

Journey to the grave: signaling events regulating removal of apoptotic cells

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Summary

Programmed cell death is critical both for organ formation during development and during adult life, when billions of cells must be removed every day. The culmination of the apoptotic process is the specific recognition and engulfment of the apoptotic cell by a phagocyte. A number of recent studies have revealed a series of evolutionarily conserved proteins that link corpse recognition to membrane movement, facilitating the internalization of the target and its subsequent degradation. Two potential signaling modules have been identified: one involving the CED-12/ELMO and CED-5/Dock180 proteins, which function as a bipartite guanine nucleotide exchange factor (GEF) for Rac1, and a second involving CED-1/LRP1 (a potential

engulfment receptor) and the adaptor protein CED-6/GULP. Recognition of the apoptotic cell modulates cytokine secretion by the phagocyte, resulting in an anti-inflammatory state distinct from that induced by necrotic cells. The recent molecular delineation of the phagocytic process and the identification of novel signaling proteins involved in engulfment have provided an exciting new platform for future studies into this biologically important process.

Key words: Engulfment, Apoptosis, GULP, ELMO, Doc 180, Rac, *C. elegans*

Introduction

Programmed cell death (apoptosis) represents a multi-step process for the elimination and subsequent removal of cells in response to environmental insult or as part of organismal development. Initiation of the death of a cell and its subsequent engulfment by a phagocyte are intimately associated; indeed, unengulfed apoptotic cells are rarely seen in vivo. In addition to performing a purely 'garbage disposal' function, recognition of the apoptotic cell (by both professional and nonprofessional phagocytes) results in enhanced production of anti-inflammatory mediators and upregulation of diverse genes in the phagocyte, potentially rendering it more competent to engulf and/or dispose of the apoptotic-cell-derived debris. Defects in removal of apoptotic cells have been associated with onset of disease states; for instance, macrophages in atherosclerotic plaques show delayed kinetics of apoptotic cell engulfment (Schrijvers et al., 2005). Phagocytic defects have also been linked to autoimmune conditions; macrophages from patients with systemic lupus erythematosus (SLE) show defects in clearance of apoptotic cells (Tas et al., 2006). Current dogma is that defects in clearance lead to exposure of autoantigens, which subsequently results in autoimmune disease; the engulfment process is thus a potential therapeutic target.

The process of corpse removal occurs via a series of recognizable steps. Initially, a phagocyte binds to the apoptotic cell, leading to specific recognition of the target, phagocytic cup formation and sustained signaling that induces actin rearrangement and phagocytosis. Ultimately, the apoptotic cell is completely internalized, and its corpse is processed for degradation (Fig. 1). Ultrastructural studies of engulfment have identified two different methods by which the phagocyte extends membrane around the apoptotic cell. In one, the

membrane is extended around the apoptotic cell corpse in a 'zipper-like' manner, resulting in the formation of a tight-fitting phagolysosome (Giles et al., 2000; Krysko et al., 2006) (Fig. 1C). Necrotic cells, by contrast, seem to be internalized in 'spacious', fluid-filled phagosomes similar to macropinosomes (Gardai et al., 2005; Krysko et al., 2006). However, both apoptotic and necrotic cells are suggested to use the same signaling apparatus for cell recognition (Chung et al., 2000). Further work is clearly needed to define the molecular events that dictate the type of phagocytic cup formed in response to recognition, because this could have important implications for subsequent processing of the engulfed target in the phagocyte (Patel et al., 2006).

The key to this conundrum may be the different signals found on the apoptotic or necrotic cell surface. After initiating apoptosis, the dying cell exposes 'eat-me' signals, which are recognized by specific receptors on a nearby phagocyte, resulting in phagocytosis and removal of the corpse (Fig. 2A). Exposure of phosphatidylserine (PtdSer) on the outer plasma membrane during apoptosis appears to be one of the key eat-me signals. Other eat-me signals include changes in glycosylation of certain proteins, changes in cell-surface charge, as well as the preferential binding of certain plasma-derived proteins to the surface of the dying cell. Exposed PtdSer can be recognized by the phagocyte either directly by receptors (for example, a PtdSer-specific receptor) or indirectly after the binding of soluble bridging molecules or 'opsonins', such as milk-fat globule EGF-factor 8 (MFG-E8), calreticulin (CRT), or C1q (complement), that are then recognized by additional receptors. The biological importance of PtdSer exposure has been firmly established by experiments using in vivo systems (Asano et al., 2004; Nera et al., 2000; van den

