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THE ENGULFMENT PROCESS OF PROGRAMMED CELL DEATH IN *CAENORHABDITIS ELEGANS*

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■ **Abstract** Programmed cell death involves the removal of cell corpses by other cells in a process termed engulfment. Genetic studies of the nematode *Caenorhabditis elegans* have led to a framework not only for the killing step of programmed cell death but also for the process of cell-corpse engulfment. This work has defined two signal transduction pathways that act redundantly to control engulfment. Signals expressed by dying cells probably regulate these *C. elegans* pathways. Components of the cell-corpse recognition system of one of the *C. elegans* pathways include the CED-7 ABC transporter, which likely presents a death ligand on the surface of the dying cell; the CED-1 transmembrane receptor, which recognizes this signal; and the CED-6 adaptor protein, which may transduce a signal from CED-1. The second *C. elegans* pathway acts in parallel and involves a novel Rac GTPase signaling pathway, with the components CED-2 CrkII, CED-5 DOCK180, CED-12 ELMO, and CED-10 Rac. The cell-corpse recognition system that activates this pathway remains to be characterized. In *C. elegans*, and possibly in mammals, the process of cell-corpse engulfment promotes the death process itself. The known mechanisms for cell-corpse engulfment leave much to be discovered concerning this fundamental aspect of metazoan biology.

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INTRODUCTION

The widespread naturally occurring process of cell death is characterized by the condensation of cytoplasm and nuclei, nuclear fragmentation, chromatin condensation, membrane blebbing, retention of intact organelles, vacuole formation, and DNA fragmentation (Kerr et al. 1972, Wyllie 1980). These morphological characteristics define the process of apoptosis. Genetic analyses of naturally occurring or programmed cell death in the nematode *Caenorhabditis elegans* have led to the identification of genes that control apoptosis. Mammalian counterparts of the components of the *C. elegans* cell-death pathway have integral roles in cell death (Metzstein et al. 1998), demonstrating that the process of programmed cell death in *C. elegans* is similar to that in humans. In *C. elegans*, the EGL-1 (BH3-only family) protein initiates cell death (Conradt & Horvitz 1998) by antagonizing the cell-death inhibitory function of the CED-9 protein (BCL-2 family) (Hengartner & Horvitz 1994). CED-9 inhibits death by antagonizing CED-4 (APAF-1-like) (Yuan & Horvitz 1992, Zou et al. 1997), which can promote death by activating the CED-3 protease (caspase) (Yuan et al. 1993).

Apoptotic cells are removed by other cells. Phagocytosis, as defined by Metchinkoff in 1883, involves the uptake of large particles or cells by other cells (Rabinovitch 1995). The removal of apoptotic cells is distinct from other types of phagocytosis in that the particle being taken up carries self-antigens and does not elicit an inflammatory response, which occurs with other types of phagocytosis, such as pathogen clearance (Ren & Savill 1998). Because the removal of cells dying by programmed cell death might occur by mechanisms distinct from those of phagocytosis in the immune system, the term engulfment sometimes has been used as a general descriptor of the cell-death removal process (Ellis et al. 1991a). In *C. elegans* there are no professional phagocytic cells, and cells dying by programmed cell death are engulfed by neighboring cells of differing types (e.g.,

hypodermis, pharyngeal muscle, gonadal sheath cells) (Robertson & Thomson 1982, Sulston et al. 1983, Gumienny et al. 1999).

Programmed cell death is important for many aspects of development and homeostasis, including (*a*) the removal of structures with transient functions, (*b*) tissue sculpting and morphogenesis, (*c*) adjustment of cell numbers, and (*d*) removal of damaged and immune cells that have the potential to harm the organism (Glücksmann 1951, Jacobson et al. 1997). Engulfment is critical for cell removal. An estimated 10^{10} cells die every day in each of us (Heemels 2000). The consequent cell corpses and debris can result in autoimmunity, tissue damage, and/or inflammation if spilled into surrounding tissue (Albert et al. 1998, Ren & Savill 1998, Sauter et al. 2000). Fortunately, as indicated by the observation that in vivo apoptotic cells are essentially always inside other cells, apoptotic cells are engulfed before they degenerate (Kerr et al. 1972, Wyllie et al. 1980, Robertson & Thomson 1982). In sites of inflammation, macrophages are important in killing and ingesting cells during tissue repair (Duffield 2003). Defects in such macrophage functions could lead to excessive or persistent inflammation (Savill 1997) and the production of autoantibodies, as is seen in the autoimmune disease systemic lupus erythematosus (SLE) (Ring & Lakkis 1999). In mice with perturbed engulfment Lupus-like autoimmunity occurs (Scott et al. 2001, Cohen et al. 2002), and in Lupus-prone mice impaired engulfment occurs (Potter et al. 2003). Furthermore, the macrophages of human SLE patients are defective in engulfment (Herrmann et al. 1998). Engulfment thus may be important for the prevention of disease.

Despite the wealth of knowledge about the molecular mechanisms that control the execution of cell death (Metzstein et al. 1998, Meier et al. 2000, Shi 2002, Adams 2003), comparatively little has been discovered about the control of the engulfment of dying cells. Studies of engulfment in *C. elegans* in recent years have identified molecular mechanisms that control the recognition and removal of programmed cell deaths. The mechanisms revealed so far, like those controlling the execution of programmed cell death in *C. elegans*, are conserved throughout the metazoa.

THE GENETIC PATHWAY FOR ENGULFMENT IN *C. ELEGANS*

Two Parallel Pathways Control Engulfment in *C. elegans*

Mutations in seven engulfment genes, *ced-1*, *-2*, *-5*, *-6*, *-7*, *-10*, and *-12*, have been identified that perturb the engulfment of cells dying by programmed cell death in *C. elegans* (Hedgecock et al. 1983, Ellis et al. 1991a, Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a). In these mutant animals unengulfed cell corpses persist and can easily be observed and quantitated using Nomarski optics (Figure 1). Double mutant analyses have indicated that the engulfment genes may define two partially redundant pathways (Ellis et al. 1991a, Chung et al. 2000, Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a) (Figure 2). Because no mutation in any of the seven engulfment genes causes a complete block in engulfment, animals

