lin-35 and lin-53, Two Genes that Antagonize a C. elegans Ras Pathway, Encode Proteins Similar to Rb and Its Binding Protein RbAp48

Xiaowei Lu and H. Robert Horvitz*
Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The Ras signaling pathway for vulval induction in Caenorhabditis elegans is antagonized by the activity of the synthetic multivulva (synMuv) genes, which define two functionally redundant pathways. We have characterized two genes in one of these pathways. lin-35 encodes a protein similar to the tumor suppressor Rb and the closely related proteins p107 and p130. lin-53 encodes a protein similar to RbAp48, a mammalian protein that binds Rb. In mammals, Rb and related proteins act as regulators of E2F transcription factors, and RbAp48 may act with such proteins as a transcriptional corepressor. We propose that LIN-35 and LIN-53 antagonize the Ras signaling pathway in C. elegans by repressing transcription in the vulval precursor cells of genes required for the expression of vulval cell fates.

Introduction

Extracellular signals can promote or inhibit cell growth and differentiation via distinct signaling systems. The proper integration of multiple signals allows a cell to respond appropriately to its environment and is vital for homeostasis. Inappropriate activity of one or more signaling pathways can lead to tumorigenesis. Receptor tyrosine kinase (RTK)/Ras pathways define one class of oncogene signaling pathways (reviewed by Cantley et al., 1991; Hunter, 1997). RTK/Ras pathways function during the normal development of many organisms, including the nematode Caenorhabditis elegans (reviewed by Dickson and Hafen, 1994).

In C. elegans, the formation of the hermaphrodite vulva is induced by an RTK/Ras signaling pathway. The vulva is generated from six multipotent ventral ectodermal blast cells, P3.p-P8.p (Sulston and Horvitz, 1977; Sulston and White, 1980). Each of these six P(3-8).p cells can potentially adopt either the 1° vulval cell fate, the 2° vulval cell fate, or the 3° nonvulval cell fate (Sulston and White, 1980; Kimble, 1981; Sternberg and Horvitz, 1986). During wild-type development, a signal from the gonadal anchor cell induces the nearest P(3-8).p cell, P6.p, to adopt the 1° fate and the adjacent P5.p and P7.p cells to adopt the 2° fate. The cells furthest from the anchor cell, P3.p, P4.p, and P8.p, adopt the uninduced 3° fate. Vulval induction acts through a signaling pathway, which includes the lin-3 EGF-like ligand, the let-23 RTK, the sem-5 adaptor, let-60 Ras, the ksr-1 kinase, lin-45 Raf, mek-2 MEK, and mpk-1 MAP kinase, to regulate the activities of the ETS transcription factor LIN-1 and the winged-helix transcription factor LIN-31 (reviewed by Horvitz and Sternberg, 1991; Sundaram and Han, 1996; Tan et al., 1998).

Vulval induction is negatively regulated by the synthetic multivulva (synMuv) genes (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989). Loss-of-function mutations in these genes result in a multivulva (Muv) phenotype as a consequence of the expression of vulval cell fates by the P3.p, P4.p, and P8.p cells. The Muv phenotype of these mutants requires mutations in two genes. Specifically, these synMuv mutations fall into two classes, referred to as A and B. Animals carrying a class A and a class B mutation have a Muv phenotype, while animals carrying one or more mutations of the same class have a wild-type vulval phenotype. These mutations appear to define two functionally redundant pathways that negatively regulate the expression of vulval cell fates.


To elucidate the molecular mechanism by which the synMuv genes inhibit vulval induction, we have characterized two class B synMuv genes, lin-35 and lin-53. Our findings indicate that in C. elegans vulval development an Rb-mediated pathway antagonizes the RTK/Ras pathway of vulval induction.

