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C. elegans CED-12 Acts in the Conserved CrkII/DOCK180/Rac Pathway to Control Cell Migration and Cell Corpse Engulfment

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Summary

We have identified and characterized a novel *C. elegans* gene, *ced-12*, that functions in the conserved GTPase signaling pathway mediated by CED-2/CrkII, CED-5/DOCK180, and CED-10/Rac to control cell migration and phagocytosis of apoptotic cells. We provide evidence that *ced-12* likely acts upstream of *ced-10* during cell migration and phagocytosis and that CED-12 physically interacts with CED-5 and forms a ternary complex with CED-2 in vitro. We propose that the formation and localization of a CED-2-CED-5-CED-12 ternary complex to the plasma membrane activates CED-10, leading to the cytoskeletal reorganization that occurs in the polarized extension of cell surfaces in engulfing cells and migrating cells. We suggest that CED-12 counterparts in higher organisms regulate cytoskeleton dynamics, as CED-12 does in *C. elegans*.

Introduction

Programmed cell death (apoptosis) is an important cellular process that has been conserved through evolution (Jacobson et al., 1997; Horvitz, 1999). Once cells undergo programmed cell death, their corpses are swiftly recognized and phagocytosed by macrophages or neighboring cells. The impaired clearance of apoptotic cells has been implicated in autoimmune diseases (Botto et al., 1998; Taylor et al., 2000).

Several molecules on the surface of engulfing cells have been identified to be important for the phagocytosis of apoptotic cells in flies and mammals (Chimini and Chavrier, 2000; Messmer and Pfeilschifter, 2000). For example, mammalian lectin-like proteins (Duvall et al., 1985) vitronectin/CD36/thrombospondin complex (Savill et al., 1990, 1991, 1992; Stern et al., 1996; Fadok et al., 1998a), integrin (Albert et al., 1998, 2000), CD68 (Oka et al., 1998), ABC1 (Luciani and Chimini, 1996; Hamon et al., 2000), CD14 (Devitt et al., 1998; Gregory, 2000), phosphatidylserine receptor (Fadok et al., 1998b, 2000), and *Drosophila* croquemort (Franc et al., 1996, 1999) have been shown to recognize moieties on the surfaces of apoptotic cells and initiate engulfment of apoptotic cells in vitro. The signaling pathways utilized by most of these receptors are not defined.

During the development of a *C. elegans* hermaphrodite, 131 somatic cells and over 300 germ cells undergo programmed cell death (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983; Gumienny et al., 1999). These cell corpses are swiftly recognized and phagocytosed by their neighboring cells within approximately an hour (Sulston and Horvitz, 1977; Albertson and Thomson, 1982). Genetic studies have identified six genes, *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10* (*ced*, cell death abnormal) that are important for the engulfment of cell corpses (Hedgecock et al., 1983; Ellis et al., 1991a). Mutations in any of these genes block the engulfment of many cell corpses and cause the phenotype of persisting cell corpses, which are readily distinguishable using Nomarski optics by their refractile button-like appearance. Genetic analysis suggests that the six engulfment genes may fall into two groups: *ced-1*, *ced-6*, and *ced-7* in one and *ced-2*, *ced-5*, and *ced-10* in the other. Double mutants between two groups have more persistent cell corpses than double mutants within the same group or single mutants (Ellis et al., 1991a). Mutants of the *ced-2*, *ced-5*, and *ced-10* group are also defective in the specific migration of DTCs (Wu and Horvitz, 1998a; Reddien and Horvitz, 2000), which are located at the leading tips of the two gonadal arms and guide the extension of each growing arm during gonadogenesis (Kimble, 1981; Hedgecock et al., 1987).

All six *C. elegans* engulfment genes have been cloned, and their homologs have been identified in mammals. CED-1 is similar to the human scavenger receptor SREC and likely acts as a phagocytic receptor that recognizes cell corpses (Zhou et al., 2001). CED-7 is similar to mammalian ABC1 (Wu and Horvitz, 1998b) and is required for clustering of CED-1 around neighboring cell corpses (Zhou et al., 2001). CED-6 contains a phosphotyrosine binding (PTB) site, a potential proline-rich motif, and a leucine zipper region for homodimerization and may act downstream of CED-1 and CED-7 during phagocytosis of cell corpses (Liu and Hengartner, 1998, 1999; Su et al., 2000). The genes *ced-2*, *ced-5*, and *ced-10* encode homologs of mammalian CrkII, DOCK180, and Rac, respectively (Wu and Horvitz, 1998a; Reddien and Horvitz, 2000). Genetic studies suggest that *ced-10* acts downstream of *ced-2* and *ced-5* in the genetic pathway that controls the engulfment of cell corpses (Reddien and Horvitz, 2000). Later studies in mammalian cells showed that CED-2/CrkII, CED-5/DOCK180, and CED-10/Rac are components of a conserved pathway responsible for the engulfment of apoptotic cells and membrane ruffling of migrating cells (Cheresh et al., 1999; Albert et al., 2000).

In addition to the engulfment of apoptotic cells, these six engulfment genes *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10* also mediate the elimination of necrotic cell corpses induced by *mec-4*(*u231*), *deg-1*(*u38*), or *deg-3* (*u662*) mutations or overexpression of $G\alpha_s$(Q208L) (Chung et al., 2000). In a screen for mutations blocking the elimination of *mec-4*(*u231*)-induced corpses, a *ced-12* mutation was isolated (Chung et al., 2000).

We isolated a novel *ced-12(tp2)* allele in a genetic screen for mutants defective in DTC migration. Further phenotypic characterization revealed that *ced-12(tp2)*

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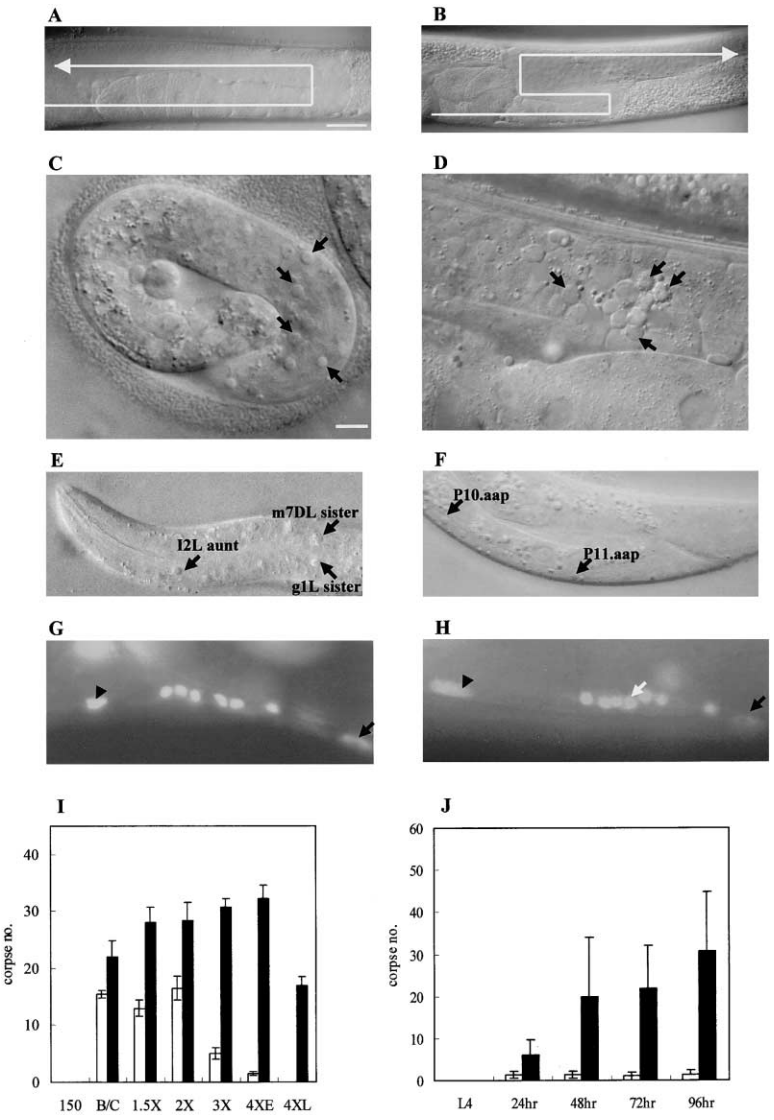


Figure 1. Defects in DTC Migration and Cell Corpse Engulfment in *ced-12* Mutants Observed using Nomarski Microscopy

In (A), (B), (D), (E), and (F), dorsal is up and anterior is to the left.

