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The *C. elegans* PH Domain Protein CED-12 Regulates Cytoskeletal Reorganization via a Rho/Rac GTPase Signaling Pathway

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Summary

The *C. elegans* gene *ced-12* functions in the engulfment of apoptotic cells and in cell migration, acting in a signaling pathway with *ced-2* CrkII, *ced-5* DOCK180, and *ced-10* Rac GTPase and acting upstream of *ced-10* Rac. *ced-12* encodes a protein with a pleckstrin homology (PH) domain and an SH3 binding motif, both of which are important for *ced-12* function. CED-12 acts in engulfing cells for cell corpse engulfment and interacts physically with CED-5, which contains an SH3 domain. CED-12 has *Drosophila* and human counterparts. Expression of CED-12 and its counterparts in murine Swiss 3T3 fibroblasts induced Rho GTPase-dependent formation of actin filament bundles. We propose that through interactions with membranes and with a CED-2/CED-5 protein complex, CED-12 regulates Rho/Rac GTPase signaling and leads to cytoskeletal reorganization by an evolutionarily conserved mechanism.

Introduction

During programmed cell death, or apoptosis, the process of phagocytosis facilitates tissue remodeling and prevents dying cells from releasing potentially harmful contents, which might trigger inflammatory or autoimmune responses (reviewed by Platt et al., 1998). The mechanisms by which engulfing cells swiftly recognize, ingest, and degrade apoptotic cells are poorly understood.

During the development of the *Caenorhabditis elegans* hermaphrodite, 131 somatic cells and approximately 300 germ cells undergo programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983; Gumienny et al., 1999). In *C. elegans* engulfing cells produce thin cytoplasmic extensions that surround and internalize dying cells (Robertson and Thomson, 1982), a process similar to that observed in mammals (Wyllie et al., 1980). However, in *C. elegans* dying cells are engulfed by neighboring cells (Sulston and Horvitz, 1977; Robertson and Thomson, 1982; Sulston et al., 1983) rather than by mobile phagocytes, such as mammalian macrophages (Platt et al., 1998).

Six *C. elegans* genes, *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10*, were identified in screens for mutants containing unengulfed cell corpses, which are distinguishable using Nomarski microscopy as highly refractile disks (Hedgecock et al., 1983; Ellis et al., 1991). Genetic and molecular studies revealed that these six genes act in two distinct and partially redundant signal transduction pathways to control cell corpse engulfment. The CED-1, CED-6, and CED-7 proteins are components of a signaling pathway essential for cell corpse recognition. CED-1 is similar to the human scavenger receptor SREC and acts as a phagocytic receptor that recognizes and clusters around cell corpses (Zhou et al., 2001). CED-6 is similar to mammalian adaptor proteins that contain phosphotyrosine binding (PTB) domains and acts downstream of *ced-1* within engulfing cells (Liu and Hengartner, 1998; Zhou et al., 2001). CED-7 is similar to ABC (ATP binding cassette) transporters (Wu and Horvitz, 1998a) and promotes cell corpse recognition by CED-1, possibly by exposing a phospholipid ligand on the surfaces of cell corpses (Zhou et al., 2001).

The CED-2, CED-5, and CED-10 proteins are conserved components of a Rac GTPase signaling pathway. CED-2 is similar to the mammalian adaptor protein CrkII, an SH2 and SH3 domain-containing protein implicated in transmembrane receptor-mediated signaling pathways (Reddien and Horvitz, 2000). CED-5 is similar to human DOCK180, a Crk-interacting protein, and to *Drosophila* Mbc (myoblast city) (Wu and Horvitz, 1998b). CED-10 is a *C. elegans* homolog of mammalian Rac (Reddien and Horvitz, 2000). The Rho/Rac family of small GTPases regulates the actin cytoskeleton and acts in dynamic processes such as phagocytosis, cell motility, axonal guidance, and cytokinesis (Hall and Nobes, 2000). Mutations in *ced-2*, *ced-5*, and *ced-10* but not in *ced-1*, *ced-6*, or *ced-7* result not only in defects in cell corpse engulfment but also in defects in cell migration (Wu and Horvitz, 1998b; Reddien and Horvitz, 2000). CED-2, CED-5, and CED-10 form a signaling pathway that controls polarized cell extension and CED-2 and CED-5 act upstream of CED-10 in this pathway (Reddien and Horvitz, 2000). Similarly, mammalian CrkII, DOCK180, and Rac1 act in a conserved signaling pathway mediating the phagocytosis of apoptotic cells (Albert et al., 2000).

Here we report the identification and molecular characterization of a new gene, *ced-12*, that encodes a component of the CED-10 Rac pathway that leads to cytoskeletal reorganization.

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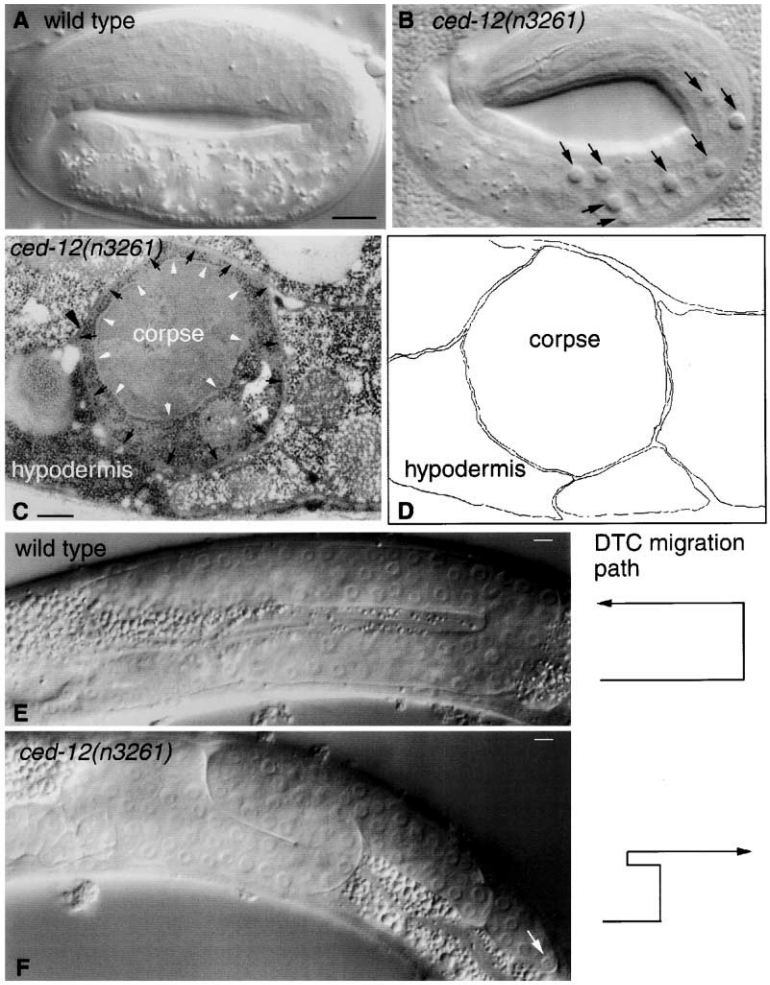

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Genotype	No. persistent cell corpses			% Abnormal DTC migration	
	L1 head	L3 PVC	Adult gonad	Length	Morphology
wild type	0.0 ± 0.0	0.0 ± 0.0	3.9 ± 1.6	0%	3.0%
ced-12(n3261)	23.9 ± 3.5	4.9 ± 1.1	15.5 ± 4.6	5.5%	50.0%

Figure 1. *ced-12* Mutant Animals Are Defective in Cell Corpse Engulfment and Distal Tip Cell Migration

(A and B) Nomarski images of embryos before hatching. Arrows indicate persistent cell corpses. Scale bar: 5 μm.

(C and D) Transmission electron micrograph of an unengulfed cell corpse in the posterior ventral cord of an L3 stage *ced-12(n3261)* larva. Black arrows indicate the plasma membrane of the corpse. White arrowheads indicate the nuclear envelope. The hypodermis, which normally engulfs this corpse, is labeled. A black arrowhead indicates a partially extended pseudopod around the corpse. The membranes of the corpse and its neighboring cells are traced and shown in (D).

(E and F) Nomarski images of one gonadal arm in wild-type or *ced-12(n3261)* L4 larvae, respectively, indicating the migration paths of the distal tip cells (DTC), as diagrammed to the right. Ventral, bottom; anterior, left. Scale bar: 5 μm. In (E) the DTC is out of focus; in (F) the DTC is marked by an arrow.

(G) Quantitation of *ced-12(n3261)* mutant defects in engulfment and DTC migration. Numbers of persistent cell corpses were scored in 15 animals for each category as described in Experimental Procedures. Data are presented as mean ± standard deviation. PVC: posterior ventral cord. Defects in DTC migration patterns were scored as percent of gonadal arms with abnormal length or morphology; the latter aspect includes abnormal turns, twists, or positions. At least 100 gonadal arms were scored for each genotype.

