clr-1 encodes a receptor tyrosine phosphatase that negatively regulates an FGF receptor signaling pathway in *Caenorhabditis elegans*

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Receptor tyrosine phosphatases have been implicated in playing important roles in cell signaling events by their ability to regulate the level of protein tyrosine phosphorylation. Although the catalytic activity of their phosphatase domains has been well established, the biological roles of these molecules are, for the most part, not well understood. Here we show that the *Caenorhabditis elegans* protein CLR-1 (CLeaR) is a receptor tyrosine phosphatase (RTP) with a complex extracellular region and two intracellular phosphatase domains. Mutations in *clr-1* result in a dramatic Clr phenotype that we have used to study the physiological requirements for the CLR-1 RTP. We show that the phosphatase activity of the membrane-proximal domain is essential for the in vivo function of CLR-1. By contrast, we present evidence that the membrane-distal domain is not required to prevent the Clr phenotype in vivo. The Clr phenotype of *clr-1* mutants is mimicked by activation of the EGL-15 fibroblast growth factor receptor (FGFR) and is suppressed by mutations that reduce or eliminate the activity of *egl-15.* Our data strongly indicate that CLR-1 attenuates the action of an FGFR-mediated signaling pathway by dephosphorylation.

Protein tyrosine phosphorylation plays an integral role in cellular signaling events and functions in basic processes such as cell division, differentiation, and cell fate determination. The phosphorylation state of specific tyrosines is regulated by the opposing actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs; Sun and Tonks 1994; Hunter 1995). PTPs are a diverse family of molecules characterized by the presence of at least one phosphatase domain (Charbonneau and Tonks 1992; Walton and Dixon 1993). This domain spans ~250 amino acids and contains the core consensus sequence (I/V)HCXAGXXR(S/T)G. Among these conserved residues, the cysteine and arginine have been shown to be essential for phosphatase activity (Streuli et al. 1990; Guan and Dixon 1991; Pot et al. 1991; Cho et al. 1992). The cysteine acts as a nucleophile on the phosphotyrosine-containing substrate and forms a thiophosphate intermediate during the dephosphorylation reaction (Guan and Dixon 1991; Cho et al. 1992; Pot and Dixon 1992). The arginine is also found at the active site and appears to aid in both binding and catalysis via its guanidinium group (for review, see Denu et al. 1996).

PTPs are broadly classified into transmembrane or receptor PTPs (RTPs) and intracellular PTPs (Fischer et al. 1991). The RTPs are further classified on the basis of their extracellular regions (Fischer et al. 1991; Brady-Kalnay and Tonks 1995). Type II isoforms contain amino-terminal immunoglobulin domains followed by fibronectin type III (FN III) repeats. These domains are characteristic of the N-CAM family of cell adhesion molecules, and some type II RTPs have been shown to mediate adhesive interactions (Brady-Kalnay et al. 1993; Gebbink et al. 1993; Sap et al. 1994).

Most RTPs contain two intracellular phosphatase domains. The first domain (D1, membrane-proximal) is catalytically active (Streuli et al. 1989, 1990), whereas the function of the second domain (D2, membrane-distal) is not understood. Some second phosphatase domains are predicted to be catalytically inactive based on the absence of specific conserved residues in their catalytic cores. For example, RPTPβ and HPTPγ are missing the catalytic cysteine known to be essential for phosphatase activity (Krueger et al. 1990; Kaplan et al. 1990). Other second phosphatase domains contain these conserved residues in the catalytic core and can show phosphatase activity (e.g., see Wang and Pallen 1991), but the importance of this domain to the function of the RTP is not clear.

The D1 and D2 domains of RTPs appear to differ struc-

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turally as well as functionally. Recent data based on multiple crystal forms of RPTPα identified an amino-terminal segment, termed the amino-terminal wedge, that may be involved in the dimerization of D1 domains (Bilwes et al. 1996). The primary sequence that forms this amino-terminal wedge appears to be found only in D1 domains and not in D2 domains (Bilwes et al. 1996), suggesting that D1 and D2 are inherently different.

To address the physiological functions of RTPs and the roles of their noncatalytic domains, in vivo systems in which RTPs have easily assayable functions are invaluable. The identification of mutations in several *Drosophila* RTPs has allowed an initial analysis of the roles of RTPs in the nervous system (Desai et al. 1996, 1997; Krueger et al. 1996). Here we identify a receptor tyrosine phosphatase, CLR-1, in the nematode *Caenorhabditis elegans.* Mutations that compromise the function of CLR-1 confer a distinctive and highly penetrant phenotype. Our analysis of CLR-1 has enabled us to define its role in a fibroblast growth factor receptor (FGFR) signaling pathway in *C. elegans* and to study the functions of its two intracellular phosphatase domains.

Results

Mutations in clr-1 *are isolated as suppressors of* egl-15(n1477ts)

egl-15 encodes a *C. elegans* FGFR that is involved in sex myoblast migration guidance and an essential function (DeVore et al. 1995). Whereas *egl-15* mutants homozygous for putative null alleles display a larval arrest phenotype, certain hypomorphic mutations in *egl-15* can confer a Scrawny (Scr) body morphology that may be a weaker manifestation of the null phenotype. To identify genes that interact with *egl-15,* we isolated mutations that could suppress the temperature-sensitive scrawny body morphology of *egl-15(n1477ts)* animals (Fig. 1B). Eighteen suppressor strains were isolated from animals representing ∼51,000 mutagenized haploid genomes. When observed in a wild-type background, 12 of the 18 suppressor mutations caused various degrees of the distinctive Clr (clear) phenotype originally defined by the temperature-sensitive recessive mutant *clr-1(e1745ts)* (Fig. 1C; Hedgecock et al. 1990). Consistent with their being *clr-1* alleles, all 12 suppressor mutations showed linkage to the *dpy-10(e128)* marker, which lies 2.2 map units right of *clr-1* on linkage group II, and failed to complement *clr-1(e1745ts)* for the Clr phenotype (data not shown). In addition, we found that *clr-1(e1745ts)* also suppressed the Scr phenotype of *egl-15(n1477ts)* animals (Fig. 1D). We conclude that these 12 suppressors are alleles of *clr-1.* Of the remaining six suppressors, three are likely to be intragenic revertants of *egl-15(n1477ts)* (see Materials and Methods) and three have not been characterized further.

From a number of direct visual screens for Clr animals, we collected 15 additional mutations that confer a recessive Clr phenotype in a wild-type background. These mutations all failed to complement *clr-1(e1745ts),* suppressed the Scr phenotype conferred by *egl-15(n1477ts),* and were suppressed by *egl-15(n1477ts).* Because all 15 mutations appeared to be *clr-1* alleles, *clr-1* is likely to be