(A) A Nomarski photomicrograph of a wild-type posterior gonadal arm. The DTC migrated posteriorly along the ventral side, then turned dorsally, and finally migrated anteriorly along the dorsal side. The bar represents 25 μm. The same scale applies to (B).

(B) A Nomarski photomicrograph of a posterior gonadal arm in a *ced-12* mutant. The DTC migrated posteriorly along the ventral side, made a premature dorsal turn, then migrated anteriorly, and finally reversed its direction to migrate posteriorly along the dorsal side. The arrow shows the migration pattern of the posterior DTC in (A) and (B).

(C) A *ced-12* embryo. Unengulfed cell corpses are indicated by arrows. No cell corpses are observed in wild-type embryos at the same stage. The bar represents 5 μm; the same scale applies to (D)–(F).

(D) The germline of a *ced-12* adult. Unengulfed cell corpses are indicated by arrows. Less than three cell corpses are observed in wild-type germline.

(E) The head region of a *ced-12* L1 larva. Arrows indicate persistent pharyngeal cell corpses generated during embryogenesis.

(F) A tail region of a *ced-12* L3 larva. Arrows indicate persistent cell corpses generated during the L1 stage.

(G) A wild-type adult stained with DAPI.

(H) A *ced-12* adult stained with DAPI. The extra DAPI staining corresponding to the persistent P10.aap cell corpse is indicated by white arrow. The P9.p and P10.p nuclei are indicated by arrow head and arrow, respectively, in (G) and (H).

(I) Cell-corpse numbers of wild-type (white columns) and *ced-12* (black columns) embryos at different developmental stages. The y axis represents the average number of corpses visible in the whole embryos (150), (B/C), and (1.5X) or in the head region of embryos (2X), (3X), (4XE), and (4XL). Stages of embryos examined: 150 min after fertilization (150), bean and comma stage embryos (B/C); 1.5-fold embryos (1.5X); 2-fold embryos (2X); 3-fold embryos (3X); early 4-fold embryos, which have a well-developed pharynx but do not pump (4XE); and late 4-fold embryos, which have a pumping pharynx (4XL). At least 20 embryos of each stage were examined.

(J) Cell-corpse numbers in the germline of wild-type (white columns) and *ced-12* (black columns) animals at different times after entering to adulthood. The y axis represents the average number of corpses visible in each gonadal arms of worms at different stages. Stages of animals examined: L4 larvae (L4) and animals at approximately 24 hr (24 hr), 48 hr (48 hr), 72 hr (72 hr), or 106 hr (106 hr) after entering to the adulthood. Error bars in (G) and (H) indicate one standard error of the mean.

mutants are also defective in phagocytosis of apoptotic cells. We have cloned and characterized the *ced-12* gene.

Results

The *ced-12* Gene Is Important for the Migration of DTCs and the Engulfment of Cell Corpses

The migration of two DTCs generates two symmetrical U-shaped gonadal arms in adults (Figure 1A). We isolated the *ced-12(tp2)* allele in a genetic screen for mutants with abnormal gonad shapes caused by defective DTC migration. Approximately 75% of *ced-12(tp2)* adults (n = 101) show abnormal gonad shapes. The most frequent defect observed in the gonad of *ced-12* mutants is an extra turn of gonadal arm, caused by the reversal in the direction of DTC migration along the dorsal body muscles (Figure 1B).

In the course of characterizing *ced-12* mutants, we observed extra cell corpses in late embryos (Figure 1C). We counted the number of cell corpses in embryos at different developmental stages and found that *ced-12* mutants have more cell corpses than wild-type throughout embryogenesis after the onset of programmed cell death (Figure 1I). To understand if the extra corpses in *ced-12* mutants are generated by programmed cell

Table 1. Numbers of Persistent Cell Corpses in *ced-12(tp2)* and Other *ced* Mutant Backgrounds

A. Persistent Cell Corpses in *ced-12(tp2)* Mutants Are Generated by Programmed Cell Death

Strains	No. of Persistent Corpses (n = 20)[a]
ced-12(tp2)	17.9 ± 3.0
ced-3(n717)	0.0 ± 0.0
ced-4(n1162)	0.0 ± 0.0
ced-12(tp2); ced-3(n717)	0.0 ± 0.0
ced-12(tp2); ced-4(n1162)	0.0 ± 0.0

B. *ced-12(tp2)* Greatly Enhances the Persistent Corpse Numbers in *ced-1*, *ced-6* and *ced-7* Alleles

Strains	No. of Persistent Corpses (n = 20)[a]
ced-1(n2089)	23.3 ± 4.9
ced-12(tp2) ced-1(n2089)	40.7 ± 5.1
ced-1(e1735)	30.4 ± 1.6
ced-12(tp2) ced-1(e1735)	41.4 ± 3.3
ced-6(n1813)	21.3 ± 2.9
ced-12(tp2); ced-6(n1813)	36.3 ± 4.8
ced-6(n2095)	22.3 ± 2.4
ced-12(tp2); ced-6(n2095)	38.5 ± 4.8
ced-7(n1892)	20.2 ± 1.3
ced-12(tp2); ced-7(n1892)	62.1 ± 6.5
ced-7(n2094)	36.1 ± 3.0
ced-12(tp2); ced-7(n2094)	58.8 ± 5.2
ced-2(e1752)	16.2 ± 4.0
ced-12(tp2); ced-2(e1752)	28.5 ± 3.7
ced-2(n1994)	35.3 ± 4.4
ced-12(tp2); ced-2(n1994)	43.7 ± 4.0
ced-5(n2002)	37.7 ± 5.9
ced-12(tp2); ced-5(n2002)	37.8 ± 3.7
ced-5(n1812)	42.8 ± 4.5
ced-12(tp2); ced-5(n1812)	41.7 ± 4.0
ced-10(n1993)	18.3 ± 3.0
ced-12(tp2); ced-10(n1993)	29.1 ± 4.6
ced-10(n3246)	24.7 ± 5.3
ced-12(tp2); ced-10(n3246)	31.4 ± 6.6

[a] Cell corpses in the head region of L1 larvae within 30 min of hatching were counted. Mean ± SEM.

death, we counted cell corpses in *ced-12*; *ced-3* and *ced-12*; *ced-4* mutants as mutations in *ced-3* or *ced-4* gene block programmed cell death (Ellis and Horvitz, 1986). No cell corpses were observed in *ced-12*; *ced-3* or *ced-12*; *ced-4* mutants (Table 1A), indicating that extra corpses in *ced-12* mutants are derived from cells that die by programmed cell death.

To determine the identities of cells that generate extra cell corpses, we examined the cell corpses in the pharynx and the ventral cord of *ced-12* mutants. Nuclei of the cells in the pharynx are easily recognized as rippled indents in the smooth wall of the pharynx (Albertson and Thomson, 1976). All pharyngeal cells including those that are destined to die are generated during embryogenesis (Albertson and Thomson, 1976; Sulston et al., 1983). In contrast, deaths in the ventral cord occur postembryonically (Sulston, 1976). Since the cell lineage in the developing *C. elegans* is essentially invariant, cells destined to die do so at specific times and locations (Sulston, 1976; Sulston et al., 1983). We found that locations of extra cell corpses in the pharynx and in the ventral cord of *ced-12* mutants correspond to those cells that are genetically programmed to die (Figures 1E and 1F). Further time-course analyses of deaths in the ventral cord showed that cells destined to die do so at the right times but lingered in the refractile disk-like appearance for hours (Figure 1F). These results altogether indicate that the extra cell corpses in *ced-12* mutants are persisting cell corpses generated by programmed cell death. Moreover, we found that DNA from persisting cell corpses in the ventral cord also persists in *ced-12* mutants when we stained *ced-12* mutants with DAPI or Syto 11 (Figures 1G and 1H). Similar staining patterns have been observed in other mutants defective in the engulfment process of programmed cell death (Hedgecock et al., 1983; Wu et al., 2000). Altogether, our data indicate that *ced-12* is important for the engulfment of somatic cell corpses.