Results

ced-12 Is Required for Cell Corpse Engulfment

We identified the mutation *n3261* in a genetic screen for mutants defective in cell corpse engulfment (Z.Z. and H.R.H., unpublished data). This mutation caused the persistence of cell corpses in late-stage embryos (Figures 1A, 1B, and 1G). In wild-type animals, 93 cells undergo programmed cell death in the head, all during embryogenesis (Sulston et al., 1983). In the posterior ventral nerve cord, seven cells undergo programmed cell death during the late L1 and early L2 stages of larval development (Sulston and Horvitz, 1977). In *n3261* mutant larvae, cell corpses persisted in both the head and the posterior ventral cord (Figure 1G). We also observed persistent cell corpses in the gonads of adult hermaphrodites, in which a large number of germ cells undergo programmed cell death (Gumienny et al., 1999) (Figure 1G). Thus, the gene defined by *n3261* is broadly required for the efficient removal of cell corpses.

We used transmission electron microscopy (EM) to examine whether the persistent cell corpses seen in *n3261* mutant animals reflected a defect in corpse engulfment or in corpse degradation within the engulfing cells. We analyzed cell corpses produced by the ventral cord precursor cells P9 through P12 at the L3 stage, at which point these corpses had persisted for at least 12 hr (Sulston and Horvitz, 1977). Persistent corpses in *n3261* mutants displayed the morphology typical of cells undergoing programmed cell death: each appeared electron dense with a condensed cytoplasm and a dilated nucleus (Figures 1C and 1D), as seen in both wild-type (Robertson and Thomson, 1982) and engulfment-defective animals (Ellis et al., 1991). Of the 10 ventral cord corpses we analyzed by EM, nine were not engulfed, and one had been engulfed by the hypodermal

syncytium (hypodermis), which is normally responsible for engulfing these corpses (Robertson and Thomson, 1982). All nine unengulfed corpses were in close contact with the hypodermis, which partially extended pseudopodia around the corpses (Figures 1C and 1D). By contrast, in wild-type animals, dying cells are found within engulfing cells (Robertson and Thomson, 1982). We conclude that *n3261* mutant animals are defective in the engulfment rather than the degradation of cell corpses and have severely reduced, but not abolished, engulfment activity. This defect is similar to that observed in mutants of all six previously described engulfment genes (Hedgecock et al., 1983; Ellis et al., 1991; Zhou et al., 2001).

The *n3261* mutation had a recessive effect on engulfment and was complemented by *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10* mutations (data not shown). *n3261* thus defined a new gene required for cell corpse engulfment. During the course of our studies, another group identified a mutation, *bz187*, that prevented the engulfment of cells undergoing necrotic cell death; this group noted that *bz187* also affected the engulfment of cells undergoing programmed cell death (Chung et al., 2000). We found that *n3261* failed to complement *bz187* (data not shown). The gene affected by both mutations is named *ced-12*.

ced-12, like *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10* (Ellis et al., 1991), displayed a maternal-effect rescue of the engulfment of somatic cell corpses: homozygous *ced-12(n3261)* progeny (n = 20) of a *ced-12(n3261)* mother all had unengulfed somatic cell corpses, but homozygous *ced-12(n3261)* progeny (n = 20) of a *ced-12(n3261)/+* mother appeared wild-type. However, homozygous *ced-12(n3261)* progeny (n = 20) of a *ced-12(n3261)/+* mother had persistent germ-cell corpses in the adult hermaphrodite gonad. These observations suggest that maternal wild-type *ced-12* gene product is sufficient to allow the engulfment of cell corpses generated during embryogenesis and larval development but does not persist to adulthood.

ced-12 Affects Pathfinding of the Distal Tip Cells

The two gonadal distal tip cells (DTCs) are located at the two ends of the hermaphrodite gonad primordium. During larval development, each DTC migrates in a U-shaped pattern along the basement membrane of the body wall, and together the two DTCs direct the extension of the growing bilobed gonad. The migration paths of the DTCs are reflected by the shapes of the extended gonadal arms (Figure 1E) (Kimble and Hirsh, 1979).

ced-12(n3261) animals were abnormal in the migration pattern of the DTCs (Figures 1E–1G), and this abnormality cosegregated with the engulfment defect (data not shown). The most common abnormality (46.4% of all arms) was a premature dorsal turn of the DTC, followed by an extra turn along the dorsal musculature, causing the distal gonad arm to extend away from, instead of toward, the midbody (Figure 1F). Mutations altering DTC migration patterns in a variety of ways have been identified in *C. elegans* (Su et al., 2000, and references therein; Reddien and Horvitz, 2000). The abnormalities displayed by *ced-12* mutant animals are similar specifically to the defects observed in *ced-2*, *ced-5*, or *ced-10* mutants (Reddien and Horvitz, 2000), suggesting that *ced-12* may act in the genetic pathway defined by *ced-2*, *ced-5*, and *ced-10* for the control of DTC pathfinding. The DTC migration defect of *ced-12(n3261)* animals was not rescued by maternal gene product.

Table 1. *ced-12* Acts in the *ced-2*, *ced-5*, *ced-10* Pathway

Genotype	No. Persistent Corpses in L1 Heads
Wild-type	0.0 ± 0.0
ced-1(e1735)	27.4 ± 2.6
ced-6(n1813)	23.7 ± 4.4
ced-7(n1996)	28.6 ± 2.7
ced-2(e1752)[1]	20.3 ± 3.0
ced-5(n1812)[2]	33.3 ± 2.2
ced-10(n1993)[3]	19.8 ± 2.4
ced-12(n3261)[4]	23.0 ± 3.1
ced-1(e1735) ced-12(n3261)	47.6 ± 2.4
ced-6(n1813); ced-12(n3261)[5]	39.9 ± 3.7
ced-7(n1996); ced-12(n3261)[5]	44.8 ± 3.2
ced-1(e1735); ced-5(n1812)[a]	43.7 ± 3.0
ced-2(e1752) ced-5(n1812)[6a]	30.0 ± 3.8
ced-5(n1812) ced-10(n1993)[a]	30.6 ± 4.1
ced-5(n1812); ced-12(n3261)[7]	29.6 ± 1.7
ced-2(e1752); ced-12(n3261)[8]	30.6 ± 3.9
ced-10(n1993); ced-12(n3261)[9]	32.4 ± 3.2
ced-2(e1752) ced-10(n1993)[9b]	32.7 ± 2.6
ced-2(e1752) ced-10(n1993); ced-12(n3261)[9]	33.2 ± 3.1

For each strain, 15 animals were scored. Data are presented as mean ± standard deviation.

[a] These double mutants were constructed by Ellis et al. (1991).

[b] This double mutant was constructed by P. Reddien (personal communication).

Additional mutations in the backgrounds: [1] *dpy-9(e12)*, [2] *dpy-20(e1282)*, [3] *dpy-13(e184)*, [4] *sem-4(n1378)*, [5] *sem-4(n1378)*; *dpy-17(e164)*, [6] *dpy-9(e12) unc-30(e191)*, [7] *sem-4(n1378)*; *dpy-20(e1282)*, [8] *sem-4(n1378)*; *dpy-9(e12)*, [9] *sem-4(n1378)*; *dpy-13(e184)*.

ced-12 Acts in a Genetic Pathway with *ced-2*, *ced-5*, and *ced-10* for Cell Corpse Engulfment

The six previously identified engulfment genes appear to define two pathways based on genetic interactions: a *ced-1*, *ced-6*, and *ced-7* pathway and a *ced-2*, *ced-5*, and *ced-10* pathway (Ellis et al., 1991). In general, a mutation in an engulfment gene does not enhance the phenotype caused by a null mutation in a gene in the same pathway but does enhance the phenotype caused by a null mutation in a gene in the other pathway. We analyzed the genetic interactions between *ced-12* and each of the six previously known engulfment genes.

The *ced-12(n3261)* mutation, like a *ced-5(n1812)* null mutation (Wu and Horvitz, 1998b), strongly enhanced the engulfment defects caused by null mutations in *ced-1* and *ced-7* and a strong loss-of-function mutation in *ced-6* (Table 1) (Liu and Hengartner, 1998; Wu and Horvitz, 1998a; Zhou et al., 2001). *ced-12(n3261),* like mutations in *ced-2* and *ced-10*, did not enhance the engulfment defect caused by a *ced-5* null mutation. We conclude that *ced-12* acts in the same genetic pathway as *ced-5* and in parallel to the pathway defined by *ced-1*, *ced-6*, and *ced-7*.

The severity of the engulfment defect in *ced-12; ced-2* and *ced-12*; *ced-10* double mutants was similar to that of *ced-5* null mutants (Table 1). However, both of these

ced-12 double mutants had a more severe defect than *ced-2*, *ced-10*, or *ced-12* single mutants (Table 1). This enhancement suggests two possibilities. First, *ced-12* could act in a branched pathway partially redundant with *ced-2* and *ced-10*. Alternatively, *ced-12* could act in a linear pathway with *ced-2* and *ced-10*, with combinations of partial loss-of-function mutations causing the additive effects. The *ced-2* and *ced-10* alleles we analyzed are likely only partial loss-of-function alleles (Reddien and Horvitz, 2000). To distinguish between these two possibilities, we compared the engulfment defects of *ced-2 ced-10* double mutants and *ced-12; ced-2 ced-10* triple mutants. In the first case, we would anticipate a greater engulfment defect to be displayed by the triple mutant. However, the two strains displayed defects of equal severity ($p = 0.6$, unpaired t test), about that of *ced-5* null mutants (Table 1; $p > 0.4$ for both comparisons, unpaired t test). We conclude that *ced-12* is likely to act in a linear pathway with *ced-2* and *ced-10*.