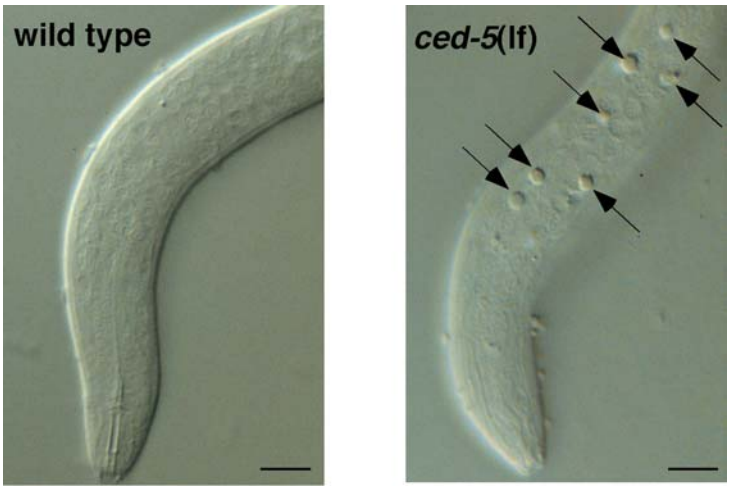


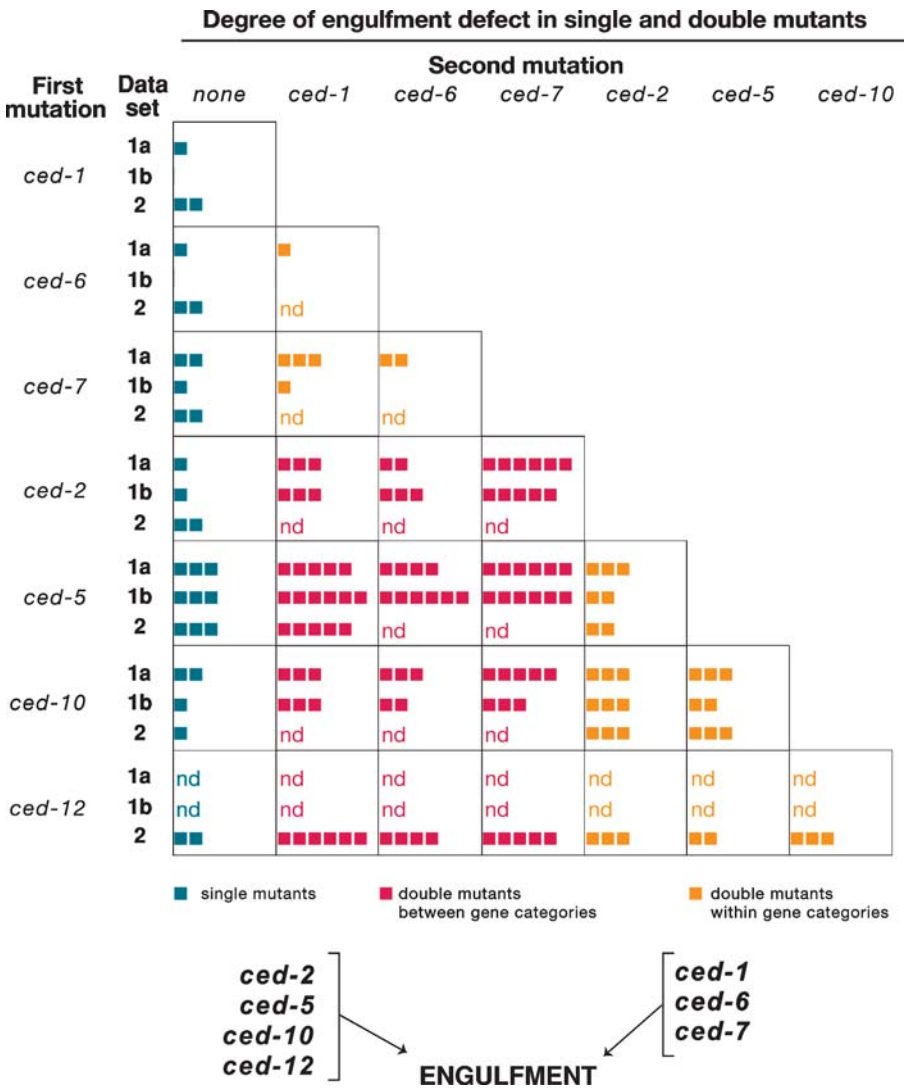
Figure 1 Cell corpses persist in *C. elegans* engulfment mutants. Left, young wild-type larvae at the four-cell gonad first larval (L1) stage. Right, *ced-5*(*n1812*) mutant at the four-cell gonad L1 larval stage. Persistent unengulfed corpses are indicated with arrows. Nomarski optics. Bar = 10 μm.

carrying mutations in two genes that control different engulfment processes could have a stronger engulfment defect than animals carrying a mutation in only one of these genes. For example, the degree of the engulfment defect in *ced-1*; *ced-2* and in *ced-1*; *ced-5* double mutants is greater than that in *ced-1*, *ced-2*, or *ced-5* single

Figure 2 Two partially redundant pathways control engulfment in *C. elegans*. Three sets of data (1a, 1b, 2) from two different publications are summarized to indicate the reported interactions among the engulfment genes. Data sets 1a and 1b are the original data that grouped engulfment genes into two pathways (Ellis et al. 1991a). Boxes represent the degrees of the engulfment defects in single and double mutants; nd, not determined. Data set 1a, the average number of corpses in the pharynges of L1 larvae (Ellis et al. 1991a): <1.5 = ■, 1.6–3.0 = ■■, 3.1–4.5 = ■■■, 4.6–6.5 = ■■■■, 6.6–8.5 = ■■■■■, >8.5 = ■■■■■■. Data set 1b, the percentage of NSM sister corpses that persist as unengulfed corpses following cell death. There are two NSM sister corpses normally produced and engulfed per animal in the wild-type. (Ellis et al. 1991a): 0% = (-), 1–10% = ■, 11–20% = ■■, 21–30 = ■■■, 31–40 = ■■■■, 41–50% = ■■■■■, and >50% = ■■■■■■. Data set 2, the average number of corpses in the heads of L1 larvae (Zhou et al. 2001a): 10.1–20.0 = ■, 20.1–30.0 = ■■, 30.1–35.0 = ■■■, 35.1–40.0 = ■■■■, 40.1–45.0 = ■■■■■, >45.0 = ■■■■■■. Other publications corroborate the trends of data set 2 (Gumienny et al. 2001, Wu et al. 2001). The alleles used in these studies are *ced-1(e1735)*, *ced-6(n1813)*, *ced-2(e1752)*, *ced-5(n1812)*, *ced-10(n1993)*, and *ced-12(n3261)*. *ced-7(n1892)* was used in data sets 1a and 1b, and *ced-7(n1996)* was used in data set 2. (*bottom*) The *ced-1*, *-6*, and *-7* genes may act together and partially redundantly with the *ced-2*, *-5*, *-10*, and *-12* genes.

mutants. Therefore, *ced-1* likely controls a different process than does *ced-2* or *ced-5*. In contrast, because the degree of the engulfment defect in *ced-2*, *ced-5* double mutants is similar to that of the *ced-2* and *ced-5* single mutant animals, *ced-2* and *ced-5* probably do not control distinct processes during engulfment. These data suggest that *ced-1* controls one and *ced-2* and *ced-5* control a second engulfment pathway.

The interpretation of such double-mutant analyses in general can be complicated if the alleles used do not completely eliminate gene function. For instance, double mutants with mutations that only partially eliminate the functions of two genes in



the same pathway could have more severe defects than either of the single mutants. In this case, the two genes could be incorrectly assigned to different pathways. For example, *ced-12*; *ced-2* double mutants have a stronger engulfment defect than do *ced-2* and *ced-12* single mutants, yet *ced-2* and *ced-12* are believed to act in the same pathway (Figure 2) (see below for explanation). In the double-mutant analyses summarized in Figure 2, alleles that may cause incomplete loss-of-function were used for the genes *ced-2* (Reddien & Horvitz 2000), *ced-6* (Liu & Hengartner 1998), *ced-10* (Reddien & Horvitz 2000), and *ced-12* (Zhou et al. 2001a) for data sets 1a, 1b, and 2. An allele that may cause incomplete loss-of-function of *ced-7* (Wu & Horvitz 1998a) was used for data sets 1a and 1b only. Nonetheless, double mutants involving the genes *ced-2*, *-5*, *-10*, and *-12* have defects of approximately the same degree as or less than that of animals with the presumptive complete loss-of-function *ced-5* allele *n1812* (Wu & Horvitz 1998b) (Figure 2). Therefore, because the *ced-5(n1812)* mutation should completely eliminate the activity of the pathway in which *ced-5* acts, *ced-2, ced-10*, and *ced-12* do not appear to control a pathway distinct from that controlled by *ced-5*. In contrast, *ced-5(n1812)* (as well as mutations in *ced-2, -10, -12*) can cause increased engulfment defects with mutations in *ced-1*, *-6*, or *-7* (Figure 2). Therefore, the genes *ced-1, ced-6*, and *ced-7* do not control the same process as *ced-5*. Mutations in *ced-2*, *-5*, *-10*, or *-12* but not in *ced-1*, *-6*, or *-7* also cause abnormal migrations of the gonadal distal tip cells (DTCs) (Wu & Horvitz 1998b, Reddien & Horvitz 2000, Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a). This observation further supports the grouping of *ced-2*, *ced-5*, *ced-10*, and *ced-12* into a single pathway separate from *ced-1*, *ced-6*, and *ced-7* and the hypothesis that at least two pathways control engulfment.

Several double mutants carrying mutations in *ced-1*, *ced-6*, and *ced-7* have somewhat greater defects than corresponding single mutants, making the grouping of *ced-1*, *-6*, and *-7* into a single pathway more tentative (Ellis et al. 1991a). However, defects conferred by *ced-1*, *-6*, or *-7* mutations are typically enhanced to a greater degree by mutations in *ced-2*, *-5*, *-10*, or *-12* than by mutations in each other (Figure 2). That a single pathway involving CED-1, CED-6, and CED-7 proteins exists is partially supported by molecular analyses (see below). Ellis et al. (1991a) interpreted their data by stating: "Thus, we can conclude that there are at least two processes that are important in engulfing dead cells, one that involves *ced-2*, *ced-5*, and *ced-10*, and one or more that involve the other *ced* genes." This view still seems valid.