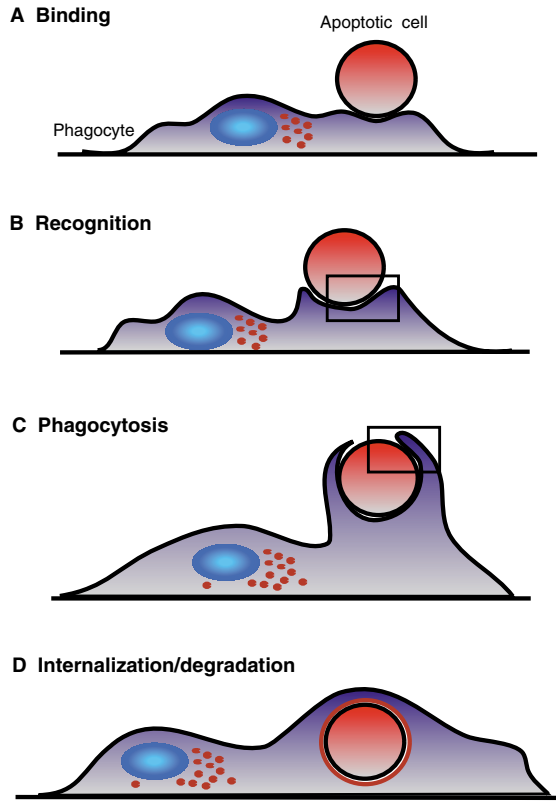


Fig. 1. Engulfment of an apoptotic cell can be broken down into four phases. Binding of the apoptotic cell to the phagocyte (A) leads to recognition (B) via a host of different receptors. Engagement of some of these leads to phagocytosis of the apoptotic cell (C) and release of anti-inflammatory cytokines. Corpse engulfment occurs via at least two evolutionarily conserved signaling modules, ultimately activating the Rac1 GTPase and actin rearrangement. Following engulfment, the corpse enters lysosomal structures (D), where components such as apoptotic-cell-derived DNA are degraded.

Eijnde et al., 1998; Wang et al., 2007); recent studies have also revealed a link between autophagy and PtdSer exposure, suggesting this molecule plays a widespread role in clearance of dying cells (Qu et al., 2007).

Other potential eat-me signals include altered sugar moieties and other phospholipids, such as lyso-PC (Gardai et al., 2006). Recent studies have also identified potential 'don't-eat-me' signals (such as CD31 and CD47), engagement of which inhibits the phagocytic process (Brown et al., 2002; Gardai et al., 2005). The receptors and molecules involved in recognition of apoptotic cells are only covered here in the context of phagocytic signaling, because these have been discussed in several excellent reviews recently (Gardai et al., 2006; Grimsley et al., 2004; Kinchen and Hengartner, 2005; Mangahas and Zhou, 2005).

Despite the identification of numerous ligands and receptors, the signaling events leading to removal of apoptotic cells remain unclear. Recent studies have shed light on some of the important details of how recognition of an apoptotic cell leads to corpse removal and anti-inflammatory signaling. Here, we cover recent insights into intracellular signaling during engulfment, specifically mechanisms of Rho-family GTPase activation, and degradation of the corpse within the phagocyte.