Overexpression of *ced-10* Rescued the Engulfment and DTC Migration Defects of *ced-12* Mutants

To test whether *ced-12* defects, like the defects of the *ced-2* or *ced-5* mutants, can be rescued by *ced-10* overexpression, we first used P_{ced-1}, the promoter for the cell corpse receptor CED-1, which is specifically expressed in cell types that act as engulfing cells (Zhou et al., 2001), to drive the expression of *ced-10* in transgenic arrays. P_{ced-1} *ced-10* partially rescued the engulfment defect of *ced-12(n3261)* animals (Figure 2A). We also assayed a P_{ced-1} *ced-10(G12V)* construct, which encoded a glycine-12-to-valine mutation in CED-10. An identical mutation caused constitutive activation of human Rac1 (Ridley et al., 1992). This construct rescued the *ced-12* engulfment defect to the same level as did P_{ced-1} *ced-10* (Figure 2A). We then found that P_{hsp} *ced-10* (Reddien and Horvitz, 2000), which expresses *ced-10* under the *C. elegans* P_{hsp} promoters (activated in somatic cells by heat shock; Stringham et al., 1992), rescued the engulfment and DTC migration defects of *ced-12(n3261)* mutants in a heat shock-dependent manner (Figures 2B and 2C and data not shown). These results indicate that a high level of the CED-10 protein can bypass the requirement for CED-12 function in the regulation of both engulfment and DTC migration.

Cloning of *ced-12*

We mapped *ced-12* to chromosome I, between *fog-3* and *lin-11* (see Supplemental Data [http://www.developmentalcell.com/cgi/content/full/1/4/477/DC1]). We then determined that *ced-12* corresponds to the gene encoding the predicted open reading frame Y106G6E.5 (see Supplemental Data). For example, we identified in *ced-12(n3261)* animals a C-to-T transition mutation predicted to result in the premature termination of Y106G6E.5 (Figure 3A) and found that expression of a Y106G6E.5 cDNA under the control of *C. elegans* promoters P_{hsp} or P_{ced-1} (see Experimental Procedures) fully rescued the engulfment defect of *ced-12(n3261)* animals (Figures 2B and 2D–2F). The P_{hsp} or P_{ced-1} *ced-12* expression constructs failed to rescue the engulfment defects of *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, or *ced-10* mutants (Z.Z. and H.R.H., unpublished data).

We determined the gene structure of *ced-12* by comparing Y106G6E genomic DNA sequence with the sequence of a full-length *ced-12* cDNA (see Supplemental Data). *ced-12* consists of eight exons and is likely a downstream gene in an operon that also encodes Y106G6E.6 (Figure 3A).

ced-12 Encodes an Evolutionarily Conserved Protein Containing a Putative PH Domain and a Proline-Rich Motif

ced-12 encodes a protein of 731 amino acids most similar to CG5336, a *Drosophila* protein predicted from the genomic sequence of chromosome 2L (GenBank accession: AAF53164); to bA394O2.2, a human protein predicted from the genomic sequence of chromosome 20 (GenBank accession: CAC00660); and to KIAA0281 and BAB14712.1, two human proteins encoded by cDNAs identified from testis and thyroid gland tissue, respectively (GenBank accession: CAB66721 and BAB14712, respectively) (Figure 3B). The C-terminal half of CED-12 (residues 365–731) is 22%, 22%, 21%, and 21% identical and 44%, 42%, 43%, and 43% similar to CG5336, KIAA0281, bA394O2.2, and BAB14712.1, respectively. We cloned CG5336 and bA394O2.2 cDNAs (see Experimental Procedures) and named them *Dced-12* and *Hced-12A*, respectively.

A pleckstrin-homology (PH) domain in the C-terminal regions of DCED-12, KIAA0281, HCED-12A, and BAB14712 was predicted by SMART, a computational domain prediction program (Schultz et al., 2000) (Figures 3B and 3C). In addition, a PSI-Blast search (Altschul et al., 1997) showed that the predicted PH domains of these proteins are similar to the PH domains of rat phospholipase C δ1 (PLC δ1) (Suh et al., 1988), human PLC ϵ; (GenBank accession: XP002720) and mouse PLC-L2 (Otsuki et al., 1999). The PH domain is a region of about 100 amino acids found in many proteins involved in signal transduction or cytoskeletal reorganization and is believed to target proteins to membranes via its binding to membrane phosphoinositides or its interaction with other proteins (reviewed by Shaw, 1996). PH domains are very conserved in secondary and tertiary structures: they all contain seven β strands connected by highly variable loops, which form two nearly orthogonal antiparallel β sheets; these β strands are followed by an amphipathic C-terminal α helix, which closes off one corner of this β sandwich (reviewed by Lemmon and Ferguson, 2000).

We aligned the predicted PH domains in CED-12-related proteins and the corresponding region in CED-12 to the PH domain of rat PLC δ1, the secondary structure of which was solved by X-ray crystallography (Ferguson et al., 1995) and is indicated in Figure 3C. CED-12 and its related proteins have relatively low sequence similarity to rat PLC δ1 over the entire predicted PH domain region; however, higher similarity occurs within the regions in which the seven β strands and the C-terminal α helix should form (Figures 3C and 3D), suggesting that the secondary structure elements critical for the formation of the tertiary structure core are conserved. In addition, although multiple insertions were found in

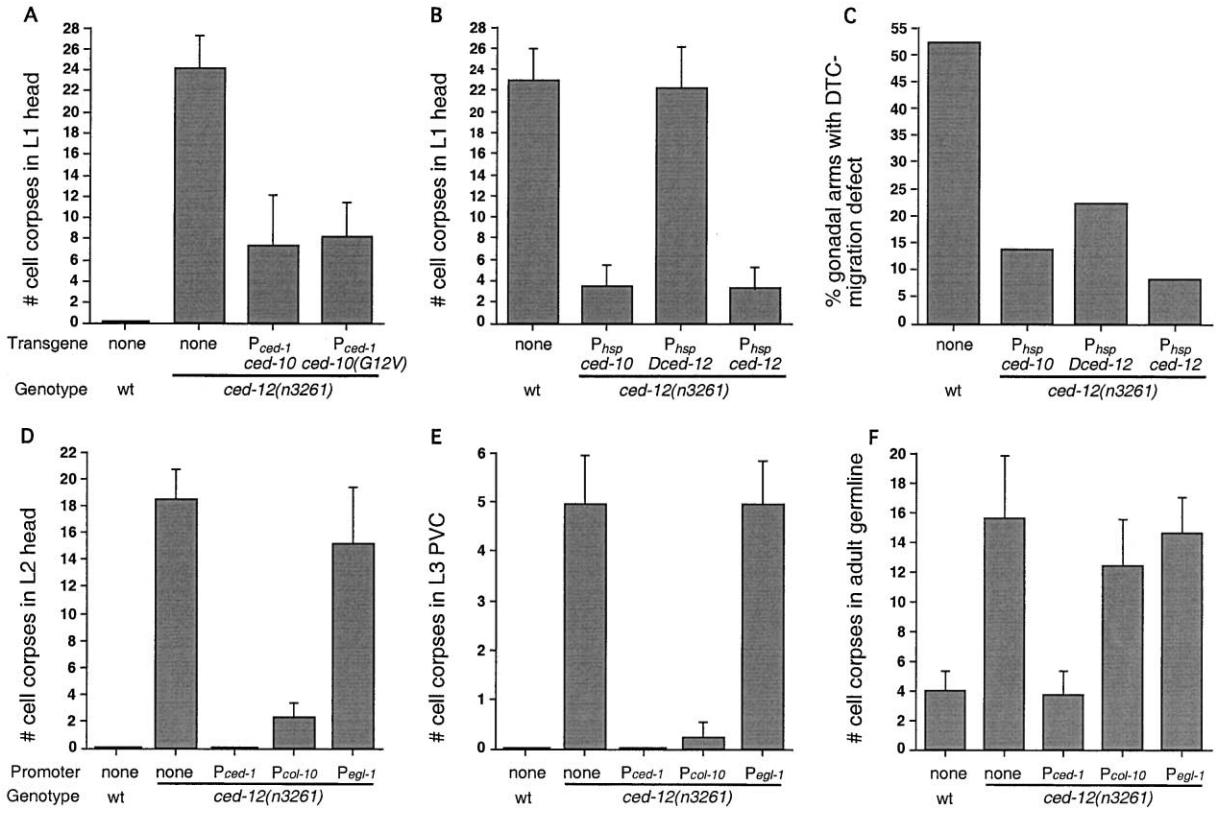


Figure 2. Rescue of *ced-12* Mutant Defects by Transgene Constructs

For each transgene, two independent lines were scored and similar results were obtained. The results of one line are presented. Data are presented as mean ± standard deviation of persistent cell corpses (A and B, D–F) or as percent of gonadal arms defective in DTC migration (C). (A and B, D–F) At least 15 animals were scored for each data point.

