Analysis of dominant-negative mutations of the Caenorhabditis elegans let-60 ras gene

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The let-60 gene of Caenorhabditis elegans controls the choice between vulval and hypodermal differentiation in response to an inductive signal from the gonad. let-60 encodes a ras protein that acts downstream of the let-23 receptor tyrosine kinase in a signal transduction pathway. Dominant-negative mutations of let-60 [let-60(dn)] cause a reduction of the gene activity in let-60(dn)/+ heterozygotes and a vulva-less mutant phenotype. We have found that nine let-60(dn) mutations cause replacements of conserved residues. Four are in two novel positions; others are in positions known previously to cause dominant-negative mutations in mammalian cells. The locations of these lesions suggest that they disrupt the ability of the ras protein to bind guanine nucleotides. Four let-60(dn) mutant genes were introduced into wild-type animals in the form of extrachromosomal arrays and were found to generate three dominant phenotypes—lethality, vulva-less, or multivulva—depending on gene dose and alleles. The dominant lethality caused by high-dose transgenic let-60(dn) genes suggests a toxic effect of these mutant genes in early development. The dominant-negative effects of these mutations in heterozygotes are likely to be caused by competition between let-60(dn) and let-60(+) protein for a positive regulator. All let-60(dn) mutations interfere with let-60(+) activity, but some alleles have partial constitutive activity, suggesting that the ability to interact with the activator is separable from the ability to exert a physiological effect (stimulation of vulval differentiation). These dn mutations might be useful for interfering with ras-mediated signal transduction pathways in other multicellular organisms.

[Key Words: ras, Caenorhabditis elegans, dominant-negative mutations, vulval induction]

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Prior to vulval differentiation in Caenorhabditis elegans hermaphrodites, each of six vulval precursor cells (VPCs, posterior daughter cells of six ectodermal P cells, P3.p–P8.p) has the potential to generate either vulval cells or hypodermal cells. According to the current model, during vulval induction, three of the six precursor cells are triggered to generate vulval cells by an inductive signal from the anchor cell in the gonad (for review, see Horvitz and Sternberg 1991). In the absence of the inductive signal, the vulval cell fates for these precursor cells are repressed by an inhibitory signal from the large syncytial hypodermis (hyp?) mediated by the lin-15 gene (Ferguson et al. 1987, Herman and Hedgecock 1990).

Genetic analysis indicated that let-60 activity controls the choice of differentiation between the vulval and hypodermal cell fates in response to the intercellular signals [Fig. 1; Beitel et al. 1990, Han et al. 1990]. Specifically, low let-60 activity results in fewer than three of the six VPCs differentiating into vulval tissue (the vulva-less or Vul phenotype), while high let-60 activity results in extra VPCs differentiating into vulval tissue (the multivulva or Muv phenotype). let-60 encodes a ras protein with 83% of its first 164 amino acids (of a total 184) identical to those in the human N-ras protein (Han and Steinberg 1990). Genetic epistasis experiments suggest that let-60 ras acts downstream of let-23, a member of the epidermal growth factor (EGF) receptor family of tyrosine kinases (Aroian et al. 1990), in a signal transduction pathway (Han et al. 1990; Fenster and Sternberg 1990). Genetic epistasis experiments suggest that let-60 ras acts downstream of let-23, a member of the epidermal growth factor (EGF) receptor family of tyrosine kinases (Aroian et al. 1990), in a signal transduction pathway (Han et al. 1990; Fenster and Sternberg 1990). Genetic epistasis experiments suggest that let-60 ras acts downstream of let-23, a member of the epidermal growth factor (EGF) receptor family of tyrosine kinases (Aroian et al. 1990), in a signal transduction pathway (Han et al. 1990; Fenster and Sternberg 1990). Genetic epistasis experiments suggest that let-60 ras acts downstream of let-23, a member of the epidermal growth factor (EGF) receptor family of tyrosine kinases (Aroian et al. 1990), in a signal transduction pathway (Han et al. 1990; Fenster and Sternberg 1990). Genetic epistasis experiments suggest that let-60 ras acts downstream of let-23, a member of the epidermal growth factor (EGF) receptor family of tyrosine kinases (Aroian et al. 1990), in a signal transduction pathway (Han et al. 1990; Fenster and Sternberg 1990). Genetic epistasis experiments suggest that let-60 ras acts downstream of let-23, a member of the epidermal growth factor (EGF) receptor family of tyrosine kinases (Aroian et al. 1990), in a signal transduction pathway (Han et al. 1990; Fenster and Sternberg 1990).

Gain-of-function mutations in let-60 ras [let-60(gf)] produce constitutively high let-60 activity and a Muv phenotype [Ferguson and Horvitz 1985; Beitel et al. 1990, Han et al. 1990, G. Jongeward and P. Sternberg, unpubl.]. The five independently isolated let-60(gf) mutations have the same DNA lesion at codon 13 [Beitel et al. 1990], such a change in mammalian ras leads to a decrease in the GTPase activity of the ras protein (for review, see Barbacid 1987). The gain-of-function (Muv) mutant phenotype can also be caused by extra copies of extrachromosomal wild-type let-60 ras gene (Han and

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Figure 1. The role of let-60 ras in the control of vulval development. The level of let-60 ras activity controls the decision between vulval and hypodermal fates for each of the six vulval precursor cells in response to upstream intercellular signals (Beitel et al. 1990; Han et al. 1990). Specifically, low let-60 activity results in hypodermal fate specification, whereas high let-60 activity leads to vulval fate specifications. Let-60 activity, normally regulated by the upstream signals, is rendered constitutively low by let-60 if or dn mutations, or constitutively high by let-60 gf mutations. Let-60 ras has been proposed to act downstream of let-23 (Han and Sternberg 1990; Han et al. 1990), which encodes a receptor tyrosine kinase (Aroian et al. 1990).

