variance from repeated images of the puncta in the absence of stimulation. The coefficient of variation of measurement, c_m, was found to be roughly constant (0.05) as the mean intensity increased. As each ΔF measurement was a difference between two images (initial and final), the variance is the sum of the variance for each image. The residual fluorescence was typically <10 units (which is comparable to fluorescence of a single vesicle), and was similar across different boutons. Therefore, the measurement variance for successive peaks is given by:

$$c_m^2((\mu_k + r)^2 + r^2)$$

where μ_k is the centre of the kth peak and r is the residual fluorescence. The variance in fluorescence due to vesicle-size variability is given by:

$$c_v^2 \mu_k^2 k$$

where c_v is the coefficient of variation of vesicle surface area (0.2, estimated from ultrastructure[9]). Therefore, the total variance for the kth peak is:

$$\sigma_k^2 = c_m^2((\mu_k + r)^2 + r^2) + c_v^2 \mu_k^2 k \quad (1)$$

The height of the peaks can be obtained from the release probability distribution $w(p)$. For a given synapse with release probability p, the distribution of the number of releases is given by a binomial distribution. For a population of synapses with a release probability distribution $w(p)$, this distribution (for five stimuli) becomes:

$$g(k) = \int_0^1 dp \binom{5}{k} p^k (1-p)^{5-k} w(p) \quad (2)$$

The release-probability distribution $w(p)$ has been measured for these synapses[7]:

$$w(p) = \frac{\lambda^2}{2} p e^{-\lambda p} \qquad \lambda = 8;$$

Therefore, $g(k)$ can be determined exactly, and will give the areas under each of the peaks in the ΔF distribution. Knowing the height and the variance of each peak (as a function of the mean), we can fit the staining distribution with only one free parameter, μ_k.

$$P(f) = \sum_{k=1}^{5} g(k) e^{-\frac{1}{2}\left(\frac{f-\mu_k}{\sigma_k}\right)^2} \quad (3)$$

Maximum-likelihood fits gave a peak spacing of 8.4 fluorescence units for the staining histogram (Fig. 2b) and 8.2 fluorescence units for the partial destaining histograms (Fig. 4a, b).

Received 19 September; accepted 5 January 1997.

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Acknowledgements. We thank C. Boyer for preparation of cultures. V.N.M. is a Helen Hay Whitney Foundation fellow. This research was supported by grants from the NIH and HHMI to C.F.S.

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C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180

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During programmed cell death, cell corpses are rapidly engulfed[1]. This engulfment process involves the recognition and subsequent phagocytosis of cell corpses by engulfing cells[1–4]. How cell corpses are engulfed is largely unknown. Here we report that *ced-5*, a gene that is required for cell-corpse engulfment in the nematode *Caenorhabditis elegans*[5], encodes a protein that is similar to the human protein DOCK180 and the *Drosophila melanogaster* protein Myoblast City (MBC), both of which have been implicated in the extension of cell surfaces[6]. *ced-5* mutants are defective not only in the engulfment of cell corpses but also in the migrations of two specific gonadal cells, the distal tip cells. The expression of human DOCK180 in *C. elegans* rescued the cell-migration defect of a *ced-5* mutant. We present evidence that *ced-5* functions in engulfing cells during the engulfment of cell corpses. We suggest that *ced-5* acts in the extension of the surface of an engulfing cell around a dying cell during programmed cell death. We name this new family of proteins that function in the extension of cell surfaces the CDM (for CED-5, DOCK180 and MBC) family.

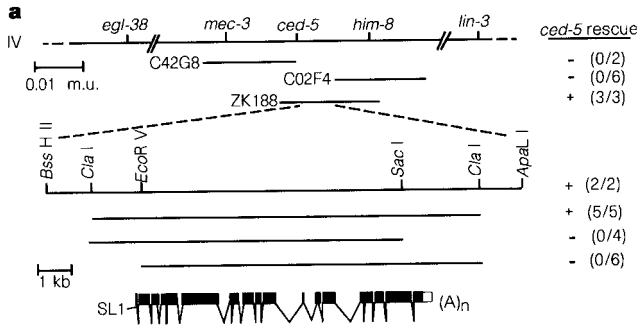
In *C. elegans*, the engulfment of cell corpses is regulated by at least six genes, *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7* and *ced-10* (*ced*, for cell death abnormal)[5,7]. Mutations in any of these genes block the engulfment of many cell corpses and hence cause a mutant phenotype that is characterized by the persistence of cell corpses. Ultrastructural studies revealed that these mutations prevent extension of the membranes of the engulfing cells around the dying cells[5,7]. We observed that *ced-2*, *ced-5* and *ced-10* mutants, but not *ced-1*, *ced-6* or *ced-7* mutants, are also defective in the specific migration of the gonadal distal tip cells (DTCs). This defect has been independently observed by K. Nishiwaki and M. Hengartner (personal communications). The DTCs are located at the tips of the two gonadal arms and guide the extension of each growing gonadal arm during larval development[8,9]. In *ced-2*, *ced-5* and *ced-10* mutants, the DTCs frequently make extra turns or stop prematurely, which results in abnormally shaped gonads. For example, 77 per cent of *ced-5(n1812)* mutant animals ($n = 120$) showed abnormal DTC migration.