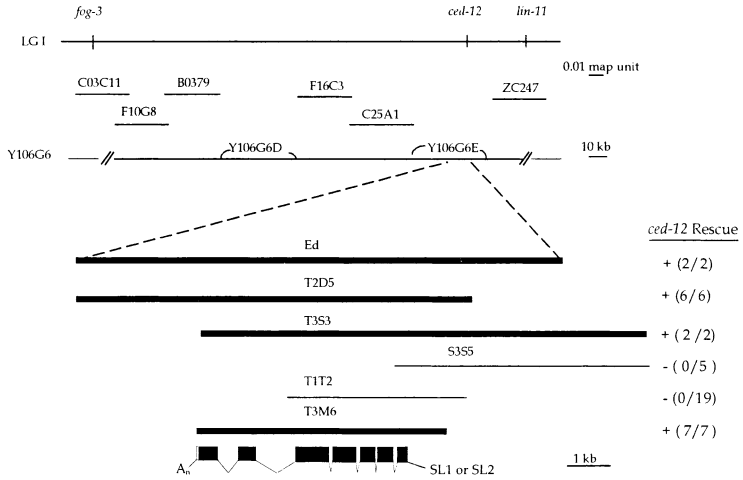
The *ced-12* gene shows a maternal rescue effect in the elimination of somatic cell corpses: homozygous *ced-12* progeny of a homozygous *ced-12* hermaphrodite have persistent somatic cell corpses, but homozygous *ced-12* progeny of a *ced-12*/+ hermaphrodite appear wild-type, as do all *ced-12*/+ animals themselves. Like somatic cells, germline cells also undergo programmed cell death (Gumienny et al., 1999). We found that germline cell corpses also persist in the gonad of *ced-12* mutants (Figures 1D and 1J). Therefore, *ced-12* is required for the removal of both somatic and germline cell corpses.

ced-12 Acts in the Pathway Mediated by *ced-2*, *ced-5*, and *ced-10* to Control Cell Corpse Engulfment

To determine if *ced-12* may act in a partially redundant manner with previously identified engulfment genes during cell corpse engulfment, we constructed *ced-12 ced-1*, *ced-12*; *ced-2*, *ced-12*; *ced-5*, *ced-12*; *ced-6*, *ced-12*; *ced-7*, and *ced-12*; *ced-10* double mutants and scored persisting cell corpses in the double mutants and compared them with those of *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10*, and *ced-12* single mutants. The *ced-5* and *ced-7* alleles used in this study are likely null (Wu and Horvitz, 1998a, 1998b). Specifically, we counted the number of persistent cell corpses in the head region of L1 larvae within 30 min of hatching. *ced-12 ced-1*, *ced-12*; *ced-6* and *ced-12*; *ced-7* double mutants showed many more cell corpses than their respective single mutants, indicating that *ced-12* acts partially redundantly with *ced-1*, *ced-6*, and *ced-7* during cell corpse engulfment (Table 1B). In contrast, *ced-12*; *ced-5* and *ced-5* mutants exhibited similar numbers of cell corpses, suggesting that *ced-5* and *ced-12* act in the same genetic pathway that controls cell corpse engulfment (Table 1B). *ced-12*; *ced-2* and *ced-12*; *ced-10* double mutants show more persistent cell corpses than their respective single mutants (Table 1B). This may be caused by the partial loss-of-function nature of *ced-2*, *ced-10* and *ced-12* (see below) alleles.

In addition to cell corpse engulfment, *ced-2*, *ced-5* and *ced-10* mutants are also defective in DTC migration (Wu and Horvitz, 1998b; Reddien and Horvitz, 2000). The gonad of these mutants frequently shows an abnormal shape, albeit with normal size, indicating that DTCs of these mutants are capable of migration but frequently

A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Y106G6D
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10 kb
ced-12 Rescue
Ed
+ (2/2)
T2D5
+ (6/6)
T3S3
+ (2/2)
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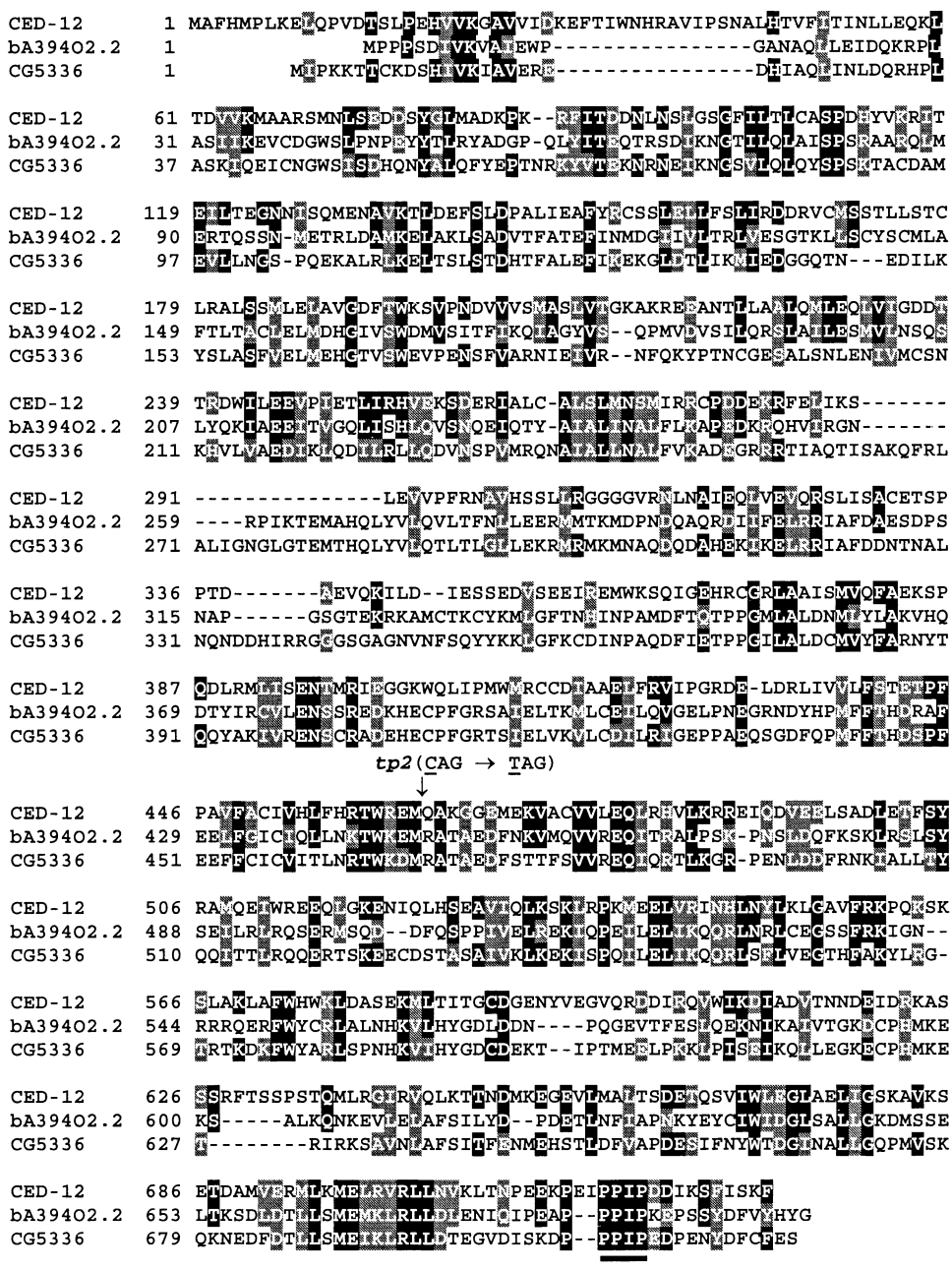
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CG5336 1 MIPKKTICKDSHIVKIAVERG----------DHIAQLINLDQRHPL
CED-12 61 TDVVKMAARSMNLSEDDSYGLMADKEK--RFITDDNLNSIGSGFILTLCASEDHYVKRIT
bA394O2.2 31 ASIIKEVCDGWSLPNPEYYTLRYADGP-QLYITEQTRSDIKNGTILQLAISPSRAARQLM
CG5336 37 ASKIQEICNGWSISDHQNYALQFYEETNRKYVTEKNRNEIKNGSVIQLQYSPSKTACDAM
CED-12 119 EILTEGNNISQMENAVKTLDEFSLDPALIEAEYRCSSLELFSLIRDDRVCMSSTLLSTC
bA394O2.2 90 ERTQSSN-METRLDAMKELAKLSADVTFATEFINMDGIIVLTRLVESGTKLISCYSCMLA
CG5336 97 EVLLNCS-PQEKALRLKELTSLSTDHTFALEFIKEKGLDTLIKMIEDGGQTN---EDILK
CED-12 179 LRALSSMLELAVGDFTWKSVPNDVVVSMASLVTGKAKREEANTLLAALQMLEQEVIGDDT
bA394O2.2 149 FTLTTCIELMDHGIVSWDMVSITFIKQIAGYVS--QPMVLVSILQRSLAILESMVLNSQS
CG5336 153 YSLASFVELMEHGTVSWEVPENSFVARNIELVR--NFQKYPTNCGESALSNLENLVMCSN
CED-12 239 TRDWILEEVPIETLIRHVSKSDERIALC-ALSLMNSMIRRCPDDEKRFEIIKS-------
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CG5336 391 QQYAKIVRENSCRADEHECPFGRTSIELVKVLCDILRIGEPPAEQSGDFQPMFFTHDSPF
tp2(CAG → TAG)
CED-12 446 PAVFACIVHLFHRTWREMQAKCGEMEKVACVVLEQLRHVLKRREIQDVEELSADLETFSY
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CG5336 679 QKNEDFDTLLSMEIKLRLLDTEGVDISKDP--PPIPEDPENYDFCFES