Sternberg 1990), suggesting that endogenous gene expression might be controlled quantitatively. The opposite mutant phenotype, Vul, is caused by loss-of-function (lf) and dominant-negative (dn) mutations of let-60 ras (Beitel et al. 1990; Han et al. 1990). The nine let-60(dn) mutations cause a dominant Vul-less phenotype in hermaphrodites and a dominant defect in male tail morphology and mating ability. Eight of these mutations are recessive lethal at an early larval stage, consistent with the conclusion that let-60 is also essential for larval growth based on analysis of let-60(lf) mutations. The dominant-negative effect in let-60(dn)/+ heterozygotes is caused presumably by an interference of let-60(+) protein by the let-60(dn) protein.

Previously, a number of mutations in guanine nucleotide-binding regions of ras proteins have been constructed or selected in vitro and found to have dominant-negative effects in mammalian or in yeast cells (Sigal et al. 1986; Feig and Cooper 1988; Powers et al. 1989). However, it is of interest to compare the in vivo-selected dominant-negative mutations of the C. elegans let-60 ras gene with those mutations studied previously.

In this study we determined the molecular lesions caused by the nine let-60(dn) mutations. We also determined the DNA alteration for two let-60(dn) intragenic revertants and found that one is an excellent candidate for a null mutation. Our results suggest that the dn mutations likely disrupt the ability of let-60 ras protein to bind guanine nucleotides and, therefore, prevent its activation. To understand the mechanism of the dominant-negative effect caused by these let-60(dn) alleles in heterozygotes, we further analyzed transgenic animals carrying exogenous let-60(dn) genes and examined the extent of vulval differentiation for two representative let-60(dn) mutations with various ratios of mutant to wild-type genes. We discuss the implications of these results as well as a model for competition between the let-60(dn) and let-60(+) proteins.

Results

All dominant-negative mutations of let-60 ras likely cause defective guanine nucleotide binding

We have determined the sequence alterations of all nine let-60(dn) mutations, after obtaining let-60 DNA fragments from mutants by polymerase chain reaction (PCR) amplification (Table 1). For example, let-60(sy101) results in a substitution of arginine for glycine at position 10; we refer to this as let-60(Arg-10). Figure 2 shows the three-dimensional crystal structure of the GTP-bound form of a truncated mammalian p21 ras protein (Pai et al. 1989, 1990, Tong et al. 1991). Because there are no gaps in the alignment between let-60 ras and mammalian ras proteins until after residue 179, the positions of let-60(dn) mutant residues likely correspond to those in

Table 1. DNA and protein lesion of let-60(dn) and revertant mutations

<table>
<thead>
<tr>
<th>Class</th>
<th>Allele</th>
<th>Codon</th>
<th>nucleotide</th>
<th>amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant-negative [dn]#</td>
<td></td>
<td></td>
<td>wild type</td>
<td>mutant</td>
</tr>
<tr>
<td></td>
<td>sy99</td>
<td>10</td>
<td>GGA</td>
<td>AGA</td>
</tr>
<tr>
<td></td>
<td>sy101</td>
<td>10</td>
<td>GGA</td>
<td>AGA</td>
</tr>
<tr>
<td></td>
<td>n2301</td>
<td>15</td>
<td>GGT</td>
<td>AGT</td>
</tr>
<tr>
<td></td>
<td>n1531</td>
<td>15</td>
<td>GGT</td>
<td>GAT</td>
</tr>
<tr>
<td></td>
<td>sy94</td>
<td>16</td>
<td>AAA</td>
<td>AAT</td>
</tr>
<tr>
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<td>sy92</td>
<td>89</td>
<td>TCT</td>
<td>TTT</td>
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<td></td>
<td>sy95</td>
<td>89</td>
<td>TCT</td>
<td>TTT</td>
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<td>sy100</td>
<td>89</td>
<td>GAT</td>
<td>AAT</td>
</tr>
<tr>
<td></td>
<td>sy93</td>
<td>119</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Revertant of dn#</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sy163</td>
<td>110</td>
<td>CCT</td>
<td>TCT</td>
</tr>
<tr>
<td></td>
<td>sy127</td>
<td>123</td>
<td>CGA</td>
<td>TGA</td>
</tr>
</tbody>
</table>

#Genetic characterization of these dn mutations was described by Han et al. (1990) for alleles named sy and by Beitel et al. (1990) for alleles named n. Phe-89 dn is equivalent to the intragenic revertant [n1981] of an activated let-60 mutant protein [n1046 or Glu-13 gf]. Glu-13 Phe-89 protein does not act as an activated ras protein but has some dominant-negative activity (Beitel et al. 1990).