Figure 1. Antagonistic roles of *egl-15* and *clr-1.* Bright-field micrographs of adult hermaphrodites. (*A*) Wild type. (*B*) *egl-15(n1477ts)* animal, which displays a temperature-sensitive Scr body morphology. (*C*) *clr-1(e1745ts)* animal, which displays a temperature-sensitive Clr phenotype. (*D*) *clr-1(e1745ts); egl-15(n1477ts)* double mutant, which displays neither the Clr nor the Scr phenotype, demonstrating a mutual suppression between non-null alleles of *clr-1* and *egl-15.* Because *clr-1(e1745ts)* is not a potent suppressor of the egg-laying and SM mispositioning defects of *egl-15(n1477ts),* the animal is beginning to accumulate eggs in her uterus. (*E*) Clr transgenic animal bearing an extrachromosomal array made with the *neu**-activated *egl-15* construct. (*F*) Non-Clr transgenic animal bearing an extrachromosomal array made with a kinase-defective *neu**-activated *egl-15* construct [*egl-15(neu*K672A)*]. Animals in *A–D* were raised at 25°C; transgenic animals shown in *E* and *F* were raised at 20°C. Transgenic animals contain the transformation marker plasmid pRF4 that confers a Roller phenotype (Mello et al. 1991). Scale bar (*A–F*), 300 μm.
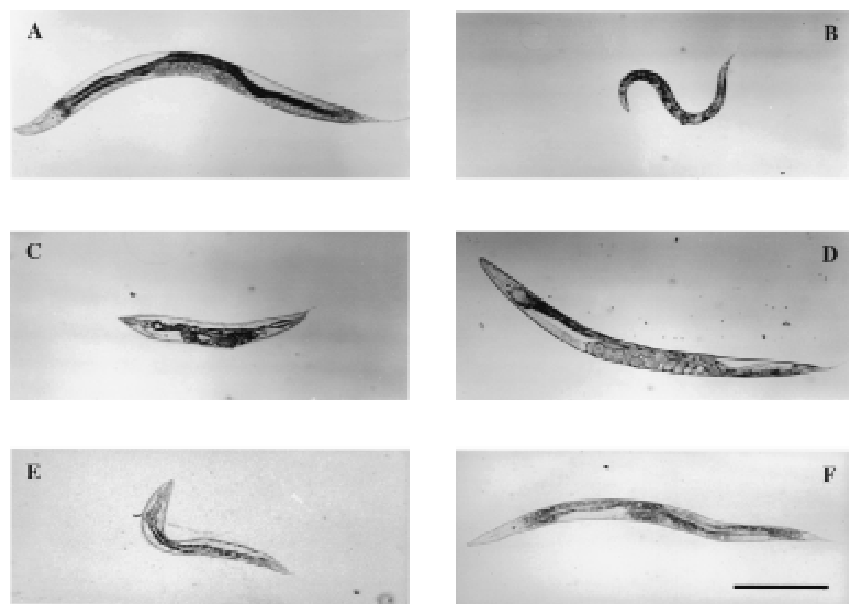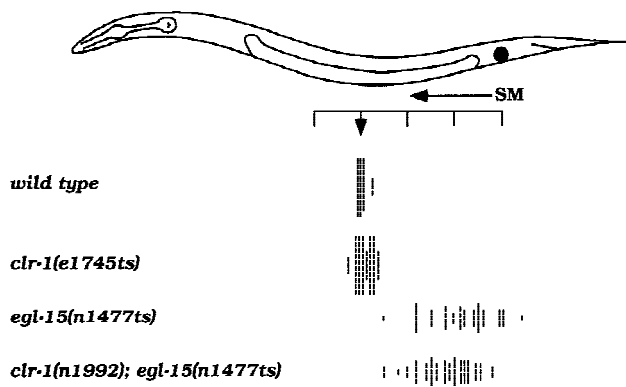
Figure 2. *clr-1* mutations have minor effects on SM migration. The positions of individual SMs are indicated by hash marks and are scored relative to the animal diagrammed above, as previously described (Thomas et al. 1990). The ruler indicates positions in units of the distance between Pn.p cells; the arrow marks their normal positions flanking the precise center of the gonad. Many other alleles of *clr-1* have similar effects on the SM distribution of *egl-15(n1477ts)* animals, and *clr-1(e1745ts)* has similar weak effects on all other *egl-15* mutants tested (data not shown). SMs were scored at 20°C for all strains shown.

the only gene that is easily mutable to result in a viable Clr phenotype. These results also provide evidence that the interaction observed between *clr-1* and *egl-15* does not require a specific type of *clr-1* mutation. Although mutations conferring the weakest Clr phenotypes were isolated only as suppressors of *egl-15(n1477ts)*, mutations conferring the stronger Clr phenotypes were obtained in both screens. Certain mutations among those that confer the strongest Clr phenotype appeared to eliminate *clr-1* function by a variety of genetic and molecular criteria (see Materials and Methods).

clr-1 appears to play a minor role in guiding the migrations of the hermaphrodite sex myoblasts (SMs). In a wild-type background, mutations in *clr-1* do not dramatically affect the ability of the SMs to migrate to their precise final positions (Fig. 2). Furthermore, whereas mutations in *clr-1* dramatically suppress the body morphology defects conferred by *egl-15(n1477ts)* (e.g., see Fig. 1D), they do not rescue the mispositioning of the SMs found in *egl-15(n1477ts)* mutants (Fig. 2). Nonetheless, *clr-1* mutations subtly shift the range of the displaced SMs closer to their wild-type positions and correspondingly decrease the penetrance of the egg-laying defect caused by certain *egl-15* mutations, suggesting a minor role in SM migration guidance.

The Clr phenotype

Animals homozygous for the *clr-1(e1745ts)* mutation appear completely wild-type at the permissive temperature of 15°C but display a severe Clr phenotype when raised at the nonpermissive temperature of 25°C (Fig. 1C). Clr animals are extremely short, immobile, and infertile, and their pharynx and intestine appear to float within the pseudocoelom. When viewed using Nomarski microscopy, the cells and cell processes in these animals appear to be separated from each other, a phenotype that has been used extensively to visualize neuronal processes and cell boundaries in *C. elegans* (e.g., see Way and Chalfie 1988; Hedgecock et al. 1990). These abnormalities all appear to result from clear fluid accumulating in the pseudocoelom. Whereas some animals homozygous for the most severe alleles of *clr-1* die during larval development with hypodermal ruptures, most remain alive and display an extreme Clr phenotype. The biological basis of the Clr phenotype is not understood, although it mimics the phenotype observed when cells in the *C. elegans* excretory system (Nelson and Riddle 1984) or the CAN neurons (Forrester and Garriga 1997) are destroyed by laser microsurgery.

Relationship between clr-1 *and* egl-15

The mutual suppression observed between *egl-15(n1477ts)* and mutations in *clr-1* indicates that these genes act antagonistically. To clarify the nature of the interactions between *clr-1* and *egl-15,* double mutants using putative null alleles of both *clr-1* and *egl-15* were constructed. Complete loss of *egl-15* function results in early larval arrest (DeVore et al. 1995). The *clr-1(e2530); egl-15(n1454)* double mutant also displays this larval arrest phenotype but does not display the Clr phenotype of similarly staged *clr-1(e2530)* mutants (Fig. 3). Thus, the Clr phenotype of *clr-1* mutants requires *egl-15* activity. The finding that both hypomorphic and null alleles of *egl-15* can suppress the Clr phenotype conferred by *clr-1*
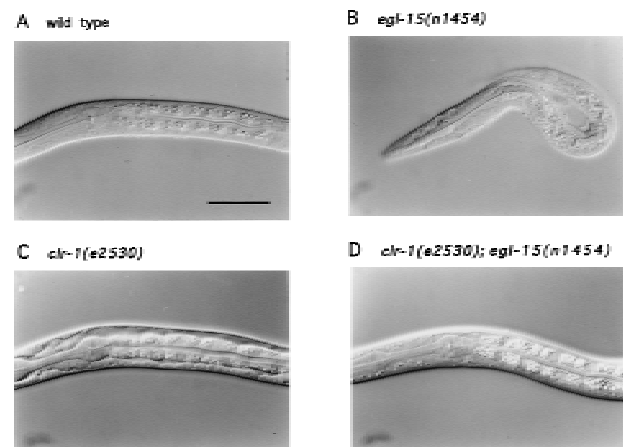

Figure 3. Interaction between null alleles of *egl-15* and *clr-1.* Nomarski photomicrographs of the pharyngeal and anterior body regions of first larval stage hermaphrodites. (*A*) Wild type. (*B*) *egl-15(n1454),* a null mutant. (*C*) *clr-1(e2530),* a null mutant. (*D*) *clr-1(e2530); egl-15(n1454)* double mutant. The Clr phenotype is seen only in *C,* which shows the intestine separating from the body wall. Anterior is *left;* dorsal is *up.* The close proximity of the intestine and body wall demonstrates suppression of the Clr phenotype by loss of *egl-15.* Scale bar (*A–D*), 30 µm.

null mutations suggests that CLR-1 acts as a negative regulator of EGL-15 function and that the Clr phenotype may be caused by hyperactivity of this FGFR-mediated signaling pathway.

