td>51</td>
<td>574</td>
</tr>
<tr>
<td>n2525; lin-15(lf)×</td>
<td>58</td>
<td>373</td>
</tr>
<tr>
<td>n1761; lin-15(lf)×</td>
<td>70</td>
<td>504</td>
</tr>
<tr>
<td>n1790; lin-15(lf)×</td>
<td>78</td>
<td>550</td>
</tr>
<tr>
<td>lin-31(lf)×</td>
<td>64</td>
<td>490</td>
</tr>
<tr>
<td>n2515; lin-31(lf)×</td>
<td>71</td>
<td>577</td>
</tr>
<tr>
<td>n2525; lin-31(lf)×</td>
<td>75</td>
<td>507</td>
</tr>
<tr>
<td>n1761; lin-31(lf)×</td>
<td>83</td>
<td>207</td>
</tr>
<tr>
<td>n1790; lin-31(lf)×</td>
<td>98</td>
<td>428</td>
</tr>
</tbody>
</table>

We scored all the adult hermaphrodites on several Petri dishes for the Muv phenotype, one or more ventral protrusions displaced from the position of the vulva.

These strains also contained dpy-13.

Figure 2.—lin-1(gf) mutations prevent Pn.p cells from adopting vulval fates. Photomicrographs of Nomarski images of L4 hermaphrodites at the “Christmas tree” stage of vulval development. In these lateral views, anterior is left and ventral is down. (A) let-60(n1046gf) mutant with a normal vulval invagination (bracket) and four ectopic invaginations (arrows) that resulted from the adoption of vulval fates by P3.p, P4.p, and P8.p. (B) lin-1(n2515) let-60(n1046gf) mutant with a normal vulval invagination (bracket) and no ectopic invaginations, which suggests that they are gain-of-function alleles that produce constitutively active protein that cannot be negatively regulated.

Two observations suggest that at least some of these lin-1 alleles do more than simply increase lin-1 activity. First, although many n1790 and n1761 mutants displayed a weak Vul phenotype, we occasionally observed a mutant animal that displayed a Muv phenotype, suggesting those mutant animals had a reduced amount of lin-1 activity. Second, a comparison of the penetrance of the larval-lethal, abnormal-vulva, and suppression-of-Muv phenotypes shows that these lin-1 alleles cannot be arranged in a simple allelic series. For example, n1761 caused the highest penetrance of larval lethality but only partially suppressed the let-60(gf) Muv phenotype (Tables 1 and 2). By contrast, n2515 and n2525 caused the lowest penetrance of larval lethality but completely suppressed the let-60(gf) Muv phenotype. As described in the discussion, these observations suggest that these alleles affect more than one aspect of lin-1 function.

The novel lin-1 mutations cause gain-of-function phenotypes and result in altered gene activity: To understand how these mutations affect the activity of the lin-1 gene, we compared these lin-1 alleles to lin-1(sy254), a null mutation by genetic criteria and by molecular criteria, since exons 3 and 6 are deleted and exons 4 and 5 are rearranged (Beitel et al. 1995). We also analyzed the effects of varying lin-1 gene dosage using mDp1, a free duplication that contains lin-1 and genes positioned right and left of lin-1 (Rogalski and Riddle 1988). By comparing n1761/lin-1 null animals (73% lethal, 31% abnormal vulva) with n1761/null animals (10% lethal, 10% abnormal vulva), we conclude that larval lethality and abnormal vulva formation are gain-of-function phenotypes, since their penetrance was reduced in trans to aln-1(null) mutation (Table 3). Similarly, n1790/lin-1790 animals (17% lethal, 54% abnormal vulva) displayed a higher penetrance of these defects than n1790/null animals (0% lethal, 12% abnormal vulva). These findings also show that the gain-of-function phenotypes caused by n1761 and n1790 are dosage sensitive, since two mutant alleles caused more severe defects than one mutant allele. By contrast, the Muv phenotype caused by n1790 was more severe in n1790/null animals (75%) than in n1790/n1790 animals (11%) (Table 3). Thus, the Muv phenotype appears to be a loss-of-function phenotype, which is consistent with previous analyses of lin-1 (Beitel et al. 1995). The finding that the n1790...
mutation caused both a gain and loss of gene activity suggests this mutation affects more than one aspect of lin-1 gene function.

The larval lethality and vulval abnormalities caused by n1761 and n1790 were reduced by lin-1(+/+) (Table 3, compare n1761/null with n1761/+, n1761/n1761 with n1761/n1761/+, n1790/null with n1790/+, and n1790/n1790 with n1790/n1790/+). If these defects resulted from an increase in the amount of wild-type lin-1 activity, then an additional copy of lin-1(+) should have enhanced the severity of these defects. The finding that lin-1(+) reduced the severity of these defects suggests that these mutations result in an altered lin-1 activity that can be suppressed by lin-1(+). These findings are consistent with the model that these mutant lin-1 alleles cannot be negatively regulated and thus are active in cells in which lin-1(+) is normally inactivated. Although lin-1(+) is functionally inactive in such cells, it might still compete with mutant lin-1 for limiting cofactors.