Results

Molecular Identification of lin-35

We mapped lin-35 between unc-40 and the Tc1 polymorphism stp124 (Williams et al., 1992) on LGI. We identified two overlapping cosmids from this interval, C03E6 and C32F10, each of which rescued the Muv phenotype of a lin-35; lin-15A strain in germline transformation experiments (Figure 1A). The smallest subclone that retained rescuing activity was a 9.3 kb XhoI-EcoRV fragment from the region of overlap between C03E6 and C32F10. The C. elegans genome consortium (Wilson et al., 1994) had determined the DNA sequence of this fragment. Using the 9.3 kb minimal rescuing fragment as a probe, we detected a single 3.2 kb transcript in RNA on a Northern blot (Figure 1C), and we isolated cDNA clones from an embryonic cDNA library and determined the complete sequence of the longest cDNA (3.2 kb). We deduced the gene structure by comparing the genomic and the cDNA sequences (Figure 1B). The cDNA contains a single open reading frame (ORF) of 961 amino
The predicted LIN-35 protein shares significant sequence similarity with the mammalian pocket proteins, which include the tumor suppressor Rb (Friend et al., 1986), p107 (Ewen et al., 1991), and p130 (Hannon et al., 1993; Li et al., 1993) (Figure 2). The similarity extends across the entire lengths of the proteins, including the “A/B pocket” domains, which mediate interactions with proteins important for cell cycle regulation, such as viral oncoproteins and E2F (reviewed by Taya, 1997). Overall, LIN-35 is 20% identical to p130, 19% to p107, 15% to Rb, and 16% to RBF, an Rb-related protein in Drosophila (Du et al., 1996). Regions important for pocket protein function are more conserved. For example, the N-terminal region of the B pocket domain of LIN-35 (amino acid residues 744-839) is 34% identical to p130, 34% to p107, 29% to Rb, and 30% to RBF. The spacer region that separates the A and B pockets of LIN-35 is not related in sequence to those of the pocket proteins. This spacer is short in length, like that of Rb. By contrast, p130 and p107 have much longer spacer regions, which are conserved between them and mediate their stable association with cyclin/cyclin-dependent kinase (CDK) complexes (reviewed by Zhu et al., 1994). Because LIN-35 is not particularly similar in sequence to any one of the three mammalian pocket proteins, lin-35 may have diverged from an ancestor of the three mammalian genes before these three genes diverged from each other.

LIN-35 Protein Is Present in Vulval Cells

We raised polyclonal antibodies against a peptide from the N-terminal region of LIN-35. Affinity-purified antisera recognized a single protein of about 110 kDa present in both embryonic (E) and mixed-staged (M) poly(A)-RNA (3 μg in each lane) from wild-type C. elegans as detected using the 9.3 kb XhoI-EcoRV minimal rescuing fragment as a probe. The trans-spliced leader 5′SL1, the initiation and stop codons, and the poly(A) tail are indicated. Solid boxes indicate coding sequences, and open boxes indicate noncoding sequences. The positions of the eight lin-35 mutations are indicated by the vertical lines above the boxes.

Northern blot analysis of lin-35. lin-35 message is present in both embryonic (E) and mixed-staged (M) poly(A)-RNA (3 μg in each lane) from wild-type C. elegans as detected using the 9.3 kb XhoI-EcoRV minimal rescuing fragment as a probe. Each predicted mutant protein is represented schematically by a box labeled with its length in amino acids. Wild-type LIN-35 is shown on top. The A/B pockets are indicated by solid boxes. The region required for E2F binding includes the A and B pockets and the hatched box.

acids and appears to be full-length by three criteria: its size matches that of the transcript detected on the Northern blot, it contains the last 11 nucleotides of the 5′SL1 trans-spliced leader sequence (Krause and Hirsh, 1987) at its 5′ end and a poly(A) tail at its 3′ end, and expression of this cDNA under control of the C. elegans heat shock promoters (Stringham et al., 1992) rescued the lin-35 mutant phenotype (data not shown). To confirm that the rescuing activity observed was indeed lin-35 activity, we identified the molecular lesions associated with the eight existing lin-35 alleles (Figure 1D). Six alleles are nonsense mutations, and two alleles have an identical splice-acceptor mutation in the fourth intron despite their independent isolation. The allele n745 contains an early nonsense mutation predicted to eliminate the C-terminal 84% of LIN-35. This allele may completely eliminate lin-35 function.