(A) Expression of *ced-10* or *ced-10(G12V)* in engulfing cells rescues the engulfment defect of *ced-12* mutants.

(B) Heat shock-induced expression of *ced-10* or *ced-12* but not *Dced-12* rescues the engulfment defect of *ced-12* mutants. A mixed population of worms was incubated for 1.5 hr at 33°C and then allowed to recover for 8 hr at 20°C. Young L1 larvae were scored.

(C) Heat shock-induced expression of *ced-10*, *Dced-12*, or *ced-12* rescues or partially rescues the DTC migration defect of *ced-12* mutants. L2 larvae were collected and subjected to three 1.5 hr heat shocks at 33°C, separated by three 10.5 hr periods of recovery at 20°C. After this 36 hr period, animals were adults or late-stage L4 larvae. Defective DTC pathfinding was scored as gonadal arms with abnormal migration patterns. At least 75 gonadal arms were scored for each line.

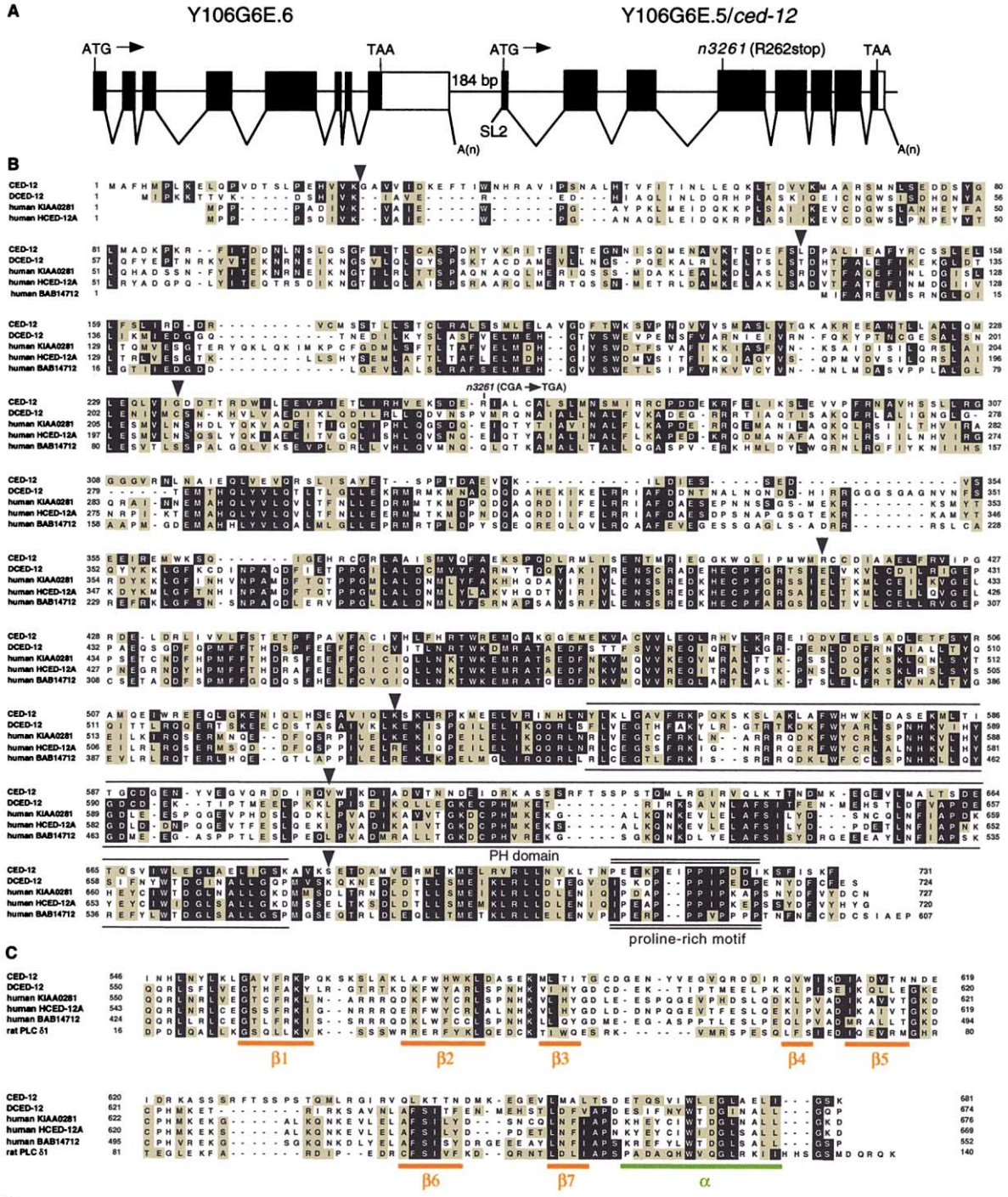
(D–F) Expression of *ced-12* in engulfing but not dying cells rescued the engulfment defect of *ced-12* mutants. Promoter: the promoter that drove *ced-12* expression; PVC: posterior ventral cord.

the variable loops between the β strands, no insertions or deletions were found within the β strands or the C-terminal α helix, further suggesting that the secondary structure elements that form the PH domain are intact.

Although PH domains display a low level of sequence similarity to each other, conserved sequence features do exist (Shaw, 1996). The N-terminal region containing the seven β strands and the variable loops is rich in large hydrophobic, turn-promoting, and charged residues and has a few well-conserved residues, such as G25 and F97 in rat PLC δ1 (Shaw, 1996). The C-terminal α helix exhibits negative charges, contains a nearly invariant tryptophan residue (W120 in rat PLC δ1), and the fourth residue C-terminal of this tryptophan is an invarible large hydrophobic residue (Shaw, 1996). The sequences of CED-12 and its related proteins fit these primary sequence features; in particular, in all but one case, the conserved glycine, phenylalanine, and tryptophan residues are present in these proteins (Figure 3C). These features suggest that the CED-12 family of proteins contains sequences characteristic of a PH domain.

Near the C termini of CED-12 (residues 711–724) and its related proteins, there is a proline-rich region bearing a core consensus sequence identical to the consensus sequence for SH3 (Src-homology-3) domain binding sites, X-P-P-X-P (P: proline, X: aliphatic residue) (reviewed by Pawson, 1995) (Figure 3B). SH3 domains are present in many signal transduction proteins and cytoskeletal components (Pawson, 1995). The sequence similarities among CED-12 and its related proteins suggest that these five proteins may define a new protein family conserved in structure and possibly in function.

The *n3261* mutation is a C-to-T transition that changes arginine codon 262 to a TGA stop codon (Figure 3B), presumably generating a truncated protein missing two-thirds of the CED-12 polypeptide. *n3261/qDf5* and *n3261/qDf7* heterozygous animals displayed an engulfment defect similar to that of *n3261* homozygous animals (see



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protein	% similarity to the PH domain of rat PLC δ1	
	entire region	β strands and α helix
CED-12	34	49
DCED-12	44	60
human KIAA0281	43	59
human HCED-12A	43	60
human BAB14712	39	55

Figure 3. CED-12 Gene Structure and Protein Sequence Alignments

(A) Gene structures of *ced-12* and Y106G6E.6. Solid boxes indicate coding sequences, open boxes indicate noncoding sequences in the cDNAs, and lines between the solid boxes represent introns.

(B) Sequence alignment of CED-12 family members. Numbers indicate amino acid positions. Residues identical between CED-12 and other proteins or identical among all four CED-12-related proteins are shaded in black. Residues similar between CED-12 and other proteins or similar among all four CED-12-related proteins are shaded in gray. Arrowheads indicate the positions of introns. The predicted PH domain and proline-rich motif are indicated by single and double lines on top and bottom, respectively.

Supplemental Data [http://www.developmentalcell.com/cgi/content/full/1/4/477/DC1]), consistent with the prediction that *n3261* causes a severe loss of *ced-12* function.

CED-12 Functions in Engulfing Cells

To explore whether *ced-12* acts in the engulfing or dying cells or both for cell corpse engulfment, we used three cell-type-specific promoters, P_{ced-1}, P_{col-10}, and P_{egl-1}, to drive the expression of a *ced-12* cDNA. P_{ced-1} is active in cell types that act as engulfing cells for both somatic and germline cell corpses but not in dying cells (Zhou et al., 2001). P_{col-10}, the promoter for the collagen gene *col-10* is specific for hypodermal cells, which are the major engulfing cells for somatic cell corpses but not for germ-cell corpses (Sulston et al., 1983; Zhou et al., 2001). P_{egl-1}, which includes the promoter and downstream regulatory sequences of the cell-death activator *egl-1* (Conradt and Horvitz, 1999), is active predominantly in somatic dying cells (B. Conradt and H.R.H., unpublished data). We fused the gene for the green fluorescent protein (*gfp*) (Chalfie et al., 1994) in-frame to the C terminus of CED-12 to generate P_{ced-1} *ced-12::gfp*. Expression of P_{ced-1} *ced-12::gfp* fully rescued the defects of *ced-12(n3261)* mutants in the engulfment of somatic and germ cell corpses (Figures 2D–2F). Expression of *ced-12* specifically in hypodermal cells (P_{col-10} *ced-12*) resulted in the complete engulfment of somatic cell corpses in the posterior ventral cord and efficient engulfment in the head but no significant engulfment of germ-cell corpses (Figures 2D–2F). These results suggest that *ced-12* acts in engulfing cells. By contrast, we observed no significant rescue of the engulfment defects of either somatic or germ-cell corpses following P_{egl-1} *ced-12* expression (Figures 2D–2F), suggesting that *ced-12* expression in dying cells is not sufficient for engulfment.