To analyze n2515 and n2525, we measured the suppression of the let-60(gf) Muv phenotype. The let-60 (gf) mutation caused 64% of the animals to be Muv (Table 4). In trans to a wild-type lin-1 allele, n2515 and n2525 reduced the penetrance of this Muv phenotype to 3% (n2515/+) and 15% (n2525/+) (Table 4). By contrast, a heterozygous lin-1(null) allele enhanced the let-60(gf) Muv phenotype to 98% (null/+) (Table 4). Thus, the suppression of let-60(gf) Muv is a gain-of-function phenotype. n2515/n2515 and n2525/null animals both displayed strong suppression of the let-60(gf) Muv phenotype, suggesting the n2515 suppression of let-60(gf) Muv phenotype is not dosage sensitive (Table 4, lines 4 and 5). n2525 caused a phenotype that was slightly dosage sensitive—whereas two mutant copies (n2525/n2525) reduced the let-60(gf) Muv phenotype to 0%, one mutant copy reduced the penetrance to 12% (n2525/null) (Table 4). The suppression of let-60(gf) Muv by n2515 and n2525 resulted from an altered lin-1 activity rather than from a simple increase of lin-1 activity, since the phenotype was not enhanced but rather was reduced somewhat by an extra copy of wild-type...

**TABLE 3**

Dosage studies with n1761 and n1790

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage dead larvae</th>
<th>Percentage abnormal vulva</th>
<th>Percentage Muv</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>n1761/null</td>
<td>10</td>
<td>10</td>
<td>1</td>
<td>490</td>
</tr>
<tr>
<td>n1761/+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>553</td>
</tr>
<tr>
<td>n1761/n1761</td>
<td>73</td>
<td>31</td>
<td>9</td>
<td>359</td>
</tr>
<tr>
<td>n1761/n1761/+</td>
<td>ND</td>
<td>16</td>
<td>0</td>
<td>183</td>
</tr>
<tr>
<td>smg-1; n1761/n1761</td>
<td>64</td>
<td>28</td>
<td>8</td>
<td>181</td>
</tr>
<tr>
<td>n1790/null</td>
<td>0</td>
<td>12</td>
<td>75</td>
<td>139</td>
</tr>
<tr>
<td>n1790/+/+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td>n1790/n1790</td>
<td>17</td>
<td>54</td>
<td>11</td>
<td>285</td>
</tr>
<tr>
<td>n1790/n1790/+</td>
<td>ND</td>
<td>13</td>
<td>1</td>
<td>163</td>
</tr>
<tr>
<td>smg-1; n1790/n1790</td>
<td>43</td>
<td>81</td>
<td>12</td>
<td>168</td>
</tr>
<tr>
<td>smg-1/®</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>303</td>
</tr>
</tbody>
</table>

For homozygous strains, we placed each egg on a separate Petri dish and observed development. Heterozygous animals were generated in mating experiments using marker mutations to distinguish self-progeny from cross-progeny. Columns are as defined in Tables 1 and 2.

1 These n1761/lin-1(sy254) unc24 hermaphrodites were non-coordinated (non-Unc) cross-progeny of n1761 males and lin-1(sy254) unc24/nT1 n754 hermaphrodites. The nT1 n754 chromosome causes a recessive lethal and dominant Unc phenotype.

2 These hermaphrodites were Unc non-Dpy cross-progeny of wild-type males and n1761; dpy-11 unc-41 hermaphrodites.

3 These n1761 dpy-13; mDp1 hermaphrodites were non-Dpy self-progeny of hermaphrodites with the same genotype. mDp1 contains lin-1(+) and dpy-13(+).

4 ND, not determined. We could not reliably determine the percent dead larvae of animals of this genotype, because mDp1 is not transmitted to every progeny animal, and mDp1 rescue of the Dpy-13 phenotype cannot be scored in dead larvae.

5 These strains also contained unc54(r293).

6 These animals were genotype n1790/lin-1(sy254) unc24; we picked non-Unc cross-progeny of n1790/+/ males and lin-1(sy254) unc24/nT1 n754 hermaphrodites, scored their phenotype, and confirmed their genotype by examining self-progeny.