#These two alleles were isolated as intragenic revertants of let-60[sy101 dn] (Han et al. 1990) and appear to eliminate function of let-60.
mammalian p21ras. All of the let-60(dn) mutations alter amino acids that are invariant throughout evolution (Barbacid 1987; Han and Sternberg 1990) and appear to be in the region required for guanine nucleotide binding. Residues 10–17 form a loop in which main-chain amide hydrogens of several amino acids, plus the amino group of Lys-16, form hydrogen bonds with the α- and β-phosphates of GTP or GDP (Pai et al. 1990; Tong et al. 1991). Thus, Arg-10, Ser-15, Asp-15, and Asn-16 might disrupt the interaction of this loop with guanine nucleotides. Asp-119 is also required for GTP/GDP binding, as its carboxyl oxygens form hydrogen bonds with the guanine ring. Asn-119 is likely to disrupt these bonds. Ser-89 is not in direct contact with the guanine nucleotide, but its proximity to the binding site suggests that it might be important for stable binding. Phe-89 might disrupt such a role. Mutations in residues 16 and 119 of the mammalian ras protein cause a drastic (~100-fold) decrease in GTP/GDP-binding ability of the ras protein (Barbacid 1987, Sigal et al. 1986). We conclude that all of the dn mutations are likely to cause defective guanine nucleotide binding.

An intragenic let-60(dn) revertant causes a premature termination codon and a null phenotype

Previously, we have isolated two intragenic revertants of let-60(sy101) [named sy127 and sy163; Han et al. 1990]. let-60(sy101 sy127) or let-60(sy101 sy163) double mutants are recessive lethal but have no dominant phenotypes: The let-60(dn, revertant)/+ heterozygotes have wild-type vulvae and normal male mating. The sequence changes of these two let-60(dn) intragenic revertants have been determined (Table 1). sy163 converts residue 110 from the absolutely conserved proline to serine [Ser110] while sy127 produces presumably a carboxy-termi-

nal-truncated protein by introducing a stop codon [TGA] at residue 123 [Stop-123]. The sequence deleted in this truncated protein contains many conserved amino acids, including a region required for guanine nucleotide binding (Bourne et al. 1991), as well as the sites required for post-translational modifications and membrane attachment (Barbacid 1987; Gibbs 1991). Therefore, Stop-123 is most likely a null mutation. let-60(Arg-10 Stop-123) homozygotes hatch but die during the early part of the first larval (L1) stage. Specifically, we observed eight animals under Nomarski optics and found that the gonad of an arrested animal consists only of the four primordial cells; the M mesoblast divided once at most, the P ectoblasts had not migrated to the ventral cord, and L1-specific alae are present in all arrested animals (Sulston and Horvitz 1977, Kimble and Hirsh 1979). On the basis of the phenotype under the dissecting microscope, Stop-123 also produces the most severe recessive lethal phenotype among all of the loss-of-function mutations of let-60 ras; they have the smallest size on plates, and there are no "escapers" that survive to later stages, as is the case with other mutations (Clark et al. 1988; Beitel et al. 1990; Han et al. 1990). Therefore, animals with no zygotic let-60 function arrest at the young L1 stage. It is possible that a maternal function of let-60 ras is required for animals to complete embryogenesis.

let-60(dn) transgenic animals exhibit mutant phenotypes

To analyze further the nature of these dominant-negative mutations, we reintroduced four different let-60(dn) genes into wild-type animals along with a dominant marker, resulting in the production of extrachromosomal DNA arrays (Table 2). The extrachromosomal arrays in transgenic animals are typically >1000 kb long.
The structure of the arrays and the gene copy numbers in individual transforming lines remain relatively stable through numerous generations of growth (Mello et al. 1991). The ratio of DNAs in such extrachromosomal arrays is similar to the ratio of DNAs in the solution injected, so that the approximate number of copies of a test gene relative to marker and carrier DNA can be controlled (Mello et al. 1991).

When each of three let-60(dn) mutant genes (Arg-10, Asn-16, and Asn-119) were injected into wild-type animals at 1 μg/ml (~70 μg/ml of total DNA; see Materials and methods), viable transformants were obtained and the expected Vul phenotype was observed. However, if the concentrations were increased to 10 μg/ml, we were unable to obtain germ-line transformants from injection of any of the three mutant genes, indicating a dominant-lethal effect. In contrast, injection of a truncated let-60 gene at 50 μg/ml does not cause lethality or any dominant phenotype (Han and Sternberg 1990). Therefore, it is possible that the dn mutations also affect dominantly ras-mediated pathways during early larval growth. The fact that a low dose of let-60(dn) causes a Vul, but not a lethal, phenotype suggests that let-60 ras function during vulval development is more sensitive to the change in its activity level than its essential functions during early development. At the same concentration (10 μg/ml), injection of let-60(Phe-89) produces viable transformants containing extrachromosomal let-60(Phe-89), although a dominant Vul phenotype is associated with these transformants. This exception is probably the result of residual ras activity associated with the mutant protein, because the extrachromosomal let-60(Phe-89) DNA can rescue the lethality of let-60(Phe-89)/let-60(Phe-89) homozygotes. Partial vulval differentiation is also observed in let-60(Phe-89) dn homozygotes carrying high copy numbers of the extrachromosomal let-60(Phe-89) gene. The residual activity of let-60(Phe-89) could be different qualitatively from the residual activity of let-60(Asn-119) or let-60(Asn-16), because it does not cause a Muv phenotype, even when injected at high concentrations.

Surprisingly, when injected at 1 μg/ml, two let-60(dn) mutant genes also generated a dominant gain-of-function Muv phenotype in addition to the Vul phenotype (Table 2). Among a population of animals derived from the same transgenic line, some are multivulva, while others are vulvaless. The Muv phenotype of these transgenic animals might be caused by the residual activity of let-60(Asn-119) and let-60(Asn-16) proteins expressed from the extrachromosomal genes. Whether a transgenic animal displays the Vul or Muv phenotype might depend on relative levels of the dominant-negative and residual activities. This hypothesis is supported by the fact that the let-60(Asn-119) allele is recessive viable and allows partial vulval differentiation [see below and Table 3].