To test directly whether EGL-15 hyperactivity can confer a Clr phenotype, we replaced sequences encoding the transmembrane domain of EGL-15 with those encoding the transmembrane domain of the constitutively activated oncogenic form of the *neu* proto-oncogene (Bargmann et al. 1986). This domain can cause the constitutive dimerization and activation of the Neu receptor tyrosine kinase (Weiner et al. 1989). Introduction of this *egl-15(neu*)* construct into wild-type animals by germline transformation could confer a Clr phenotype similar to that observed in *clr-1* mutants (Fig. 1E). By contrast, introduction of a similar construct containing an additional mutation that is predicted to abolish kinase activity had no effect [*egl-15(neu*K672A);* Fig. 1F]. Thus, we conclude that hyperactivation of the kinase activity of EGL-15 can confer a Clr phenotype.

clr-1 is predicted to encode a receptor tyrosine phosphatase

Cosmids from the region of the *C. elegans* physical map most likely to contain *clr-1* were tested for their abilities to rescue the Clr phenotype of *clr-1(e1745ts)* animals following germ-line transformation. This assay was used to show that *clr-1* activity is contained on cosmid F43G3 and to narrow rescuing activity to a 7.8-kb genomic fragment (data not shown). cDNAs with sequences that correspond to portions of the 7.8-kb rescuing fragment were isolated and analyzed. These cDNAs provide evidence for two alternatively spliced *clr-1* transcripts, termed cDNA1 and cDNA2 (see Materials and Methods).

The *clr-1* transcript corresponding to cDNA1 contains a single, large ORF that is predicted to encode a protein of 1409 amino acids (Fig. 4A). Within this protein are two stretches of hydrophobic amino acids (residues 1–19 and 719–738) predicted to correspond to a signal sequence and a transmembrane domain. This protein also contains an amino-terminal region of 72 amino acids (residues 64–135) of which 15% are cysteines. Comparative sequence analysis revealed the presence of two phosphatase domains in the presumptive intracellular portion of CLR-1 (Fig. 5), indicating that CLR-1 is a receptor tyrosine phosphatase. In addition, our searches suggested the presence of two immunoglobulin-like domains and one FN III repeat in the presumptive extracellular region of CLR-1 (data not shown). The combination of these domains is characteristic of the type II family of RTPs (Fischer et al. 1991); however, no previously known RTP has the same number and arrangement of domains found in CLR-1. Based on this difference in overall organization and the fact that the CLR-1 sequence has similar levels of identity to the sequences of several RTPs, we conclude that CLR-1 is not the specific homolog of any known RTP.

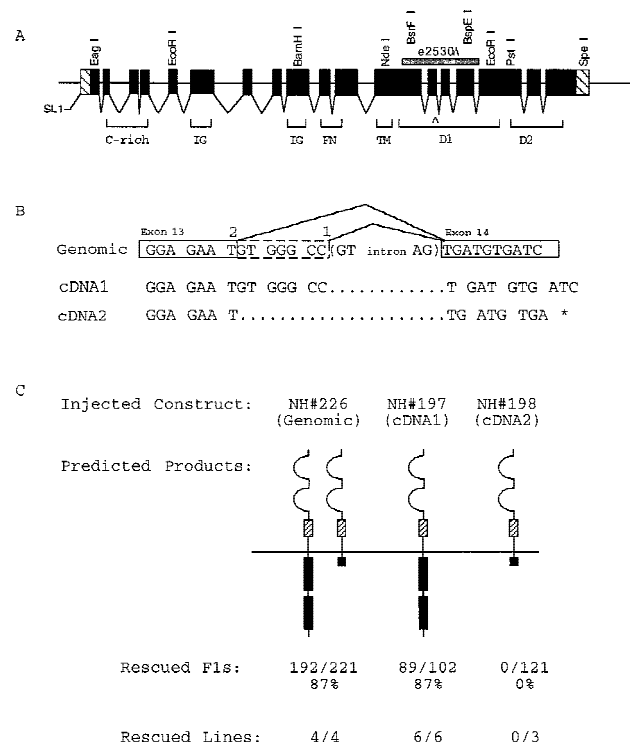The *clr-1* transcript corresponding to cDNA2 is identical to that corresponding to cDNA1 except for seven

Figure 4. Two alternatively spliced *clr-1* transcripts. (*A*) Genomic organization of the 9-kb (*Stu*I–*Stu*I) *clr-1* genomic rescuing fragment. (Solid boxes) Coding sequence; (hatched boxes) untranslated regions of cDNA1. The SL1 *trans*-spliced leader sequence is found at the 5′ end of *clr-1* transcripts. Domains identified on the basis of sequence similarity are labeled: (C-rich) Cysteine-rich; (IG) immunoglobulin-like; (FN) fibronectin type III repeat; (TM) transmembrane; (D1,D2) phosphatase domains 1 and 2. The deletion associated with *clr-1(e2530)* is indicated above the *clr-1* genomic structure. (ˆ) Alternate splice-donor sites. (*B*) The two transcripts corresponding to *clr-1* differ in the use of a single splice–donor site. Genomic sequence surrounding the alternative splice is shown on the first line. Exons are boxed; the intron is in parenthesis. (Dashed box) Additional seven nucleotides found in cDNA1, which maintains an ORF that extends beyond the phosphatase domains. The splice that generates cDNA2 creates a UGA stop codon (*) just downstream of the splice–acceptor site. (*C*) *clr-1*-rescuing activity of the individual alternatively spliced *clr-1* forms. Diagrams of the predicted protein products of the constructs introduced by germ-line transformation are shown. (Horizontal line) Cell membrane; (half circles) immunoglobulin domains; (hatched boxes) FN III repeats; (solid boxes) phosphatase domains. DNA constructs were tested for rescue in both F_1 transformants (Rescued F1s) and stable lines (Rescued lines).

fewer nucleotides at the 3′ end of exon 13 (Fig. 4B). This difference appears to result from the use of an alternative splice–donor site seven nucleotides upstream of the site used in cDNA1. As a consequence of the use of this alternative splice site, cDNA2 contains a stop codon that truncates its protein product early within the first phosphatase domain. Northern analysis revealed a single band that corresponds to the 4.4-kb transcript size predicted for both cDNAs (data not shown).

To determine whether either cDNA1 or cDNA2 could provide *clr-1* rescuing activity, we subcloned the unique portion of each cDNA into the genomic rescuing fragment and tested the abilities of the resulting constructs to rescue *clr-1(e1745ts).* As shown in Fig. 4C, the construct encoding only the full-length protein provided *clr-1* rescuing activity, whereas the construct encoding only the truncated product did not. Thus, the full-length form of CLR-1 appears to be necessary for *clr-1* function in vivo.

The role of the phosphatase domains in CLR-1 function

The core consensus sequence of tyrosine phosphatase domains, (I/V)HCXAGXXR(S/T)G, lies at the active site and contains the cysteine and arginine residues that play essential roles in catalysis (Streuli et al. 1990; Denu et al. 1996). In CLR-1, the membrane-proximal domain (D1) contains this core consensus sequence (Fig. 5). In the distal domain (D2), however, the arginine is replaced with glutamic acid, suggesting that this domain is not catalytically active (Fig. 5). As in other RTPs (Bilwes et al. 1996), only the D1 domain of CLR-1 appears to contain the amino-terminal wedge sequences.