7 These hermaphrodites were Unc non-Dpy cross-progeny of wild-type males and n1790; dpy-11 unc-41 hermaphrodites.

8 These n1790 dpy-13; mDp1 hermaphrodites were non-Dpy self-progeny of hermaphrodites of the same genotype.
TABLE 4
Dosage studies with n2515 and n2525

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage Muv</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-60(gf)</td>
<td>64</td>
<td>637</td>
</tr>
<tr>
<td>null/+ let-60(gf)</td>
<td>98</td>
<td>315</td>
</tr>
<tr>
<td>n2515/+ let-60(gf)</td>
<td>3</td>
<td>360</td>
</tr>
<tr>
<td>n2515/n2515 let-60(gf)</td>
<td>0</td>
<td>515</td>
</tr>
<tr>
<td>n2515/null let-60(gf)</td>
<td>1</td>
<td>392</td>
</tr>
<tr>
<td>n2515/null n2515/+ let-60(gf)</td>
<td>22</td>
<td>296</td>
</tr>
<tr>
<td>n2525/+ let-60(gf)</td>
<td>15</td>
<td>287</td>
</tr>
<tr>
<td>n2525/n2525 let-60(gf)</td>
<td>0</td>
<td>516</td>
</tr>
<tr>
<td>n2525/null let-60(gf)</td>
<td>12</td>
<td>509</td>
</tr>
<tr>
<td>n2525/n2525/+ let-60(gf)</td>
<td>41</td>
<td>340</td>
</tr>
</tbody>
</table>

For homozygous strains, we scored all adult hermaphrodites on several Petri dishes for the Muv phenotype. Heterozygous animals were either cross-progeny from mating experiments, in which case marker mutations were used to distinguish self-progeny from crossprogeny, or they were self-progeny of heterozygous animals, in which case marker mutations were used to distinguish homozygous from heterozygous progeny. Columns are as defined in Table 2.

lin-1 (Table 4, compare line 4 to 6 and line 8 to 10). The effect of mdp1 was greater than the effect of lin-1(+) contained on an intact chromosome IV; this effect may result from genes other than lin-1 present on mdp1.

A smg mutation enhanced the lin-1(n1790) gain-of-function phenotype: C. elegans contains a surveillance system that degrades transcripts that contain a premature stop codon (Pulak and Anderson 1993). Therefore, a nonsense mutation results in both a truncated protein and the production of less protein than normal because the mutant mRNA is relatively unstable. It is possible to eliminate the second effect of a nonsense mutation by creating a double mutant with a smg mutation; the smg genes are required for the function of the surveillance system, and mRNAs with premature stop codons accumulate to essentially wild-type levels in smg(-) mutants (Pulak and Anderson 1993).

Of the four different lin-1(gf) mutations, only n1790 is a nonsense change. Our results indicated that the larval lethality and abnormal vulva caused by n1790 are dose-dependent, gain-of-function phenotypes. If this model is correct, then we predict that stabilizing the mutant lin-1(n1790) mRNA and producing more LIN-1(Arg352stop) protein would enhance these phenotypes. Table 1 shows that smg-1; lin-1(n1790) double mutants displayed significantly more larval lethality (43% versus 17%) and abnormal vulvae (81% versus 54%) than did n1790 single mutants. To investigate the cause of the vulval defects, we determined the number of P5.p, P6.p, and P7.p descendants in the vulval invaginations of L4 hermaphrodites. smg-1; n1790 animals had an average of 15 descendants, compared to an average of 20 descendants for n1790 mutants and 22 descendants for wild-type animals (Table 1 and Figure 2C). Thus, the smg-1 mutation enhanced the n1790 Vul phenotype. In control experiments, the smg-1 mutation alone did not cause significant vulval defects, and it did not enhance the defects caused by n1761, a mutation in a splice site (Table 3, lines 4, 6, and 12). These observations support the model that LIN-1(Arg352stop) protein encoded by n1790 prevents P6.p from adopting a vulval fate and causes larval lethality in a dose-dependent manner. Furthermore, lin-1(n1790) mRNA appears to be less stable than lin-1(+) mRNA, suggesting the loss-of-function Muv phenotype caused by n1790 may be a consequence, at least in part, of reduced protein levels caused by mRNA instability.

lin-1 acts parallel to or upstream of lin-31: A lin-31(If) mutation causes a partially penetrant Muv phenotype, suggesting lin-31 activity prevents P3.p, P4.p, and P8.p from adopting vulval fates (Ferguson et al. 1987). The predicted LIN-31 protein contains an HNF-3/ forkhead DNA-binding domain and presumably functions as a transcription factor (Miller et al. 1993). Genetic epistasis experiments suggest lin-31 functions downstream of mpk-1 ERK MAP kinase, if these genes act in a linear pathway (Lackner et al. 1994). Previously, it had not been possible to use genetic epistasis experiments to investigate the order of action of lin-31 and lin-1, because a loss-of-function mutation in either gene causes a Muv phenotype. We used the lin-1(gf) mutations, which suppressed the Muv phenotype caused by let-60(gf) and lin-15(If) mutations, to explore the relationship between lin-1 and lin-31. Table 2 shows that none of the lin-1(gf) mutations suppressed the lin-31 Muv phenotype. These results suggest that if lin-1 and lin-31 act in a linear pathway, lin-1 acts upstream of lin-31. These data are also consistent with the possibility that lin-1 and lin-31 function in parallel. We favor this possibility, since both genes encode predicted transcription factors.