How might partial redundancy between the engulfment pathways be explained? The complete and essentially invariant cell lineage of *C. elegans* has been described, and consequently the location and developmental origin of every cell that normally dies is known (Sulston & Horvitz 1977, Sulston et al. 1983). The two pathways can affect the engulfment of the same cells, so the apparent genetic redundancy cannot be explained by distinct sets of persisting corpses (Ellis et al. 1991a). In engulfment mutants some cell corpses can be engulfed normally (Hoeppner et al. 2001), and some cell corpses can persist from embryogenesis into adulthood, i.e., approximately three days (Ellis et al. 1991a). Therefore,

engulfment defects do not appear to reflect the slow engulfment of all cell corpses, and the additive engulfment defect in animals with mutations in both pathways cannot be explained by additive decreases in the engulfment rate of all cell corpses. These observations suggest that mutations in engulfment genes decrease the likelihood that engulfment will be initiated and that this likelihood is further decreased by combining mutations in partially redundant engulfment genes.

The Same Genes Function in the Engulfment of Apoptotic and Necrotic Cells

Cells that would otherwise live can be induced to die in *C. elegans* either by apoptosis (Shaham & Horvitz 1996) or by a nonapoptotic mechanism reminiscent of necrotic cell death in mammals (Hall et al. 1997). For example, cell death can be induced by certain alleles of *mec-4* that cause the MEC-4 amelioride-sensitive sodium channel to be hyperactive (Driscoll & Chalfie 1991). Mutations in genes that control programmed cell death (e.g., *ced-3*, *ced-4*, *ced-9*) do not affect these necrosis-like deaths (Ellis & Horvitz 1986, Korswagen et al. 1997, Berger et al. 1998, Chung et al. 2000), indicating that the molecular mechanisms of programmed cell death and necrosis-like cell death are largely distinct. *C. elegans* necrotic cells are removed from the organism, but much more slowly than are cells dying by programmed cell death (Chalfie & Sulston 1981, Hall et al. 1997). All seven genes involved in the engulfment of cells dying by programmed cell death also act in the removal of necrotic corpses (Chung et al. 2000). This observation indicates that some common mechanisms are used for the recognition and removal of cells dying by programmed cell death and necrosis-like cell deaths, a conclusion with important implications concerning the molecular mechanisms of the cell-corpse recognition controlled by engulfment genes (see below).

DYING CELLS SIGNAL TO ENGULFING CELLS

The CED-1 Receptor and the CED-7 ABC Transporter Function in the Cell-Corpse Recognition Step of the *C. elegans* CED-1, -6, and -7 Pathway

C. elegans CED-1 is a large transmembrane protein with many extracellular EGF-repeats and a short novel intracellular candidate signaling domain. CED-1 functions in engulfing rather than in dying cells, and a CED-1::GFP fusion protein clusters around cell corpses, indicating CED-1 probably acts as a receptor for dying cells (Zhou et al. 2001b). Because the CED-1 intracellular domain is not necessary for CED-1::GFP to cluster around dying cells, CED-1 probably accumulates around the dying cell simply by binding to it. The intracellular domain of CED-1 is necessary for CED-1 function in vivo, indicating it may play a role in transducing

signals (Zhou et al. 2001b). Both an NPXY (Asn-Pro-any amino acid-Tyr) motif and a YXXL (Tyr-any amino acid-any amino acid-Leu) motif in the CED-1 intracellular domain are important for engulfment (Zhou et al. 2001b). Because the tyrosine residues of NPXY and YXXL motifs can be phosphorylated (Pawson & Scott 1997), CED-1 may transduce signals through a cytoplasmic factor that interacts with phosphorylated tyrosines (Zhou et al. 2001b).

The identity of the CED-1 mammalian ortholog remains unclear. The extracellular domain of CED-1 is most similar to the mammalian SREC (scavenger receptor from endothelial cells) protein (Zhou et al. 2001b), but SREC lacks similarity to the intracellular domain of CED-1. The LDL receptor-related protein (LRP), also known as CD91, may have a role in the engulfment of apoptotic cells (Ogden et al. 2001) and shares some similarity to CED-1, including NPXY and YXXL motifs in an intracellular domain (Su et al. 2002). CD91(LRP) and CED-1 can physically interact with the same proteins (see discussion of the CED-6 protein below) (Su et al. 2002). However, CD91(LRP) does not have an extracellular domain similar to that of CED-1. CED-1 contains a candidate cysteine-repeat domain in its extracellular region; this domain is known as an EMI domain because of its presence in a protein family named EMILIN (Callebaut et al. 2003). Other proteins, such as mEGF10, have been identified as candidate CED-1 orthologs because they have a similar predicted domain structure, including an EMI domain (Callebaut et al. 2003).

CED-7 encodes an ABC transporter similar to mammalian ABC1 (Wu & Horvitz 1998a). ABC transporters control the ATP-dependent translocation across cell membranes of a variety of substrates, including sugars, proteins, and phospholipids (Higgins 1992), and can affect membrane polarity (Smit et al. 1993, Ruetz & Gros 1994). ABC1 has been implicated in Tangier disease, which is characterized by abnormal lipoprotein production and lipid trafficking defects (Orso et al. 2000). ABC1 is expressed in macrophages engaged in engulfment (Luciani & Chimini 1996) and is needed for apoptotic cell removal in the mouse (Hamon et al. 2000). ABC1 may affect the distribution of phosphatidylserine (PS) on cell surfaces (Hamon et al. 2000). PS exposure at the outer surface of cell membranes is a well-established attribute of apoptotic cells (Fadok et al. 1992, Martin et al. 1995, van den Eijnde et al. 1998) and is important for engulfment (Fadok et al. 2001). By analogy to known functions of ABC transporters, CED-7 might affect membrane phospholipid asymmetry. CED-1::GFP fails to cluster around cell corpses in *ced-7* mutants, indicating that CED-7 may transport a ligand recognized by CED-1 (Zhou et al. 2001b). These results are consistent with a model proposed by Zhou et al. in which CED-7 normally promotes the exposure of PS on the surface of a dying cell, and CED-1 recognizes PS (Zhou et al. 2001b). This hypothesis needs direct testing. Mosaic analysis indicates that *ced-7* is required in both the dying and the engulfing cell (Wu & Horvitz 1998a). In mammalian cell culture, engulfing cells, as well as dying cells, can expose PS on their outer membranes (Marguet et al. 1999, Callahan et al. 2000). Perhaps CED-7 is needed in *C. elegans* in both engulfing and dying cells to regulate PS exposure.

The PSR-1 Phosphatidylserine Receptor and NEX-1 Annexin I May Promote Removal of Corpses in *C. elegans*