To determine whether CLR-1 has intrinsic tyrosine phosphatase activity, we tested CLR-1 for its ability to release inorganic phosphate from a tyrosine phosphorylated substrate in vitro. This experiment was performed in two different ways. First, full-length CLR-1 was expressed in COS cells, immunoprecipitated using anti-CLR-1 antibodies, and assayed for tyrosine phosphatase activity (Fig. 6A). By this assay, full-length CLR-1 has significant phosphatase activity when compared to the vector control. Because only the first phosphatase domain was predicted to be catalytically active, we similarly tested a CLR-1 construct, CLR-1(C1013S), in which

we mutated the active-site cysteine in the first phosphatase domain to serine. The activity of CLR-1(C1013S) was indistinguishable from the vector control, providing strong evidence that CLR-1 itself has phosphatase activity rather than the activity being caused by another molecule that coimmunoprecipitates with CLR-1. Our second assay for CLR-1 phosphatase activity assessed the specific activity of the phosphatase domains of CLR-1 (Fig. 6B). A portion of the intracellular domain of CLR-1 containing both phosphatase domains was expressed as a fusion protein with glutathione *S*-transferase (GST), purified, and assayed in vitro for phosphatase activity. The phosphatase domains from wild-type CLR-1 showed significant phosphatase activity in this assay, whereas the identical protein containing the C1013S mutation showed no activity. These data support the hypothesis that CLR-1 can function as a tyrosine phosphatase and that only the D1 domain has significant catalytic activity.

To test whether the observed phosphatase activity of the D1 domain is essential for the physiological function of CLR-1, we introduced the C1013S mutation into the genomic rescuing fragment. This alteration abolished *clr-1*-rescuing activity (Fig. 7), supporting the hypothesis that CLR-1 functions as a phosphatase in vivo. Further evidence that the catalytic activity of CLR-1 is necessary for function comes from the finding that several endogenous *clr-1* mutations affect conserved residues in the D1 domain (Table 1). Three of these mutations affect residues in the catalytic core consensus sequence. These three mutations are among those that confer the strongest Clr phenotypes, consistent with the importance of this region for catalysis.

The failure of the C1013S construct to rescue *clr-1(e1745ts)* in germ-line transformation rescue experiments could be a consequence of global effects on CLR-1 rather than the intended specific alteration in the function of the D1 phosphatase domain. To monitor the lo-
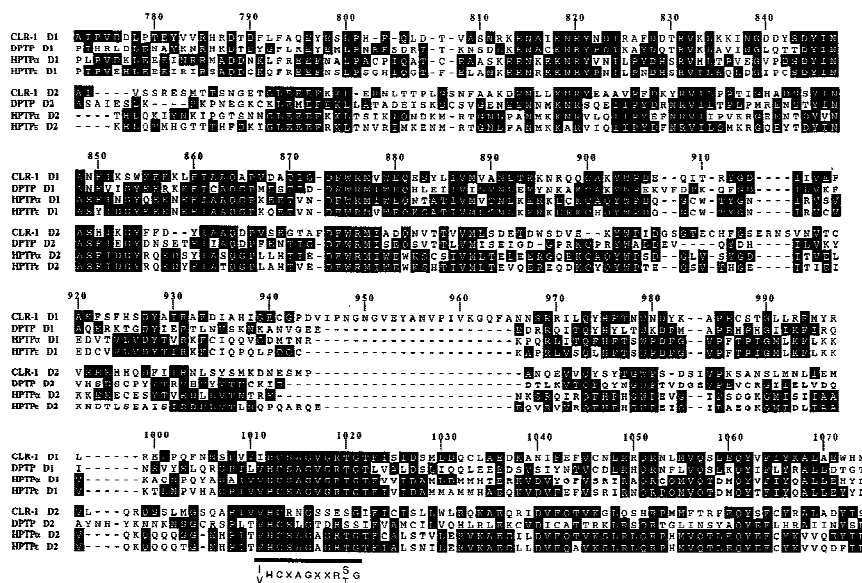

Figure 5. Comparison of the tyrosine phosphatase domains from CLR-1, DPTP (Streuli et al. 1989), HPTPα (Krueger et al. 1990), and HPTPε (Krueger et al. 1990). (Solid boxes) Identities among at least two of the eight domains; (dashes) gaps introduced by the alignment. The membrane-proximal (D1) and membrane-distal (D2) domains are grouped separately. The 11 amino acid core consensus sequence is underlined. Numbers are according to residues in the D1 domain of CLR-1. The amino-terminal wedge of the CLR-1 D1 domain corresponds to amino acids 772–802.
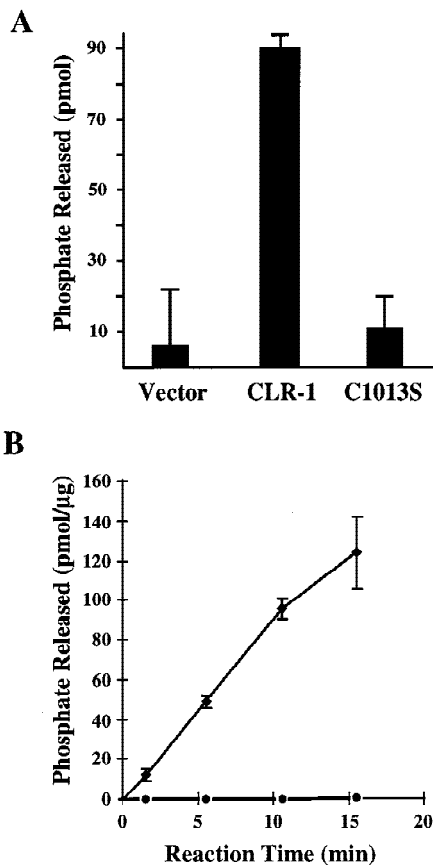
A

B

Figure 6. Tyrosine phosphatase activity of CLR-1. (*A*) Full-length CLR-1 expressed in COS cells. (Solid bars) Amount of phosphate released from a Tyr-^{32}P-labeled RCML substrate by CLR-1 immunoprecipitates from COS cells transiently transfected with the wild-type *clr-1* cDNA1 (CLR-1), the *clr-1* cDNA1 harboring the C1013S mutation in the membrane-proximal phosphatase domain (C1013S), or the expression vector alone (Vector). (*B*) The phosphatase domain region of the intracellular domain of CLR-1 expressed in *E. coli*. Phosphate released was measured for GST fusions to the wild-type phosphatase domains (♦) and the identical protein bearing the C1013S mutation in the D1 domain (●). Error bars represent the standard errors of the mean.

calization and steady-state levels of expression of altered *clr-1* constructs introduced by germ-line transformation, we created a construct encoding a protein in which the green fluorescent protein (GFP) is fused to the amino terminus of the predicted mature CLR-1 product. This construct (*clr-1::gfp*) rescued *clr-1(e1745ts),* albeit at slightly lower efficiency than the wild-type genomic rescuing fragment (data not shown). In lines transformed with this construct, GFP fluorescence was detected at the periphery of body muscles, consistent with localization of the CLR-1 RTP to the plasma membrane (Fig. 8). Although the C1013S mutation abolished rescuing activity, it did not affect either the localization or expression levels of GFP when introduced into the *clr-1::gfp* construct (Fig. 7). Thus, the inability of CLR-1(C1013S)

to rescue *clr-1(e1745ts)* is likely caused by the lack of phosphatase activity of the D1 domain.