lin-35 Encodes a Protein Similar to the Tumor Suppressor Rb

The predicted LIN-35 protein shares significant sequence similarity with the mammalian pocket proteins, which include the tumor suppressor Rb (Friend et al., 1986), p107 (Ewen et al., 1991), and p130 (Hannon et al., 1993; Li et al., 1993) (Figure 2). The similarity extends across the entire lengths of the proteins, including the "A/B pocket" domains, which mediate interactions with proteins important for cell cycle regulation, such as viral oncoproteins and E2F (reviewed by Taya, 1997). Overall, LIN-35 is 20% identical to p130, 19% to p107, 15% to Rb, and 16% to RBF, an Rb-related protein in Drosophila (Du et al., 1996). Regions important for pocket protein function are more conserved. For example, the N-terminal region of the B pocket domain of LIN-35 (amino acid residues 744-839) is 34% identical to p130, 34% to p107, 29% to Rb, and 30% to RBF. The spacer region that separates the A and B pockets of LIN-35 is not related in sequence to those of the pocket proteins. This spacer is short in length, like that of Rb. By contrast, p130 and p107 have much longer spacer regions, which are conserved between them and mediate their stable association with cyclin/cyclin-dependent kinase (CDK) complexes (reviewed by Zhu et al., 1994). Because LIN-35 is not particularly similar in sequence to any one of the three mammalian pocket proteins, lin-35 may have diverged from an ancestor of the three mammalian genes before these three genes diverged from each other.
pleiotropic effects (data not shown). It is possible that other functions of lin-35, like those involved in vulval development, are redundant.

lin-53 Encodes a Protein Similar to RbAp48

Based on the finding that lin-35 encodes a protein similar to Rb, we reasoned that other genes in the class B synMuv pathway might also be evolutionarily conserved. We identified from the C. elegans sequence database genomic sequences and expressed sequence tags (ESTs) (Wilson et al., 1994) predicted to encode proteins similar to proteins known to interact with Rb, and we compared their map positions with those of known class B synMuv genes. The cosmid K07A1 contained two predicted genes, named K07A1.12 and K07A1.11, both similar to RbAp48 (p48) and RbAp46 (p46), two closely related proteins that bind to Rb in vivo (Huang et al., 1991; Qian et al., 1993; Qian and Lee, 1995). This cosmid mapped near the class B synMuv gene lin-53 (Ferguson and Horvitz, 1989; J. H. Thomas and H. R. H., unpublished data) between unc-29 and lin-11 on LGI. K07A1.11 lies about 100 bp 3' to K07A1.12.

To confirm that K07A1 indeed contained lin-53 activity, we determined the sequence of K07A1.12 and K07A1.11 from the two independently isolated lin-53
alleles, n833 and n2978, and found they carried an identical mutation in K07A1.12 (see below) and had no mutation in K07A1.11. We therefore conclude that K07A1.12 is lin-53. We used EST clones (Kohara et al., unpublished results) that correspond to each gene as probes to screen for additional cDNAs. The longest lin-53 cDNA contained at its 5′ end the last seven nucleotides from the C. elegans trans-spliced leader SL1 followed by three nucleotides upstream of the first ATG and at its 3′ end a poly(A) tail. The longest K07A1.11 cDNA contained at its 5′ end the last ten nucleotides from the C. elegans trans-spliced leader SL2 (Huang and Hirsh, 1989) followed by six nucleotides upstream of the first ATG and at its 3′ end a poly(A) tail (Figure 4A). The tandem arrangement of these two genes in close proximity, with the message of the 3′ gene SL2 trans-spliced, suggests that they form a complex locus and are cotranscribed (Szipeti et al., 1993).

The LIN-53 protein is 72% identical to p48 and 70% to p46, while K07A1.11 is 53% identical to p48 and 52% to p48 and p46 are 7 WD-repeat proteins, which are regulatory proteins that mediate protein-protein interactions (Neer et al., 1994; Neer and Smith, 1996). Several p48-related proteins have been identified in different organisms, including the p55 subunit of the Drosophila melanogaster chromatin assembly factor 1 (Tyler et al., 1996) and the Saccharomyces cerevisiae proteins Msi1p (Ruggieri et al., 1989) and Hat2p (Parthun et al., 1996). LIN-53 is 72%, 27%, and 25% identical to these proteins, respectively. The mutation in lin-53(n833) and lin-53(n2978) animals causes a leucine-to-phenylalanine change at a conserved leucine in the fifth WD domain (Figure 4B).

Since K07A1.11 is 54% identical to LIN-53, we tested whether expression of K07A1.11 could rescue the lin-53 mutant phenotype. Driven by the col-10 gene promoter, which is strongly expressed in hypodermal and hypodermal blast cells including the P(3-B).p cells during larval development (P. Olsen and V. Ambros, personal communication), expression of the lin-53 cDNA but not of the K07A1.11 cDNA partially rescued the Muv phenotype. This result suggests that K07A1.11 cannot substitute for wild-type lin-53 function.