Two candidate *C. elegans* engulfment genes, *psr-1* and *nex-1*, were identified on the basis of predicted protein sequence homology to proteins implicated in engulfment in mammals. The *C. elegans* PSR-1 protein is similar to the putative human phosphatidylserine receptor, PSR (Wang et al. 2003). Human PSR is a novel transmembrane protein that specifically recognizes PS (Fadok et al. 2000) and can cluster around cell corpses (Arur et al. 2003). However, it has been noted that PSR has some characteristics of a nuclear protein (Clissold & Ponting 2001, Savill et al. 2003, Cui et al. 2004), and subcellular localization studies are needed to confirm the presence of PSR on engulfing cell membranes and its action in engulfment. Consistent with the idea that PSR acts in engulfment, PSR knockout mice have accumulations of unengulfed apoptotic cells in the lungs and brain and produce macrophages with partially impaired engulfment capabilities in cell culture (Li et al. 2003). *C. elegans psr-1* mutants have been reported to have a subtle increase in the number of cell corpses present at multiple time points during embryonic development, possibly as a consequence of a slight increase in the longevity of those corpses (Wang et al. 2003). Rescue experiments indicate that *psr-1* functions in engulfing cells and that human PSR may rescue the slight defect in engulfment in *psr-1(lf)* animals (Wang et al. 2003). Together, these observations suggest a minor role for PSR-1 as an engulfment receptor in *C. elegans*. The loss of *psr-1(lf)* function causes a subtle enhancement of the engulfment defects conferred by mutations in *ced-1*, *-6*, *-7*, or *-10* but not by mutations in *ced-2*, *-5*, or *-12* (Wang et al. 2003). Overexpression of wild-type *ced-2*, *-5*, *-10*, or *-12* but not of *ced-1*, *-6*, or *-7* can rescue the engulfment defect of *psr-1*(*lf*) animals. These findings suggest that *psr-1* may act together with and upstream of *ced-2*, *-5*, *-10*, and *-12* and in parallel to *ced-1*, *-6*, and *-7*. However, if PSR-1 does activate the CED-2, -5, -10, and -12 pathway, it cannot be the major receptor in this pathway because the engulfment defects of *psr-1*(*lf*) animals are far weaker than those of animals defective in *ced-2*,*-5*,*-10*, or *-12*. Furthermore, it has been proposed that CED-7 exposes PS on the surface of dying cells and that this PS is recognized by CED-1 (see above), so PS might be expected to act in a pathway with CED-7 and CED-1. In any event, the primary receptor for the CED-2, -5, -10, -12 pathway remains to be identified.

The *C. elegans* NEX-1 protein is similar to the human PS-binding protein annexin I (Arur et al. 2003). annexin I translocates from the cytosol to the surface of apoptotic cells and can colocalize in patches with exposed PS (Arur et al. 2003). Inhibition of *annexin I* function in Jurkat cells by siRNAs perturbs their engulfment when induced to die in a cell culture engulfment assay. These observations indicate that annexin I may act as a signal from dying cells triggering their engulfment. RNA-mediated genetic interference (RNAi) (Fire et al. 1998) of the *C. elegans nex-1* gene has been reported to perturb engulfment (Arur et al. 2003), and the *nex-1*(*RNAi*) phenotype is fairly robust, with an expressivity about one

third to one half of that caused by strong loss-of-function mutations in *ced-7* and *ced-5*, respectively. However, animals with a putative *nex-1* deletion appear to display no engulfment defect (B. Galvin & H. R. Horvitz, unpublished observations; L. Neukomm & M. Hengartner, unpublished observations). Perhaps *nex-1*(*RNAi*) has an effect in addition to or other than reducing *nex-1* activity or perhaps the putative *nex-1* deletion allele fails to eliminate *nex-1* function.

Caenorhabditis elegans CED-3 May Generate Engulfment Signals

Whether caspases directly generate engulfment signals is central to our understanding of engulfment yet remains unanswered and not extensively tested in any organism. To illustrate the issues involved and identify observations that call for further experimentation, we discuss below observations that relate to the question of whether the CED-3 caspase in *C. elegans* and caspases in other organisms generate engulfment signals.

Are engulfment signals generated by a specific process or as a nonspecific consequence of cell death? In other words, do cells triggered to die activate a process, such as caspase cleavage of a specific protein, that generates engulfment signals? Or, does some cellular feature change after death by an inactive process that indirectly allows engulfment, as would be the case, for example, if the asymmetry of membrane lipid types prevents engulfment and requires energy-dependent maintenance? That engulfment initiates early in the death process in many organisms suggests that engulfment signals are specifically generated (Kerr et al. 1972). In *C. elegans*, for instance, engulfment can initiate, as determined using electron microscopy, before the completion of the cell division that generates a cell that will die (Robertson & Thomson 1982). Cells dying by programmed cell death in *C. elegans* are therefore becoming engulfed long before they are dead and likely before they are grossly disrupted.

Assuming that engulfment signals are specifically generated, we can consider whether such engulfment signals depend upon CED-3 caspase activity. In *ced-3* mutants, most cells that normally die instead survive; such surviving cells have been referred to as undead, a word introduced by L. Avery (personal communication). Undead cells are not engulfed and can differentiate and function as normal cells (Ellis & Horvitz 1986, Avery & Horvitz 1987, White et al. 1991, Reddien et al. 2001), indicating that *ced-3* may be necessary for the generation of one or more engulfment signals. However, there may be something about live cells, such as contacts with other cells, cell shape, contact with extracellular matrix, or membrane structure, that prevents them from being engulfed even if they still send engulfment signals. Because there are alleles of *ced-3* that perturb CED-3 function only weakly (*weak lf*), it is possible to ask whether cells that die with a partial reduction of CED-3 are engulfed normally. In *ced-3*(*weak lf*) animals most but not all cells that should die by programmed cell death still die, albeit more slowly than in the wild-type, and a single dead cell has been observed to persist unengulfed (Hoeppner et al. 2001).

This observation is consistent with the idea that there is an impaired generation of engulfment signals as a consequence of reduced CED-3 function. No engulfment of dying cells is observed in *ced-3*(*weak lf*); *ced-7*(*lf*) double mutants, whereas some engulfment is observed in *ced-7*(*lf*) animals (Hoeppner et al. 2001). Perhaps there is an additive defect in engulfment caused by the impaired generation of engulfment signals that results from reduced CED-3 function and the loss of the CED-7 ABC transporter.

In contrast, other findings suggest that CED-3 is not needed to generate engulfment signals. For instance, a low level of cell death occurs in mutants with a *ced-3* deletion allele that completely removes the protease-encoding region of *ced-3*. These dying cells are typically removed in *ced-3*(*lf*) animals but persist unengulfed in *ced-3*(*lf*) animals also carrying a mutation in the engulfment gene *ced-1* (Shaham et al. 1999). Thus the CED-1 engulfment receptor can recognize CED-3 caspase-independent signals for engulfment. More importantly than whether CED-3 per se is needed for the generation of engulfment signals is whether caspase activity, in general, is needed. Although CED-3 is considered the main caspase involved in cell death in *C. elegans*, other caspase-like proteins are encoded by the *C. elegans* genome: *csp-1*, *csp-2*, and *csp-3* (Shaham 1998). Genetic studies of *csp-1*, *csp-2*, and *csp-3* are needed to determine whether a low level of cell death and engulfment can be entirely caspase independent. Additionally, because *ced-1* (as well as the other six established engulfment genes) is involved in the engulfment of necrotic cell deaths, and *ced-3* has no apparent role in necrosis (see above), the CED-1 receptor probably can recognize at least some dying cell signals generated independently of CED-3. It has been noted that CED-1 might recognize more than one type of signal, one generated downstream of CED-3 for apoptotic cells and a distinct signal exposed on necrotic cells, or that CED-1 might recognize the same signal on apoptotic and necrotic cells, with the latter signal generated nonspecifically (e.g., by loss of membrane integrity) in necrotic cells (Hengartner 2001).

It is unresolved whether mammalian caspases directly promote engulfment. Because PS exposure is likely important for engulfment, mammalian cell culture data concerning whether caspases are needed for PS exposure during apoptosis have relevance to this issue. Some data argue that cells in culture can produce engulfment signals in the absence of caspase function. For instance, delayed PS exposure occurs coincident with delayed abnormal death in *Caspase-3* knockout cells (Woo et al. 1998) and in *Apaf-1*, *Caspase-9*, and *Caspase-3* knockout cells induced to die by BH3-only proteins (Cheng et al. 2001). In addition, *Caspase-3*-defective cells induced to die by etoposide can be engulfed by macrophages (Turner et al. 2003). Notable caveats concerning these data include (*a*) there are 13 mouse caspases (Thornberry & Lazebnik 1998), so other caspases might produce engulfment signals and (*b*) cell culture assay conditions may not reflect in vivo situations. For instance, macrophages may need to chemotax to apoptotic cells in vivo for engulfment (see below). Other data argue that caspases are important for engulfment signals. For example, Jurkat cells inhibited for caspase function by ZVAD-fmk fail to expose annexin I (Arur et al. 2003). Additionally,

normal apoptotic cells can trigger the chemotaxis of engulfing cells in culture (Lauber et al. 2003), and *Caspase-3*-deficient cells induced to die with UV irradiation, staurosporine, and mitomycin C are defective in this macrophage chemotaxis (Lauber et al. 2003). Caspases may be necessary in vivo for the generation of chemotactic signals required for engulfment.