To understand how CED-5 functions in both the engulfment of cell corpses and the migration of the DTCs, we cloned the gene. *ced-5* maps between *mec-3* and *him-8* on chromosome IV (ref. 5). These two genes define an approximately 100-kilobase (kb) region on the physical map[10,11] (P. Meneely, personal communication; Fig. 1a). We used genomic DNA clones from this interval to rescue the Ced-5 mutant phenotype of persisting cell corpses in germline transformation

Table 1 Expression of DOCK180 rescues the DTC-migration defect in *ced-5* mutants

hsp transgene*	*ced-5*	DOCK180	GFP	none
Animals with DTC-migration defect (%)	8 ± 2	29 ± 5	69 ± 4	72 ± 5

* The heat-shock constructs were injected into *ced-5(n1812); unc-76(e911)* animals (see Methods). All data were obtained after heat shock. The DTC-migration defect was scored on the basis of the shape of the gonad in early adults. Wild-type animals ($n = 181$) show no defect in DTC migration following heat shock. Data are means ± s.e.m. from at least two stably transmitting lines. At least 80 animals were scored from each line. GFP, green fluorescent protein[17].



experiments and localized the rescuing activity to a 10.6-kb genomic DNA fragment (Fig. 1a). We isolated *ced-5* complementary DNAs using this fragment and defined the 5′ end of the *ced-5* messenger RNA using the RACE (for rapid amplification of cDNA ends) method. The sequences of these cDNAs revealed a 1,781-amino-acid open reading frame, an SL1 *trans*-spliced leader sequence found at the 5′ end of many *C. elegans* transcripts[12], and a 3′-end poly(A) tract, indicating that we had identified a complete *ced-5* transcription unit. Northern blot analysis, using a *ced-5* cDNA as probe, showed a single band of 5.6 kb (data not shown), consistent with the size of the full-length *ced-5* cDNA. The expression of a *ced-5* DNA in *ced-5(n1812)* animals under the control of *C. elegans* heat-shock promoters (*hsp*)[13] rescued the defects in both DTC migration (Table 1) and cell-corpse engulfment (Table 2; see below), indicating that the *ced-5* cDNA encodes a functional

Figure 1 Molecular cloning of the *ced-5* gene. **a**, Rescue of the phenotype responsible for persisting cell corpses in *ced-5* mutant animals by germline transformation using genomic DNA clones. The genetic map of the *ced-5* region of chromosome IV is shown. Cosmid clones and subclones were tested for rescue of the *ced-5* engulfment defect. +, Rescue; –, no rescue. Numbers in parentheses indicate the number of rescued lines and the total number of transgenic lines. A partial restriction map of one subclone with *ced-5*-rescuing activity is shown. The structure of the *ced-5* gene was deduced by comparing the sequences of genomic DNA and cDNAs. A 5′ SL1 *trans*-spliced leader and a 3′ poly(A) tail are indicated at the ends of the transcript. Boxes represent exons: black boxes indicate the *ced-5* open reading frame; the white box indicates the untranslated region. m.u., Map unit **b**, CED-5 protein sequence and alignment with the human DOCK180 (ref. 6) and *Drosophila* MBC protein[14]. Black boxes indicate amino acids that are identical in CED-5 and DOCK180 or MBC. Grey boxes indicate amino acids that are identical in only DOCK180 and MBC. Arrows indicate the positions of nonsense mutations or a frameshift mutation, and vertical bars indicate the positions of splice-site mutations found in *ced-5* mutant alleles. Codon changes are indicated in parentheses.