take aberrant paths. Similarly, almost all gonadal malformations in *ced-12* mutants result from defects in pathfinding rather than in movement per se (Figure 1B). Therefore, like *ced-2*, *ced-5* and *ced-10* genes, *ced-12* may primarily function to establish the polarity of migrating DTCs toward the migration cue. Similarly, *ced-12* may act to establish the polarity of an engulfing cell toward the apoptotic cell during the phagocytosis process.

Molecular Cloning of *ced-12*

To clone *ced-12*, we mapped *ced-12* between *fog-3* and *lin-11* on chromosome I (Figure 2A). This interval corresponds to an ~230 kb region on the physical map and has been completely sequenced by the *C. elegans* Genome Sequencing Consortium (Coulson et al., 1986; *C. elegans* Genome Sequencing Consortium, 1998). We tested genomic DNA from this region for their abilities to rescue the Ced-12 mutant phenotype of persisting cell corpses using germline transformation experiments (Mello et al., 1992). We found that none of the cosmids tested in this region were able to rescue. We further tested various genomic fragments amplified by long-range polymerase chain reaction from regions Y106G6D and Y106G6E, which are not represented by cosmid clones (Figure 2A). We found that the 10 kb genomic fragment Ed amplified from the Y106G6E region was able to rescue the Ced-12 phenotype (Figure 2A). Sequence analysis by the *C. elegans* genome sequencing consortium predicted three open reading frames in this fragment. By testing various genomic fragments amplified from this region, we restricted the *ced-12* rescuing fragment in an ~6 kb region containing a single open reading frame, Y106G6E.5.

CED-12 Is Evolutionarily Conserved among Species

We obtained several cDNA clones from Y. Kohara corresponding to the Y106G6E.5 open reading frame. Sequences of these clones revealed an open reading frame of 731 amino acids with a 3′ poly(A) tract. We further defined the 5′ end of the *ced-12* message using the reverse transcriptase-polymerase chain reaction (RT-PCR) method. We found that *ced-12* transcripts can be either SL1 or SL2 *trans*-spliced, consistent with the hypothesis that *ced-12* is a downstream gene in an operon, since messages from downstream genes in *C. elegans* operons usually carry SL2 *trans*-spliced leader sequences (Spieth et al., 1993). The upstream gene of the operon is similar in sequence to casein kinase (*C. elegans* Genome Sequencing Consortium, 1998). Northern analysis using the *ced-12* cDNA as a probe revealed a single band of 2.4 kb (data not shown), consistent with the size of the full-length *ced-12* cDNA. Since the expression of a *ced-12* cDNA under the control of *C. elegans* heat shock promoters (*hsp*; Stringham et al., 1992) rescues the defects in both DTC migration (Table 2A) and cell corpse engulfment (Tables 2B and 2C; see below) in *ced-12* mutants, the *ced-12* cDNA encodes a functional CED-12 protein. We identified a nonsense mutation in the exon 5 of the *ced-12* locus in *ced-12(tp2)* mutants (Figure 2B). The premature stop codon (TAG) eliminates ~36% of the CED-12 protein.

A search of protein databases with the predicted CED-12 protein sequence revealed that CED-12 is similar to human bA394O2.2 and *Drosophila* CG5336 with 19% and 18% identities, respectively, throughout their entire lengths (Figure 2B). CED-12 and its putative *Drosophila* and human homologs each contain a proline-rich motif with PPIP sequence in the C-terminal region (Figure 2B). Many proline-rich domains have been shown to interact with SH3 domains (Ren et al., 1993; Yu et al., 1994; Grabs et al., 1997). *Drosophila* CG5336 (amino acids 555–674) and human bA394O2.2 (amino acids 531–648) also contain a PH domain in the C-terminal region prior to the PPIP motif. In addition to similarity to human bA394O2.2 and fly CG5336, CED-12 also shows significant sequence similarity to human KIAA0281 and BAB14712.1, suggesting that at least three different CED-12-like proteins exist in humans.

ced-12 Probably Functions within Engulfing Cells during the Engulfment Process

The engulfment of cell corpses requires the recognition and subsequent phagocytosis of dying cells by engulfing cells. To determine whether *ced-12* acts within dying or engulfing cells, we ectopically expressed *ced-12* in *ced-12* mutants using *C. elegans* heat shock promoters (*hsp*) long after programmed cell death had occurred and cell corpses had persisted for hours. It has been shown that cells that have been dead and fail to be engulfed do not express transgene under the control of *C. elegans* heat shock promoters and likely have no active transcription and/or translation machinery (Wu and Horvitz, 1998a; Reddien and Horvitz, 2000). Specifically, we heat-shocked the *ced-12* transgenic embryos at ~3 hr before hatching. By this time, embryonic cell death has completed, and most apoptotic cells

Figure 2. Molecular Cloning of *ced-12*

(A) Rescue of the phenotype responsible for persisting cell corpses in *ced-12* mutant animals by germline transformation using genomic DNA clones. The genetic map near the *ced-12* locus on chromosome I is shown above. The cosmid clones and the Y106G6D and Y106G6E regions on the YAC clone Y106G6 were tested for the rescue of the *ced-12* engulfment defect. None of cosmid clones were found to rescue. Plus sign, rescue; minus sign, no rescue. Numbers in parentheses indicate the number of rescued lines and the total number of transgenic lines. The structure of the *ced-12* gene was deduced by comparing the sequences of genomic DNA and cDNAs. A 5′ SL1 or SL2 *trans*-spliced leader and a 3′ poly(A) tail are indicated at the ends of the transcript. Boxes represent exons. Solid boxes indicate the *ced-12* open reading frame; open boxes indicate the untranslated region. The transcription direction is from right to left.

(B) CED-12 protein sequence and alignment with human bA394O2.2 and *Drosophila* CG5336. Black boxes indicate amino acids identical between CED-12 and bA394O2.2 or CG5336. Gray boxes indicate similar amino acids between CED-12 and bA394O2.2 or CG5336. The putative SH3 interacting domain PPIP is underlined. The position of a point mutation identified in *ced-12(tp2)* is indicated. The alignment was performed using the ClustalW algorithm.