The CED-12::GFP fusion protein expressed from P_{ced-1} was detected in engulfing cell types, including hypodermal, intestinal (Figures 4A and 4B), pharyngeal, and gonadal sheath cells (data not shown). CED-12::GFP was excluded from the nuclei in all cells in which it was expressed and was predominantly localized to the cytoplasm in a punctate pattern (Figures 4A and 4B). Since CED-12 lacks transmembrane domains or signal sequences that target protein to intracellular organelles, the punctate cytoplasmic localization and the presence of a PH domain together suggest that some CED-12::GFP molecules associate with intracellular membranes.

The Predicted PH Domain and Proline-Rich Motif Are Important for CED-12 Function

We expressed truncated CED-12 forms that eliminated regions including the predicted PH domain and proline-rich motif under the control of P_{col-10} and assayed for their abilities to rescue the engulfment defect of *ced-12* mutants. Deleting the C-terminal seven residues (aa 725–731) [P_{col-10} *ced-12(Δ7)*] prior to the proline-rich motif did not affect the rescuing ability of CED-12 significantly (Figure 4D). However, a further deletion including the proline-rich motif [P_{col-10} *ced-12(ΔProΔ7)*] (deleting aa 710–731) caused an approximately 40% loss of CED-12 rescuing activity, and a larger deletion including both the PH domain and the proline-rich motif (eliminating aa 551–731) [P_{col-10} *ced-12(ΔPHΔProΔ7)*] caused a nearly complete loss of CED-12 rescuing activity (Figure 4D). In addition, an internal deletion of the putative PH domain (aa 551–681) [P_{col-10} *ced-12(ΔPH)*] resulted in a loss of 67% of rescuing activity (Figure 4D). These results indicate that both the putative PH domain and the proline-rich motif are important for the engulfment function of *ced-12*.

The subcellular localization pattern of CED-12(ΔProΔ7)::GFP expressed from P_{ced-1} was similar to that of CED-12::GFP (data not shown). However, a CED-12(ΔPHΔProΔ7)::GFP fusion protein expressed from P_{ced-1} was not excluded from nuclei and failed to maintain a punctate staining pattern in the cytoplasm (Figure 4C). Instead, this protein was distributed evenly throughout the whole cell, indicating that the deleted region is required for punctate cytoplasmic localization and suggesting that the putative PH domain mediates an association with intracellular membranes.

CED-12 Interacts with CED-5

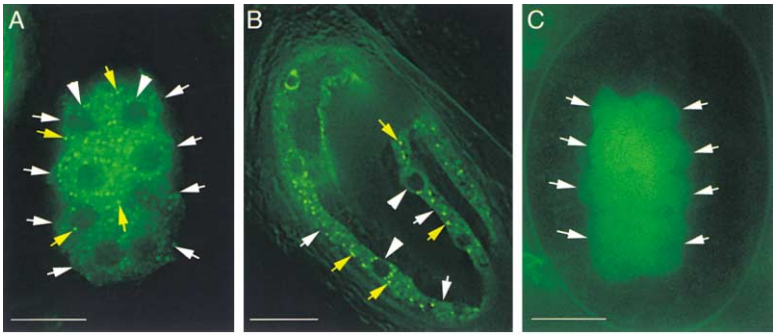
We used the yeast two-hybrid system based on the DNA binding (DB) and transactivation (AD) domains of the yeast GAL4 protein (Bai and Elledge, 1997) to assay pairwise interactions between DB-CED-12 and AD fusions of CED-1 (cytoplasmic domain), CED-2, CED-5, CED-6, and CED-10 (Experimental Procedures), and found that only the DB-CED-12/AD-CED-5 combination allowed activation of reporter gene expression and yeast cell growth on selection medium (Figure 5A and data not shown), indicating that CED-12 and CED-5 can specifically interact in yeast.

We immobilized a bacterially expressed GST-CED-12 fusion protein on glutathione-sepharose beads and observed that it interacted with in vitro translated ^{35}S-methionine-labeled full-length CED-5 protein but not with luciferase (Figure 5B). In a control experiment, ^{35}S-methionine-labeled CED-5 did not interact with an excessive amount of GST protein (Figures 5B and 5C). We conclude that CED-12 and CED-5 can interact specifically in vitro as well as in yeast.

CED-12 and Its *Drosophila* and Human Homologs Induced Remodeling of the Actin Cytoskeleton in Swiss 3T3 Fibroblasts

We investigated whether CED-12 and related proteins could induce cytoskeletal reorganization in murine Swiss 3T3 fibroblasts. Serum starvation of quiescent Swiss 3T3 cells causes the loss of almost all recognizable actin structures, including actin stress fibers, lamellipodia,

(C) Alignment of the PH domains predicted for the CED-12 family members to that of rat PLC δ1. Numbers indicate amino acid positions. Residues identical or similar to PLC δ1 are shaded in black or gray, respectively. The β strands (β1–β7) and the C-terminal α helix identified in PLC δ1 by X-ray crystallography, which are defining features of the PH domain, are underlined in red and green, respectively.
(D) Percentage of similarity of each predicted PH domain of CED-12 family of proteins to the PH domain of rat PLC δ1. Data are based on (C).



D

Genotype	Transgene (P_{col-10})	# corpses in L2 heads
wild type	none	0.0 ± 0.0
ced-12	none	18.3 ± 2.6
ced-12	*ced-12*	2.1 ± 1.6
ced-12	*ced-12(Δ7)*	2.7 ± 1.2
ced-12	*ced-12(ΔProΔ7)*	6.9 ± 2.2
ced-12	*ced-12(ΔPHΔProΔ7)*	17.0 ± 2.4
ced-12	*ced-12(ΔPH)*	10.8 ± 2.0

Figure 4. The PH Domain and the Proline-Rich Motif Are Important for CED-12 Function

(A–C) The GFP signal produced from P_{ced-1} *ced-12::gfp* (A and B) or from P_{ced-1} *ced-12(ΔPHΔProΔ7)::gfp* (C) in *ced-12(n3261)* embryos were visualized by epifluorescence. Yellow arrows indicate puncta with higher GFP intensity. White arrowheads indicate nuclei. Anterior is to the top. Scale bar: 10 μm.

(A) A 200–250 cell stage embryo. White arrows indicate the cell boundary of eight intestinal precursor cells.

(B) An embryo before hatching. White arrows indicate the cell boundary of the fused hypodermis.

(C) A 200–250 cell stage embryo. White arrows indicate the cell boundary of eight intestinal precursor cells.

(D) Transgenes expressing truncated forms of CED-12 under the control of the *col-10* promoter (P_{col-10}) were assayed for rescue of the engulfment defect of *ced-12(n3261)* mutants. For each transgene, at least two independent lines were scored, and similar results were obtained. The results of one line are presented. Data are presented as mean ± standard deviation. Fifteen animals were scored for each data point.

and membrane ruffles (Ridley and Hall, 1992; Ridley et al., 1992). This cell system has proved useful in investigating signaling (e.g., small GTP binding proteins) and effector molecules in the induction and regulation of actin-based cytoskeletal structures (Ridley and Hall, 1992; Ridley et al., 1992; Caron and Hall, 1998).

We injected constructs expressing the CED-12, DCED-12, and HCED-12A proteins with an N-terminal HA epitope tag and human KIAA0281 (cloned and named as ELMO1 and kindly provided by K. Ravichandran) with a C-terminal FLAG tag into the nuclei of subconfluent, quiescent serum-starved 3T3 cells. Strikingly, the expression of these proteins, which was confirmed by immunostaining using antibodies against the tagged epitopes (Figures 6E–6G and data not shown), all induced assembly of actin filaments, detectable by phalloidin staining (Figures 6B–6D and 6K). This effect was absent from control dextran-injected cells, which showed very few if any organized actin structures (Figure 6A). The actin filament assembly induced by these proteins resembled actin bundling and stress fibers induced by expression of an active form of Rho (Ridley et al., 1992). Interestingly, this assembly was distinct from that induced by active human Rac1 or CED-10 [Rac1G12V or CED-10(G12V), respectively], both of which caused the formation of membrane ruffles in over 75% of the expressing cells (Figures 6D, 6J, and 6K). A low-level actin bundling was induced by Rac1G12V (Figures 6J and 6L) as a result of activation of endogenous Rho, as previously reported (Ridley et al., 1992).