TWO SIGNAL TRANSDUCTION PATHWAYS ACTIVATE ENGULFMENT

The CED-6 Adaptor Protein May Transduce Signals from CED-1

CED-6 may define a novel type of adaptor protein and has a human counterpart hCED-6 (Liu & Hengartner 1998, Su et al. 2002). Overexpression of hCED-6 promotes engulfment in mammalian cell culture (Smits et al. 1999) and can partially replace *ced-6* functionally in *C. elegans* (Liu & Hengartner 1999). CED-6 and hCED-6 each have a candidate phosphotyrosine-binding (PTB) domain; PTB domains can bind tyrosine-phosphorylated NPXY (Asn-Pro-any amino acid-Tyr) motifs to mediate protein-protein interactions (Kavanaugh et al. 1995). An NPXY motif is found in the intracellular domain of CED-1 (Zhou et al. 2001b). Both CED-6 and hCED-6 also have a leucine zipper domain that mediates homodimerization (Su et al. 2000).

Mosaic analysis indicates CED-6 functions within engulfing cells (Liu & Hengartner 1998). Overexpression experiments indicate that CED-6 can partially bypass the requirement for CED-1 and CED-7 (Liu & Hengartner 1998). Experiments with CED-1::GFP indicate that CED-6 is not required for the clustering of CED-1 around cell corpses (Zhou et al. 2001b). These data indicate that CED-6 may act downstream of CED-1 and CED-7 to transduce, directly or indirectly, signals from the CED-1 receptor (Figure 3*a*). Both hCED-6 and CED-6 can bind to CED-1, and these interactions depend on the CED-1 NPXY PTB-binding motif (Su et al. 2002). hCED-6 directly binds a possible CED-1 functional counterpart in mammalian cells, CD91(LRP), in a manner dependent upon the CD91(LRP) NPXY phosphorylation motif (Su et al. 2002). Thus, CED-6-like proteins are candidates to directly transduce signals from phosphorylated CED-1-like proteins during engulfment in *C. elegans* and other organisms. The nature and functional relevance of the interaction between CED-6 proteins and CED-1 or CD91(LRP) proteins require further exploration for at least two reasons. First, because CD91(LRP) can bind multiple PTB-containing proteins (e.g., Shc and Dab1) (Howell et al. 1999, Gotthardt et al. 2000, Barnes et al. 2001), the interaction between CD91(LRP) and hCED-6 could be nonspecific. Second, interactions between hCED-6 and CED-1 or CD91(LRP) apparently do not require phosphorylation of the receptor (Su et al. 2002), which does not fit the model that the PTB domain of CED-6-like proteins binds a phosphorylated CED-1-like receptor.

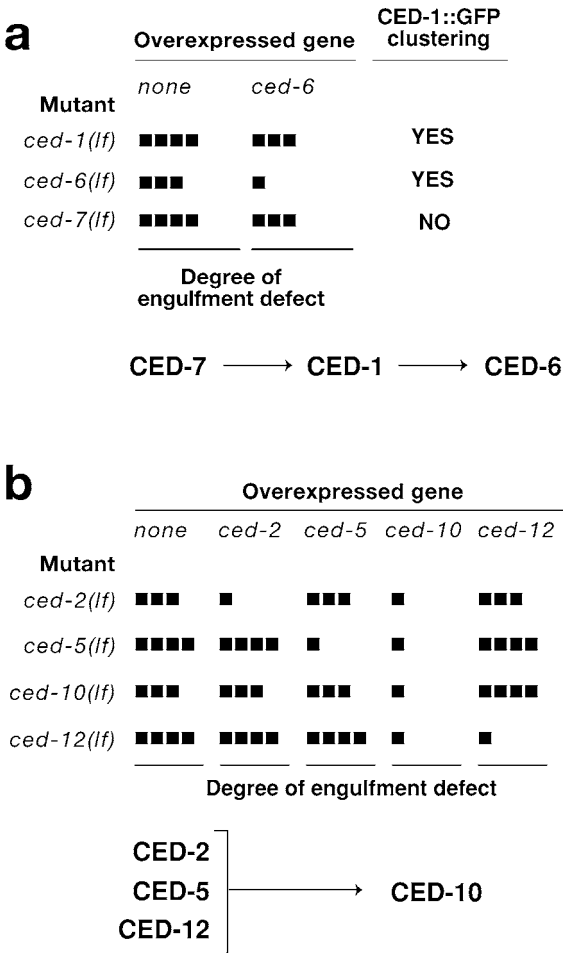


Figure 3 (*a*) The overexpression of *ced-6* partially bypasses the requirement for *ced-1* and *ced-7*. Boxes represent the numbers of corpses in the heads of L1 larvae summarized from Liu & Hengartner (1998): <5 = ■, 10–15 = ■■■, 15–25 = ■■■■. *ced-7* is required for CED-1::GFP to cluster around cell corpses (Zhou et al. 2001b). (*bottom*) The molecular genetic pathway in which CED-7 acts at a step upstream of the CED-1 receptor and CED-6 acts downstream of CED-1. (*b*) The overexpression of *ced-10* bypasses the requirement for *ced-2*, *ced-5*, and *ced-12*. The numbers of corpses in the heads of L1 larvae are summarized from Reddien & Horvitz (2000), Gumienny et al. (2001), Wu et al. (2001), and Zhou et al. (2001a): <5 = ■, 20–30 = ■■■, >30 = ■■■■. (*bottom*) CED-10 may act downstream of CED-2, -5, and -12.

CED-2 CrkII, CED-5 DOCK180, and CED-12 ELMO Interact in Engulfing Cells

The *C. elegans* CED-2 protein is similar to the human adaptor protein CrkII (Reddien & Horvitz 2000). CrkII can affect cell migration, suggesting CED-2 and Crk proteins might function to regulate cell shape and motility (Klemke et al. 1998). Because CED-2 likely acts within engulfing cells, CED-2 may regulate the extension of engulfing cell membranes (Reddien & Horvitz 2000). CED-2 and CrkII each have one N-terminal SH2 (Src-homology 2) domain followed by two SH3 (Src-homology 3) domains (Matsuda et al. 1992, Reddien & Horvitz 2000). SH2 domains bind phosphotyrosines, and SH3 domains bind proline-rich sequences (PXXP; where X is any amino acid) (Koch et al. 1991).

CED-5 was one of the founding members of a protein family that includes human proteins DOCK180 and DOCK4, and *Drosophila* Myoblast City (Wu & Horvitz 1998b). DOCK180 was identified on the basis of its physical interaction with Crk (Hasegawa et al. 1996) and can functionally replace *ced-5* for the migration of the distal tip cells (Wu & Horvitz 1998b). DOCK180 can localize with CrkII to focal adhesions and affect cell spreading (Kiyokawa et al. 1998). CED-5 likely acts within engulfing cells (Wu & Horvitz 1998b) and can bind CED-2 (Reddien & Horvitz 2000), suggesting Crk-like and DOCK180-like proteins interact in engulfing cells to mediate engulfment in vivo. The human gene DOCK4, found as deleted during tumorigenesis, can functionally replace *ced-5* for the engulfment of germline cell corpses in *C. elegans* (Yajnik et al. 2003). Myoblast City controls myoblast fusion and dorsal closure in *Drosophila*, indicating CED-5-like proteins control a variety of events that, similar to engulfment, involve changes in cell shape (Erickson et al. 1997). Both CED-5 and DOCK180 contain an N-terminal SH3 domain, a large central region with a DOCKER domain (see below for description) (Brugnera et al. 2002), and a C-terminal proline-rich region that likely binds the first SH3 domain of CED-2 and CrkII, respectively.