CED-5 protein. We identified molecular lesions in six *ced-5* mutant alleles: three, *n1812*, *n2002* and *n2691*, have nonsense mutations (at codon positions 28, 962 and 1,145, respectively); one allele, *mu57*, has a single-base deletion (in codon 216); two other alleles, *n2098* and *n2099*, have single-base changes (in the splice-acceptor sequences of the sixth and eighth introns, respectively) (Fig. 1b). The allele *ced-5(n1812)* is likely to be null as this premature stop codon (UAA) eliminates more than 98% of the CED-5 protein and the engulfment defect of *n1812* homozygotes is indistinguishable from that of *n1812/sDf2* heterozygous animals[5] (*sDf2* is a deletion that spans the *ced-5* locus).

The 1,781-amino-acid sequence of CED-5 is most similar to the sequence of the human protein DOCK180. These proteins share 26% identity over their entire lengths (Fig. 1b). CED-5 and DOCK180 both share significant sequence similarity with the amino-acid sequences predicted from the *Drosophila* gene *mbc*[14] (Fig. 1b), the human cDNA clone KIAA0209 (ref. 15), the yeast open reading frame L9576.7 (GenBank accession number 664878) and a mouse expressed sequence tag (EST) (GenBank accession number AA110899).

To determine whether human DOCK180 and CED-5 are functionally related as well, we tested the ability of *hsp*::DOCK180 to rescue the Ced-5 mutant phenotype. Inducing the expression of DOCK180 rescued the DTC-migration defect (Table 1) but not the corpse-engulfment defect (data not shown). This partial rescue by DOCK180 of the Ced-5 mutant phenotype could indicate either a need for a higher level of gene activity for cell-corpse engulfment than for DTC migration or a bifunctional role for CED-5, such that DOCK180 can supply the function needed for DTC migration but not that for corpse engulfment. As DOCK180 rescued the CED-5 defect in DTC migration, DOCK180 and CED-5 are at least in this respect functionally interchangeable.

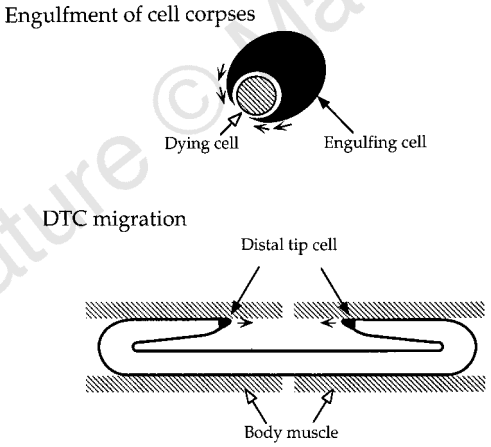


Figure 2 Cell-corpse engulfment and DTC migration are similar processes. In each case, the surface membrane of a cell (black) extends along the surface of another cell (hatched). The small arrows near the black cells indicate the directions of cell-surface extension. Only the relevant parts of body muscles are shown.

DOCK180 was isolated on the basis of its interaction with the cytoskeleton-associated adaptor protein CRK[6], which consists mainly of SH2 and SH3 domains[16] and has been implicated in integrin-mediated signalling and cell movement[17]. Vitronectin, a member of the integrin superfamily, has been implicated in the engulfment of cell corpses in mammals[18]. The expression of DOCK180 in 3T3 fibroblasts can cause these cells to extend their surfaces and adopt flat and polygonal shapes, indicating that DOCK180 can regulate the extension of cell surfaces[6]. *Drosophila* MBC is necessary for myoblast fusion and for the migrations of some epithelial cells, both of which require the extension of cell surfaces, presumably through reorganization of the cytoskeleton[14,19]. We propose that CED-5, DOCK180 and MBC all function to mediate the extension of cell surfaces and that CED-5 does so by acting in engulfing cells during the phagocytosis of cell corpses as well as in migrating DTCs. *ced-5* mutants appear to be normal in the migrations of other cells (for example, the P1–P12 precursor cells and the HSN neurons) as well as in axonal outgrowth and in cell fusions involved in the development of the hypodermal syncytium (ref. 5; and data not shown). Thus, CED-5 is likely to be involved in a specific type of membrane extension that is common to cell-corpse engulfment and DTC migration.