The ability to use *clr-1::gfp* to distinguish specific mutational effects versus more global effects allowed us to perform a structure/function analysis of the D2 phosphatase domain of CLR-1. Several lines of evidence indicate that this phosphatase domain is not essential for *clr-1*-rescuing activity, although mutational perturbations carboxy-terminal to domain D1 can destabilize the entire protein. First, a frameshift mutation predicted to result in a CLR-1 product truncated at the very beginning of the D2 phosphatase domain (Pst FS) could confer partial rescuing activity (Fig. 7). The reduced rescuing activity of this construct appears to be caused by reduced levels of steady-state product, since no detectable GFP fluorescence was visible in any of the transformed lines (Fig. 7). Second, the temperature-sensitive mutation *clr-1(e1745ts)* was found to correspond to a nonsense mutation affecting the very carboxyl terminus of the D1 phosphatase domain (W1072–opal; Table 1). We postulated that the same truncated product is encoded at both temperatures. Since *clr-1(e1745ts)* appears wild-type at the permissive temperature, this product must be functional. However, at the nonpermissive temperature, the

Injected Construct	*clr-1* Rescue		GFP +
	F1s	Lines	Lines
Wild Type	192/221 (87%)	4/4	2/2
C1013S	0/145 (0%)	0/8	2/2
Pst FS	10/175 (6%)	0/2	0/5
ΔD2	48/149 (32%)	9/9	5/5

Figure 7. Role of CLR-1 phosphatase domains in *clr-1*-rescuing activity. Injected constructs are diagrammed as in Fig. 4. C1013S has the catalytic cysteine of the membrane-proximal phosphatase domain mutated to serine. Pst FS has a frameshift mutation at a *Pst*I site at the beginning of the region encoding the membrane-distal phosphatase domain. ΔD2 deletes the region between the site of the nonsense mutation in *clr-1(e1745ts)* and the normal *clr-1* stop codon. The rescue results shown are from constructs lacking the GFP insert; similar results were obtained with constructs bearing the *clr-1::GFP* fusion. Localization and steady-state levels of CLR-1 products from the injected construct were determined by measuring GFP expression in lines obtained from constructs bearing the *clr-1::GFP* fusion. At least 10 animals from each line were observed for GFP expression. Lines were scored as GFP⁺ if the intensity and distribution of GFP expression was similar to that observed for transformants bearing the wild-type *clr-1::GFP* construct. Those lines scored as GFP⁻ had no detectable GFP expression in all animals observed. GFP results are shown for lines obtained in a *clr-1(e1745ts)* background, except for the ΔD2 construct in which case results were obtained in a wild-type background. Similar GFP expression was observed in both genetic backgrounds wherever tested. GFP expression levels for the ΔD2 construct were slightly greater than for the wild-type *clr-1::GFP* construct.

Table 1. clr-1 *mutations in phosphatase domain 1*

Allele	Protein alteration	Sequence		Phenotypic strength
		wild type	mutant	
e1745ts	W1072–Opal	TGG	TGA	15°C non-Clr 20°C intermediate 25°C strong
n1635	P985S	CCG	TCG	intermediate
n1641	A1015V	GCT	GTT	strong
n1649	A862V	GCT	GTT	weak
n1660	G1021R	GGA	AGA	strong
gm30	C1013Y	TGC	TAC	strong

Sequence alterations found in *clr-1* mutants are underlined. Altered amino acids are abbreviated in the single-letter code and are numbered as in Fig. 5. The phenotypic strength of the alleles was judged in homozygous animals according to the following criteria: (non-Clr) Animals are indistinguishable from the wild type; (weak) the only observable defect is a subtle filling of the pseudocoelom; (intermediate) animals display an obvious fluid-filled pseudocoelom but are predominantly non-Dpy and the strain can be propagated; (strong) animals are severely Clr, infertile, Dpy, and immobile.

premature translational termination might result in destabilization of either the *clr-1* message or encoded protein, and steady-state levels of the product could drop below a level critical for normal function. The temperature-sensitivity of steady-state levels of CLR-1(e1745ts) protein was tested by introducing the W1072–opal mutation into the *clr-1::gfp* construct. Consistent with our model, all lines obtained with this construct displayed a temperature-sensitive GFP fluorescence pattern (Fig. 8).

To test whether a CLR-1 construct completely lacking the D2 phosphatase domain could provide efficient *clr-1*-rescuing activity, we deleted *clr-1* sequences between the site of the *e1745ts* mutation and the endogenous *clr-1* stop codon (ΔD2). This construct was capable of providing *clr-1*-rescuing activity following germ-line transformation. In addition to rescuing the temperature-sensitive mutant *clr-1(e1745ts)* (Fig. 7), this construct could also rescue the null mutant *clr-1(n1992)* (see Materials and Methods), eliminating the possibility of any contribution from the chromosomal copies of *clr-1.* On the basis of these data, the D2 phosphatase domain of

CLR-1 does not appear to be required to prevent the Clr phenotype in vivo. The quantitative decrease in rescuing activity observed for animals transiently transformed with this construct might reflect slightly compromised activity caused by the deletion of the D2 domain or truncation of the final α helix of the D1 domain. It is interesting that the ΔD2 construct apparently produces a temperature-stable product, given that its encoded product is essentially identical to that encoded by *clr-1(e1745ts).* In fact, levels of GFP fluorescence from the ΔD2 *clr-1::gfp* construct are more robust than those from the wild-type *clr-1::gfp* construct (data not shown). It is possible that the additional untranslated portion of *clr-1(e1745ts)* is responsible for destabilizing the message at the nonpermissive temperature.

Discussion

We have discovered that *clr-1* encodes an RTP comprising a large extracellular region containing two immunoglobulin-like domains and a FN III repeat and an intra-
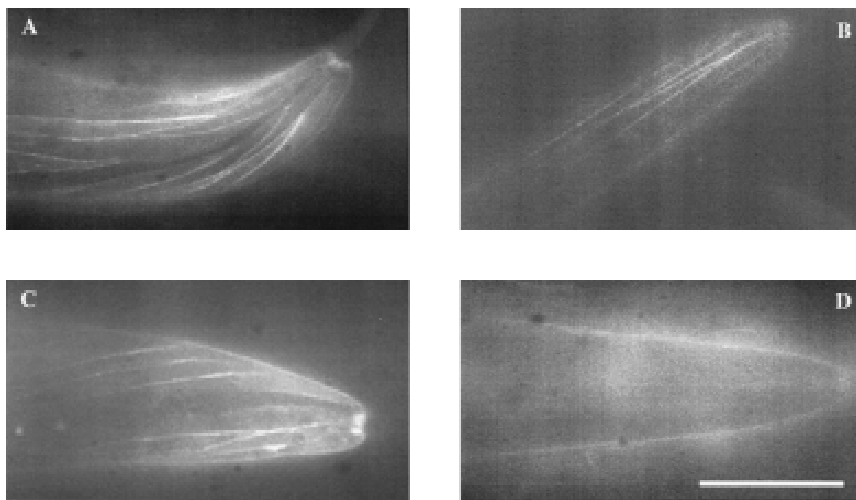

Figure 8. GFP expression in animals containing *clr-1::GFP* transgenes. Wild-type *clr-1::GFP* at 15°C (*A*) and 25°C (*B*); *clr-1(e1745ts)::GFP* at 15°C (*C*) and 25°C (*D*). All lines were generated in a wild-type background using *clr-1::GFP* constructs injected at 150 ng/μl; additional lines behaved similarly.

cellular region containing two phosphatase domains. Mutations that compromise or eliminate the function of the CLR-1 protein confer a dramatic translucent or Clr phenotype. The Clr phenotype has provided an assay for CLR-1 function that we have used to assess the roles of its phosphatase domains and to implicate CLR-1 in regulating an FGFR signaling pathway in *C. elegans.*

The membrane-proximal phosphatase (D1) domain of CLR-1 appears to be catalytically active and essential for its in vivo function. The full-length CLR-1 protein showed in vitro phosphatase activity when expressed in COS cells, as did a bacterially expressed fragment of CLR-1 containing both phosphatase domains. Both activities appeared to be completely dependent upon the catalytic activity of the D1 phosphatase domain, since all phosphatase activity was abolished by mutating the nucleophilic cysteine of this domain to serine. This alteration also destroyed *clr-1*-rescuing activity without changing the expression or localization of the CLR-1 product. Thus, CLR-1 phosphatase activity appears to be essential for its physiological function. This conclusion is supported by the identification of alterations of key residues of the D1 phosphatase domain in *clr-1* mutants.