Wild-Type lin-53 Activity Is Required for the Class B synMuv Pathway

To investigate further the role of lin-53 in the class B synMuv pathway, we used RNA-mediated interference (RNAi), which has been shown to produce a specific phenocopy of the loss-of-function phenotype of a targeted gene by an unknown mechanism (Fire et al., 1998). We assayed the phenotypes of the progeny of wild-type, lin-15A, or lin-15B mothers injected with antisense RNA derived from a lin-53 cDNA clone. In all cases, antisense RNA injection caused embryonic lethality, suggesting that lin-53 is required during embryogenesis. Similar observations have been reported by Shi and Mello (1998), who studied the role of the gene we have now identified as lin-53 in embryonic development.

Because of this lethality, we were unable to use the RNAi technique to address the role of lin-53 during vulval induction. Instead, we used the col-10 promoter to drive expression of the antisense strand of a lin-53 cDNA in hypodermal and hypodermal blast cells. About 18% of lin-15A animals carrying the Pcol-10 antisense lin-53 construct were Muv, and this Muv phenotype was dependent on the presence of the lin-15A mutation (Table 1). Neither antisense expression of the K07A1.11 cDNA nor sense expression of the lin-53 or K07A1.11 cDNAs had any effect in similar experiments. These experiments suggest that wild-type lin-53 activity is required for the class B synMuv pathway.

lin-53(n833) Is Likely to Have a Dominant-Negative Effect

Unlike other synMuv mutations, lin-53(n833) and lin-53(n2978) cause a semidominant class B synMuv phenotype (e.g., lin-53(n833)/+; lin-15A animals have an incompletely penetrant Muv phenotype [Table 2]). Since loss of lin-53 function causes a class B synMuv phenotype, as indicated by our antisense experiments, lin-53 might be a haplo-insufficient locus; alternatively, lin-53(n833) might be a dominant-negative mutation. To distinguish between these two possibilities, we examined the phenotypes of animals of genotype +/Df; lin-15A (the Df chromosome was deleted for the lin-53 locus, see Table 2 for details). Five of 413 +/Df; lin-15A
Figure 4. lin-53 Structure and Similarity to Human p48

(A) Genomic organization of the lin-53/K07A1.11 locus. The top horizontal line indicates cosmid K07A1, which is missing the presumptive regulatory region and part of the first exon of lin-53. Solid boxes indicate coding sequences, and open boxes indicate noncoding sequences. SL1 and SL2 trans-spliced leaders, initiation and stop codons, and poly(A) tails are indicated.

(B) Sequence alignment of LIN-53, K07A1.11, human p48 and p46, Drosophila p55, and S. cerevisiae Hat2p and Msl1p. Identities with LIN-53 are shaded in black, and identities among other proteins are shaded in gray. The signature residues of each WD repeat are indicated by asterisks. The two identical lin-53 L292F mutations (n833 and n2978) are indicated by the arrow.

animals examined were Muv (Table 2). This penetrance of the Muv phenotype is much lower than in a lin-53(n833);+; lin-15A strain, indicating that a 2-fold reduction in wild-type lin-53 activity only occasionally causes a synMuv phenotype and that lin-53(n833) is unlikely to simply reduce or eliminate lin-53 function. Rather, lin-53(n833) is probably a dominant-negative mutation.

Table 2. lin-53(n833) May Act as a Dominant-Negative Allele

<table>
<thead>
<tr>
<th>lin-53 Genotype</th>
<th>Percent Muv (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n833/n833</td>
<td>100 (many)</td>
</tr>
<tr>
<td>n833/+;qDf9</td>
<td>12 (500)</td>
</tr>
<tr>
<td>n833/+;Ex[n33]</td>
<td>17 ± 6 (504)</td>
</tr>
<tr>
<td>n833/+;qDf9</td>
<td>1 (413)</td>
</tr>
<tr>
<td>+/+;Ex[n33]</td>
<td>0 (many)</td>
</tr>
</tbody>
</table>

All strains tested were homozygous for lin-15A. Muv. animals with at least one pseudovulva on their ventral sides; n, number of animals scored.