Arg-10 and Asn-119 have distinct properties

Because the transgene studies indicate two classes of dominant-negative mutations—those that cause a Muv phenotype (Asn-16 and Asn-119) and those that do not cause a Muv phenotype (Arg-10 and Phe-89)—we selected one of each class for further study. Vulval differentiation in strains containing the Arg-10 and Asn-119 mutations was analyzed further with Nomarski optics to observe the extent of vulval differentiation. As mentioned earlier, let-60(Arg-10) homozygotes are inviable.
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Table 3. Dosage effect of two let-60(dn) alleles on vulval differentiation

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Vulval differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% average (n)b</td>
</tr>
<tr>
<td>(Arg-10,Stop-123)/ (Arg-10,Stop-123)/ + 4</td>
<td>100 (13)</td>
</tr>
<tr>
<td>+/+/+ 4</td>
<td>100 (20)</td>
</tr>
<tr>
<td>(Arg-10)/ +</td>
<td>100 (20)</td>
</tr>
<tr>
<td>(Arg-10)/ (Arg-10)/ +</td>
<td>4 (17)</td>
</tr>
<tr>
<td>(Arg-10)/ (Arg-10) lethal</td>
<td></td>
</tr>
<tr>
<td>(Asn-119)/ +/+</td>
<td>33 (18)</td>
</tr>
<tr>
<td>(Asn-119)/+</td>
<td>2 (22)</td>
</tr>
<tr>
<td>(Asn-119)/(Asn-119)/+</td>
<td>79 (28)</td>
</tr>
<tr>
<td>(Asn-119)/(Asn-119)</td>
<td>29 (28)</td>
</tr>
<tr>
<td>(Asn-119)/(Stop-123) lethal</td>
<td></td>
</tr>
</tbody>
</table>

*Only the let-60 genotype is indicated. (The complete genotype of each strain is described in Materials and methods.) A duplication covering part of chromosome IV [nDp5; Beitel et al. 1990] was used for the additional wild-type copy of let-60.

†Percentage of vulval precursor cells generating vulval cells relative to wild type (100%). Examined under Nomarski optics (defined by Han et al. 1990; see Materials and methods). The numbers of animals examined are indicated [n]. Two strains listed show an early larval lethal phenotype; their vulval cell differentiation cannot be examined. Animals of genotype let-60(Arg-10 Stop-123)/ + or Df/+ have been shown previously to have wild-type vulval development [Han et al. 1990; Beitel et al. 1990]. In strains heterozygous for let-60(Asn-119) and let-60(+), both mutant and wild-type genes contribute to the total activity, although let-60(+) activity is greatly reduced by the strong dominant negative effect of let-60(Asn-119) dn. It is also possible that stability of the mutant proteins is different from that of the wild-type protein such that the quantity of the mutant proteins are also different from wild type.

Number of examined animals having <100% vulval differentiation, 100% vulval differentiation, or >100% vulval differentiation, respectively. Animals having 100% vulval differentiation do not necessarily have wild-type vulva.

4Stop-123 is a putative null mutation [see text].

Three of these 28 let-60(Asn-119)/let-60(Asn-119) +/+ animals showed >100% vulval cell differentiation [more than three vulval precursor cells generate vulval cells]. In these animals, in addition to P5.p, P6.p, and P7.p, P4.p [one animal] or half of the Pn.p progeny [P4.pa, two animals] differentiated into vulval cells.

while let-60(Asn-119) homozygotes are viable. A chromosomal duplication [nDp5; Beitel et al. 1990] was used to alter the ratio between mutant and wild-type gene dose [Table 3]. We found that changes in the endogenous gene ratio between let-60(+)/+ and let-60(Arg-10 dn) genes cause drastic changes in the extent of vulval differentiation. In particular, an additional wild-type gene in dn/+/+ animals suppresses the dn toxic effect of let-60(Arg-10 dn) completely in dn/+ animals [from 41% vulval differentiation to 100%]. An additional mutant gene reduces the average differentiation to 4% in dn/ dn/+ animals [Table 3]. Therefore, let-60(Arg-10 dn) contains no or little activity by itself but interferes with the function of the wild-type gene product.

The Asn-119 dn mutation has more complex effects on let-60 ras activity [Table 3]. First, let-60(Asn-119 dn) exerts a stronger dominant-negative effect than let-60(Arg-10 dn): Average vulval differentiation for Asn-119/ + and Asn-119/+/+ animals is 2% and 33%, respectively. Second, let-60(Asn-119 dn) has residual activity: let-60(Asn-119 dn) homozygous animals are recessive viable and have an average of 29% vulval differentiation. Third, the lethal phenotype of let-60(Asn-119 dn) in trans to the presumptive null mutation [let-60(Arg-10 Stop-123)], see above] suggests that the residual activity is dose dependent. Two copies of let-60(Asn-119 dn) are sufficient for viability, but one copy is not.