As already noted, we rescued the engulfing defect of a *ced-5* mutant using heat shock to induce the expression of a wild-type *ced-5* transgene (Table 2). We believe that this rescue was effected by the expression of *ced-5* in engulfing cells rather than in cell corpses, for two reasons. First, the defect in the mutant animals subjected to heat-shock was rescued even at late stages of larval development. The finding that expression of *ced-5*, even at a time when cell corpses have presumably long been dead (for hours in L1 and L2 larval stages, or even days in L3 and L4 larval stages), still rescued the defect (Table 2; larvae column) suggests that *ced-5* functions not in cell corpses but rather in engulfing cells. Second, using the green fluorescent protein (GFP) as a reporter[20], we found that *ced-5(n1812)* animals carrying a GFP transgene under the control of these *C. elegans* heat-shock promoters did not express GFP in persisting cell corpses following heat shock, but they did express GFP in other somatic cells, including engulfing cells, throughout larval development (data not shown). This finding indicates that the transcriptional and/or translational machineries are probably inactive in persisting cell corpses. We conclude that rescue of the *ced-5* engulfment defect by the heat-shock-inducible *ced-5* transgene is caused by the expression of this transgene in engulfing cells.

We used a similar strategy to explore whether *ced-5* functions in engulfing cells during the removal of germline cell corpses in addition to its function in engulfing cells during the removal of somatic cell corpses. Germline cell corpses are engulfed by the gonadal sheath cells in a *ced-5*-dependent manner (M. Hengartner, E. Hartwieg and H.R.H., unpublished observations). After heat shock of *ced-5* animals carrying an *hsp*::GFP transgene, we detected GFP expression in gonadal sheath cells but not in the germ line (data not shown). This finding is consistent with previous observations that these *C. elegans* heat-shock promoters do not drive the expression of transgenes in the germline[21] (A. Fire, personal communication). *ced-5* animals carrying an *hsp*::*ced-5* transgene

Table 2 Expression of a *ced-5* cDNA rescues the engulfing defect of *ced-5* mutants

hsp transgene*	Heat shock	No. of cell corpses in head†					No. of cell corpses in germ line‡
		4-fold embryo	L1	L2	L3	L4	
ced-5	+	0.8 ± 2	6 ± 4	3 ± 2	3 ± 2	2 ± 2	4 ± 5
	–	34 ± 2	30 ± 5	27 ± 3	21 ± 4	15 ± 3	27 ± 4
GFP	+	33 ± 2	30 ± 3	28 ± 2	20 ± 2	16 ± 4	24 ± 6

* The heat-shock constructs were injected into *ced-5(n1812)*; *unc-76(e911)* animals (see Methods). Mixed-staged transgenic progeny were subjected to heat shock (+) or left at 20 °C (–).
† Transgenic animals were scored for the number of head cell corpses, which were generated during embryogenesis, and for developmental stage 10 h after heat shock.
‡ Number of cell corpses in each gonadal arm of the transgenic animal was scored 24 h after heat shock (see Methods). Data shown are means ± s.e.m. from two independent stably transmitting lines. More than 20 animals were scored from each line. GFP, green fluorescent protein[17].

had significantly fewer germline cell corpses persisting after heat shock compared with heat-shocked *ced-5* animals carrying an *hsp*::GFP transgene (Table 2). This observation further suggests that *ced-5* functions in engulfing cells and not in persisting cell corpses during programmed cell death.

We have shown that *ced-5* is likely to act in engulfing cells during cell-corpse engulfment and also is required for the normal migration of DTCs. Both the engulfment of cell corpses[5,22] and cell migration require a cell to extend its surface in a polarized fashion (Fig. 2), which is consistent with our hypothesis that CED-5, like DOCK180 and MBC, functions in cell-surface extension. On the basis of this consideration and our finding that DOCK180 rescued the DTC-migration defect of *ced-5* mutant animals, we propose that CED-5, DOCK180 and MBC define a new evolutionarily conserved gene family involved in the extension of cell surfaces. We call this family CDM. We propose that *ced-5* acts in phagocytosis in response to the recognition of dying cells during programmed cell death. By analogy to MBC, *ced-5* could mediate the cytoskeletal reorganization that occurs as an engulfing cell extends its cell surface around a dying cell. We suggest that, like other proteins involved in programmed cell death[23–25], CED-5 and the proteins with which it interacts during the engulfment of cell corpses are evolutionarily conserved. □

Methods

Germline transformation experiments. For genomic rescue experiments, DNA was co-injected into *ced-5(n1812)* animals at concentrations of 25–50 μg ml^{-1} with the dominant roller marker pRF4 (50 μg ml^{-1}) as previously described[26]. We scored persistent cell corpses in the head regions of L2 or L3 roller animals from stably transmitting transgenic lines using Nomarski optics as previously described[26]. Non-rollers or non-rescued rollers had about 20–25 cell corpses. Roller animals containing 0–5 corpses were scored as rescued for the *ced-5* engulfment defect.