The CED-12 protein contains a PH domain (Pleckstrin Homology) and defines a novel protein family that has been found to have mammalian and *Drosophila* members (Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a). The two mammalian CED-12-like proteins have been named ELMO1 and ELMO2 (Gumienny et al. 2001). PH domains can target interacting proteins to cell membranes (Shaw 1996), and ELMO1 can indeed facilitate the recruitment of DOCK180 to cell membranes (Gumienny et al. 2001). CED-12 likely acts within engulfing cells and can physically interact with CED-5 (Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a), possibly as part of a CED-2 CrkII-CED-5 DOCK180-CED-12 ELMO ternary complex (Gumienny et al. 2001, Wu et al. 2001). CED-12 has a proline-rich candidate SH3-binding domain that may mediate interaction with the N-terminal SH3 domain of CED-5 DOCK180. Both the proline-rich and PH domains are needed for CED-12 function in vivo (Zhou et al. 2001a).

The primary receptor that regulates CED-2, CED-5, and CED-12 remains to be identified. CED-5 and CED-12 have been reported to bind the candidate *C. elegans* engulfment receptor PSR-1, consistent with the hypothesis that PSR-1 may have

a minor effect on the CED-2, -5, -10, -12 pathway (Wang et al. 2003). Integrin receptors are also candidate regulators of this pathway (Albert et al. 2000) but have as yet no established role in *C. elegans* engulfment (Gumienny et al. 2001, Wu et al. 2001).

CED-10 Is a Rac GTPase that Regulates Engulfment, Migration, and Axon Outgrowth

CED-10 is 84% identical to the human Rac GTPase (Reddien & Horvitz 2000). Rac GTPases are members of a Ras superfamily subgroup that includes Rho, Rac, and Cdc42 and that controls cell morphology via regulation of the cytoskeleton (Van Aelst & D'Souza-Schorey 1997). The actin cytoskeleton is important for the uptake of apoptotic cells (Chimini & Chavrier 2000). GTPases act as molecular switches that regulate the activities of a variety of signal transduction pathways: When bound to GTP, the protein is active for signal transduction and when bound to GDP is typically inactive (Bourne et al. 1991). In culture, Rho is known to affect focal adhesions and stress fiber formation (Ridley & Hall 1992), Rac to affect lamellipodia formation (Ridley et al. 1992), and Cdc42 to affect filopodia formation (Nobes & Hall 1995). CED-10 can act outside of dying cells for engulfment (Reddien & Horvitz 2000) and cell autonomously for axon guidance (Lundquist et al. 2001), consistent with the notion that CED-10 acts within engulfing cells to control the cytoskeleton. Rac, as well as Cdc42, has been implicated in the phagocytosis of opsonized particles, suggesting that because CED-10 Rac is involved in engulfment, Rac GTPase function may be a conserved feature in the phagocytosis of distinct objects (Caron & Hall 1998, Massol et al. 1998). Mammalian Rac is capable of mediating lamellipodia extension and phagocytic cup closure, critical processes for the engulfment of apoptotic cells (Ridley et al. 1992, Massol et al. 1998). The finding that CED-10 Rac is important for engulfment in *C. elegans* defines an in vivo role for Rac proteins in the removal of apoptotic cells.

Overexpression of *ced-10* can bypass the requirement for *ced-2*, *ced-5*, or *ced-12*, whereas overexpression of *ced-2*, *ced-5*, or *ced-12* cannot bypass the requirement for *ced-10* (or for each other), indicating that *ced-10* likely acts downstream of the other three genes (Reddien & Horvitz 2000, Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a) (Figure 3*b*). CED-2, -5, and -12 are thus probably activators of CED-10. Proteins that facilitate the exchange of GTP for GDP bound to GTPases and thus act as activators of signal transduction are known as guanine nucleotide exchange factors (GEFs) (Kaibuchi et al. 1999). Although neither DOCK180 nor ELMO1 has clear homology to established GEFs, a domain of DOCK180, termed DOCKER, can act as a novel type of RacGEF in vitro (Brugnera et al. 2002), and DOCK180 and ELMO1 can synergize to promote GTP loading of Rac in mammalian cell culture (Gumienny et al. 2001, Brugnera et al. 2002). DOCK180 and ELMO proteins appear to act together as a newly identified type of GEF. Cell culture experiments support the idea that CED-12-like proteins can regulate cell-shape changes through Rac-Rho-Cdc42 family members (Gumienny et al. 2001, Zhou et al. 2001a, Katoh & Negishi 2003). The CED-2 CrkII-CED-5

DOCK180-CED-12 ELMO-CED-10 Rac pathway thus defines a novel signaling pathway and a novel mechanism for the activation of GTPases.

In *C. elegans*, *ced-10* is one of three *rac*-like genes, together with *mig-2* and *rac-2* (Lundquist et al. 2001). CED-10 is the primary Rac involved in the engulfment of apoptotic cells (Lundquist et al. 2001), which may reflect a specificity of the interaction with the upstream regulator complex CED-2/5/12 rather than distinct expression patterns of the *rac* genes (Zipkin et al. 1997, Lundquist et al. 2001). *mig-2* and *rac-2* may have subtle contributing roles in engulfment (Lundquist et al. 2001). CED-10 acts with CED-2, CED-5, and CED-12 to control at least one type of cell migration during the development of the somatic gonad (Wu & Horvitz 1998b, Reddien & Horvitz 2000, Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a). CED-10 has a key role in controlling axon guidance and neuronal migration, and in contrast to engulfment, is not activated by CED-2 or CED-5 for these processes (Lundquist et al. 2001). UNC-73, a *C. elegans* Rac GEF similar to mammalian Trio (Steven et al. 1998), likely affects CED-10 signaling during axon guidance but has no apparent role in engulfment (Lundquist et al. 2001). These findings indicate that distinct Rac proteins can be differentially utilized and act in different pathways for multiple developmental events.

CED-2, CED-5, CED-12, and CED-10 Define an Evolutionarily Conserved Signaling Pathway that Controls Cell-Corpse Engulfment

CED-2, -5, -12, and -10 define components of a signal transduction pathway involved in cell-corpse engulfment, predicted to act as follows (Figure 4): Engagement of an unidentified ligand with an unidentified receptor leads to the tyrosine phosphorylation of that receptor or of a receptor-associated protein that interacts with the SH2 domain of CED-2 CrkII (Reddien & Horvitz 2000). An SH3 domain in CED-2 binds the C-terminal proline-rich region of CED-5 DOCK180 and recruits CED-5 to cell membranes (Reddien & Horvitz 2000). CED-10 Rac is localized to cell membranes by a geranylgeranylation modification (Reddien & Horvitz 2000). The proline-rich region of CED-12 ELMO binds the N-terminal SH3 domain of CED-5 (Gumienny et al. 2001, Wu et al. 2001, Zhou et al. 2001a), which then promotes the GTP loading of CED-10 Rac (Brugnera et al. 2002). GTP-bound CED-10 Rac drives reorganization of the actin cytoskeleton and extension of lamellipodia around cell corpses via an undiscovered effector.

Mammalian counterparts of the members of this *C. elegans* engulfment pathway have begun to be examined for homologous function in the engulfment of apoptotic cells in cell culture. CrkII, DOCK180, and Rac indeed act together to promote the phagocytosis of apoptotic mammalian cells (Albert et al. 2000, Leverrier & Ridley 2001, Tosello-Trampont et al. 2001). The CED-12 counterpart, ELMO1, interacts with CrkII, DOCK180, and Rac to regulate cell shape (Gumienny et al. 2001) and cell migration (Grimsley et al. 2004) but has not yet been extensively tested for function with other pathway components in engulfment. That CrkII, DOCK180,