Also, one additional copy of let-60(Asn-119 dn) increases vulval differentiation: let-60(Asn-119 dn)/let-60(Asn-119 dn) homozygous animals have more vulval differentiation [29%] than do let-60(Asn-119 dn)/+ heterozygotes [2%], and dn/dn/+ animals have more vulval differentiation [79%] than do dn/+/+ animals [33%]. Finally, some multivulva animals [i.e., >100% vulval differentiation] were observed among the let-60(Asn-119)/let-60(Asn-119) +/+ animals. We found that vulval differentiation in these animals is partially signal independent. The gonads of nine animals of this genotype were ablated at the L1 stage with a laser microbeam to eliminate the source of inductive signal. Three of the animals had a total of five and one-half VPCs [for definition, see Materials and methods] differentiating into vulval tissue, suggesting that let-60 activity in the strain is partially constitutive. Therefore, the let-60(Asn-119 dn) gene product has a very strong dominant-negative effect on wild-type activity but has some constitutive activity by itself to trigger vulval differentiation.

The dominant-lethal effect of high-dose transgenic let-60(Arg-10 dn) is suppressed by the presence of the genomic let-60(Glu-13 gf) gene

The dominant-negative effects of dn alleles are suppressed completely by gf alleles in let-60(dn)/let-60(gf)
animals (Beitel et al. 1990; Han et al. 1990), indicating that dn protein cannot compete with gf protein when there is one copy of the dn and one copy of the gf mutant genes. Because we can drastically alter the relative dose, transgenic animals carrying exogenous let-60(Arg-10 dn) allow a more stringent test of the hypothesis that dn protein cannot interfere with activated ras.

The DNA array carrying high copy numbers of let-60(Arg-10 dn) genes was generated in a let-60(gf) background, and the segregants of hermaphrodites of genotype let-60(gf)/+; Array were examined for the production [and proportion] of lethal and nonlethal transgenic progeny. Table 4 lists the genotypes and phenotypes of transgenic animals segregating from a single let-60(Glu-13 gf)/+ heterozygote carrying a let-60(Arg-10 dn) extrachromosomal array injected at 10 μg/ml [see Table 2]. As described above, the injected let-60(Arg-10 dn) genes cause a dominant-lethal phenotype in a wild-type background: None of the 22 transgenic progeny from a single transgenic animal show the marker phenotype b (gf/+, an average ratio of 1 gf/ + : 2 +/+ : 1 +/+). The Vul phenotype caused by let-60(Arg-10 dn) is also suppressed completely. Suppression of the dn mutant phenotype by gf mutations has been reported previously in yeast cells with either yeast or mammalian ras [dn] genes (Sigal et al. 1986; Feig and Cooper 1988; Powers et al. 1989). The constitutive activity of activated ras (gf) mutant proteins is most likely the result of a decrease in GTPase activity (Barbacid 1987). Therefore, failure of let-60(dn) protein to compete with let-60(gf) protein suggests that the dominant-negative effect of the dn mutant proteins on wild-type activity is probably the result of disruption of the activation of wild-type ras, rather than disruption of its interaction with an effector protein.

Discussion

We have demonstrated that nine dn mutations in C. elegans let-60 ras gene [let-60(dn)] cause changes in five conserved amino acids that are required for guanine nucleotide binding in mammalian ras proteins. Two of these positions are new. Because gf [or "activated"] and some of the dn mutations of let-60 ras correspond to positions that result in gf and dn mutations in mammalian and yeast ras, it is likely that let-60 ras has many of the biochemical functions of these other ras proteins (e.g., GTP/GDP binding and GTP hydrolysis). Disruption of these functions leads to specific developmental defects in C. elegans. We have also demonstrated that these let-60(dn) mutant genes can cause various dominant mutant phenotypes in transgenic animals. These phenotypes suggest that different properties are associated with some of these dn mutations even though each mutation interferes with let-60(+) function in vulval development. Our further dose analysis shows that the dominant-negative effect of a mutant gene is separable from its ability to be activated, because let-60(Asn-119) has the strongest toxic effect on let-60(+) but, nonetheless, has partial constitutive activity to induce vulval differentiation as well as support larval growth.

Disruption of the essential roles of let-60 ras by dn mutations

Because the putative let-60 null mutation causes animals to arrest during the early part of the first larval stage, let-60 is required for postembryonic growth and development. Similar larval lethality is seen for most let-60(dn) homoyzogotes, suggesting that they are impaired severely in the essential function of this gene. However, in let-60(dn)/+ heterozygotes, let-60 function during vulval development, but not early larval growth, is disrupted: Viable dn/+ animals often have no vulval differentiation (Beitel et al. 1990; Han et al. 1990). Therefore, it is possible that the dominant-negative effect is tissue specific. The results described here suggest that such an explanation is unlikely because dominant le-
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Lethality can also be produced in wild-type animals by the presence of a high copy number of let-60(dn) genes in transgenic animals. At least for let-60(Arg-10 dn), this lethality is likely caused by a strong dominant-negative effect that leads to a decrease in let-60(+) activity. Therefore, the selective dn effects (disrupting vulval development but not growth) in let-60(+/+) animals are the result of differences of sensitivity to the decrease in let-60 ras activity between these two aspects of development. A similar observation has been made for the gain-of-activity mutant phenotype caused by extra copies of let-60(+) genes in transgenic animals [Han and Sternberg 1990]. Early larval growth thus appears to be less sensitive to the changes in let-60 activity than is vulval development.