Is CED-12-induced formation of filamentous actin structures dependent on endogenous Rho family GTPase(s)? C3 transferase, a specific inhibitor of Rho (Ridley and Hall, 1992), abolished the induction of actin filament assembly by CED-12 (Figures 6H and 6L). By contrast, coexpression with HA-CED-12 of a dominant-negative form of Rac1 (Rac1S17N, Ridley et al., 1992) did not affect the ability of CED-12 to induce actin filament assembly (Figures 6I and 6L). These results suggest that CED-12 expression promoted the reorganization of the actin cytoskeleton in Swiss 3T3 fibroblasts in a manner dependent on endogenous Rho but not Rac1.

Drosophila ced-12 Rescued the *C. elegans ced-12* DTC Migration Defect

Heat shock-induced expression of *Dced-12* cDNA (P_{hsp} *Dced-12*) partially rescued the DTC migration but not the engulfment defect of *ced-12(n3261)* mutants (Figures 2B and 2C). This result indicates that *Dced-12* and *ced-12* are functionally interchangeable for the control of DTC pathfinding.

Discussion

CED-12 May Function by Associating with Membranes and with CED-5 DOCK180

CED-12 is a component of a *C. elegans* Rho/Rac family GTPase signaling pathway. Where does CED-12 act in this pathway? The overexpression of *ced-10* Rac rescued the *ced-12* mutant defects in both engulfment and DTC migration, just as it rescued the defects of *ced-2* and *ced-5* mutants (Reddien and Horvitz, 2000). By contrast, the overexpression of *ced-12* did not suppress the engulfment defects of *ced-2*, *ced-5*, or *ced-10* mutants (Z.Z. and H.R.H., unpublished data). The simplest interpretation of these observations is that CED-12 acts together with CED-2 CrkII and CED-5 DOCK180 as an upstream regulator of CED-10 Rac.

The presence of a putative PH domain suggests that CED-12 may associate with membranes. However, CED-12::GFP was mostly cytoplasmic, displaying many puncta of high GFP intensity. What does this localization pattern mean? PH domains can selectively associate with certain types of phosphatidylinositols (PIs), some

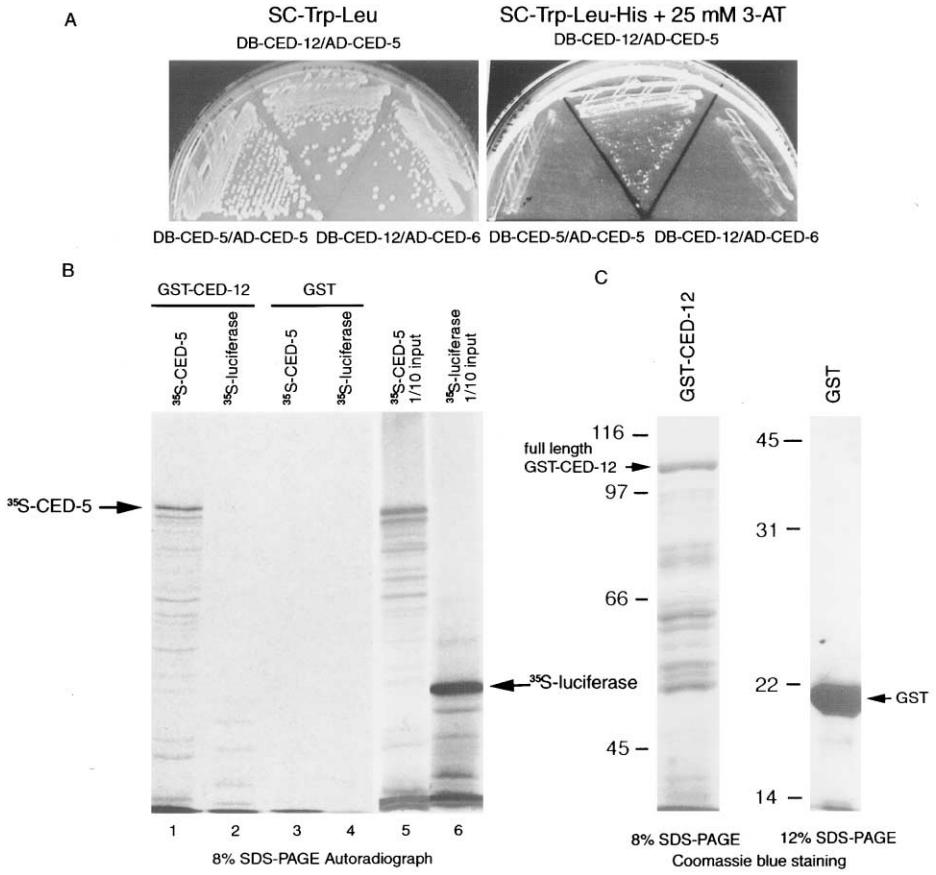


Figure 5. CED-12 Interacts with CED-5

(A) CED-12 and CED-5 interaction detected in the yeast two-hybrid system. The plasmids cotransformed into *Saccharomyces cerevisiae* strain Y190 are marked as DB/AD. 3-AT (3-aminotriazole) inhibits yeast growth on SC-Histidine plates due to basal level expression of the *HIS3* reporter. Growth on plates is indicated by the formation of single colonies.

(B) GST-CED-12 interacted with in vitro translated ^{35}S-methionine-labeled CED-5. Radiolabeled proteins were separated on 8% SDS-PAGE and detected by autoradiography. (Panel 1) Glutathione-sepharose beads bound with GST-CED-12 or GST protein were incubated with in vitro translated ^{35}S-methionine-labeled CED-5 or luciferase. (Panel 2) Ten percent of the input radiolabeled proteins used in panel 1. The minor bands that migrated faster than the major in vitro translated CED-5 band (arrow) seen in lanes 1 and 5 are likely to be proteins initiated from internal methionines or degradation intermediates of full-length CED-5.

(C) GST-CED-12 or GST proteins bound to glutathione-sepharose beads were separated on SDS-PAGE and detected with Coomassie blue staining; the same amount of proteins were used in (B). Western blot using polyclonal anti-GST antibodies (data not shown) indicated that the proteins that migrated faster than the full-length GST-CED-12 (arrow) were likely degradation products.

of which, for example, PI(3, 4, 5)P_3 or PI(3, 4)P_2, are not usual components of the plasma membranes (reviewed by Lemmon and Ferguson, 2000). In fact, a number of PH domain-containing proteins have been observed in the cytoplasm (Lemmon and Ferguson, 2000). However, many PH domains also display low affinity to other types of PIs or even to other kinds of phospholipids (Lemmon and Ferguson, 2000). The punctate localization pattern may indicate that in the absence of a CED-12-activating signal, some CED-12::GFP molecules interact with intracellular membranes, probably with low affinity. Deleting a region that includes the putative PH domain resulted in the loss of the punctate localization pattern of CED-12::GFP, consistent with our hypothesis that this region is responsible for membrane interactions. Furthermore, deleting the PH domain caused a 67% loss of CED-12 engulfment activity, establishing a strong correlation between the membrane-interaction capability and engulfment activity.

A number of PH domains drive signal-dependent membrane targeting of their host proteins by binding to specific types of PIs. For example, PKB (protein kinase B), a downstream effector of PI-3 kinase signaling, is targeted to the plasma membrane and activated by the high-affinity association of its PH domain to PI(3, 4, 5)P_3 and PI(3, 4)P_2, products of activated PI-3 kinase in growth factor stimulated cells (Lemmon and Ferguson, 2000). PI-3 kinases are also activated during phagocytosis and result in the rapid accumulation of PI(3, 4, 5)P_3 on the macrophage plasma membrane within the phagosomal cup (Marshall et al., 2001). This accumulation was proposed to recruit a variety of PH domain-containing proteins to the phagosomal cup, some of which may activate signaling pathways that lead to local cytoskeletal reorganization (Marshall et al., 2001). We speculate that the putative PH domain acts to localize CED-12 to the plasma membrane in response to signals. In the presence of neighboring apoptotic cells, CED-12 in the