It is noteworthy that all the lin-1(gf); lin-31(If) double mutant strains displayed a higher penetrance Muv phenotype than the lin-31(If) single mutant strain (Table 2). We interpret these results as an indication that these lin-1 mutations partially reduce lin-1 activity, resulting in...
Beitel et al. The ETS domains of LIN-1 and Aop are somewhat less similarly positioned in the N-terminal region. LIN-1 is phosphorylated by ERK MAP kinase.

Lin-1 gain-of-function mutations affect a conserved motif in the C box: The vertebrate proteins Elk-1, SAP-1a, and Net/ERP/SAP-2 are classified as members of the Elk subfamily of ETS proteins because they share three regions of significant sequence conservation: an N-terminal ETS domain, a centrally positioned B box, and a C-terminal C box (reviewed by Wasylk et al. 1993; Giovane et al. 1994; Treisman 1994). Based on the positions and sequences of their ETS domains and the positions and sequences of regions similar to the C box, we propose that LIN-1 and Drosophila Aop are members of the Elk subfamily. The ETS domain of LIN-1 shares more sequence identity with the ETS domain of human Elk-1 (67% identity) and human SAP-1a (61% identity) than with any other ETS domain (Beitel et al. 1995). Likewise, the ETS domain of Aop is most similar to the ETS domain of Elk-1 (51% identity; Lai and Rubin 1992). The ETS domains of LIN-1 and Aop are somewhat less similar (41% identity; Beitel et al. 1995). LIN-1 (441 residues), Elk-1 (428 residues), SAP-1a (453 residues) and Net (409 residues) are similarly sized and have ETS domains similarly positioned in the N-terminal region (Figure 3A). By comparison, Aop (688 residues) is larger and has more residues N-terminal to the ETS domain, which is located near the center of the protein. However, the number of residues C-terminal to the ETS domain is similar in Aop and the other four proteins (Figure 3A).

Further evidence that ETS proteins are members of a subfamily is sequence similarity outside the ETS domain. By studying the C termini of these proteins, we found that LIN-1, Elk-1, SAP-1a, and Net each have the sequence FQFP, while Aop has the sequence FQFHP (Figure 3B). In Elk-1, SAP-1a, and Net, the FQFP sequence is at the end of the C box (Giovane et al. 1994; Treisman 1994). The C boxes of ELK-1, SAP-1a, and Net are characterized by five or six S/TP sequences, which are potential MAP kinase phosphorylation sites. In the corresponding regions, LIN-1 has five S/TP sequences and Aop has three (Figure 3B). Elk-1, SAP-1a, and Net have additional identities in the C box that are not conserved in LIN-1 and Aop. These observations suggest that LIN-1 and Aop contain divergent C boxes. Thus, lin-1, aop, elk-1, sap-1, and net appear to be derived from an ancestral gene that encoded a protein with an N-terminal ETS domain and a C-terminal C box.

All the lin-1(gf) mutations result in changes in the FQFP motif. Lin-1(n1790) and lin-1(n1761) encode truncated proteins that lack the FQFP motif. Lin-1(n2525) and lin-1(n2515) result in a change to FQFS or FQFL, respectively (Figure 3B). Our genetic experiments indicate that these mutations impair the negative regulation of lin-1, suggesting the conserved FQFP motif may be important for this negative regulation.

LIN-1 is phosphorylated by ERK MAP kinase in vitro.
To investigate whether LIN-1 is phosphorylated by ERK MAP kinase, we used the lin-1 cDNA to express in E. coli full-length LIN-1 protein fused to GST. We partially purified this protein by affinity chromatography and assayed its ability to be phosphorylated by purified, recombinant, murine Erk2 MAP kinase. The GST::LIN-1(1–441) fusion protein was a high-affinity substrate for Erk2 with a $K_m$ of 0.18 μm (Figure 4). This $K_m$ is about 18-fold lower than the 3.3 μm $K_m$ of myelin basic protein (MBP), a protein frequently used to assay ERK activity. The relative acceptor ratio ($V_{max}/K_m$) is an overall measure of the ability of a protein to function as a substrate. The relative acceptor ratio of GST::LIN-1(1–441) was 7-fold higher than the value for MBP (Figure 4).

To investigate the regions of LIN-1 that are important for phosphorylation, we generated and assayed fragments of LIN-1 fused to GST. GST::LIN-1(1–379) includes the residues predicted to be encoded by lin-1(n1761). This protein lacks the C-terminal 62 amino acids and the FQFP motif but contains the S/TP motifs in the C box. GST::LIN-1(1–379) had a $K_m$ of 1.1 μm, which is about 6-fold higher than the $K_m$ of full-length LIN-1, and a $V_{max}$ that was similar to the $V_{max}$ of full-length LIN-1 (Figure 4). The difference in $K_m$ values suggests that the deleted region is important for the high-affinity interaction between LIN-1 and Erk2, while the similarity in $V_{max}$ values suggests that this LIN-1 fragment contains the phosphorylation site(s).