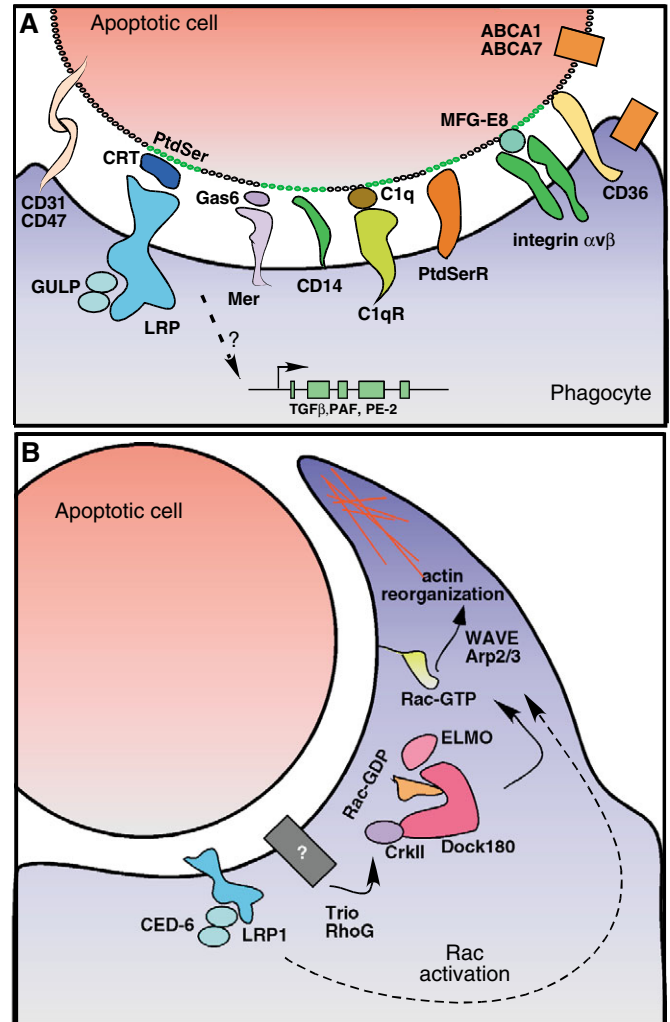


Fig. 2. Recognition of the apoptotic cell and subsequent engulfment utilize a host of evolutionarily conserved proteins. (A) Some of the cell surface receptors on phagocytes that mediate the recognition of ligands (eat-me signals) on apoptotic cells and potentially lead to induction of anti-inflammatory mediators. Not all of the receptors need to be expressed or utilized by a phagocyte. Current evidence suggests that a subset of these receptors may engage a subset of the ligands on an apoptotic cell, which is sufficient to generate the signals necessary for the engulfment of the corpse. (B) Intracellular signaling driving extension of the phagocyte around the apoptotic cell involves two potentially redundant, evolutionarily conserved pathways consisting of CED-1/LRP, CED-6/GULP and UNC-73/Trio, MIG-2/RhoG, CED-2/CrkII, CED-5/Dock180, CED-12/ELMO, which may function coordinately in the regulation of CED-10/Rac1 activation.

Recognition of the apoptotic cell leads to activation of an evolutionarily conserved signaling machinery

The removal of apoptotic cells involves an almost unprecedented complexity of cell signaling (Fig. 2). Numerous cell surface receptors – such as the scavenger receptors LRP1/CD91 and CD36, lectins, integrins, CD14 (a plasma membrane glycoprotein), the C1q Receptor (C1qR), ATP-binding cassette (ABC) proteins ABCA1 and ABCA7, and the receptor tyrosine kinase MER, to name a few – have been shown to engage the apoptotic cell and promote phagocytosis

(Grimsley and Ravichandran, 2003). How the majority of these surface proteins induce intracellular signaling is poorly understood. Induction of anti-inflammatory signaling has been shown to involve 'recognition' of the apoptotic cell, but does not require internalization of the apoptotic cell, which suggests that receptors mediate discrete sets of responses to the apoptotic cell (Lucas et al., 2006; Voll et al., 1997).

Genetic studies in *C. elegans* were critical in the identification of two partially redundant pathways that function upstream of the Rho family GTPase CED-10/Rac1, a key regulator of the actin cytoskeleton during internalization of the apoptotic cell (Conradt, 2001; Horvitz, 2003; Kinchen et al., 2005). Since many of these molecules appear to play a similar role in mammals, these early experiments have been instrumental in the characterization of signaling events regulating engulfment.

The first pathway: CED-1/LRP1/MEGF10, CED-6/GULP and CED-7/ABCA1/ABCA7

The first pathway identified in the nematode consists of CED-1, CED-6 and CED-7, whose mammalian orthologues are the transmembrane scavenger receptor LRP1/MEGF-10, an adaptor protein (GULP), and an ABC transporter (ABCA1 or ABCA7), respectively (Hamon et al., 2006; Liu and Hengartner, 1998; Su et al., 2002; Wu and Horvitz, 1998a; Zhou et al., 2001b). Both CED-1 and CED-6 have been shown to cluster simultaneously around the apoptotic cell, which suggests they function at similar times during corpse recognition. Indeed, the two proteins physically interact and are required for actin reorganization around the apoptotic cell, which depends on CED-10/Rac1 (Kinchen et al., 2005; Su et al., 2002). LRP1 and GULP have been shown to play a role in phagocytosis in mammals (Gardai et al., 2005; Liu and Hengartner, 1998; Liu and Hengartner, 1999); LRP1 directly interacts with GULP (Su et al., 2002), potentially leading to Rac activation (Gardai et al., 2005). GULP, similarly to CED-6, appears to function as a homodimer (Su et al., 2000). To date, no proteins have been identified that function downstream of CED-1 and CED-6 to mediate corpse removal or Rac activation.