**Mechanism of the dominant-negative effect of let-60(dn) mutations**

The amino acid changes caused by let-60(dn) mutations likely disrupt the normal interaction between let-60 ras protein and GDP or GTP, resulting in loss of function. Such a defect easily explains the recessive-lethal phenotype. How do these mutant ras proteins, with presumably a decreased ability to bind to guanine nucleotides, inactivate the wild-type let-60 ras protein in a let-60(+/+) heterozygote? Two common models can be used to explain a dominant-negative mutant effect [Herskowitz 1987]: Either the mutant protein exerts a toxic effect in a heteromultimer or the mutant protein competes with the wild-type protein for another factor. According to the first model, let-60 ras protein would form multimers through direct or indirect interactions. For example, multiple ras protein molecules may form a functional protein complex by interacting with another factor. In this model the dominant toxic effect of a dn mutant protein is exerted by inactivating a complex containing both the wild-type and dn ras proteins. If such a complex contained more than two let-60 ras molecules, this model would be consistent with all of our molecular genetic data on let-60(dn), including the Vul phenotype of let-60(Asn-119 dn)/+/+ animals (Table 3). However, there is no compelling evidence that ras proteins form multimeric complexes.

The second model proposes a competition between the wild-type and mutant protein for another positive factor (Fig. 3). This competition results in reduction of the let-60(+)+ activity in let-60(60)/+ heterozygous animals. To explain the severe Vul phenotype generated by let-60(dn) mutations in let-60(heterozygotes [especially in the case of let-60(Asn-119), in which vulval differentiation in Asn-119+/+ animals is only 33%], two assumptions need to be made. First, the cellular concentration of this positive factor must be limiting relative to a target protein, let-60 ras. Second, let-60(+/+) proteins form a more stable complex with the factor than the wild-type let-60 ras protein. This positive factor could either be an upstream activator or a downstream target protein. Because the effect of a dominant-negative mutant can be suppressed by the presence of constitutively active ras protein [let-60(Glu-13 gf)], the let-60(+/+) proteins likely compete for an activator rather than a target protein. ras protein is activated through a change from the GDP-bound state to the GTP-bound state and is deactivated by GTP hydrolysis (Fig. 2; Bourne et al. 1991). Therefore, this activator could be a guanine nucleotide exchange factor [GNEF; Downward et al. 1990; Jones et al. 1991; Kaibuchi et al. 1991]. We speculate that binding of ras(dn) mutant protein (presumably the guanine nucleotide-free protein) to GNEF prevents the release of the GNEF and depletes the pool.
available to interact with, and hence activate, wild-type ras protein.

A competition model has been used previously to explain the dominant-negative effect of ras mutations in mammalian cells and yeast [e.g., Asn-16, Ala-17, Asn-119 of mammalian ras p21 protein and Pro-22 and Ala-25 of yeast RAS2 protein] (Sigal et al. 1986; Feig and Cooper 1988; Powers et al. 1989). Particularly, the work done by Powers et al. (1989) suggested that CDC25 of Saccharomyces cerevisiae, a yeast GNEF [Créchet et al. 1990; Jones et al. 1991], is the limiting factor and is titrated by binding to ras\(^+\) or ras\(^dn\) proteins. Increased expression of the CDC25 gene in yeast can suppress the toxic effect caused by a dominant-negative mutation in either the yeast RAS2 gene (Ala-22) or the mammalian H-ras gene [Ala-15] in the presence of wild-type yeast ras protein. Furthermore, because increased dosage of the wild-type RAS2 gene neither causes a dominant phenotype nor obviously suppresses the dominant-negative effect of one \(dn\) mutation in yeast, there is no evidence that ras is a limiting factor in yeast.

Although the competition model is consistent with our molecular and genetic data on let-60\(^dn\) mutants, so far there is no direct evidence for it in either C. elegans or mammalian cells. Also, a downstream effector protein is not excluded from being the limiting factor that is titrated out by some, if not all, of the dominant-negative let-60 ras mutant proteins: Suppression of let-60\(^dn\) by let-60(Glu-13 \(gf\)) may be the result of an even higher affinity of let-60(Glu-13 \(gf\)) for the effector. In yeast, suppression of the \(dn\) phenotype by increasing the level of CDC25 might simply be the result of increase in the amount of ras–GTP, which can compete with ras\(^dn\) for the effector.

**Negative regulation of let-60 ras**

Our molecular genetic analyses of the let-60 gene suggest that its product is a limiting factor in signal transduction. The extent of vulval differentiation is sensitive to the number of copies of wild-type or mutant let-60 genes [Table 3; Beitel et al. 1990; Han and Sternberg 1990]. In mammalian cells, an increased dose of wild-type ras genes causes malignant transformation [Chang et al. 1982; Pulciani et al. 1985; McKay et al. 1986; Quaife et al. 1987]. The dose effect of the let-60 ras protein may be the result of its interaction with a negative regulator. For example, ras activity is known to be negatively regulated by GAP [GTPase-activating protein], which catalyzes the GTP hydrolysis of the ras–GTP complex [for review, see McCormick 1989; Bourne et al. 1991]. The cellular ras concentration might be limited relative to the GAP activity. An increase in dose of the wild-type ras gene might overwhelm this negative activity of GAP or decrease the rate of GAP-stimulated GTP hydrolysis and thus cause a \(gf\) mutant phenotype. This hypothesis is consistent with the observation that overexpression of GAP can suppress c-ras-mediated transformation in mammalian cells [Zhang et al. 1990].