* Number obtained from three transgenic lines.
Consistent with this hypothesis, expression of a lin-53 cDNA carrying the n833 mutation (L292F) driven by the col-10 promoter caused a partially penetrant Muv phenotype in lin-53(–); lin-15A animals (Table 2). The lin-53(n833) mutation appears to affect only vulval development, while the lin-53 null phenotype may be embryonic lethality. One explanation for the tissue-specific effect of the lin-53(n833) allele could be that vulval development is particularly sensitive to a decreased dosage of wild-type lin-53. Alternatively, the dominant-negative mutant LIN-53 protein may be titrating a factor that becomes limiting specifically in vulval tissue.

A GFP::LIN-53 Transgene Is Expressed in the Vulval Cell Nuclei
A transgene containing the lin-53 cDNA tagged with GFP (Chalfie et al., 1994) at its N terminus and under the control of the endogenous lin-53 promoter was capable of partially rescuing the Muv phenotype of lin-53(n833); lin-15A animals. We examined the GFP expression pattern in animals carrying an integrated array of this transgene and observed GFP expression in most if not all nuclei during embryogenesis and in newly hatched L1s (Figures 5A and 5B). During larval development, we observed fluorescence in many nuclei in the head and tail regions, similar to the LIN-35 staining pattern seen with the anti-LIN-35 peptide antibody. GFP was also present in hypodermal cells throughout development (data not shown). At the time of vulval induction, GFP was visible in all P(3–8);p cells and persisted until after the P(3–8);p cell divisions and vulval morphogenesis were complete (Figures 5C and 5D).

LIN-35 Rb, LIN-53 p48, and HDA-1 Interact In Vitro
To determine whether LIN-35 Rb can directly bind LIN-35 p48 or HDA-1, as predicted by their sequences, we performed GST pull-down experiments. Bacterially produced glutathione S-transferase (GST) or GST::LIN-53 or GST::HDA-1 fusion proteins immobilized on beads were incubated with different in vitro translated 35S-methionine-labeled proteins. GST::LIN-53 interacted with a fragment of LIN-35 Rb that contains the entire A/B pocket (LIN-35BX) but not with LIN-35 fragments that lack the intact histone deacetylase homologs, hda-1, hda-2, and hda-3 (Shi and Mello, 1998), might be involved in the synMuvc pathway. RNAi of hda-1 caused embryonic lethality, while RNAi of hda-2 and hda-3 produced no obvious phenotypic abnormality in either a lin-15A or lin-15B background (data not shown). We then tested whether tissue-specific antisense expression of hda-1 driven by the col-10 promoter can cause a synMuv phenotype. Similar to lin-53 antisense expression, hda-1 antisense expression caused a Muv phenotype in a lin-15A background but not in a wild-type background (i.e., a class B synMuv phenotype). Antisense expression of the closely related hda-3 gene had no effect (Table 3). This result suggests that the histone deacetylase gene hda-1 activity is required for the lin-35 Rb-mediated synMuvc pathway.

Table 3. hda-1 Tissue-Specific Antisense Expression Causes a synMuvc Phenotype

<table>
<thead>
<tr>
<th>Construct</th>
<th>Percent Muvs (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unc-76; lin-15A</td>
<td></td>
</tr>
<tr>
<td>P.col-10 hda-1 antisense</td>
<td>21 ± 9% (462)*</td>
</tr>
<tr>
<td>P.col-10 hda-3 antisense</td>
<td>0 (many)</td>
</tr>
<tr>
<td>ND, not determined.</td>
<td></td>
</tr>
</tbody>
</table>

* Number obtained from three transgenic lines.
A schematic of LIN-35 Rb fragments used. (A) Vulval phenotype was assayed at 25°C using in the experiments shown in the left panel. The molecular weights of marker proteins are indicated.

Figure 6. LIN-35 Rb, LIN-53 p48, and HDA-1 In Vitro Interactions (A) Schematic of LIN-35 Rb fragments used. (B) GST::LIN-53 interaction with LIN-35 Rb and HDA-1. (Left) GST::LIN-53 beads were incubated with in vitro translated 35S-methionine-labeled LIN-35 fragments, luciferase, CED-4, EGL-1, or HDA-1. (Right) Twenty percent of the input radiolabeled proteins used in the experiments shown in the left panel. The molecular weights of marker proteins are indicated.