To construct *hsp*::*ced-5*, we did a three-component ligation reaction: we ligated the *Kpn*I–*Xho*I fragment from the plasmid pC5OKBA, which contains the 5′ half of the *ced-5* cDNA, with the *Xho*I–*Apa*I fragment from the pC583 construct, which contains the 3′ half of the *ced-5* cDNA, to the *Kpn*I–*Apa*I fragment from *hsp* vectors pPD49.78 or pPD49.83 (from A. Fire). To construct *hsp*::GFP, we excised the *Xba*I–*Apa*I fragment from the plasmids pPD96.04 (from A. Fire) and Tu#61 (from M. Chalfie); these plasmids contain the GFP gene with and without a nuclear localization signal, respectively. We cloned the fragments into the vectors pPD49.78 and pPD49.83, previously digested with *Nhe*I and *Apa*I. To make *hsp*::DOCK180 constructs, we excised the *Xho*I fragment from plasmid pBlDOCK180, which contains a DOCK180 cDNA[6], blunt-ended the fragment and cloned it into the vectors pPD49.78 and pPD49.83 through their *Eco*RV sites. The heat-shock constructs were co-injected into *ced-5(n1812);unc-76(e911)* animals at 50 μg ml^{-1} with the *unc-76*-rescuing plasmid p76-16B[27] to establish transgenic lines and with the *egl*-5:GFP plasmid pSC212 to identify transgenic embryos (50 μg ml^{-1} each) (A. Chisholm and H.R.H., unpublished results).

Heat-shock experiments. To determine the extent of rescue of the *ced-5* engulfment defect in somatic cell death, we heat-shocked mixed-staged transgenic animals for 1.5 h at 33 °C, followed by 10 h recovery at 20 °C. We counted persisting cell corpses in the heads of transgenic animals at different stages. To test for rescue of the engulfment defect in germline cell death, transgenic animals were given two pulses of heat shock over 36 h. In brief, we picked L4 transgenic animals and exposed them for 1.5 h to 33 °C. Following heat shock, animals that entered adulthood were selected and transferred to an incubator at 20 °C for recovery. After 10.5 h, we exposed these animals for 1.5 h to 33 °C. We counted germline cell corpses in each gonadal arm 22.5 h after this treatment. To test for rescue of the DTC-migration defect, L2 transgenic animals were selected and exposed to three 1.5-h heat-shock treatments at 33 °C, separated by two 10.5-h recovery intervals at 20 °C to span an approximately 25-h period during DTC migration. The DTC-migration pattern was inferred from the gonadal morphology of young adults.

Characterization of *ced-5* genomic and cDNA structure. The 10.6-kb *Cla*I genomic fragment containing *ced-5* rescuing activity was used to screen a mixed-staged cDNA library[28] and an embryonic cDNA library[29]. We determined the sequences of *ced-5* genomic DNA and three overlapping *ced-5* cDNAs, using a Sequenase 2.0 kit (USB). We identified the 5′ end of *ced-5* mRNA using a 5′ RACE system (GIBCO-BRL) and identified mutations in *ced-5* alleles by determining the sequences of genomic regions produced by amplification with the polymerase chain reaction.

Received 15 October 1997; accepted 6 January 1998.

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Acknowledgements. We thank members of H.R.H.'s laboratory and C.-L. Wei for their comments, D. Hall and E. Hedgecock for sharing unpublished results regarding DTC migration, R. Barstead and P. Okkema for cDNA libraries, A Coulson for cosmids, H. Hasegawa and M. Matsuda for the DOCK180 cDNA clone, E. James for determining DNA sequences of the RACE product, S. Glass for the *ced-5* alleles *n2098* and *n2099* and J. Harris and C. Kenyon for *mu57*. H.R.H. is an Investigator of the Howard Hughes Medical Institute. The accession number for the *ced-5* sequence is AF038576.

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