**Mechanism of let-60(Asn-119 \(dn\)) functions**

The let-60(Asn-119 \(dn\)) gene product has the strongest dominant-negative effect on let-60\(^+\) activity but contains some constitutive activity itself to trigger vulval differentiation and to support larval growth (Table 3). The residual activity of let-60(Asn-119 \(dn\)) protein in the vulval signaling pathway is consistent with the properties of mammalian ras proteins with changes in residue 119. For example, ras proteins with residue 119 changed from Asp to Ala or His can cause malignant transformation in mammalian cells, although these mutant proteins cause a drastic decrease in affinity for GDP/GTP (Der et al. 1986; Sigal et al. 1986). The constitutive nature of the residual activity associated with changes in residue 119 may be due to a decrease in affinity of ras for guanine nucleotides that leads to an increase in the exchange rate from ras–GDP to ras–GTP (i.e., causes GNEF-independent ras activity; Sigal et al. 1986). Therefore, the complicated phenotypes of let-60(Asn-119 \(dn\)) may be the result of two separable and abnormal biochemical functions of the protein: On one hand, let-60(Asn-119) protein [possibly free of guanine nucleotides] might form a very stable complex with an activator [e.g., GNEF], preventing it from interacting with the let-60(+) protein [see Fig. 3]; on the other hand, let-60(Asn-119) has its own low activity that does not require the activation by GNEF and is less sensitive to the deactivation by GAP.

**Regulation of let-60 by the inductive signal**

During vulval induction, how is let-60 ras activity regulated by the upstream inductive signal and the receptor tyrosine kinase encoded by the let-23 gene [Aroian et al. 1990]? The upstream signal may either activate a GNEF that promotes the exchange of ras from GDP- to GTP-bound form or down-regulate the activity of GAP, which promotes GTP hydrolysis by ras. In the absence of upstream kinase activity, the activation step would be limiting, but a high dose of wild-type ras might reverse the situation. In the presence of the signal, the deactivation step would be limiting, but dominant-negative ras mutations might reverse the situation. This model predicts that overexpression of GNEF in wild-type C. elegans will cause a multiculva phenotype.

It is also possible that the in vivo regulation of ras activity in these let-60\(^dn\) heterozygotes is more complicated, perhaps involving the combination of a number of positive and negative-regulatory factors or a protein complex that exists in a number of distinct states (see legend to Fig. 3). Understanding how these dominant-negative mutations act should provide insights into the mechanism by which ras proteins exert their signal-transducing functions.

**Utility of dominant-negative mutations**

Dominant-negative mutations can be a useful tool for study in vivo of functions of cloned genes [Herskowitz
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1987]. For example, dn mutations of the Xenopus fibroblast growth factor (FGF) receptor have suggested a role of this protein in the patterning of the early Xenopus embryo [Amaya et al. 1991]. We have shown that particular ras dn mutations (e.g., Arg-10) disrupt the function of ras during vulval development in transgenic animals. Such simple dn mutations might be useful to elucidate the function of ras in the development of other organisms.

Materials and methods

General methods

Methods for culturing, handling, and genetic manipulation of C. elegans were as described by Brenner [1974]. All genetic experiments were performed at 20°C. Methods for analysis of vulval defects under dissecting microscope and under Nomarski optics were as described previously [Han and Sternberg 1990; Sulston and Horvitz 1977]. The genetic nomenclature used was as described [Horvitz et al. 1979]. let-60(dn) strains were described by Han et al. [1990] and Beitel et al. [1990]. Other strains were constructed according to standard methods.

Determining the nucleotide changes in let-60 mutants

DNA fragments containing let-60-coding regions were obtained by polymerase chain reaction (PCR) amplification from homozygous mutant animals [which are dead larvae, in most cases]. The method for PCR amplification of DNA from dead larvae was as described by Beitel et al. [1990]. Sequences of the mutant DNA were determined by one or both of the two methods: In most cases, PCR-amplified DNA fragments were directly sequenced after gel purification [Kretz et al. 1989], in some cases (exon 1 of sy94 and sy101, exons 2 and 3 of sy92, sy93, sy95, and sy100), PCR fragments were subcloned [see below] and then sequenced. In later cases, the DNA lesion of each mutation was confirmed by sequencing multiple clones, or in the case of sy94 and sy101, by also sequencing gel-purified amplified fragments. All four exons have sequenced for all 11 alleles listed in Table 1. Four determined DNA lesions [representing seven genetically isolated dn alleles] were also tested functionally for their dn effects by microinjection experiments (Table 2). There had been ambiguity of the dn allele [either sy101 or sy94] used to generate sy127 [Han and Sternberg 1990]. Our sequence analysis showed it to be sy101, and we will thus refer it as sy101 sy127 [or Arg-10 Stop-123].

In vitro construction of let-60(dn) genes

New plasmids were constructed to facilitate the construction of mutant let-60 genes. pMH105 was first generated by deleting part of the linker region [between EcoRI and Apal] of vector Bluescript (SK +). This deletion was achieved by digesting the Bluescript (SK +) with EcoRI and Apal and then self-ligating the large fragment after end-filling with T4 polymerase. A 6.8-kb BamHI-XhoI genomic fragment containing the entire let-60 gene [Han et al. 1990] was inserted into the BamHI site of pMH105. The resulting plasmid, called pMH106, was used subsequently for subcloning let-60(dn) fragments. For alleles located in exon 1, the HindIII–Apal fragment of pMH106 was replaced by mutant DNA fragments, for alleles located in exon 2 or 3, the Apal–EcoRV fragment of pMH106 was replaced by mutant DNA fragments.

Microinjection transformation

Each construct was injected into dpy-20(e1282) [Hosono et al. 1982] hermaphrodites together with pMH86 (containing the dpy-20 gene) [15–25 μg/ml]. To keep total DNA concentration approximately the same, Bluescript (SK +) plasmid was co-injected at 50 μg/ml. Because of the large number of F1 transformants [non-Dpy animals] produced, the number of F1 transgenic animals listed in Table 2 is approximate. For some experiments described in Table 2, about four to eight F1 transformants were pooled on a single plate for screening for stable lines. Each plate containing F2 transformants was scored as one stable line, thus, the number of stable lines listed in Table 2 is a minimum.