In contrast, the membrane-distal phosphatase (D2) domain of CLR-1 does not appear to have tyrosine phosphatase catalytic activity and is not essential for *clr-1*-rescuing activity in vivo. The D2 domain is missing a residue present within the catalytic core of active phosphatase domains and is thought to be essential for activity. Furthermore, the D2 domain did not appear to have any in vitro tyrosine phosphatase activity. The nonessential nature of the D2 phosphatase domain for CLR-1 function is supported by a variety of CLR-1 mutant forms that lack the D2 domain but appear to be functional. These include a temperature-sensitive mutant, *clr-1(e1745ts),* that is phenotypically wild-type at the permissive temperature. The molecular lesion associated with *e1745ts* is predicted to truncate CLR-1 prior to the second phosphatase domain at all temperatures, and yet is non-Clr at the permissive temperature. This result is corroborated by the function of a construct that eliminates *clr-1* sequences between the position of the *e1745ts* nonsense mutation and the endogenous *clr-1* stop codon. This construct is capable of rescuing even a putative null allele of *clr-1.* These data demonstrate that CLR-1 can function essentially normally without its D2 phosphatase domain.

The role of the D2 phosphatase domains found in most RTPs has been one of several unresolved issues concerning the functions of RTPs. D2 domains have been found to mediate associations with other molecules (Serra-Pagès et al. 1995; Debant et al. 1996) and to be able to alter the in vitro substrate specificity of the D1 domain (Streuli et al. 1990; Wang and Pallen 1991). Furthermore, domains having striking sequence similarity to tyrosine phosphatase domains, but lacking phosphatase activity, have also been found to have other catalytic activities, suggesting that D2 domains might similarly have other activities (Takagi et al. 1997). Nonetheless, the physiological roles of these domains have been difficult to

establish. Using CD45-defective cell lines, the physiological roles of the two intracellular phosphatase domains of the CD45 RTP have been studied. CD45, which is necessary for antigen receptor signal transduction (Trowbridge and Thomas 1994), appears to require the tyrosine phosphatase activity of its D1 domain but not that of its D2 domain (Desai et al. 1994). A single membrane-tethered tyrosine phosphatase domain can even restore some CD45-like activity to CD45-defective cell lines. However, this activity is clearly suboptimal, leaving open the possibility of other important roles for the D2 domain of CD45 (Motto et al. 1994). We have several lines of evidence that suggest that the D2 domain of CLR-1 is not essential for in vivo *clr-1*-rescuing activity; the absence of this domain appears to confer no obvious phenotypic consequences in otherwise wild-type animals.

CLR-1 affects an FGFR signaling pathway

The isolation of *clr-1* alleles as suppressors of hypomorphic mutations affecting the EGL-15 FGFR implicates the CLR-1 RTP in FGF signaling pathways. The mutual suppression observed between *clr-1* and *egl-15* mutants indicates that the CLR-1 phosphatase and the EGL-15 kinase have opposing actions. Genetic analysis using putative null alleles of *egl-15* and *clr-1* demonstrated that complete loss of CLR-1 activity produces a phenotype that is dependent upon the functional integrity of the EGL-15 FGFR. In contrast, complete loss of EGL-15 FGFR activity produces a phenotype that is unaffected by CLR-1 RTP function. Based on these observations, we propose that CLR-1 acts as a negative regulator of an EGL-15-mediated signaling pathway. Thus, the Clr phenotype of *clr-1* mutants may result from the hyperactivity of this FGFR. Consistent with this hypothesis, an EGL-15 construct bearing a mutation known to constitutively activate a variety of receptor tyrosine kinases could confer a Clr phenotype when introduced into an otherwise wild-type background. Taken together with the requirement for the activity of the D1 phosphatase domain of CLR-1, CLR-1-mediated dephosphorylation seems likely to reduce signaling through the EGL-15 pathway.

Stimulation of FGFRs, like other receptor tyrosine kinases, results in the activation and tyrosine phosphorylation of both the receptor itself and additional proteins thought to act as downstream signaling components (for review, see van der Geer and Hunter 1994). Some of these components include phospholipase C-γ (Burgess et al. 1990), Shc (Spivak-Kroizman et al. 1994; Vainikka et al. 1994; Wang et al. 1994), and the p90 phosphoprotein FRS2 (Coughlin et al. 1988; Kouhara et al. 1997). Although these components have not yet been identified in FGF signaling pathways in *C. elegans,* the EGL-15 FGFR contains regions that are conserved among many FGFRs and include some of the tyrosine residues known to be phosphorylated in response to FGF in the human type 1 FGFR (Mohammadi et al. 1991, 1996). Although these residues are possible targets of CLR-1 phosphatase activ-

ity, our genetic data do not establish that CLR-1 dephosphorylates EGL-15 directly. Rather, CLR-1 could act on any molecule activated by tyrosine phosphorylation in response to the stimulation of EGL-15.

The complete suppressibility of the Clr phenotype by mutations in *egl-15* does not necessarily indicate that CLR-1 acts only on a component of the FGFR signaling pathway. It is possible that CLR-1 has roles in other pathways as well, but that its failure to function in these pathways does not result in an obvious phenotypic effect in a wild-type background. In fact, we have observed (Chen et al. 1997; M.J. Stern, unpubl.) that mutations in *clr-1* can partially suppress the effect of mutations that compromise the vulval induction pathway mediated by the epidermal growth factor receptor (EGFR) homolog LET-23 (Aroian et al. 1990). Thus, CLR-1 may partially antagonize signaling mediated by both FGFR and EGFR tyrosine kinases. Such other roles of CLR-1 might not be revealed by *clr-1* mutations in a wild-type background if the other pathways affected by CLR-1 have different primary negative regulatory elements.

Other genes associated with clr-1

RTPs, including CLR-1, have large regions that are not necessary for tyrosine phosphatase catalytic activity and that might play important regulatory roles by mediating interactions with other components. Such components might be identified by mutations that confer a similar Clr phenotype. We have characterized 15 mutants that were isolated on the basis of their Clr phenotypes, and all have mutations in *clr-1.* This result indicates that positively acting factors, if they exist, are not easily mutable to give this specific phenotype. Conversely, components that negatively regulate CLR-1 activity might be identified as *clr-1* suppressors that restore activity to a partially inactivated *clr-1* mutant. We have isolated a large number of *clr-1(e1745ts)* suppressors that define four genes (DeVore et al. 1995). These genes do not appear to encode components that negatively regulate CLR-1, as representative alleles of each of these four genes suppress the putative null mutant *clr-1(e2530)* (M. Kokel and M.J. Stern, unpubl.). The behavior of these mutants is more consistent with their requirement for the EGL-15 signal transduction pathway. The identification of *sem-5* among these suppressors is consistent with this hypothesis.

The discovery of the large class of protein tyrosine phosphatases led to great excitement in part because it was expected that they would play instrumental roles in the regulation of pathways controlled by tyrosine phosphorylation. Many important developmental processes involve FGF signaling pathways whose activation is mediated by early tyrosine phosphorylation events. Thus, the regulation of these pathways is critical to many aspects of development and might be important in understanding the basis of various human diseases. In fact, a number of human skeletal disorders are associated with the hyperactivation of FGF receptors (De Moerlooze and Dickson 1997). The discovery of the CLR-1 RTP that attenuates the activity of an FGF signaling pathway in *C. elegans* provides a mechanism that might help control these important pathways as well.