Table 2. Ectopic Expression of *ced-12* Rescues Ced-12 DTC Migration and Cell Corpse Engulfment Defects

A. Overexpression of *ced-12* Rescues the DTC Migration Defect in *ced-12* Mutants

Transgene[a]	Heat Shock	Percent of Animals Defective in DTC Migration (n = 100)[b]
hsp::gfp	−	65.2
hsp::gfp	+	56.8
hsp::ced-12	−	61.2
hsp::ced-12	+	8.6
lag-2:ced-12	−	4.8

B. Overexperession of *ced-12* before Cell Deaths Occur Rescues the Ced-12 Engulfment Defect

		Number of Unengulfed Corpses (n = 20)	
Transgene[a]	Heat Shock	Four-fold Embryo[c]	Germline[d]
hsp::gfp	−	32.2 ± 2.4	27.4 ± 8.7
hsp::gfp	+	29.4 ± 3.2	22.3 ± 6.3
hsp::ced-12	−	28.8 ± 2.6	28.7 ± 9.2
hsp::ced-12	+	1.8 ± 0.5	0.9 ± 1.4

C. Overexpression of *ced-12* after Cell Deaths Have Occurred Can Induce the Engulfment of Persistent Cell Corpses in *ced-12* Mutants

		Number of Persistent Corpses (n = 20)	
Transgene[a]	Heat Shcok	L1 larvae[e]	Germline[f]
hsp::gfp	+	18.4 ± 2.4	33.2 ± 15.8
hsp::ced-12	+	1.1 ± 0.7	2.5 ± 2.8

[a] The heat shock constructs were injected into *ced-12(tp2)* animals. Transgenic progeny were subjected to heat shock (+) or left at 20°C (−).
[b] The DTC migration defect was scored on the basis of the shape of the gonad in early adult.
[c] Transgenic animals were scored for the number of head cell corpses, which were generated during embryogenesis 11 hr after heat shock.
[d] The L4 transgenic animals were heat-shocked and scored for germline cell corpses 24 hr after heat shock.
[e] Transgenic animals were scored for the number of head cell corpses, which were generated during embryogenesis and had been dead for approximately 5 hr prior to the heat shock treatment.
[f] Transgenic animals that have entered to adulthood for 50 hr were heat shocked and scored for germline cell corpses 24 hr after heat shock.
Data of the corpse numbers are means ± SEM from two independent stably transmitting lines. gfp, green fluorescent protein.

in embryos have been dead for ~5 hr. Therefore, *ced-12* expression would be induced in all somatic cells but not persisting cell corpses. We found that such a heat shock treatment induces the engulfment of persisting cell corpses in *ced-12* mutants (Table 2C, L1 larvae column). The rescue of the *ced-12* engulfment defect by a *hsp::ced-12* transgene may be caused by the expression of this transgene in engulfing cells.

We used a similar strategy to examine whether *ced-12* acts within engulfing cells during the elimination of germline cell corpses. Ultrastructural studies show that germline cell corpses are engulfed by somatic sheath cells that contact the germline (Gumienny et al., 1999). It has been known that the *C. elegans* heat shock promoters do not drive the expression of transgenes in germline (Wu and Horvitz, 1998a; Reddien and Horvitz, 2000). We found that much fewer germline cell corpses persisting in *ced-12* animals carrying a *hsp::ced-12* transgene after heat shock compared with heat-shocked *ced-12* animals carrying a *hsp::gfp* transgene (Table 2B, germline column). We also tested if overexpression of *ced-12* could induce the engulfment of persisting germline cell corpses in *ced-12* mutants. We found that germline cell corpses that have persisted for hours or even days can still be engulfed by heat shock-induced expression of *ced-12* in *ced-12* animals carrying a *hsp::ced-12* transgene (Table 2C, germline column). These results further suggest that *ced-12* probably functions in the engulfing cells rather than cell corpses during the engulfment of cell corpses.

ced-12 Likely Acts within a Migrating DTC to Control DTC Migration along a Defined Path

Since *ced-12* is required not only for cell corpse engulfment but also for DTC migration, we further explored if *ced-12* functions in a migrating DTC during its migration along body wall musculature. We placed a wild-type *ced-12* gene under the control of the *lag-2* promoter, which drives the transgene expression in DTCs but not in muscle cells (Henderson et al., 1994), and examined the effect of transgene on DTC migration in *ced-12* mutants. Only 4.8% of *ced-12* animals carrying a *lag-2::ced-12* transgene showed a defect in DTC migration (Table 2A; 9/9 lines, about 50 animals from each line were examined), whereas ~75% of *ced-12* animals without the transgene (n = 101) have a defect in DTC migration. This result suggests that *ced-12* acts in a migrating DTC during DTC migration along body musculature.

ced-12 Likely Acts Upstream of *ced-10* to Control Cell Corpse Engulfment and DTC Migration

We utilized the *hsp::ced-12* transgene to examine whether overexpression of *ced-12* could bypass the functional requirement for the other known engulfment genes. In these experiments, we crossed the *hsp::ced-12* transgene into the genetic backgrounds of *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, or *ced-10* mutants and counted persisting cell corpses after heat shock. We

Table 3. Overexpression of *ced-12* Does Not Rescue the Engulfment Defect in *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, or *ced-10* Mutants

		Number of persistent corpses[b]					
Transgene	Heat Shock[a]	*ced-1(e1735)*	*ced-2(n1994)*	*ced-5(n1812)*	*ced-6(n1813)*	*ced-7(n2094)*	*ced-10(n3246)*
hsp::ced-12	−	24.5 ± 1.8	26.3 ± 2.4	41.3 ± 2.5	27.2 ± 3.3	30.2 ± 2.1	31.8 ± 2.9
hsp::ced-12	+	22.4 ± 2.9	27.1 ± 2.8	39.8 + 1.6	29.8 ± 1.3	33.0 ± 3.4	33.9 ± 2.4

[a] Transgenic animals were subjected to heat shock (+) or left at 20°C (−).
[b] Transgenic embryos were scored for the number of head cell corpses 11 hr after heat shock (see Experimental Procedures).
Data shown are means ± SEM from two independent stably transmitting lines. More than 20 animals were scored from each line.

found that none of the engulfing defect in these mutants could be rescued by *ced-12* overexpression upon the heat shock treatments (Table 3). We also performed reciprocal experiments to overexpress *ced-2*, *ced-5*, *ced-6*, *ced-7* and *ced-10* in a *ced-12* background and scored persisting cell corpses. We found that overexpression of *ced-10* but not *ced-2*, *ced-5*, *ced-6*, or *ced-7* rescues the engulfment defect in *ced-12* mutants after heat shock (Table 4). Overexpression of *ced-1* also does not rescue the engulfment defect in *ced-12* mutants (Z. Zheng and R.H. Horvitz, personal communication). In addition, we found that overexpression of a constitutively activated *ced-10* (*G12V*) cDNA that carries a mutation converting the twelfth amino acid from glycine to valine also rescues the *ced-12* engulfment defect after heat shock (Table 4). These results suggest that *ced-12* genetically acts upstream of *ced-10* during the engulfment of cell corpses.

We also examined the phenotype of DTC migration in transgenic animals. Approximately 73% *ced-12* animals carrying *hsp::ced-10* (n = 100) without heat shock exhibited a DTC migration defect; in contrast, only 8% *ced-12* animals carrying *hsp::ced-10* (two lines, n = 50 each line) were defective in DTC migration after heat shock. Taken together, our results are consistent with the model that *ced-12* acts upstream of *ced-10* in the genetic pathway for DTC migration and cell corpse engulfment.

CED-12/KIAA0281 Interact with CED-5/DOCK180 In Vitro

Our bypass experiments and the previous observations (Reddien and Horvitz, 2000) together showed that overexpression of one of *ced-2*, *ced-5*, *or ced-12* genes does not compensate for the loss of any of other two genes during cell corpse engulfment, consistent with the model that *ced-2*, *ced-5* and *ced-12* acts in the same step during the phagocytosis of cell corpses. CED-2 and CED-5 have been shown to interact in vitro (Reddien and Horvitz, 2000). We thus tested if CED-12 may interact with CED-2 or CED-5 using the GST pulldown assay. We divided and expressed *ced-5* cDNA in two separate fragments (CED-5N: amino acids 1–1414 and CED-5C: amino acids 1415–1781) because of the large size of CED-5 protein (~203.7 kDa) and tested the interaction of each fragment with CED-12. We found that ^{35}S-methionine-labeled CED-5C interacts with the C-terminal region of CED-12 tagged with glutathione-S-transferase (GST) (Figure 3A) and less strongly with the N-terminal