The lin-35 and lin-53 synMuv Phenotypes Require a Functional RTK/Ras Signaling Pathway
To determine how the lin-35 and lin-53 synMuv genes interact with the Ras signaling pathway during vulval development, we analyzed the vulval phenotype of triple mutants carrying either a lin-35 or a lin-53 mutation, a mutation in a class A synMuv gene, and a vulvaless (Vul) mutation in a Ras signaling gene (Table 4). The synMuv phenotype was epistatic to the Vul phenotype caused by a mutation in the lin-3 gene, which encodes the inductive signal (Table 4). This observation suggests that the lin-35 and lin-53 genes act downstream of or in parallel to the RTK/Ras signaling pathways.

By contrast, the Vul phenotypes of mutations in let-23 RTK, sem-5, let-60 Ras, lin-45 Raf, and mpk-1 genes were epistatic to the synMuv phenotype (Table 4). These observations suggest that the lin-35 and lin-53 genes act upstream of or in parallel to the RTK/Ras signaling pathways. In other words, the expression of vulval cell fates by the P3.p, P4.p, and P8.p cells in synMuv mutants requires a functional RTK/Ras signal transduction pathway. These observations concerning lin-35 and lin-53 genes are equivalent to previous observations concerning other synMuv genes (Ferguson et al., 1987; Huang et al., 1994; J. H. Thomas and H. R. H., unpublished data).

Discussion
lin-35 encodes a protein related to Rb, and lin-53 encodes a protein with striking similarity to an Rb-binding protein, p48 (72% identity). lin-35, lin-53, and a C. elegans histone deacetylase gene act in the same genetic pathway to antagonize a Ras signal transduction pathway in C. elegans. We propose that in mammals Rb,
Table 5. Gene Interactions with lin-2, lin-7, and lin-10

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Vulval Phenotype</th>
<th>Percent Induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>synMuvv</td>
<td>Muv</td>
<td>100</td>
</tr>
<tr>
<td>lin-2(lf)</td>
<td>Vul</td>
<td>0</td>
</tr>
<tr>
<td>lin-2(lf); synMuv</td>
<td>Muv, Muv/Vulb</td>
<td>64</td>
</tr>
<tr>
<td>lin-7(lf)</td>
<td>Vul</td>
<td>0</td>
</tr>
<tr>
<td>lin-7(lf); synMuv</td>
<td>Muv, Muv/Vul</td>
<td>49</td>
</tr>
<tr>
<td>lin-10(lf)</td>
<td>Vul</td>
<td>0</td>
</tr>
<tr>
<td>lin-10(lf); synMuv</td>
<td>Muv, Muv/Vul</td>
<td>58</td>
</tr>
</tbody>
</table>

*Two synMuv genotypes were studied: lin-35(n745); lin-15A (shown) and lin-53(n833); lin-15A (data not shown, but similar); comparable observations have been made of lin-36(n766); lin-15A and lin-15(e1763) animals (J.H. Thomas and H.R.H., unpublished data).

*a Muv/Vul, animals with both ectopic vulval tissues and nonfunctional vulvae.

p48, and histone deacetylase genes act in a tumor suppressor pathway that involves mechanisms and molecules similar to those of the synMuv pathway in C. elegans and that may well antagonize a mammalian Ras pathway.

lin-35 Rb, lin-53 p48, and hda-1 May Act To Repress Transcription in the P(3–8).p Cells

Rb, a key regulator of the mammalian cell cycle (reviewed by Weinberg, 1995; Wang, 1997), acts mainly through E2F transcription factors (composed of E2F and DP heterodimers), which regulate the expression of genes required for entry into S phase (reviewed by Dyon, 1998; Nevins, 1998). Rb, p107, and p130 can interact with DNA-bound E2F either to abolish transcription or to exert active repression of transcription from target promoters. One mechanism by which Rb represses transcription is to bind and recruit the histone deacetylase HDA1, presumably to remodel chromatin structure on a target promoter and thereby limit access of the transcriptional machinery to the DNA (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Julin et al., 1998). p48 has been found together with HDA1 in a large corepressor complex required for Mad-Max-mediated transcriptional repression (Hassig et al., 1997; Laherty et al., 1997).