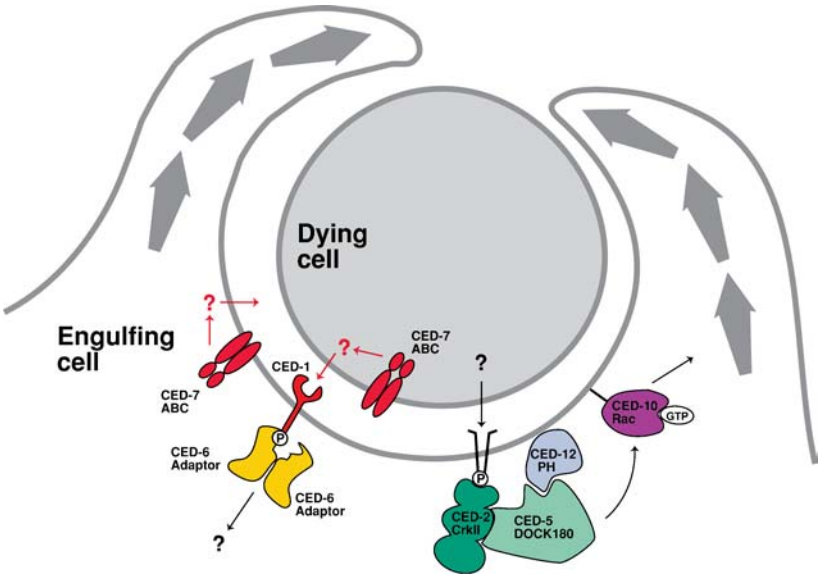


Figure 4 Molecular model for cell-corpse engulfment in *C. elegans*. The CED-7 ABC transporter transports an unknown molecule(s) (*red arrows*) in both the engulfing and dying cells and in dying cells generates a signal recognized by the CED-1 receptor. CED-1 binds directly to CED-6, a PTB domain adaptor-like protein. CED-6 can homodimerize and transduce signals from CED-1. The targets of CED-6 are unknown. In the second pathway, an unknown signal from the dying cell stimulates the phosphorylation of an unknown receptor or receptor-associated protein, which activates CED-2 CrkII. CED-2 CrkII and CED-12 ELMO (PH domain-containing) both bind CED-5 DOCK180. One or both of these proteins recruit CED-5 DOCK180 to the cell membrane. This complex facilitates the exchange of GTP for GDP bound to CED-10 Rac. CED-10 Rac is present at cell membranes as a consequence of a geranylgeranylation, and GTP-bound CED-10 Rac signals a reorganization of the actin cytoskeleton.

and Rac act in the engulfment of apoptotic mammalian cells demonstrates that, similar to the genes that control the execution of cell death, the genes that control the removal of cell deaths in *C. elegans* define pathways conserved in humans.

ENGULFMENT PROMOTES PROGRAMMED CELL DEATH

Engulfing Cells Facilitate Cell Death in *C. elegans*

That cell corpses are formed in *C. elegans* mutants defective in engulfment indicates that cells can die without being engulfed (Hedgecock et al. 1983). However, several studies indicate that engulfment promotes cell death rather than simply eliminating dead cells. For instance, mutations in engulfment genes can enhance

the survival of many cells normally fated to die in animals partially impaired in cell death, e.g., in *ced-3*(*weak lf*) animals (Hoeppner et al. 2001, Reddien et al. 2001). Thus engulfment genes can, at least in certain circumstances, promote cell death. That mutations in any of the known engulfment genes can cause such enhancement suggests the engulfment process itself promotes cell death, rather than that engulfment genes have separate functions in dying cells to enhance cell killing. This view is supported by the finding that expression of the cell-corpse receptor encoding gene *ced-1* (Zhou et al. 2001b) in engulfing cells rescues the cell-killing defect of *ced-1* mutants (Reddien et al. 2001).

In animals perturbed for engulfment, but with an intact core cell-killing pathway, most cell deaths occur, but some fail, indicating that engulfment is a normal component of the killing process (Reddien et al. 2001), i.e., engulfment mutations do not only cause a reduction in cell death when cell death is partially impaired by *ced-3* mutations. Cells fated to die occasionally fail to display the morphological characteristics of dying cells in engulfment-defective mutants, whereas cells fated to die in wild-type animals always display characteristic and dramatic morphological changes. This observation indicates that engulfment actively promotes the progression of morphological changes during cell death, and thus presumably the death process itself, rather than passively eliminating the chance for recovery from the death process (Reddien et al. 2001). In other words, if engulfment passively eliminated the chance for the recovery of dying cells, then one would expect to observe normal cell death-related morphological changes in engulfment mutants followed by recovery, rather than to observe defects in the death process.

Some cell deaths in *C. elegans* are largely dependent upon engulfment. For example, the linker cell, which directs the development of the male gonad, is subsequently killed by engulfing cells. A cell death in the male proctodeum [B.a(l/r)rapaav] is dependent upon the engulfing cell, P12.pa, and upon engulfment genes (Hedgecock et al. 1983, Reddien et al. 2001). Mutations in two genes exist that cause cells that normally live instead to die in a manner partially dependent upon engulfment genes, further supporting the idea that engulfment can promote cell death: *lin-24* and *lin-33* mutations can dominantly cause particular cells of the ventral ectoderm that normally survive (Pn.p cells) to die a necrotic-like *ced-3*-independent death (Ferguson & Horvitz 1985, Ferguson et al. 1987, Ellis et al. 1991b) that appears to be partially suppressed by mutations in multiple engulfment genes (Ellis et al. 1991b, Wu et al. 2001). *lin-24* and *lin-33* mutations may cause Pn.p cells to become sick and recognized by engulfing cells, thus triggering engulfment and promoting their deaths.

DNA degradation is a hallmark of programmed cell death (Wyllie 1980). Some engulfment genes can directly affect the initial aspects of the DNA degradation that occurs within dying cells. DNA persists in unengulfed cell corpses, suggesting that the completion of DNA degradation during programmed cell death occurs within engulfing cells (Hedgecock et al. 1983). However, some aspects of DNA degradation occur within dying cells in the absence of engulfment (Wu et al.

2000), as determined using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay to monitor free DNA ends (Gavrieli et al. 1992). In cells that die but fail to be engulfed, DNA is made TUNEL-positive and then, in a process dependent upon the DNase II *nuc-1* gene, TUNEL-negative. In double mutants involving *nuc-1* and *ced-2*, *-5*, *-10*, or *-6*, TUNEL-positive DNA is generated (Wu et al. 2000). Therefore, the initial aspects of DNA degradation during cell death generally seems to be normal in *ced-2*, *-5*, *-10*, and *-6* mutants. In contrast, mutations in *ced-1* and *ced-7* block the generation of TUNEL-positive DNA in dying cells (Wu et al. 2000). *ced-1* and *ced-7* have been proposed to mediate the recognition of cell corpses by engulfing cells (Zhou et al. 2001b) (see above). Such a recognition event may be necessary to trigger a signaling pathway from the engulfing cell to the dying cell to promote DNA degradation. This aspect of the *ced-1*(*lf*) and *ced-7*(*lf*) phenotypes suggests *ced-6* function is at least partially distinct from those of *ced-1* and *ced-7*. However, because the *ced-6* mutation used in this study may not be a complete loss-of-function allele, it remains possible that complete elimination of *ced-6* would cause DNA degradation defects similar to those in *ced-1* and *ced-7* mutants (Liu & Hengartner 1998).

The impairment of engulfment in *C. elegans* allows cells that initiate some morphological aspects of death to recover (Reddien et al. 2001). Therefore, at least in *C. elegans*, the activation of caspase activity does not necessarily lead to death, and blocking engulfment can promote cell survival in cases in which cells are poised between life and death. The inhibition of cell death in humans could have medical benefit in cases in which too much cell death occurs, such as in neurodegenerative disorders or in tissue surrounding areas of damage, such as that caused by a myocardial infarct (Thompson 1995, Rudin & Thompson 1997). If engulfment proves to promote cell death in humans (see below), proteins that control the engulfment process or the general functioning of engulfing cells could be considered as drug targets to reduce cell death.

Macrophages Can Promote Cell Death in Mammals

Inflammatory mediators that stimulate macrophages and macrophage-induced cytotoxicity (which is regulated by tumor necrosis factor alpha and nitric oxide) can promote the deaths of cellular targets such as tumor cells (Albina et al. 1993, van de Loosdrecht et al. 1993, Cui et al. 1994). Macrophage depletion experiments indicate that macrophages may also have roles in the promotion of developmental cell deaths, such as those that occur during tissue regression in mouse eye development (Lang & Bishop 1993, Lang et al. 1994) and those of vascular endothelial cells during capillary regression in vivo (Diez-Roux & Lang 1997). That macrophages can promote developmental cell death during tissue regression indicates that engulfment may promote cell death by perhaps an unidentified mechanism in both mammals and *C. elegans*. Mice lacking engulfment genes could be used to address the role of engulfment in promoting cell death in mammals. For instance, PSR knockout mice have brain hyperplasia reminiscent of that found in

Caspase-3 and *Caspase-9* knockout mice (Li et al. 2003), although at a lower penetrance, consistent with the idea that impaired engulfment blocks cell death to some degree in mammals. Continued genetic studies of this aspect of the $PSR^{-/-}$ mouse phenotype could help test this hypothesis.