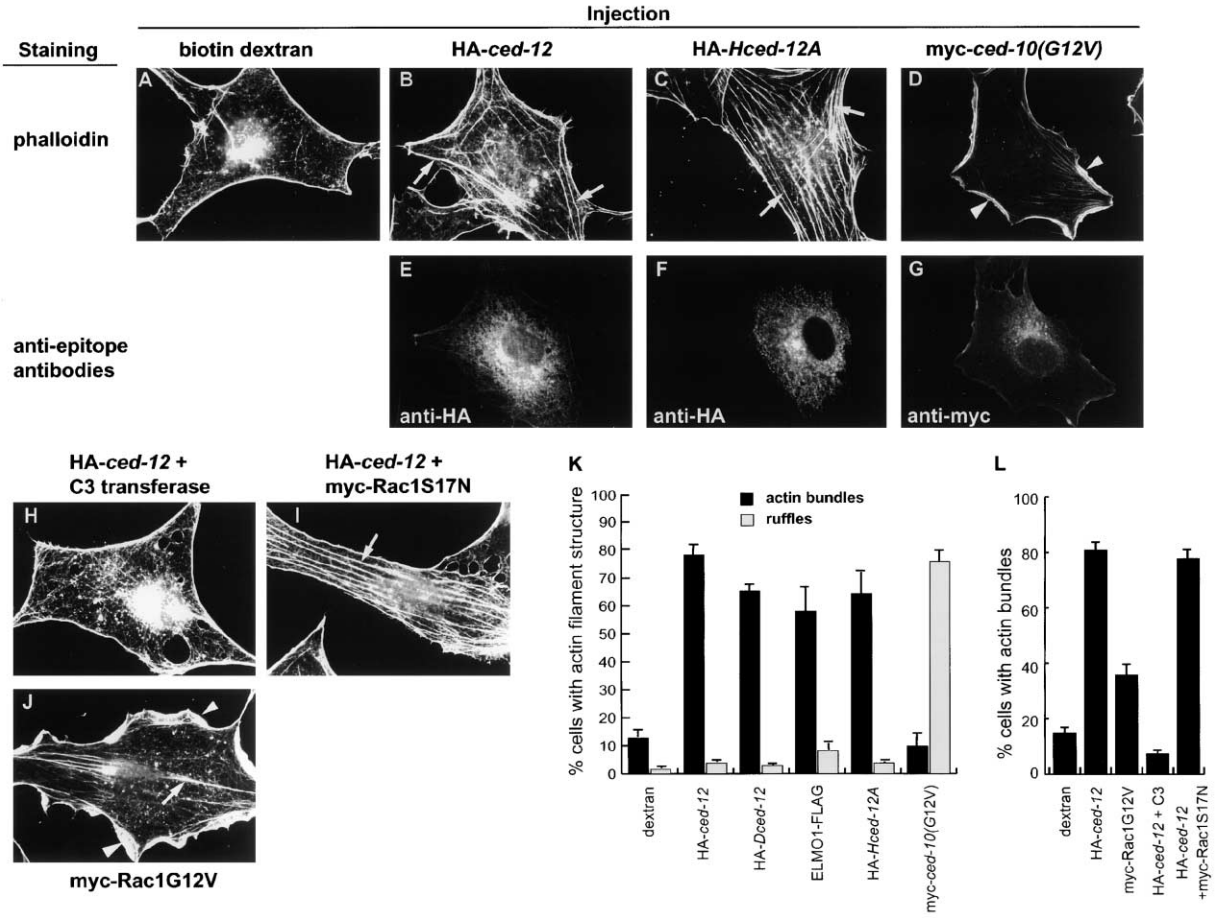


Figure 6. Induction of Cytoskeletal Rearrangements by Overexpressed CED-12 Family Proteins in Quiescent Swiss 3T3 Fibroblasts

Nuclei of 60–100 serum-starved subconfluent Swiss 3T3 cells were microinjected with expression constructs as labeled or with biotin dextran and examined for the distribution of filamentous actin (F-actin) using labeled phalloidin or the epitope-tagged proteins using immunofluorescence. Representative examples of F-actin (A–D and H–J), CED-12 (E), HCED-12A (F), and CED-10(G12V) (G) distributions are given. Arrows and arrowheads indicate examples of actin filament bundles and membrane ruffles, respectively. (K–L) The presence of actin filament structures (black box: actin bundles; gray box: membrane ruffles) in injected cells that expressed the expected proteins was quantified using fluorescence microscopy. Statistical analysis of the data (ANOVA with Fisher's PLSD post-test) shows that CED-12-related proteins did not elicit membrane ruffling ($p > 0.1$, versus biotin dextran). Data are expressed as mean ± SEM for 3–5 independent experiments, with a minimum of 60 expressing cells per experiment.

cytosol or bound to the intracellular membranes of an engulfing cell may be recruited to the plasma membrane at the site of corpse recognition.

DOCK180, a human counterpart of CED-5, has been reported to interact with Rac and increase the GTP binding activity of Rac, leading to the proposal that DOCK180 functions as a coactivator of an unknown guanine nucleotide exchange factor (GEF) for Rac (Kiyokawa et al., 1998; Nolan et al., 1998). The apparent membrane association of CED-12 and the interaction of CED-12 with CED-5 together suggest that CED-12 functions to facilitate the localization of a CED-2/CED-5/CED-12 complex to cell membranes. Since CED-5 has an SH3 domain at its N terminus (Z.Z. and H.R.H., unpublished data), we suggest that the CED-12/CED-5 interaction is mediated by the proline-rich motif of CED-12 and the SH3 domain of CED-5. Reddien and Horvitz (2000) proposed that CED-2 CrkII causes the association of a CED-2/CED-5 protein complex with an unidentified transmembrane receptor in a signal-dependent manner via an interaction between the CED-2 SH2 domain and the phosphorylated cytoplasmic domain of the receptor. Perhaps CED-12 and CED-2 act cooperatively to recruit a CED-2/CED-5/CED-12 complex to the membrane: CED-12 could recruit the complex to the plasma membrane, while CED-2 could target the complex specifically to the transmembrane receptor, after which this complex could trigger the localized remodeling of the actin cytoskeleton, possibly through the activation of CED-10 Rac, which is also membrane localized (Chen et al., 1996) (Figure 7).

CED-12 Promotes Actin Cytoskeletal Reorganization via Rho/Rac Family GTPase(s)

In quiescent Swiss 3T3 fibroblasts, the activation of Rho induces the assembly of actin into bundles and stress fibers, while the activation of Rac and Cdc42 induces actin polymerization leading to ruffles and filopodia, respectively (reviewed by Hall and Nobes, 2000). We found that the overexpression of *C. elegans* CED-12 and its

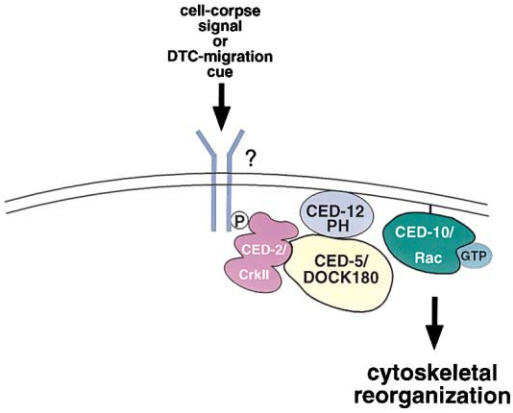


Figure 7. Model of How CED-12 Recruits the CED-2/CED-5/CED-12 Complex to the Plasma Membrane and Activates the Rac GTPase Signaling Pathway

See text for details. The thin double line indicates the plasma membrane of an engulfing cell or of a migrating distal tip cell. The question mark indicates that the transmembrane receptor(s) for the cell corpse signal or DTC migration cue is (are) of unknown identity. The SH2 domain of CED-2 may interact with the cytoplasmic tail of the activated receptor or with a component of the receptor complex. CED-12 associates with the plasma membrane via its PH domain and interacts with CED-5 via its SH3 binding motif. CED-10 is known to associate with the plasma membrane via its prenylated C terminus. CED-10 may be activated by an unknown guanine nucleotide exchange factor that associates with the CED-2/CED-5/CED-12 complex. The activation of CED-10 leads to cytoskeletal reorganization and polarized cell extension.

three homologs from *Drosophila* and humans in murine Swiss 3T3 fibroblasts induced prominent actin filament bundling. All CED-12 family members tested are therefore capable of promoting a similar cytoskeletal change, strongly supporting our hypothesis that in *C. elegans ced-12* acts to control the reorganization of the actin cytoskeleton.

Intriguingly, however, CED-12 overexpression induced actin filament bundling rather than membrane ruffling. In addition, this actin bundling was prevented by C3 transferase, a specific inhibitor of Rho (Ridley and Hall, 1992), but not by a dominant-negative Rac. It appears that in this assay CED-12 either activates endogenous Rho or in some other way induces the bundling of Rho-dependent actin filaments.

Our studies suggest that, although during *C. elegans* engulfment CED-12 activates CED-10 Rac, in murine fibroblasts CED-12 activates Rho. This difference is unlikely to be simply a consequence of expressing a nematode protein in mammalian cells, since the human CED-12 counterparts similarly appeared to activate Rho in 3T3 cells. Perhaps CED-12 family members can differentially activate distinct Rho/Rac family GTPases in different cellular contexts. Alternatively, it is conceivable the induction of actin bundling by CED-12 in 3T3 cells was mediated by Rac in addition to Rho. A similar phenomenon was described recently: Plexin B1, the membrane receptor for semaphorins, can induce actin filament bundling in a manner dependent on both Rho and Rac (Driessens et al., 2001). That our attempts to block CED-12-induced actin bundling in fibroblasts by dominant-negative Rac (Rac1S17N) were unsuccessful does not preclude a possible involvement of Rac: Rac1S17N remains as a stable GDP-bound form and sequesters GEFs for endogenous Rac1 (Ridley et al., 1992); if CED-12 regulates a Rac-activating GEF that is not sequestered by Rac1S17N, its effect on Rac1 would not have been inhibited by Rac1S17N. In *C. elegans*, while CED-10 Rac is clearly required for engulfment, it is possible that a Rho-like protein is also involved.