**Figure 4.** LIN-1 is phosphorylated by ERK MAP kinase. Each protein listed in D was analyzed using the same experimental approaches; A–C show examples of these data. (A) A Western blot of samples containing partially purified GST or GST fused to the indicated LIN-1 residues that were treated with an anti-GST::LIN-1(154–294) antiserum. Lines indicate the positions of protein standards from an adjacent lane, with sizes in kilodaltons (kD). This experiment shows that each sample contained intact fusion protein (indicated by a star) that reacted with the antiserum and migrated with the predicted molecular weight [myelin basic protein (MBP), 18 kD; GST::LIN-1(1–441), 74 kD; GST::LIN-1(281–441), 43 kD; GST::LIN-1(1–278), 58 kD; GST, 26 kD]. We adjusted the amount of total protein loaded in each lane to achieve a similar signal intensity of intact protein. Most samples also contained lower molecular weight forms that reacted with the antiserum and are likely to be fragments of fusion proteins that were generated by protease activity in E. coli. These samples also contained endogenous bacterial proteins that were visible on Coomassie blue-stained gels (data not shown) and usually did not react with the antiserum. However, the uppermost band in lane 3 did react with this antiserum and appears to be an endogenous bacterial protein, since we observed the same band in partially purified samples of unrelated GST fusion proteins (data not shown). MBP did not react with the antiserum. (B) Equal amounts of intact GST or GST::LIN-1 fusion proteins were incubated with purified, recombinant, murine Erk2 and $[^{32}P]$ATP, fractionated by SDS-PAGE, and visualized by autoradiography. GST::LIN-1(1–441) and GST::LIN-1(281–441) were strongly labeled, whereas GST alone or GST::LIN-1(1–278) showed no significant incorporation. MBP was weakly labeled. The GST::LIN-1(1–441) sample contained many proteolytic fragments that bound to the affinity column and thus appear to be an endogenous bacterial protein, since we observed the same band in partially purified samples of unrelated GST fusion proteins (data not shown). MBP did not react with the antiserum. (C) A kinetic analysis showing the amount of $[^{32}P]$ incorporated measured by filter binding and scintillation counting (counts per minute, CPM) in assays with increasing amounts of GST::LIN-1(1–441). Assays were terminated after 15 min, at which point $[^{32}P]$ incorporation was linear with respect to time. Values are the average of two samples, a bar indicates the range. The inset shows a Lineweaver-Burke plot of the data. (D) Kinetic analyses were performed as described above using seven concentrations of intact proteins, usually ranging from 0.2 $K_m$ to 2.0 $K_m$. $V_{max}$ and $K_m$ were calculated from the intercepts of Lineweaver-Burke plots in each case the data closely approximated a straight line. Values are the average and one standard deviation of three or four separate experiments. To determine $V_{max}$, we calculated total phosphate incorporated using the measured CPM and the specific activity of the $[^{32}P]$ATP, and factored in the assay time (15 min) and the amount of Erk2 used (about 0.03 pmol). Relative acceptor ratio is $V_{max}/K_m$; values were normalized by assigning a value of 1.0 to MBP.
lin-1Gain-of-Function Mutations

1(1–278) lacks the C-terminal 163 amino acids and the entire C box. This protein was an extremely poor substrate for Erk2 with a relative acceptor ratio that was 200-fold lower than the value for GST:LIN-1(1–379) (Figure 4D). These findings suggest that the region from amino acid 278 to 379 may contain the normal phosphorylation site(s). GST:LIN-1(281–441) contains the C-terminal 160 amino acids and includes the C box; it had a relative acceptor ratio that was about 300-fold higher than the N-terminal region of LIN-1 (Figure 4D). Thus, the C terminus of LIN-1 was necessary for efficient phosphorylation of LIN-1 and sufficient to function as a substrate for Erk2.

To investigate how the lin-1(gf) missense mutations affect phosphorylation by ERK, we generated full-length LIN-1 containing the change encoded by lin-1(n2515). GST:LIN-1(1–441P384L) had a \(K_m\) of 0.8 \(\mu\)m, which is about fourfold higher than the \(K_m\) of wild-type LIN-1, and a \(V_{\text{max}}\) similar to that of wild-type LIN-1. Thus, changing FQFP to FQFL reduced the binding affinity of Erk2.