Table 4. Overexpression of *ced-10* Rescues the Ced-12 Engulfment Defect

Transgene	Genotype	Heat Shock[a]	No. of Persistent Corpses[b]
hsp::ced-2	*ced-2(n1994)*	−	26.1 ± 2.3
hsp::ced-2	*ced-2(n1994)*	+	0.5 ± 0.7
hsp::ced-2	*ced-12(tp2)*	−	32.4 ± 3.4
hsp::ced-2	*ced-12(tp2)*	+	29.9 ± 4.6
hsp::ced-5	*ced-5(n1812)*	−	42.2 ± 4.2
hsp::ced-5	*ced-5(n1812)*	+	0.5 ± 0.7
hsp::ced-5	*ced-12(tp2)*	−	31.6 ± 3.7
hsp::ced-5	*ced-12(tp2)*	+	33.1 ± 3.6
hsp::ced-6	*ced-6(n1813)*	−	27.4 ± 3.4
hsp::ced-6	*ced-6(n1813)*	+	2.2 ± 0.9
hsp::ced-6	*ced-12(tp2)*	−	33.2 ± 4.2
hsp::ced-6	*ced-12(tp2)*	+	31.8 ± 2.6
hsp::ced-7	*ced-7(n2094)*	−	30.5 ± 3.4
hsp::ced-7	*ced-7(n2094)*	+	1.2 ± 0.8
hsp::ced-7	*ced-12(tp2)*	−	30.2 ± 2.1
hsp::ced-7	*ced-12(tp2)*	+	33.0 ± 3.4
hsp::ced-10	*ced-10(n3246)*	−	29.6 ± 2.7
hsp::ced-10	*ced-10(n3246)*	+	1.9 ± 0.5
hsp::ced-10	*ced-12(tp2)*	−	32.2 ± 0.4
hsp::ced-10	*ced-12(tp2)*	+	1.1 ± 1.3
hsp::ced-10(G12V)	*ced-10(n1993)*	−	19.3 ± 3.7
hsp::ced-10(G12V)	*ced-10(n1993)*	+	2.5 ± 2.9
hsp::ced-10(G12V)	*ced-12(tp2)*	−	28.2 ± 3.4
hsp::ced-10(G12V)	*ced-12(tp2)*	+	2.9 ± 3.4

[a] Transgenic animals were subjected to heat-shock (+) or left at 20°C (−).
[b] Transgenic embryos were scored for the number of head cell corpses 11 hr after heat shock (see Experimental Procedures).
Data shown are means ± SEM from two independent stably transmitting lines. More than 20 animals were scored from each line.

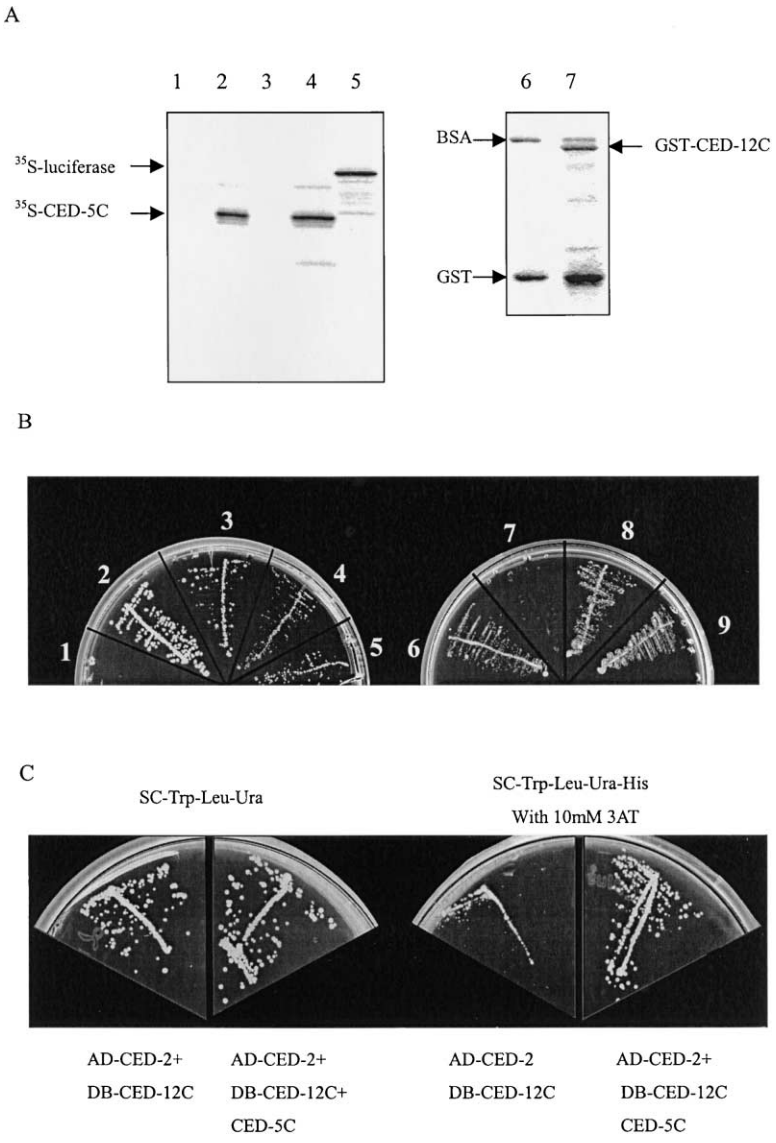

Figure 3. CED-12 Interacts with CED-5 and Forms a Ternary Complex with CED-2 In Vitro

(A) CED-12 physically interacts with CED-5 in a GST pulldown assay. In the autoradiograph (lanes 1–5), ^{35}S-labeled-CED-5C (lanes 1, 2, and 4) or ^{35}S-labeled-luciferase (lanes 3 and 5) were incubated with GST alone (lane1) or GST-CED-12C (lanes 2 and 3) and analyzed for binding by SDS-PAGE and autoradiography as described in Experimental Procedures. Lanes 4 and 5 represent 10% of the input of ^{35}S-labeled-CED-5C or ^{35}S-labeled-luciferase, respectively. In the Comassie blue staining of the gel (lanes 6 and 7), approximately equal amounts of GST alone (lane 6) or GST-CED-12C (lane 7) used in the binding reactions were loaded.

(B) CED-12/KIAA0281 interacts with CED-5/DOCK180 in the yeast two-hybrid system. Constructs expressing DB-CED-5 and AD-CED-12 or AD-CED-12C fusions are transformed into the yeast strain MaV203 to generate DB-CED-5+AD-CED-12 or DB-CED-5+AD-CED-12C transformants. Constructs expressing indicated fusion proteins were transformed in pair to the yeast strain MaV203 to generate respective transformants: (1) negative control (vectors alone), (2) positive control (AD-E2F and DB-Rb) (Vidal and Brachmann, 1996), (3) AD-CED-2+DB-CED-5, (4) AD-CED-12+DB-CED-5, (5) AD-CED-12C+DB-CED-5, (6) AD-CED12C+DB-CED-5C1, (7) AD-CED-12C+DB-CED-5C2, (8) AD-KIAA0281C+DB-DOCK180C, (9) AD-DOCK180C+DB-KIAA0281C. Resulting transformants were streaked on SC-Trp-Leu plates+15 mM 3AT. Growth on the plate indicates interaction of fusion proteins.

(C) Formation of the CED-2-CED-5 and CED-12 complex is detected in the yeast three-hybrid system. Constructs expressing AD-CED-2 and DB-CED-12C fusions are cotransformed with the construct expressing nonhybrid CED-5C or vector alone into the yeast strain MaV203 to generate CED-5C+AD-CED-2+DB-CED12C and AD-CED-2+DB-CED12C transformants, respectively. Resulting transformants were streaked on SC-Trp-Leu-Ura plates or on SC-Trp-Leu-Ura-His+10 mM 3AT. Growth on the SC-Trp-Leu-Ura-His+10 mM 3AT plate indicates interaction of fusion proteins.

region of CED-12-GST fusion (see Experimental Procedures). We also tested the interaction between CED-5 and CED-12 using the yeast two-hybrid system. In this experiment, we fused full-length CED-5 or CED-5C to the GAL4 activation domain (AD) and full length CED-12 or CED-12C to the GAL4 DNA binding domain (DB) and tested their interaction in the yeast strain MaV203 using the *HIS-3* reporter gene. Interactions between full-length CED-12 or CED-12 C with full-length CED-5 or CED-5C were observed in the yeast two-hybrid system (Figure 3B). Since CED-5C contains a few proline-rich motifs very close to the C terminus, we tested if these motifs are necessary for binding to CED-12 by dissecting CED-5C further into two fragments CED-5C1 (amino acids 1415–1689, without proline-rich motifs) and CED-5C2 (amino acids 1690–1781, with proline-rich motifs) and testing each fragment for CED-12 binding. We found that CED-12 interacts with CED-5C1 but not with CED-5C2 (Figure 3B). This result suggests that CED-5 proline-rich motifs are dispensable for binding to CED-12. No interaction between CED-12 with CED-2 or CED-10 has been detected in the GST pulldown assay or the yeast two-hybrid system.