Whereas the ligand that CED-1 recognizes in the nematode is unknown, LRP1 has been shown to bind calreticulin (a Ca²⁺-binding protein normally found in the endoplasmic reticulum) on the surface of apoptotic cells (Gardai et al., 2005); this in turn may directly interact with PtdSer on the apoptotic cell surface. In the nematode, calreticulin mutants do not show defective removal of apoptotic cells, which suggests that the function of the mammalian protein may have diverged from that of the nematode orthologue. The structure of the extracellular domain of LRP1 differs from that of CED-1, which supports this hypothesis (Su et al., 2002). Another potential mammalian orthologue, MEGF10, appears to be more similar to CED-1 in the extracellular domain and has recently been shown to play a role in corpse removal in cell culture systems (Hamon et al., 2006). However, many scavenger-receptor-related proteins seem to function in corpse removal in mammals, and further work is needed to determine the extent to which this pathway is evolutionarily conserved.

Drosophila also eliminates a number of cells during development through programmed cell death (Greenwood and Gautier, 2005). Draper, the *Drosophila* orthologue of CED-1, and Dced-6 mediate the removal of apoptotic cells both in vivo in the *Drosophila* central nervous system (CNS) and in cell

culture models (Awasaki et al., 2006; Freeman et al., 2003; MacDonald et al., 2006; Manaka et al., 2004). Draper also plays a role in axon pruning and elimination of severed axons (Awasaki et al., 2006; MacDonald et al., 2006); loss of Draper or Dced-6 results in failure of axon processes to be removed and, in some cases, unremoved processes appear to remain functional (Awasaki et al., 2006). Interestingly, this is similar to phenotypes observed in the nematode, where mutations in engulfment genes lead to increased cell survival (Hoepfner et al., 2001; Reddien et al., 2001). Croquemort, the *Drosophila* CD36 orthologue, also functions in corpse removal in the fly (Franc et al., 1999); how Croquemort cooperates with Draper/Dced-6-mediated phagocytosis remains to be determined.

CED-7 is something of an enigma, because this protein is unique in several respects. It is the only protein whose function is required both in the engulfing and in the apoptotic cell (Wu and Horvitz, 1998a). CED-7 is also the only *C. elegans* protein that appears to serve a time-limited function during embryogenesis (Ellis et al., 1991). *ced-7*-deficient worms show a strong engulfment defect during embryogenesis; however, after hatching, corpses are rapidly removed; an additional protein may therefore substitute for CED-7 following embryogenesis (Wu and Horvitz, 1998a). *ced-7*-deficient worms show decreased recruitment of CED-1 around apoptotic cells during embryogenesis (Zhou et al., 2001b). However, the experiments in which this was observed were conducted at the mid-L1 larval stage, when *ced-7* is no longer required for efficient corpse removal; if these corpses had been recently engulfed, CED-1 staining would no longer be observed, which complicates the interpretation of these experiments. In the adult hermaphrodite gonad, CED-7 appears to be dispensable for localization of CED-1 around apoptotic cells (Kinchen et al., 2005).

The identity of the functional equivalent of CED-7 in mammals is also controversial. ABCA1 (Luciani and Chimini, 1996) and ABCA7 (Jehle et al., 2006) have been implicated by different studies: both proteins have been shown to be required for removal of apoptotic cells in the mouse. ABCA1 has been shown to cooperate with MEGF10 to promote engulfment in cell culture systems (Hamon et al., 2006); CED-7/ABCA1 may therefore play a direct role in promoting corpse internalization. ABCA1 also functions in cholesterol transport and maintenance of lipid subdomains on the plasma membrane (Landry et al., 2006). Interestingly, LRP1 and GULP have been proposed to play a role in cholesterol homeostasis, which suggests a direct link between phagocytosis and corpse disposal (Kiss et al., 2006). Several studies have indicated that defects in corpse degradation may inhibit engulfment (see below); however, at this time this remains highly speculative.