**DISCUSSION**

**lin-1 activity promotes nonvulval fates and larval lethality:** We identified and characterized six mutations that define a new class of lin-1 allele. Our genetic analysis suggests that these mutations affect two aspects of lin-1 function. First, they have a major effect on the ability of lin-1 to be negatively regulated, which results in constitutively active lin-1 and causes larval lethality, a vulval phenotype, and a suppression of Muv phenotype. These are gain-of-function phenotypes. Our results suggest that negative regulation depends on the ability of LIN-1 protein to be phosphorylated by MAP kinase. Second, these mutations have a minor effect on the ability of lin-1 to control cell fates, which results in inactive lin-1 and causes a loss-of-function Muv phenotype. The LIN-1 protein presumably controls cell fates by binding DNA and regulating transcription. The missense mutations n2515 and n2525 did not significantly impair the ability of lin-1 to control cell fates, because they caused only a slight Muv phenotype. These mutations partially impaired the ability of lin-1 to be negatively regulated, because they caused weak gain-of-function phenotypes (larval lethality and Vul). The proline-to-leucine change caused by n2515 impaired negative regulation more than the proline-to-serine change caused by n2525; although the difference was small, it was observed in multiple genetic backgrounds. The alleles n1790 and n1761, which are predicted to encode truncated proteins, partially impaired the ability of lin-1 to control cell fates, because they caused a Muv phenotype of low penetrance. These mutations severely impaired the ability of lin-1 to be negatively regulated, because they caused strong gain-of-function phenotypes. The defect in the ability of lin-1 to control cell fates could be caused by a defect in the ability of the mutant protein to regulate transcription, a reduced level of mutant protein caused by mRNA or protein instability, or a combination of such defects. The n1790 nonsense mutation seems to reduce mRNA stability, which contributed to its phenotype.

Our genetic results indicate that the lin-1(gf) mutations result in altered lin-1 activity. We suggest the mutant lin-1 alleles are constitutively active in cells in which lin-1(+) is negatively regulated, and we propose that this ectopic activity can be used to infer the normal functions of lin-1. lin-1(gf) alleles have not been described previously, and the phenotype caused by these alleles considered together with the phenotype caused by lin-1(lf) alleles clarifies the functions of lin-1. lin-1(gf) mutations cause a strong Muv phenotype (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985; Ferguson et al. 1987; Beitel et al. 1995). In our experiments, lin-1(gf) mutations caused a weak Vul phenotype and caused P3.p, P4.p, and P8.p to adopt the nonvulval 3° fate in let-60(gf) mutants. These observations suggest that lin-1 activity prevents Pn.p cells from adopting vulval fates. The lin-1(gf) mutations caused larval lethality characterized by a rigid, rod-like morphology. This lethality is likely to result from a failure to establish the excretory duct cell fate and hence from defective osmoregulation (Yochem et al. 1997). A lin-1(lf) mutation can suppress the larval lethality caused by a loss of activity of the RTK-Ras-MAP kinase signaling pathway (reviewed by Kornfeld 1997). These observations suggest that lin-1 activity prevents a precursor cell from adopting the excretory duct fate. The lin-1(gf) mutations did not cause hermaphrodite sterility, and a lin-1(lf) mutation does not suppress the hermaphrodite sterility caused by a loss of the activity of the Ras-MAP kinase pathway (Kornfeld et al. 1995a). Thus, lin-1 does not appear to function in the germ cells, which require the activity of the Ras-MAP kinase pathway to exit from pachytene (Church et al. 1995).

Genetic epistasis tests with both classes of lin-1 alleles lead to similar conclusions about the position of lin-1 in the signaling pathway. The lin-1(lf) mutation (Muv phenotype) is epistatic to a let-60 ras loss-of-function mutation (Vul phenotype) (Han et al. 1990). Similarly, the lin-1(gf) mutation (Vul phenotype) was epistatic to a let-60 ras gain-of-function mutation (Muv phenotype). Both findings suggest that lin-1 functions downstream of let-60 ras, if these two genes function in a linear signaling pathway. Taken together, the genetic analyses of the lin-1(gf) and lin-1(lf) mutations strongly support the model that lin-1 is negatively regulated by the activity of the RTK-Ras-MAP kinase pathway to allow the establishment of the excretory duct cell fate and allow P6.p to adopt a 1° vulval fate.

**LIN-1 is likely to be phosphorylated and thereby regulated directly by ERK MAP kinase:** Although genetic experiments show that lin-1 and mpk-1 function in the same processes, epistasis tests do not prove that lin-1 is negatively regulated by the RTK-Ras-MAP kinase pathway, because these data are also consistent with the
possibility that lin-1 functions in a parallel signaling pathway and is negatively regulated by other molecules. Furthermore, even if lin-1 is negatively regulated by the RTK-Ras-MAP kinase pathway, the genetic data are consistent with either direct or indirect regulation of lin-1 by mpk-1 ERK MAP kinase. Here we show that murine Erk2 can phosphorylate LIN-1 in vitro. The $K_m$ for full-length LIN-1 was 0.18 $\mu$m, about 18-fold lower than the $K_m$ for myelin basic protein, showing LIN-1 is a high-affinity substrate and suggesting that phosphorylation of LIN-1 is not likely to result from promiscuous kinase activity in vitro. Vertebrate Erk2 was used because purified, active enzyme is readily available. However, it is likely that C. elegans MPK-1 also can phosphorylate LIN-1, since C. elegans mpk-1 shares more than 70% identity with vertebrate ERK, and vertebrate ERK can functionally substitute for C. elegans mpk-1 (Lackner et al. 1994; Wu and Han 1994). Taken together, the genetic experiments suggesting that mpk-1 ERK MAP kinase functions upstream of lin-1 at multiple times during development and the biochemical demonstration that Erk2 can phosphorylate LIN-1 strongly support the model that MPK-1 phosphorylation directly regulates LIN-1. This model predicts that LIN-1 is phosphorylated in vivo and that phosphorylation requires mpk-1. We are testing these predictions.