We propose that the class B synMuv genes inhibit vulval induction by a conserved mechanism: LIN-35 Rb forms a complex with a sequence-specific transcription factor (indicated as “TF” in Figure 7), presumably an E2F-like protein, and recruits a corepressor complex containing HDA1, LIN-53 p48, and other proteins (indicated as “X” in Figure 7) to turn off the transcription of vulval specification genes via E2F-binding sites (Figure 7). In the wild type, in the P3.p, P4.p, and P8.p cells, synMuv gene activity antagonizes the basal activity of the RTK/Ras pathway by repressing transcription of vulval genes. As a result, those cells adopt the nonvulval 3° fate. In P5.p, P6.p, and P7.p, on the other hand, the antagonistic effect of synMuv gene activity is inactivated or can be overcome by the activated RTK/Ras pathway, thereby releasing transcriptional repression and permitting the expression of vulval fates. In the P(3–8).p cells of a synMuv mutant, repression cannot occur and all six P(3–8).p cells express vulval fates, resulting in a Muv phenotype. The synMuv genes do not appear to exert their effects by regulating cell cycle progression of the P(3–8).p cells, since all six of these cells have very similar cell cycle profiles (Sulston and Horvitz, 1977; Euling and Ambros, 1996).

Class B synMuv Genes Act in an Intercellular Signaling Pathway to Regulate Transcription

Both lin-35 and lin-53 appear to be expressed in the P(3–8).p cells and their descendants, consistent with the hypothesis that these class B synMuv genes act in P(3–8).p. Some class B synMuv genes, specifically lin-15 and lin-37, appear to act in the hypodermal cell hyp7p to regulate an intercellular signal (Herman and Hedgecock, 1990; Hedgecock and Herman, 1993), while others, specifically lin-36 (J.H. Thomas and H.R.H., unpublished data), lin-35, and lin-53, seem to act in the P(3–8).p cells to control the response to that signal. We suggest that a LIN-35 Rb/LIN-53 p48/HDA1 nuclear complex responds to the intercellular signaling pathway encoded by other class B synMuv genes to regulate transcription during vulval development in C. elegans.

Many questions remain to be answered. For example, what are the ligand, receptor, and signal transducers in the class B synMuv pathway? So far, none of the cloned class B genes appears to encode a secreted or transmembrane protein. Also, what are the other components of the proposed repressor complex, and what functions might they serve? It is possible that components of the class B synMuv signal transduction pathway are used in biological processes in addition to vulval development, so that mutations in such components would cause pleiotropic effects and would not be isolated as synMuv mutants. Indeed, the null phenotypes of lin-53 and hda-1 appear to be embryonic lethality, as indicated by RNAi experiments (this study, Shi and Mello, 1998), and strong mutations in lin-9 cause sterility (Ferguson and Horvitz, 1989). The characterization of the remaining class B genes and the identification of genes that interact with the known class B genes should provide more insight into this signaling pathway.

How Might the synMuv Pathway Interface with the Ras Pathway?

Gene interaction experiments both by us and by others indicate that in synMuv mutants anchor cell-independent activity of the Ras pathway is necessary for the expression of vulval cell fates (Ferguson et al., 1987;
Huang et al., 1994; J. H. Thomas and H. R. H., unpublished data). Thus, the synMuv genes must act genetically upstream of or in parallel to the Ras pathway. Action in parallel would be consistent with recent findings from studies of mammalian cells: dominant-negative Ras and Ras neutralizing antibodies induced an Rb-dependent block in DNA synthesis and G1 arrest (Mittnacht et al., 1997; Peeper et al., 1997), suggesting that Rb functions to inhibit mitogenesis downstream of or in parallel to Ras.

Although we cannot yet assign the precise point of interface between the synMuv and Ras pathways in vulval development, our molecular analyses of the class B synMuv pathway take us one step closer to understanding the nature of the antagonism between the synMuv genes and the Ras pathway. As we discussed above, we propose that the class B synMuv pathway acts by repressing transcription in the P(3–8)p cells.

What might be the target genes for LIN-35 Rb-mediated transcriptional repression? Candidate genes include those in the let-23 RTK/let-60 Ras pathway. However, given the findings of Peeper et al. (1997) cited above, it seems more likely that the class B synMuv genes act to repress genes involved in vulval differentiation. Since the LIN-1 ETS protein also seems likely to act by repressing vulval differentiation genes (Beitel et al., 1995; Tan et al., 1998), LIN-35 Rb and LIN-1 may have at least partially overlapping targets. The identification of target genes for the LIN-35 Rb repressor complex and for the LIN-1 ETS protein may well help establish the nature of the antagonism between the Rb pathway and the Ras pathway both in C. elegans and in other organisms.