The second pathway: CED-2/CrkII, CED-5/Dock180, CED-12/ELMO and CED-10/Rac1

CED-2, CED-5 and CED-12 constitute a second pathway that is required for the efficient phagocytosis of apoptotic cells in the nematode (Gumienny et al., 2001; Reddien and Horvitz, 2000; Wu and Horvitz, 1998b; Wu et al., 2001; Zhou et al., 2001a). Overexpression of the mammalian homologues of these proteins, CrkII (an adaptor protein) and Dock180-ELMO – a guanine nucleotide exchange factor (GEF) complex for Rac – in mammalian cells promotes phagocytosis and Rac activation (Albert et al., 2000; Brugnera et al., 2002; Gumienny et al., 2001). In addition to playing a key role in phagocytosis,

these proteins also function during cell migration in nematodes and mammals (Grimsley et al., 2004; Gumienny et al., 2001; Wu et al., 2001; Zhou et al., 2001a). Whereas *Caenorhabditis elegans* and *Drosophila* have only one CED-12 protein, mammals possess three orthologues of ELMO (ELMO1, ELMO2 and ELMO3) and numerous Dock family proteins (Cote and Vuori, 2002; Meller et al., 2005), some of which also interact with ELMO1 (Lu et al., 2004). ELMO proteins tend to have an overlapping tissue distribution in the mouse (Gumienny et al., 2001). However, in situ studies of ELMO1 and ELMO2 in the mouse brain suggest these proteins play cell-type-specific roles (Katoh et al., 2006a).

The regulation of ELMO-Dock180 activation appears surprisingly complex. Preliminary studies led many to postulate that recruitment of ELMO-Dock180 to the membrane by CrkII is a major regulatory mechanism (Gumienny et al., 2001). However, recent studies have shown that both ELMO and Dock180 possess constitutive membrane-targeting signals (discussed further below), which suggests this mode of regulation is unlikely. The role of CrkII in this complex is thus something of a mystery; CrkII can be immunoprecipitated in an endogenous complex with ELMO-Dock180 but does not appear to play a catalytic role. siRNA-mediated-knockdown experiments suggest that ELMO, Dock180, and CrkII are required for efficient engulfment of apoptotic targets (our unpublished observations) (Tosello-Trampont et al., 2007), but overexpression studies in both cell culture and *Drosophila* suggest that direct interaction between CrkII and Dock180 is not required for the function of either proteins in engulfment or cell migration (Balagopalan et al., 2006; Tosello-Trampont et al., 2007). Thus, it is possible that CrkII mediates multiple, semi-redundant processes during engulfment, perhaps by playing a role in signaling downstream of multiple receptors. CrkII possesses two SH3 (Src homology-3) motifs, one of which has been shown to interact with Dock180. The binding partner of the other SH3 domain is unknown; identification of this interacting partner(s) may provide additional insight into the role of CrkII during phagocytosis.

Dock180 functions like other GEFs that activate small GTPases; it stabilizes the nucleotide-free transition state as Rac cycles from the GDP-bound to the GTP-bound state (Lu et al., 2005). However, Dock180 differs in that it does not contain the traditional tandem Dbl-homology and pleckstrin-homology (DH-PH) domains common to most GEFs. This structure instead appears partitioned into two proteins: ELMO, which contains the PH domain, and Dock180 itself, which contains a Docker domain, the functional equivalent to the DH motif (Brugnera et al., 2002; Cote and Vuori, 2002). Studies of Dock180 function have identified a multi-step mechanism for activation (Lu and Ravichandran, 2006). Dock180 exists in a 'closed' complex in which an intramolecular interaction between the N-terminal SH3 domain and the catalytic Docker domain sterically blocks Rac interaction (Lu et al., 2005). ELMO binds to the Dock180 SH3 domain, which suggests that ELMO enhances the GEF activity of Dock180 by relieving this intramolecular inhibition. However, further research has determined that the PH domain of ELMO stabilizes the nucleotide-free Rac transition state independently of binding to the Dock180 SH3 motif (Lu et al., 2004). Direct interaction between ELMO and Rac (either nucleotide-free or GTP/GDP bound) has yet to be demonstrated; this stabilization may

therefore be due to an as-yet-unidentified transient interaction with Dock180.

Additional studies have identified other potential regulators of ELMO-Dock180 function. The small GTPase RhoG preferentially interacts in its GTP-bound form with ELMO (Katoh and Negishi, 2003); overexpression of RhoG, or its GEF Trio, promotes uptake of apoptotic targets in an ELMO-Dock180-dependent manner (deBakker et al., 2004). RhoG has also been suggested to influence cell migration mediated by Dock180 or Dock4, which can also interact with ELMO (Hiramoto et al., 2006; Katoh et al., 2006b; Lu et al., 2004). MIG-2, the nematode orthologue of RhoG, plays a key role in cell migration, but only a minor role in corpse removal (deBakker et al., 2004; Lundquist et al., 2001). Although overexpression of RhoG induces Rac activation dependent on ELMO-Dock180, whether this is mediated solely by recruitment of ELMO-Dock180 proteins to the membrane or another means of activation has yet to be determined. One possibility is that RhoG acts as a targeting/recruitment signal, since an ELMO protein in which the RhoG-interacting motif is mutated cannot be targeted to membrane ruffles (deBakker et al., 2004). Other studies have identified ELMO-Dock180 as a point of cross-talk between the GTPases Arf6 and Rac (Santy et al., 2005); however, the significance of this observation in removal of apoptotic cells remains unclear.