Gumienny et al. (2001) report that ELMO1, one of the human homologs of CED-12, induced cell shape changes but no membrane ruffles when transfected into LR73 CHO fibroblasts. A cotransfection of ELMO1, CrkII, and DOCK180 resulted in abundant membrane ruffles in a manner that was inhibited by dominant-negative Rac. The hypothesis that CED-12 family members can differentially activate distinct Rho/Rac family GTPases in different cellular contexts could, in part, account for the differences between our observations concerning 3T3 cells and the observations reported by Gumienny et al. In addition, the serum-starved, quiescent Swiss 3T3 cells that we studied lost virtually all polymerized actin structures and thus allowed a very sensitive detection of induced actin structures, whereas the LR73 CHO fibroblasts studied by Gumienny et al. contained endogenous actin structures, which might have made the detection of induced actin bundles and stress fibers difficult.

CED-12 Is a Member of an Evolutionarily Conserved Protein Family

The *Drosophila* CED-12 counterpart, DCED-12, partially rescued the *ced-12* DTC migration defect, indicating that CED-12 and DCED-12 are functionally related. Furthermore, the finding that CED-12, DCED-12, HCED-12A, and ELMO-1 all induced actin bundle formation in quiescent Swiss 3T3 cells strongly suggests that these proteins affect the actin cytoskeleton similarly, perhaps by activating the same downstream signaling pathway. We propose that the interaction between CED-12 family members and CED-5 DOCK180 family members is evolutionarily conserved, that the PH domain in CED-12 homologs functions to facilitate membrane associations, and that CED-12 family members have conserved biological functions, for example, in the phagocytosis of apoptotic cells.

Experimental Procedures

Mutations, Strains, and Transgenic Animals

C. elegans strains were cultured at 20°C as described by Brenner (1974). N2 Bristol was the wild-type strain. Mutations used were as follows and were described by Riddle et al. (1997), except where noted otherwise: LGI, *ced-1(e1735)*, *ced-12(n3261)* (this study), *ced-12(bz187)* (Chung et al., 2000), *sem-4(n1378)*, *lin-11(n566)*, *unc-29(e1072am)*, *unc-75(e950)*; LGIII, *ced-6(n1813)*, *ced-7(n1892)*, *dpy-17(e164)*, *unc-32(e189)*; LGIV, *ced-2(e1752)*, *ced-5(n1812)*, *ced-10(n1993)*, *dpy-9(e12)*, *dpy-13(e184)*, *dpy-20(e1282)*; LGV, *unc-76(e911)*. The following deficiencies were used: *qDf5*, *qDf7*, *qDf8*, and *qDf9* (Ellis and Kimble, 1995). Germline transformation was performed as described by Mello and Fire (1995). All strains used for introducing transgenes contained the *unc-76(e911)* mutation. *ced-12* expression constructs were coinjected at 10–50 ng/μl with the *unc-76* rescuing plasmid p76-16B (Bloom and Horvitz, 1997), and lines of non-Unc transgenic animals were established.

ced-12, *Dced-12*, and *Hced-12A* Expression Constructs

We cloned the *ced-12* cDNA (see Supplemental Data [http://www.developmentalcell.com/cgi/content/full/1/4/477/DC1]) into the P_{hsp} vectors pPD49.78 and pPD49.83 (Mello and Fire, 1995) to generate P_{hsp} *ced-12*, and under the control of the *col-10* and *egl-1* promoters to generate $P_{col\text{-}10}$ *ced-12* and $P_{egl\text{-}1}$ *ced-12*, respectively. We cloned truncated forms of *ced-12* cDNA (generated by PCR) encoding CED-12 proteins missing aa 551–681, 551–731, 710–731, and 725–731 under the control of $P_{col\text{-}10}$ to generate $P_{col\text{-}10}$ *ced-12ΔPH*, $P_{col\text{-}10}$ *ced-12ΔPHΔProΔ7*, $P_{col\text{-}10}$ *ced-12ΔProΔ7*, and $P_{col\text{-}10}$ *ced-12Δ7*, respectively. To construct $P_{ced\text{-}1}$ *ced-12::gfp*, we cloned the *ced-12* cDNA into vector pPD95.75 (a gift from Dr. A. Fire) to generate a *ced-12::gfp* in-frame fusion and inserted $P_{ced\text{-}1}$ upstream of the ATG of *ced-12* cDNA.

We amplified the *Dced-12* cDNA by PCR from a *Drosophila* embryonic cDNA library (Rubin et al., 2000) using primers corresponding to the predicted CG5336 coding sequence and confirmed that this cDNA clone corresponded to the CG5336 coding sequence. We cloned the *Dced-12* cDNA into pPD49.78 and pPD49.83 to generate P_{hsp} *Dced-12* constructs. Using a Titan One Tube RT-PCR Kit (Roche Diagnostics, Indianapolis, IN), we amplified the *Hced-12A* cDNA from polyA(+) human testis RNA using primers corresponding to the predicted bA394O2.2 coding sequence. The sequence of four independent RT-PCR clones all contained an additional 51 nucleotides inserted between nucleotides 756 and 757 and an additional 12 nucleotides inserted between nucleotides 1750 and 1751, encoding a protein of 720 aa instead of 699 aa as predicted. We named this protein HCED-12A. We cloned *ced-12*, *Dced-12*, and *Hced-12A* cDNAs into the vector pRK5 as N-terminal HA-tagged constructs.

Quantitation of Engulfment Defects

We scored the number of persistent cell corpses as described by Zhou et al. (2001). For strains bearing a *sem-4(n1378)* mutation and retaining L1 larvae inside the mothers (Riddle et al. 1997), we released young L1 larvae by opening gravid mothers with a 25G needle.

Electron and Fluorescence Microscopy

Midstage L3 larvae were analyzed by transmission electron microscopy as described by Zhou et al. (2001). A Zeiss-Applied Precision DeltaVision deconvolution microscope was used to analyze the GFP fluorescence in living animals as described by Zhou et al. (2001).

Yeast Two-Hybrid System

We cloned the yk359f9 cDNA into the vector pAS1 (Bai and Elledge, 1997) to generate a DB (GAL4 DNA binding domain) fusion. We cloned cDNAs of *ced-1* (cytoplasmic domain, amino acids 931–1111), *ced-2*, *ced-5*, *ced-6*, and *ced-10* into the vector pACT2 (Bai and Elledge, 1997) to generate AD (GAL4 *trans*-activation domain) fusions. We cotransformed yeast strain Y190 with DB-CED-12 and one of the AD-CED fusions, and streaked out Trp^+ Leu^+ transformants onto SD plates lacking tryptophan, leucine, and histidine and containing 25 mM of 3-aminotriazole (3-AT), an inhibitor of the *HIS3* gene product, to test for protein-protein interactions that activated robust *HIS3* expression and resulted in yeast growth.

In Vitro Binding Assay

We cloned the *ced-12* cDNA into vector PGEX-2T (Pharmacia) and expressed this clone in bacterial BL21(DE3) cells. We purified the GST-CED-12 fusion protein or GST protein alone as suggested (Pharmacia). We cloned full-length CED-5 cDNA into vector pCITE-4a (Novagen). We generated ^{35}S-methionine-labeled proteins using a T7-based coupled reticulocyte lysate system (Promega) and tested them for binding to GST-CED-12 or GST protein immobilized on glutathione-sepharose beads as described (Reddien and Horvitz, 2000). Proteins bound to the beads were analyzed using 8% SDS-PAGE and autoradiography.

Cell Culture, Microinjection, and Immunofluorescence

Subconfluent, serum-starved Swiss 3T3 fibroblasts were prepared as described (Lamarche et al., 1996), except that cells were serum-starved for 40 hr instead of overnight. Cells were microinjected with pRK5 eukaryotic expression vectors encoding various tagged constructs (0.1 mg/ml) and biotin dextran (1 mg/ml, Molecular Probes) in PBS and incubated for 3 hr for optimal expression. Cells were fixed for 15 min in PBS containing 4% paraformaldehyde followed by incubation with 50 mM NH_4Cl for 10 min to block free amino groups, and permeabilized using 0.2% Triton X-100 in PBS. Cells were immunostained with appropriate primary and secondary antibodies or rhodamine-labeled phalloidin and examined with a Zeiss Axiophot microscope using a Zeiss 63X1.4 oil immersion objective. Fluorescence images were recorded using a Hamamatsu C5985-10 video camera. The fluorescence reagents used include mouse anti-myc 9E10, rat anti-HA 3F10 (Boehringer), mouse anti-FLAG M2 (Sigma), FITC-coupled goat anti-mouse or anti-rat immunoglobulins (Jacksons Immunoresearch Laboratories), and Alexa fluor 488 Streptavidine (Molecular Probes).

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Accession Numbers

The GenBank accession numbers for the sequences of *ced-12*, *Dced-12*, and *Hced-12A* are AF416781, AF417860, and AF417861, respectively.

Note Added in Proof

Gumienny et al. (2001) and Wu et al. (2001; this issue of *Developmental Cell*) report observations similar to those we describe in this paper.