Materials and methods

Genetic manipulations

C. elegans strains were maintained according to Brenner (1974) and grown at 20°C unless otherwise indicated. All work used strains derived from *C. elegans* var. Bristol, strain N2 (Brenner 1974). Standard genetic manipulations were used for all strain constructions (Herman 1988). *clr-1(e2530); egl-15(n1454)* double mutants were obtained from *clr-1(e2530); egl-15(n1477ts)/egl-15(n1454)* animals. Data for the genetic mapping of *clr-1* are available from the *C. elegans* Genetic Stock Center and ACeDB.

The following *clr-1* mutations were isolated in a variety of clonal screens on the basis of their abilities to confer a Clr phenotype in live animals: *e1745ts, e2530, n1946, n1992, n2151, n2152, n2153, n2158, n2169, ay4, ay67, ay72, q421, gm30,* and *gm79.* In addition to failing to complement *clr-1(e1745ts),* seven of these mutations (*e2530, n1946, n1992, ay72, q421, gm30,* and *gm79*) also showed linkage to markers tightly linked to *clr-1.*

clr-1(e2530) and *clr-1(n1992)* belong to a class of *clr-1* mutations that confer the strongest Clr phenotype observed and behave as null mutations by a number of genetic criteria. *clr-1(n1992)* is associated with an amber nonsense mutation affecting Trp6 of CLR-1 and is suppressible by the amber suppressor *sup-5(e1464sd)* (data not shown). Molecular evidence that *clr-1(e2530)* is a null allele comes from the identification of a 645-bp deletion predicted to disrupt the D1 phosphatase domain and truncate the intracellular domain encoded by this allele (Fig. 4A).

Isolation and characterization of egl-15(n1477ts) suppressors

We isolated suppressors of the Scr body morphology of *egl-15(n1477ts)* mutants by mutagenizing *egl-15(n1477ts)* animals maintained at 20°C with ethyl methanesulfonate (Brenner 1974). L4 hermaphrodites were transferred to separate Petri dishes, three per plate. After the appearance of F_2 progeny, the plates were transferred to 25°C and screened by eye for healthy animals after two to three days. True breeding non-Scr animals could be maintained at 25°C. Autosomal suppressors were separated from the X-linked *egl-15(n1477ts)* mutation by three consecutive backcrosses, and the resulting strains were observed for segregating animals with visible mutant abnormalities. The *clr-1* mutations *n1632, n1634, n1635, n1640–n1644, n1646, n1647, n1649,* and *n1650* were isolated in this screen. Three additional *clr-1* mutations, *n1657, n1660,* and *n1666,* were isolated in a similar screen.

Three suppressors (*n1633, n1639,* and *n1648*) appeared to be X-linked during the backcrossing protocol and are candidates for being intragenic pseudorevertants of *egl-15(n1477ts) X.* First, these mutations are semidominant suppressors of *egl-15(n1477ts).* Second, *n1633,* the most strongly semidominant suppressor, was mapped to the region of *egl-15* by three-factor mapping: in 58 crossover events in the 12 map unit region from *dpy-6* to *unc-9* spanning *egl-15, n1633* was inseparable from *egl-15(n1477ts).* Third, a molecular lesion that corresponds to the *n1633* mutation has been identified in *egl-15* (T692I; D. DeVore and M.J. Stern, unpubl.).

Physical mapping and germ-line transformation

A contig consisting of an overlapping series of yeast artificial chromosomes (YACs) and cosmids assembled by the *C. elegans* genome project consortium was used to clone *clr-1* (Coulson et al. 1988). The position of *clr-1* was estimated based on genetic mapping data and the physical positions of *lin-31, hlh-1, lin-4,* and *dpy-10,* and localized by germ-line transformation assays (Mello et al. 1991) that relied on the ability of *clr-1*-containing DNA fragments to rescue the Clr phenotype of *clr-1(e1745ts).* Strains bearing extrachromosomal arrays formed from cosmids from this region and the cotransformation marker plasmid pRF4 were obtained from R. Predy and J. McGhee. pRF4 confers a Roller (Rol) phenotype to animals bearing this plasmid in extrachromosomal arrays (Mello et al. 1991). An array carrying the cosmid F43G3 was shown to confer a non-Clr phenotype in *clr-1(e1745ts)* homozygotes raised at 25°C. To narrow the region containing *clr-1,* transformation mixes containing DNA fragments from cosmid F43G3 (8 ng/µl) and the cotransformation plasmid pRF4 (100 ng/µl) were injected into *clr-1(e1745ts)* hermaphrodites grown at 15°C. Transformed (Rol) progeny were either shifted to 25°C and scored for rescue of the Clr phenotype (F1 rescuing activity) or maintained at 15°C for a second generation to establish transformed lines. Rescued animals no longer displayed the Clr phenotype when viewed with a dissecting microscope after growth at 25°C. Rescued lines were sufficiently non-Clr to propagate at 25°C. A 7.8-kb *Stu*I–*Spe*I subclone of F43G3 was the smallest fragment that showed efficient rescuing activity in both F1 transformants and stable lines. This fragment was subsequently found to lack the 3′ terminus of the 3′ untranslated region of *clr-1.* For all subsequent analyses, we used derivatives of a 9-kb *Stu*I–*Stu*I genomic fragment containing the entire *clr-1* gene (shown in Fig. 4A) injected at 30 ng/µl unless otherwise noted.

Germ-line transformation rescue of the *clr-1* null mutant *clr-1(n1992)* was performed by injecting *clr-1* constructs to be tested along with pRF4 into *clr-1(n1992)+/+bli-2(e768)* balanced heterozygotes. Rol lines were obtained and checked to see whether Rol *clr-1(n1992)* homozygotes could be established. Using this protocol, for one line of three that contained the *clr-1(n1992)* mutation we could establish a strain that was homozygous for *clr-1(n1992)* and contained the ΔD2 construct. Wild-type animals from the other two lines could also be found that segregated only Clr animals, suggesting that they, too, were homozygous for *clr-1(n1992)* and rescued by the ΔD2 construct.

Constructs of activated *egl-15* [*egl-15(neu*)*] and the kinase-defective derivative [*egl-15(neu*K672A)*] were introduced into wild-type hermaphrodites at 20 ng/µl along with pRF4 as the cotransformation marker (100 ng/µl). F₁ Rol transformants were scored for displaying the Clr and other phenotypes.

Isolation of cDNA clones

Six cDNA clones corresponding to different portions of the 7.8-kb rescuing fragment were isolated from a mixed-stage cDNA library (Barstead and Waterston 1989). Two of these cDNAs contain the SL1 splice-leader sequence found at the 5′ end of many *C. elegans* mRNAs (Krause and Hirsh 1987), suggesting that these cDNAs correspond to the 5′ end of the *clr-1* message. One cDNA that does not overlap with the other five contains a poly-A tail and corresponds to the opposite end of the *clr-1*-rescuing fragment. Four pieces of evidence indicate that the two types of cDNAs represent the two ends of a single *clr-1* message: (1) A PCR product bridging these two classes of cDNAs and representing a spliced message can be amplified from a second cDNA library; (2) a single mRNA of the predicted size can be

detected on a northern blot; (3) the protein sequence predicted by joining these cDNAs as described below encodes a full-length RTP; and (4) a cDNA/genomic chimera containing a fragment of the full-length cDNA at the joint area could rescue *clr-1(e1745ts),* whereas one truncated by alternative splicing downstream of this joint failed to rescue.