We have not yet determined the precise LIN-1 residues that are phosphorylated by ERK. However, we found that the C-terminal 160 residues were necessary for phosphorylation of full-length LIN-1 and sufficient to function as an Erk2 substrate, suggesting that phosphorylation of full-length LIN-1 occurs in this region. Furthermore, each lin-1(gf) mutation is predicted to affect the C terminus of LIN-1 and these alleles appear to be constitutively active, suggesting the C terminus is necessary for LIN-1 to be negatively regulated. These findings support the model that MAP kinase phosphorylation of the LIN-1 C terminus causes negative regulation of LIN-1 activity. We have not determined how phosphorylation regulates LIN-1. Phosphorylation might decrease LIN-1 protein activity or result in a change in protein localization or stability.

The finding that LIN-1, a predicted transcription factor, is directly regulated by ERK supports the model that MAP kinase is a transition point between signaling proteins and proteins that mediate cell-fate changes. Furthermore, the finding that lin-1 is an important target of mpk-1 ERK MAP kinase during the establishment of the excretory duct cell fate and during vulval development but not in germ cells suggests that mpk-1 ERK MAP kinase does not regulate the same target proteins in each developmental context. These observations support the model that MAP kinase contributes to the specificity of different cellular responses by phosphorylating different target proteins in different cells. LIN-1 is the first C. elegans protein shown to be directly regulated by ERK MAP kinase, and many important questions about specificity remain to be answered. For example, how many different proteins are phosphorylated by mpk-1 ERK MAP kinase in vivo, how much overlap exists between mpk-1 ERK MAP kinase targets in different cells, and is target specificity controlled by target protein availability and/or differences in mpk-1 ERK MAP kinase in different cells?

C. elegans LIN-1, vertebrate Elk-1, SAP-1a, Net, and Drosophila Aop are members of the Elk subfamily of ETS proteins: Conserved structure and conserved function can be used to infer the evolutionary relationships among ETS genes. We propose that LIN-1 and Drosophila Aop are members of the Elk subfamily, which contains the vertebrate proteins Elk-1, SAP-1a, and Net, ERP, SAP-2 (Giavante et al. 1994; Treisman 1994). This model is supported by three similarities among these proteins—sequence and position of the ETS domain, sequence and position of the C box, and interactions with ERK. Other ETS proteins do not share all of these features, although a few ETS proteins share some of these features. The structural and sequence similarities are described in the results and illustrated in Figure 3.

All five proposed members of the Elk subfamily appear to be phosphorylated by ERK. Elk-1, SAP-1a, and Net are ternary complex factors that bind the serum response element present in the promoters of immediate early genes, such as c-fos (reviewed by Treisman 1994). In cultured vertebrate cells, transcription of immediate early genes is rapidly induced by activation of RTK-Ras-ERK MAP kinase pathways. This induction seems to be mediated by phosphorylation of ternary complex factors. In vitro, ERK phosphorylates multiple sites in and around the Elk-1 C box; the same sites are phosphorylated in vivo following activation of the Ras-ERK MAP kinase pathway (Janknecht et al. 1993; Marais et al. 1993). Phosphorylation stimulates the ability of the Elk-1 C terminus to function as a transcriptional activation domain. The C termini of SAP-1a and Net behave like the C terminus of Elk-1 (Price et al. 1995). Genetic analyses show that aop is an important regulator of cell fate decisions at multiple times during Drosophila development (Lai and Rubin 1992). aop appears to be negatively regulated by RTK-Ras-ERK MAP kinase pathways in flies and in cultured Drosophila cells (O'Neill et al. 1994). Aop can be phosphorylated by ERK in vitro (Brunner et al. 1994). In flies, mutant versions of Aop that lack multiple putative MAP kinase phosphorylation sites are unresponsive to negative regulation (Rebay and Rubin 1995). Phosphorylation may cause Aop protein to leave the nucleus and be degraded (Rebay and Rubin 1995). Genetic analyses suggest lin-1 is negatively regulated by RTK-Ras-ERK MAP kinase pathways, and our biochemical experiments suggest the mechanism is phosphorylation of the C terminus by ERK. These observations suggest lin-1, aop, dk-1, sap-1, and net are derived from a common ancestral gene that encoded a protein that was phosphorylated by ERK MAP
kinase. The finding that phosphorylation positively regulates Elk-1 but negatively regulates LIN-1 and Aop suggest that the effect of ERK phosphorylation has diverged during evolution.