For the experiments described in Table 4, a dominant rol-6 mutant gene [pRF4; Mello et al. 1991; 50 μg/ml] and dpy-20 gene [pMH86; 10 μg/ml] were co-injected into let-60(n1046 gfl)/dpy-20(e1282) hermaphrodites along with 10 μg/ml of let-60(sy101 dn) DNA [pMH136]. pMH86 is a Bluescript (SK +) derived plasmid that has a 6-kb XhoI DNA insert containing the entire dpy-20 gene [subcloned from a dpy-20-containing cosmid, D. Clark and D. Baillie, pers. comm.]. Because the Dpy phenotype of dpy-20 homozygotes, which normally suppresses the roller (Rol) phenotype, is rescued in the transgenic animals by the extrachromosomal dpy-20 gene, the dominant Rol phenotype is associated with all transgenic animals.

Estimation of copy numbers of extrachromosomal let-60 genes

To estimate extrachromosomal copy number, a let-60 DNA fragment was amplified by PCR from L4-stage stable transgenic animals as well as nontransgenic dpy-20 animals. To keep PCR amplification in a linear range, only 15 rounds of amplification were performed [Robinson and Simon 1991]. The PCR products were loaded on an agarose gel, and the amount of amplified DNA was detected by Southern analysis. As a control, primers were also added to each reaction to amplify a DNA fragment located in the let-23 gene [chromosome I] [Aroian et al. 1990]. For each transgenic line, three to four animals were first lysed with proteinase K in 20 μl. The lysate [2×2 μl and 2×5 μl] was then added to one of the four tubes containing the PCR mix. The relative intensity of the two DNA bands on an autoradiograph was measured by a densitometer [LKB]. The number of extrachromosomal let-60 genes listed in Table 2 is the average of the four measurements for each transgenic line.

Strain construction

To construct strains containing nDp5 and let-60(sy101 dn) dpy-20(e1362) unc-22(e66); dpy-20(e1282); let-60(n1046 gfl) unc-22(s7) hermaphrodites. Non-Dpy non-Unc F1 progeny were picked and placed individually on new plates. F2 progeny of the genotype let-60(sy101 dn) dpy-20(e1282)/let-60(n1046 gfl) unc-22(s7) hermaphrodites. Non-Dpy non-Unc F1 cross progeny were picked and placed individually on new plates. F2 progeny of the genotype let-60(sy101 dn) +/animals was obtained from screening the F2 progeny. The complete genotype for let-60(sy101 dn)+/animals is let-60(sy101 dn) dpy-20(e1282)/let-65(e159); let-60(sy101 dn) dpy-20(e1282); nDp5 was obtained from screening the F3 progeny. The complete genotype for let-60(sy101 dn)+/animals is let-60(sy101 dn) dpy-20(e1282)/let-65(e159); let-60(sy101 dn) dpy-20(e1282); nDp5; him-5(e1490) males [Beitel et al. 1990] were crossed with let-60(sy101 dn) dpy-20(e1282)/let-60(n1046 gfl) unc-22(s7) hermaphrodites. Non-Dpy non-Unc F1 cross progeny were picked and placed individually on new plates. F2 progeny of the genotype let-60(sy101 dn)+/animals was obtained from screening the F2 progeny. The complete genotype for let-60(sy101 dn)+/animals is let-60(sy101 dn) dpy-20(e1282)/let-65(e159); let-60(sy101 dn) dpy-20(e1282); nDp5; him-5(e1490) was constructed first. Males of this strain were then crossed with unc-24(e138) let-60(sy93dn)/unc-24(e138) let-60(sy93 dn) hermaphrodites [Han et al. 1990].
The F1 non-Unc cross progeny were picked and have the genotype unc-24 let-60(sy93) dn/unc-24 mec-3 dpy-20(e1282); nDp5; him-5(e1490)+. A strain of genotype let-60(Asy93) dn/unc-24 let-60(sy93) dn; nDp5 was obtained from F2 or F3 progeny.

Examining vulval differentiation with Nomarski optics

For let-60(dn)/+/+ and +/+/+ animals, self progeny (late L3 or early L4 larvae) of let-60(dn)/+/+ animals are first examined for vulval induction, and their genotypes were determined by progeny testing. Except for +/+/+ animals, vulval differentiation was determined by examining the progeny from mothers of the same genotype.

The percentage of vulval differentiation is determined as the percentage of vulval precursor cells [P3.p–P8.p] differentiating into vulval cell types relative to wild-type level of differentiation (as defined by Han et al. 1990). In a completely vulvalless animal, each of the six precursor cells divides once, and their progeny appears to fuse with the syncytial hypodermis. The vulval differentiation in such an animal is said to be 0%. In a wild-type hermaphrodite, each of the six precursor cells divide further than the first round of division, producing the progeny characteristic of vulval cell types [Sulston and Horvitz 1977, Sternberg and Horvitz 1986]. Animals with fewer than three cells differentiating into vulval cell types have <100% vulval differentiation (vulvalless); animals with more than three precursor cells differentiating to vulval cell types have >100% vulval differentiation (multivulva). Sometimes only one of the two daughters of a precursor cell divided further to generate vulval tissue, the vulval differentiation in this case is “one-half cell”. Laser ablation experiments were performed as described [Sulston and White 1980].

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