We further examined if human CED-12-like protein may interact with DOCK180 as CED-12 with CED-5. We tested the interaction of C-terminal regions of DOCK180 (DOCK180C, amino acids 1114–1865) and human KIAA0281 (KIAA0281C, amino acids 94–727) using the yeast two-hybrid system. The result that KIAA0281C interacts with DOCK180C (Figure 3B) suggests that KIAA0281 may also act through the conserved CrkII/Dock180/Rac pathway and may therefore regulate cell corpse engulfment and/or cell migration as CED-12 does in *C. elegans*.

CED-5 Interacts with CED-2 and CED-12 Simultaneously In Vitro

We further explored if CED-5 may interact with CED-2 and CED-12 simultaneously to form a CED-2-CED-5-CED-12 complex. We used the yeast three-hybrid system, a modified yeast two-hybrid system to test the formation of the ternary complex. In this experiment, we fused CED-2 to AD and CED-12C to DB and then tested their interaction in the presence or absence of the non-hybrid CED-5C protein in the yeast strain MaV203 using the *HIS-3* reporter gene (see Experimental Procedures). We found that interaction between AD-CED-2 and DB-CED-12C is detected in the presence of CED-5C but not in the absence of CED-5C (Figure 3C). One simple interpretation is that a ternary complex is formed which is composed of CED-2, CED-5C, and CED-12C, with CED-5C bridging CED-2 and CED-12C.

Discussion

Studies in *C. elegans* and mammalian cells showed that CED-2/CrkII, CED-5/DOCK180, and CED-10/Rac are components of a conserved pathway responsible for the engulfment of apoptotic cells and migration of specific cells (Cheresh et al., 1999; Albert et al., 2000). $\alpha_v\beta_5$ integrin receptor has been shown to recruit the CrkII-Dock180-Rac1 complex for phagocytosis of apoptotic cells by human dendritic cells and kidney epithelial cells (Albert et al., 2000). There are two integrin α-subunit genes *ina-1* (Baum and Garriga, 1997) and *pat-2* (Williams and Waterston, 1994) and a single integrinβ-subunit gene *pat-3* (Gettner et al., 1995) in *C. elegans*. No persistent embryonic cell corpses were observed in *ina-1*, *pat-2*, or *pat-3* mutants (Y.-C.W., unpublished data). Since *pat-2* and *pat-3* mutants arrest during early embryogenesis, function of these genes beyond early embryogenesis is difficult to determine based upon mutant analysis. However, our data suggest that integrin receptors are not absolutely required for the phagocytosis of apoptotic cells during early *C. elegans* development.

Engulfment of apoptotic cells in *C. elegans* is controlled by two partially redundant pathways: *ced-1*, *ced-6* and *ced-7* in one and *ced-2*, *ced-5* and *ced-10* in the other (Ellis et al., 1991a; Reddien and Horvitz, 2000). Several lines of evidence suggest that *ced-12* functions in the pathway defined by *ced-2*, *ced-5* and *ced-10*, but not the one defined by *ced-1*, *ced-6* and *ced-7*. First, *ced-12*; *ced-5* (null) double mutants and *ced-5* (null) single mutants exhibited similar numbers of cell corpses. In contrast, *ced-12*; *ced-7* (null) double mutants exhibited greatly enhanced numbers of cell corpses than *ced-12* or *ced-7* (null) single mutants. Second, like *ced-2* and *ced-5*, *ced-12* genetically acts upstream of *ced-10*. Third, CED-12 physically interacts with the CED-2 and CED-5 complex in vitro. Forth, mutations in *ced-2*, *ced-5* and *ced-10* but not in *ced-1*, *ced-6* and *ced-7* cause the DTC migration defect (Wu and Horvitz, 1998b; Reddien and Horvitz, 2000) and can suppress the vulvaless phenotype caused by dominant mutations in *lin-24* or *lin-33* (Ellis et al., 1991b). We found that the *ced-12(tp2)* mutation results in the DTC migration defect and can also suppress the vulvaless phenotype of *lin-24* and *lin-33* mutants (Y.-C.W. and S.-F. Han, unpublished data).

We showed that overexpression of *ced-12* cannot bypass the requirement for *ced-2* or *ced-5* and that overexpression of neither *ced-2* nor *ced-5* can bypass the requirement for *ced-12* during the engulfing process. These results in the combination with the previous finding that overexpression of *ced-2* or *ced-5* cannot rescue the loss of the other gene (Reddien and Horvitz, 2000) suggest that *ced-2*, *ced-5* and *ced-12* act in the same step in the genetic pathway that regulates cell corpse engulfment. In addition, we found that similar to *ced-2* and *ced-5* genes, *ced-12* also acts upstream of *ced-10* in a genetic pathway that controls the engulfment process. Furthermore, we showed that CED-12 physically interacts with CED-5 and forms a ternary complex with CED-2 in vitro, with CED-5 bridging CED-2 and CED-12. On the basis of these results, we propose that CED-12 forms a ternary complex with CED-2 and CED-5, which is recruited to the plasma membrane to activate CED-10/Rac, leading to the cytoskeletal reorganization in an engulfing cell as the engulfing cell extends pseudopodia around an apoptotic cell. A similar signaling event may occur as a migrating DTC moves along the body musculature.

CED-10 contains a membrane targeting signal in the C-terminal region (Chen et al., 1993) and appears localized to the plasma membrane (Chen et al., 1996). The translocation of the CED-2-CED-5-CED-12 complex to plasma membrane as suggested in our model likely plays an important role for CED-10/GTPase activation. Purified DOCK180 and Rac proteins can interact physically, suggesting that the direct association between CED-5 and CED-10 may be important for CED-10 activation (Kiyokawa et al., 1998; Nolan et al., 1998). In addition, a guanine nucleotide exchange factor (GEF) is required to activate GTPase to a GTP-bound state from an inactive GDP-bound state. UNC-73 may be a candidate GEF for CED-10 activation during DTC migration, since *unc-73* mutants have a DTC migration defect (Y.-C.W. and L.-C.C., unpublished data) and UNC-73 has a GEF activity for mammalian Rac in vitro (Steven et al., 1998).

CED-5 and DOCK180 each contain a few proline-rich motifs in the C terminus, and the interaction of DOCK180 with CrkII has been shown to be mediated by one of these motifs in vitro (Hasegawa et al., 1996). We found that CED-5 proline-rich motifs are dispensable for binding to CED-12, consistent with the finding that the delPS mutant of DOCK180 (amino acids 1–1472), deficient in proline-rich motifs, can still bind to ELMO1 in vitro (Gumienny et al., 2001 [October 5 issue of *Cell*]). These results suggest that CED-5/DOCK180 can interact with CED-12/KIAA0281 without binding to CED-2/CrkII.

We showed that CED-12 is a new component of the conserved CrkII/DOCK180/Rac signaling pathway, which controls cell migration and the engulfment of apoptotic cells in *C. elegans* and mammals. We found that KIAA0281 interacts with DOCK180 as CED-12 with CED-5. We suggest that human CED-12 counterparts, at least KIAA0281, also act in the conserved GTPase signaling pathway to regulate the cytoskeleton dynamics, as CED-12 does in *C. elegans*. Further genetic and biochemical study of CED-12 in nematodes will likely shed light on how Rac and other GTPase family proteins act to regulate cell motility, cell shape, and movement in general.

Experimental Procedures

Nematodes

C. elegans strains were grown at 20°C, except where otherwise noted. All mutations were generated in a strain Bristol N2 background, the standard wild-type strain (Brenner, 1974). The following mutations were used: LG I: *dpy-5(e61)*, *unc-29(e193)*, *lin-11(n566)*, *ced-12(tp2)*, *fog-3(q470)*, *ced-1(e1735*, *n2089)*, *unc-75(e950)*; LG III: *ced-4(n1162)*, *ced-6(n1813*, *n2095)*, *ced-7(n1892*, *n2094)*; LG IV: *ced-2(n1994*, *e1752)*, *ced-10(n3246*, *n1993)*, *ced-5(n1812*, *n2002)*, *ced-3(n717)*, *dpy-20(e1282)*. Deficiencies used were as follows: *qDf5*, *qDf8*, *qDf9*, *qDf10*, *qDf15*, and *mnDf111* (Ellis and Kimble, 1995).