A full-length *clr-1* cDNA2 was assembled from these cDNAs and a genomic *Nde*I–*Bsr*FI exon fragment that bridged these cDNAs. Two types of PCR products, corresponding to the alternatively spliced portions of cDNA1 and cDNA2, were obtained from each of two independent cDNA libraries (obtained from R. Barstead and L. Miller) using the primers 5′-CTGCCGTTCCTTCTGTTC-3′ and 5′-GGAAGTTGTCATT-GATTCTC-3′. We assembled cDNA1 by replacing a *Bsr*FI–*Eco*RI fragment in cDNA2 with the analagous fragment from a PCR product corresponding to cDNA1. The predicted amino acid sequence of cDNA1 was compared with published sequences using the program BLASTp (Altschul and Lipman 1990). The GenBank accession numbers for cDNA1 and cDNA2 are AF047880 and AF047881, respectively. The complete genomic sequence of *clr-1* was obtained from the cosmid F56D1 (accession no. U39997) by the *C. elegans* Genome Sequencing Consortium.

Constructs

DNA manipulations were performed using standard procedures (Sambrook et al. 1989). Sequences of regions subjected to mutagenesis, PCR, or extension/deletion of restriction enzyme-generated ends were confirmed by sequence determination. Mutations were identified by sequencing exons encoding the very amino-terminal portion of CLR-1 and the D1 phosphatase domain. Genomic DNA from each *clr-1; egl-15(n1477ts)* strain was used to amplify the *clr-1* coding region by PCR. Sequence was determined from PCR pools and confirmed from independently derived pools.

Genomic-cDNA chimeras (constructs NH#197 and NH#198) were constructed by inserting a 2.3-kb *Bam*HI–*Pst*I cDNA fragment spanning exons 9–16 into the corresponding sites in the 9-kb genomic rescuing fragment.

The C1013S mutation was introduced into a fragment of the full-length *clr-1* cDNA1 using the Clonetech Transformer site-directed mutagenesis kit with the mutagenic primer 5′-CTC-CAGCACTGGAATGTATCAC-3′. The *Bsp*EI–*Pst*I fragment of this portion of the cDNA was introduced into the corresponding sites of the genomic rescuing fragment. The wild-type *Bsp*EI–*Pst*I cDNA fragment did not affect *clr-1*-rescuing activity in this context (data not shown). The *e1745ts* mutation was introduced into the genomic rescuing fragment in a similar manner using the mutagenic primer 5′-CACCATACATGTGTCATTCCG-CAAGTGC-3′.

The Pst FS construct (NH#228) was made by deleting 4 bp at the *Pst*I site of the 9-kb genomic rescuing fragment by first cutting with *Pst*I and then digesting the ends with T4 DNA polymerase.

The *clr-1::GFP* construct was made by adding *Eag*I sites to both ends of GFP(S65C) from pPD95.02 (kindly provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu) using PCR and subcloning into the *Eag*I site of the genomic rescuing fragment.

The ΔD2 construct was made by first introducing an *Xba*I site in the genomic rescuing fragment to replace the codon affected by the *e1745ts* mutation using the Clonetech Transformer site-directed mutagenesis kit with the mutagenic primer 5′-CAC-CATACATTCTAGATTCCGCAAGTGC-3′. An *Xba*I site was also added just 5′ to the endogenous stop codon by PCR using the primer 5′-CCTCTAGATAGCTGTTGTTATATTTTTCC-

3′. Ligation of the *Xba*I ends of these fragments results in a construct encoding the putative *e1745ts* truncated product followed by two amino acids encoded by the inserted *Xba*I site, the endogenous TAG stop codon, and the 3′ UTR of *clr-1*.

The *egl-15(neu*)* construct was made by replacing the transmembrane domain of EGL-15 with that of the oncogenic form of NEU. Unique *Mlu*I and *Kpn*I sites were added upstream and downstream, respectively, of sequences encoding the EGL-15 transmembrane domain using the mutagenic primers 5′-GC-CATACATTTGCTCACGCGTGACGAACCG-3′ and 5′-GTT-TATGGTGTGCAAAGGTACCCTACACAAAAAAGGATTC-3′. *Mlu*I and *Kpn*I sites were added to flank sequences encoding the activated NEU transmembrane domain by using the primers 5′-GATACGCGTGGCTGCCCAGCAGAGCAGAG-3′ and 5′-GGGGTACCTCTCCTTCGTTTGATTAGGATTCCAACGA-CC-3′ to amplify this region from plasmid SRαneu* (kindly provided by C. Burke and D. Stern). The kinase-defective derivative *egl-15(neu*K672A)* was constructed using the mutagenic primer 5′-CATTTTCAATTTTGCGACAGCAACTGCG-3′.

Antibodies, COS cell expression, and phosphatase assay

A fragment encoding the amino-terminal portion of the extracellular domain of CLR-1 (the *Eag*I–*Eco*RI fragment of cDNA1, corresponding to amino acids 23–154) was subcloned into pGEX4T-1 using a short *Bam*HI–*Eag*I linker. The fusion protein was induced with 1 mM IPTG for 4 hr at 30°C, purified by binding to glutathione–agarose beads (Sigma), and used to generate polyclonal anti-CLR-1 antibodies in rabbits (anti-258).

CLR-1 and CLR-1(C1013S) were expressed in COS cells using cDNA1 derivatives in the mammalian expression vector pFLAG-CMV3 (Kodak). NH#318 [CLR-1] and NH#319 [CLR-1(C1013S)] were transfected into COS cells in 10-cm dishes using lipofectamine (GIBCO BRL). Forty-eight hours after transfection, cells were washed once in PBS and lysed for 20 min at 4°C in 1 ml of ice-cold phosphatase lysis buffer (50 mM HEPES at pH 7.4, 150 mM NaCl, 1% Triton X-100 with 5 mM DTT, 1 µg/ml aprotinin, 100 µg/ml PMSF, and 1 µg/ml leupeptin). Protein A-purified αCLR-1 antibodies (anti-258) were used to precipitate CLR-1 from transiently transfected COS cell lysates at 4°C. Immunoprecipitates were washed four times in ice cold phosphatase lysis buffer with 5 mM DTT and two additional times in ice cold phosphatase assay buffer (50 mM HEPES at pH 7.4, 0.1 mg/ml BSA, 2 mM DTT).

Phosphatase assay buffer (120 µl) containing 5 µM Tyr-^{32}P-labeled RCM-lysozyme (Charles et al. 1993) was added to each immunoprecipitate, incubated at 30°C for 20 min, and stopped by addition of 480 µl stop solution (10% suspension of Norit-A charcoal in 0.9 M HCl, 90 mM $Na_4P_2O_7$, 2 mM Na_2HPO_4). Reactions were vortexed briefly, incubated at room temperature for 5 min, and centrifuged for 5 min. Phosphate release was determined by counting 500 µl of supernatant in 2 ml of scintillation fluid. Total possible counts were determined by counting substrate added directly to the scintillation fluid; the negative control reaction was performed in the absence of immunoprecipitated material.

Phosphatase activity of domains expressed in *Escherichia coli*

The region of CLR-1 containing the phosphatase domains was amplified by PCR using the primers 5′-CGCCCGGGTG-CAATTCCAGTTGATGATCCTCC-3′ and 5′-GGCGGCCGT-ATATGTCTTAGAGATATAGTCAGCTAGGG-3′ and cDNA1 or cDNA1(C1013S) as template. These fragments were subcloned between the *Sma*I and *Not*I sites of pGEX4T-1 using

the flanking *Sma*I and *Eag*I sites in the primers. Phosphatase domain fusion proteins were induced with 20 µM IPTG for 2 hr at 30°C. Fusion proteins were purified by binding to glutathione agarose and eluting with 50 mM glutathione in 25 mM Tris at pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 5 mM DTT. Phosphatase assays were performed as described above in a 60 µl volume of assay buffer containing 2 µM Tyr-^{32}P-labeled RCM–lysozyme for the reaction times indicated. The amount of fusion protein assayed was assessed by comparison to a BSA standard.

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