Experimental Procedures

Strains and Genetics

C. elegans strains were cultured as described by Brenner (1974) and were grown at 20°C unless otherwise noted. Mutations used (Riddle et al., 1997) were as follows: LGI, unc-40(e271), bli-6(e937), dpy-5(e61), unc-29(e1072), lin-11(n566), lin-10(n299), and qd9 (Eills and Kimble, 1995); LGI, lin-7(e1413) and let-23(997); LGII, mplk-1(oz140); LGIV, lin-3(n378), let-60(n1876), and lin-45(n96); LGX, lin-15(n767) (a class A mutation), lin-15(n744) (a class B mutation), lin-2(n397), and sem-5(n2030). To map lin-35 with respect to unc-40 and stp124, we isolated Bli non-Unc recombinants from the progeny of unc-40 stp124 bli-4/lin-35(n2977); lin-15(n433) hermaphrodites and scored the progeny of these recombinants for lin-35 and stp124. Of 63 recombinants, 19 had stp124 and were non-Muv, 9 had stp124 and were Muv, and 35 did not have stp124 and were Muv.

Transgenic Animals

Germline transformation was performed as described by Mello et al. (1991). DNA (30–100 ng/µl) was co-injected with a 100–200-residue plasmid (100 ng/µl) (Bloom and Horvitz, 1997), and lines of non-Unc-76 transgenic animals were established. Chromosomal integration of extrachromosomal arrays of transgene was accomplished by y-ray irradiation of transgenic animals.

Identification of cDNAs and Sequence Analysis of cDNAs and Mutant Alleles

To identify lin-35 and lin-53 cDNAs, we screened a cDNA library made from C. elegans embryonic RNA (Okkema and Fire, 1994). The sequences of the ends of inserts of positive clones were first determined using vector primers, and the complete sequence of the longest cDNA was then determined using primers positioned within the coding sequences. The sequence of at least one additional cDNA clone for each gene was determined to confirm splicing patterns. The sequences of both strands of the coding regions and splice junctions were determined from PCR fragments amplified from the lin-35 and lin-53 mutant alleles and purified by gel electrophoresis. DNA sequences were determined using an automated ABI 373A DNA sequencer (Applied Biosystems).

In Vitro Interaction Assays

Wild-type and mutant lin-53 cDNAs were cloned into vector pgEXAT-3 (Pharmacia), expressed in E. coli strain BL21(DE3), and purified with glutathione sepharose beads as recommended by the manufacturer (Pharmacia).

lin-35, lin-53, and hda-1 cDNA fragments were cloned into the vector pCITE4(+)(Novagen) or pRK5. The resulting constructs were used as templates to synthesize [35S]-methionine-labeled proteins in the TNT coupled Reticulocyte Lysate System (Promega). Labeled proteins were incubated with equal amounts of GST fusion proteins for 2 hr at 4°C. Bound proteins were eluted with 2× SDS sample buffer and analyzed by SDS-PAGE (10%) and autoradiography.

Antibodies

A peptide from the N-terminal region of LIN-35, HSRKRKYEQEYIRR, with a cysteine added to its N terminus was coupled to keyhole limpet hemocyanin (KLH) and used to immunize rabbits and obtain antisera (Zymed). Antibodies were purified over a peptide affinity
column (Pierce). For Western blots, the LIN-35 protein was visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescent detection reagents (Pierce). Immunocytochemistry was as described by Finney and Ruvkun (1990).

RNA and RNAi Analyses

For Northern blot analysis, poly(A)+ RNA was isolated using the FAST TRACK system (Invitrogen). RNA was subjected to electrophoresis and transferred to Nytran. The filter was probed with 32P-radio-labeled LIN-35 genomic DNA. PCR fragments containing cDNA flanked by the T7 and the T3 promoters amplified from the cDNA phage lysates were used for in vitro RNA synthesis as described (Fire et al., 1998). The unmodified RNA was resuspended in H2O and injected at 1-5 μg/μl. Injected animals were moved to a new plate 12 hr after injection to enrich for progeny that had been subject to antisense injection.

Acknowledgments

We thank Sander van den Heuvel, Tyler Jacks, Yang Shi, and members of the Horvitz laboratory for critically reading this manuscript, Beth J ames for help with determining DNA sequences, Xiaolu Yang for the plasmid pRK5, Yuji Kohara for the EST cDNA clones, and the C. elegans Genetics Center for the deficiency strains used in the lin-53 gene dosage studies. H. R. H. is an Investigator of the Howard Hughes Medical Institute.

Received May 8, 1998; revised November 5, 1998.

References