SUMMARY AND PROSPECTS

In the 20 years since the first *C. elegans* engulfment mutants were identified by Hedgecock et al. (1983), the characterization of the molecular genetic pathways defined by the seven well-studied engulfment *ced* genes in *C. elegans* has provided the framework for an understanding of the recognition and removal of apoptotic cells in the metazoa. Despite substantial progress, many questions remain. We highlight below some of the issues that remain to be resolved about engulfment and suggest genetic and molecular approaches to identify more engulfment pathway components.

Some Unsolved Mysteries of *C. elegans* Engulfment

What cell surface changes occur on dying cells? How is it determined which cell engulfs a particular dying cell? What processes trigger engulfment to begin before the completion of the cell division that will generate a cell fated to die? Why do mutations in different engulfment genes affect the engulfment of specific dying cells differently (Ellis et al. 1991a)? How can some cell deaths in engulfment mutants be engulfed with relatively normal kinetics, whereas others persist for days (Ellis et al. 1991a, Hoeppner et al. 2001)? Is there a shared aspect of the death processes in apoptotic and necrotic cells that allows the same engulfment genes to mediate engulfment (Chung et al. 2000)? Do caspases trigger engulfment through the direct generation of an engulfment signal? If so, how can cells that die without *ced-3* activity be engulfed (Shaham et al. 1999)? Why are undead cells not engulfed (Ellis & Horvitz 1986)? Why is *ced-7* needed in both dying and engulfing cells (Wu & Horvitz 1998a)? Why do cell corpses in *ced-7* mutant embryos disappear after hatching (Ellis & Horvitz 1991)? What is the mechanistic nature of the redundancy between the two engulfment pathways? Do *ced-1*, *ced-6*, and *ced-7* really define one single pathway?

Some cells are more reliant upon engulfment for their deaths than others. For instance, cells that normally die in the developing pharynx essentially always die in engulfment mutants, whereas some cells that normally die in the ventral cord survive about 10% of the time in engulfment mutants (Reddien et al. 2001). In contrast, the deaths of some cells, such as B.al/rapaav in the male tail, may be entirely dependent upon engulfment (Hedgecock et al. 1983, Reddien et al. 2001). How can engulfment differentially affect the deaths of different cells? How the deaths of distinct cells vary among mutants defective in the different engulfment genes has not been thoroughly explored. For example, why do mutations in *ced-7* cause, at least for some cells, a greater defect in cell killing than do mutations

in other engulfment genes, e.g., 1.4 failed cell deaths on average (of 5 observed possible cell deaths) in the ventral cords of *ced-7*(*n1892*) mutants compared with 0.6 or fewer failed cell deaths on average for mutants in any of the other engulfment genes (Reddien et al. 2001)?

Considering only the components of engulfment that are already identified, many questions remain. For instance, what is transported by the CED-7 ABC transporter, and how is CED-7 regulated? What is the human ortholog of CED-1? Does the CED-1 receptor activate CED-6 and, if so, by what biochemical mechanism? Is CED-1 activated by phosphorylation? Does CED-6 dimerize in vivo and, if so, for what reason? What is the subcellular localization of CED-6 before and during engulfment? Given that both CED-2 CrkII and CED-12 ELMO1 can bind CED-5 DOCK180, which recruits CED-5 to engulfing cell membranes in vivo? What are the in vivo expression and localization patterns of CED-2 and CED-5? How do CED-5 DOCK180 and CED-12 ELMO proteins act together to facilitate GTP exchange with CED-10 Rac? Is the dynamic regulation of CED-10:GDP and CED-10:GTP bound states important in engulfment?

The *C. elegans* Engulfment Pathways Are Incomplete

Many molecules involved in engulfment remain to be identified. For example, what are the ligands that activate the CED-7, -1, -6 and CED-2, -5, -12, -10 pathways? What is the major receptor that regulates the CED-2, -5, -12, -10 pathway? What activates CED-1? What acts downstream of CED-6? What acts downstream of CED-10? What proteins mediate engulfment-promoted cell death? Previous genetic screens for engulfment mutants required that animals homozygous for engulfment mutations be viable. Thus genetic screens that allow the recovery of homozygous lethal mutations that confer a block in engulfment could identify essential components. Modifier screens for mutations that enhance or suppress defects conferred by partial loss-of-function alleles of engulfment *ced* genes, screens for mutations that modify phenotypes conferred by dominant-negative and dominant-active CED-10 Rac, and screens for genes that act with *ced-10* in distal tip cell migration and/or axon guidance also could identify additional engulfment-pathway components. Genetic screens for mutations that phenocopy the defects in cell killing conferred by mutations in engulfment genes could help reveal the mechanism(s) by which engulfment promotes cell death.

In addition to genetic screens, biochemical and candidate gene approaches could help identify engulfment pathway components. The generation of deletion mutants (Jansen et al. 1997) and RNAi can provide rapid means for testing the functions of such candidates. The identification of proteins that interact with CED-6 or hCED-6 may help define the signal transduction pathway mediated by the CED-1 receptor. The identification of novel CED-2, CED-5, and/or CED-12-interacting proteins could identify additional pathway regulators, such as the receptor for this pathway. Rac GTPases can interact with numerous upstream activators and downstream effectors. Understanding which of these many interactions are relevant to

engulfment will be a future challenge. Do other GTPases, such as Rho and Cdc42, contribute to engulfment? Many candidate engulfment receptors have been identified in mammals, including scavenger receptors (Krieger & Herz 1994; Hughes et al. 1995; Platt et al. 1996, 1998) such as the class B scavenger receptor CD36 (Savill et al. 1989) (the *Drosophila* counterpart of which *croquemort* is involved in engulfment) (Franc et al. 1996, 1999), CD14 (Pradhan et al. 1997, Devitt et al. 1998), integrins (Savill et al. 1990; Albert et al. 1998, 2000), and the MER receptor tyrosine kinase (Scott et al. 2001). Various mammalian soluble "bridging" molecules, such as the complement protein C1q (Botto et al. 1998, Taylor et al. 2000), GAS6 (Nagata et al. 1996, Nakano et al. 1997), and thrombospondin (Savill et al. 1992), also have been implicated in mediating interactions between apoptotic cells and engulfment receptors. Genetic analyses of functional counterparts to these mammalian classes of receptor and bridging molecules in *C. elegans* could help assign in vivo functions to these genes.

Conclusions

Two *C. elegans* engulfment pathways—the CED-7 ABC transporter, CED-1 receptor, CED-6 hCED-6 pathway and the CED-2 CrkII, CED-5 DOCK180, CED-12 ELMO1, CED-10 Rac pathway—probably define key components of evolutionarily conserved pathways that control the removal of apoptotic cells. Studies testing the roles of the human counterparts of these *C. elegans* proteins in the engulfment of human apoptotic cells are ongoing and have already identified roles for many of these human proteins in engulfment (Smits et al. 1999, Albert et al. 2000, Leverrier & Ridley 2001, Tosello-Trampont et al. 2001). The studies of the *C. elegans* CED-6 and the CED-12 proteins led to the identification of biological functions for conserved and previously uncharacterized human proteins. The identification of CED-5 and CED-12 as regulators of CED-10 Rac helped lead to the identification of a novel biochemical pathway for regulating GTPases. The analysis of engulfment in *C. elegans* promises to continue to provide insight into signal transduction in general and engulfment in particular. Studies of engulfment mutants uncovered a role for engulfment not only in removing dead cells but also in promoting normal programmed cell death. Studies of this phenomenon in *C. elegans* may well contribute to an understanding of how cells die by programmed cell death during metazoan development. The normal death and removal of cells is of fundamental importance for both development and homeostasis, and the misregulation of the death and/or removal of unwanted cells has been implicated in disease. Basic knowledge about engulfment in *C. elegans* should promote our understanding of animal development and also identify novel strategies for intervention in human disease.

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