Isolation of *ced-12(tp2)* Mutants

L4 hermaphrodites were mutagenized with ethyl methanesulfonate (EMS) as previously described (Brenner, 1974). F2 progeny were screened using Nomarski microscopy for mutants with abnormal gonadal shape. The *ced-12(tp2)* mutants were outcrossed to wild-type N2 animals at least three times.

Genetic Mapping of *ced-12*

The mutation *ced-12(tp2)* was mapped to linkage group I by linkage to *unc-29*. To better position *ced-12* on the genetic map, we mapped *ced-12* with respect to deficiencies in the region. We found that the deficiency *qDf5* takes out *ced-12* but the deficiencies *qDf8*, *qDf9*, *qDf10*, *qDf15*, and *mnDf111* do not. The deficiency-mapping data indicate that *ced-12* maps right of *fog-3*. We further confirmed this result by the following cross: from the *dpy-5(e61) ced-12(tp2) unc-75(e950)/ fog-3(q470)* hermaphrodites, 2/187 Unc Fog non-Dpy recombinants segregated *ced-12*. The following cross shows that *ced-12* maps left of *lin-11*: from *lin-11(n566)/dpy-5(e61) ced-12(tp2) unc-75(e950)* hermaphrodites, 1/512 Dpy Lin non-Unc recombinant segregated *ced-12*.

Rescue Experiments

We performed injections as previously described (Mello et al., 1992), using the pRF4 (150 μg/ml) and pTG96 plasmids (100 μg/ml) as coinjection markers. The pRF4 plasmid carries the mutated collagen gene *rol-6(su1006)* and confers a dominant roller phenotype (Mello et al., 1992). The pTG96 plasmid contains a *sur-5::GFP* fusion that is expressed in all somatic cells (Gu et al., 1998).

For genetic rescue experiments, we injected cosmids (20 μg/ml) or PCR genomic fragment (2 μg/ml) to *ced-12* mutants. To determine the extent of rescue, we counted the number of persisting cell corpses in the head region of 4-fold stage embryos from the stably transmitting lines using Nomarski optics as previously described (Ellis et al., 1991a). Nonrescued embryos have about 29 cell corpses. Embryos with few or no cell corpses were considered to be rescued.

For the heat shock experiment, we injected heat shock constructs (50 μg/ml) to wild-type animals. To express transgenes in the engulfment-defective mutant background, extrachromosomal arrays were crossed to *ced-1(e1735)*, *ced-2(n1994)*, *ced-5(n1812)*, *ced-6 (n1813)*, *ced-7(n2094)*, *ced-10(n3246)*, and *ced-12(tp2)* to generate corresponding transgenic mutant strains.

We injected the *lag-2::ced-12* plasmid at 20 μg/ml into *ced-12* mutants using only the pTG96 plasmid as coinjection marker. Transgenic animals with GFP expression were scored for the phenotype of DTC migration. The gonadal arms with inappropriate turns, twists, positions, or lengths were considered defective.

Molecular Biology

Standard molecular biological procedures were followed (Sambrook et al., 1989). For the genomic rescue experiment, DNA fragments in the Y106G6E region were amplified from *C. elegans* genomic DNA using Expand long template PCR system (Roche) as recommended. The primers 5′-CACTTGAGCCGTAGAATTGGAGCTATTAGAGCA GAGG-3′ and 5′-CCTATCTGGCCTCTCACACTGGGCACCTTCCG CAATG-3′ were used to amplify the DNA fragment Ed. Sequences of the primers used for amplification of other genomic fragments are available upon request.

To determine the 5′ end of *ced-12* message, we performed RT-PCR experiment (Roche) from *C. elegans* mixed-staged mRNA using the S5 primer 5′-CGGTTTATCAGCCATCAATCCATACGAATCATCT TCTG-3′ in combination with SL1 or SL2 primers.

Plasmid Construction

The *ced-12* cDNA was excised from the phage clone yk359f9 (from Y. Kohara) and cloned to pPD49.78 to generate *hsp::ced-12*. The *lag-2* promoter region was amplified by PCR as previously described (Henderson et al., 1994) and used to replace the heat shock promoter in *hsp::ced-12* to generate *lag-2::ced-12*. We designed 5′-GTCGTT GGTGACGTCGCCGTCGGTAAAC-3′ as forward primer and 5′-GTTTTACCGACGGCGACGTCACCAACGAC-3′ as reverse primer to generate the single mutation G12V in the *ced-10* cDNA using the Quickchange site-directed mutagenesis kit (Stratagene), and the resulting DNA was introduced into the pPD49.78 vector to generate the *hsp::ced-10G12V* construct.

Heat Shock Experiments

To overexpress *ced-12* after embryonic cell death has completed during embryogenesis, worm plates containing embryos at all developmental stages were subjected to heat shock in 33°C incubator for 1.5 hr. Freshly hatched L1 larvae were transferred to new plates one hour after the heat shock treatment to synchronize the animals that were heat-shocked 2.5 hr prior to hatching. These larvae were scored for cell corpses in the head region 5 hr after the heat shock.

To overexpress *ced-12* before germline cell death occurs, transgenic animals were heat-shocked and assayed as previously described (Wu and Horvitz, 1998a).

To overexpress *ced-12* after germline cell death has occurred and cell corpses have been generated, transgenic animals that entered adulthood for approximately 50 hr were incubated at 33°C for 1.5 hr and recovered at 20°C. Cell corpses in the gonad were counted 24 hr after the heat shock treatment.

To test for bypass of cell corpse engulfment, we heat-shocked mixed-stage embryos at 33°C for 1.5 hr and scored the number of cell corpses in 4-fold embryos 10.5 hr after the heat shock treatment.

To test for rescue of the DTC migration defect, transgenic animals were treated and scored as previously described (Wu and Horvitz, 1998a). The DTC migration pattern was inferred from the gonadal morphology of young adults.

In Vitro Binding Assay

We dissected CED-12 into two fragments to generate GST fusion proteins fused to N- or C-terminal fragments of CED-12 due to the low solubility of the GST fusion protein fused to the full-length CED-12. *ced-12* cDNA fragments corresponding to the amino acids 1–395 and 396–738 were cloned to pGEX-4T3 to generate the GST-CED-12N and GST-CED-12C fusion proteins, respectively. The resulting fusion protein was expressed and purified as manufacturer suggested (Pharmacia). The *ced-5* cDNA fragments corresponding to the amino acids 1–1414 and 1415–1781 were cloned to pCITE 4a (+) (Novagen) and transcribed and translated to obtain ^{35}S-CED-5N and ^{35}S-CED-5C as recommended (Promega). ^{35}S-labeled-CED-5N (or ^{35}S-labeled-CED-5C or ^{35}S-labeled luciferase as control) was incubated with GST-CED-12N (or GST-CED-12C or GST as control), bound to glutathione-Sepharose-4B (Pharmacia), for 1 hr at 4°C in 500 μl binding buffer (142.5 mM NaCl, 10 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 1 mM EDTA, 0.25% IGEPAL CA-630, 2.5 mg/ml BSA). Protein complexes were washed three times with 1 ml binding buffer and one time with 1 ml binding buffer without BSA and analyzed by SDS-PAGE (15%) and autoradiography.

Yeast Two- and Three-Hybrid Experiments

Related cDNAs were cloned into pPC86 and pDBLeu (GIBCO) to produce AD and BD fusion proteins, respectively. In the yeast two-hybrid assay, specific pairs of constructs expressing AD and BD fusion proteins were transformed to the yeast strain MaV203 (GIBCO). In the yeast three-hybrid assay, the *ced-5* cDNA fragment coding for CED-5C was first cloned to pVT-101U vector, and the DNA fragment containing the *ced-5* cDNA and the 3′ and 5′ regions of the alcohol dehydrogenase gene (ADH) of the resulting construct was cut out and inserted to the pRS316 vector to generate the construct pRSADHCED-5C. The AD-CED-2 and DB-CED-12C fusion constructs were cotransformed with the pRSADHCED-5C construct

or an empty pRS316 vector to the yeast strain MaV203 (GIBCO). In both two- and three-hybrid experiments, transformants were selected on synthetic complete medium lacking tryptophan and leucine (SC-Trp-Leu) or lacking tryptophan, leucine, and uracil (SC-Trp-Leu-Ura), respectively. Individual colonies were streaked on SC-Trp-Leu or SC-Trp-Leu-Ura in the presence of 3AT to test for the activation of the reporter gene *HIS3*.

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