**FOFP may be a recognition motif for ERK MAP kinases:** MAP kinases can be divided into subfamilies based on particular conserved residues. Five subfamilies are currently known in budding yeast and three in vertebrates (reviewed by Davis 1995; Treisman 1996). C. elegans MPK-1, Drosophila ERK, and vertebrate Erk-1 and Erk-2 are members of the ERK MAP kinase subfamily (Lackner et al. 1994; Treisman 1996). All MAP kinases phosphorylate SP and TP motifs (reviewed by Davis 1993). However, 90% of all proteins have one or more S/TP sequences (data not shown), and yet a particular MAP kinase does not phosphorylate all these proteins or every S/TP sequence within a target protein. The mechanisms that achieve target specificity have not been established.

We propose, based upon three pieces of evidence, that FOFP is a recognition motif that enables substrate proteins to bind ERK MAP kinase. First, FOFP is a conserved element of the C box. Comparisons of Elk-1, SAP-1a, and Net first identified the C box as a region with extensive sequence conservation (Giovanne et al. 1994; Treisman 1994). Our analysis revealed that LIN-1 and Aop contain divergent C boxes, and the only highly conserved elements among all these C boxes are multiple S/TP sequences and the FOFP motif. The S/TP sequences in the C boxes of Elk-1 and SAP-1a are the major phosphorylation sites for ERK (Janknecht et al. 1993; Janknecht and Hunter 1997; Marais et al. 1993). The conservation of the FOFP motif and its proximity to crucial phosphorylation sites implicates the FOFP motif in this process. Second, our results suggest that changing the FOFP motif affects the regulation of LIN-1 in vivo. The proteins encoded by lin-1(n1790) and lin-1(n2525) have a change in the proline of the FOFP motif and appear to be partially unresponsive to negative regulation by the RTK-Ras-MAP kinase pathway. The proteins encoded by lin-1(n1790) and lin-1(n1761) lack the FOFP motif and appear to be extremely unresponsive to negative regulation. We propose that these mutant proteins are constitutively active because alterations in the FOFP motif prevent efficient recognition and phosphorylation by MPK-1 ERK MAP kinase. Third, our in vitro biochemical experiments support this hypothesis. GST:LIN-1(1-441P384L), which has the amino acid substitution caused by lin-1(n2515), had a \( K_m \) for Erk2 that was fourfold higher than the \( K_m \) of wild-type LIN-1. GST:LIN-1(1-379), which lacks the FOFP motif and is similar to the mutant protein encoded by n1761, had a \( K_m \) for Erk2 that was sixfold higher than the \( K_m \) of full-length LIN-1. Thus, the severity of the change in the FOFP motif correlates with the ability of LIN-1 to be phosphorylated by Erk2 in vitro and the ability of lin-1 to be negatively regulated in vivo.

The role of the FOFP sequence in Elk-1, SAP-1a, and Net or the FQFHP sequence in Aop has not been investigated directly. However, the aop\(^{n52382}\) mutation is a 5 bp deletion that shifts the reading frame, thereby replacing the C-terminal 162 amino acids with a new group of 86 amino acids (Rebay and Rubin 1995); the mutant Aop protein lacks the FQFHP motif and some S/TP sequences (Figure 3) and thus resembles the proteins encoded by lin-1(n1790) and lin-1(n1761). Genetic analysis also indicates that aop\(^{n52382}\) resembles the lin-1(gf) alleles, since aop\(^{n52382}\) was isolated in a genetic screen for suppressors of activated Ras and it appears to be unresponsive to Ras-MAP kinase signaling, which negatively regulates aop (+) (Rebay and Rubin 1995; Karim et al. 1996). We propose that this mutant Aop protein is not phosphorylated efficiently by ERK, because it lacks the recognition motif and is therefore constitutively active. We are now performing biochemical experiments to test the role of the FOFP motif in mediating ERK MAP kinase target recognition.

We thank Cheri Zobel and Radhika Tripathani for scoring lin-1 phenotypes, Andrew Turk and Danielle Glossip for constructing lin-1 expression plasmids, and Jennie Liang and Yunxiang Zhu for guidance about protein analysis. Some strains were provided by the Caenorhabditis Genetics Center (St. Paul, MN), which is supported by the National Institutes of Health. This research was in part supported by the Edward Mallinkrodt, Jr. Foundation (K.K.). H.R.H. is an Investigator and G.J.B. was a predoctoral fellow of the Howard Hughes Medical Institute.

**LITERATURE CITED**


Wu, Y., and M. Han, 1994 The origin of activated Let-60 Ras protein defines a role of Caenorhabditis elegans Surf-1 MAP kinase in vulval differentiation. Genes Dev. 8: 147–159.
Communicating editor: I. Greenwald