Other mechanisms for localization of ELMO-Dock180 to the plasma membrane continue to be identified. The Dock180 DHR-1 domain specifically interacts with PtdIns(3,4,5) P_3 , which is enriched at the leading edge during migration and phagocytosis (Cote et al., 2005) and appears to be required for recruitment of Dock180 to this region. Other studies have identified a high-molecular-weight protein complex in the nucleus containing both Dock180 and ELMO, which indicates ELMO-Dock180 activation may also involve spatial segregation from the site of action (Yin et al., 2004). Further, ELMO1 is phosphorylated by the Src-family kinase Hck when the two proteins are co-overexpressed; mutation of the tyrosines phosphorylated in vitro impairs the function of ELMO in phagocytosis (Yokoyama et al., 2005). Additionally, studies have shown that cross-talk exists between ELMO-Dock180 and the Mer tyrosine kinase; ELMO-Dock180 might thus integrate signals from diverse receptors (Wu et al., 2005).

Destabilization of the ELMO-Dock180 complex may also be a key mechanism for regulation of its activity. Recent work suggests that Dock180 is ubiquitinated at the plasma membrane (Makino et al., 2006); Dock180 complexed with ELMO appears to be refractory to degradation. Overexpression of CrkII increases ubiquitylation of Dock180; the E3 ligase may therefore be recruited in a CrkII-dependent manner, either directly or indirectly through as-yet-unidentified machinery. Alternatively, association with Crk may increase cycling of Dock180, such that less is complexed with ELMO; however, this remains speculation. A cohesive regulatory scheme for ELMO-Dock180 activation during phagocytosis that accounts for all these observations will require additional investigations.

ELMO and Dock proteins have also been shown to play a role in the induction of various disease states. One report suggests a role for an ELMO-Dock2 complex in Rac activation downstream of Nef during HIV pathogenesis (Janardhan et al., 2004). Mutations within the ELMO1 locus are associated with increased susceptibility to diabetic nephropathy (kidney

disease associated with diabetes) (Shimazaki et al., 2005). Surprisingly, the NOD mouse, a popular diabetes model, has defects in phagocytosis of apoptotic cells, although this may be linked to other uncharacterized macrophage defects (Maree et al., 2005). Type I diabetes is in part due to autoimmune responses directed against the cells in the pancreas that produce insulin (Gillespie, 2006); whether this can be related to defects in engulfment of apoptotic cells, which also generates an autoimmune state, remains to be seen.

Rac-independent phagocytosis

Rac has long been known to play a role in the removal of apoptotic cells. However, work using *ced-10(null)* mutants has shown that a Rac-independent pathway might also exist in the nematode, although this 'salvage' pathway appears to be inefficient (Kinchen et al., 2005). Previous work had hinted at the existence of this pathway, since many apoptotic cell corpses are still engulfed in *ced-1;ced-5* double mutants. However, detailed studies using time-lapse microscopy have only recently begun to determine the fate of individual cells, and this third pathway remains completely uncharacterized. Studies using single-cell resolution have also shown that the two engulfment pathways do not function to the same degree in all cell types (Yu et al., 2006). Recent data from the Shaham group also show that the two engulfment pathways are not required for removal of the linker cell, which undergoes a caspase-independent death (Abraham et al., 2007). Thus, the two pathways for corpse removal in the nematode may function to different degrees in different cells and in different situations.

The roles of other Rho-family GTPases in engulfment are just beginning to be addressed. Studies using overexpressed RhoA have shown that this gene appears to function as a negative regulator of phagocytosis (see below) (Tosello-Trampont et al., 2003). Cdc42 does not appear to play a primary role in phagocytosis of apoptotic cells, although overexpression of intersectin, a Cdc42-specific GEF, promotes phagocytosis (Nakaya et al., 2006; Tosello-Trampont et al., 2003). Surprisingly, overexpression of Rab5 efficiently promotes phagocytic uptake, and dominant-negative constructs inhibit it (Nakaya et al., 2006). Rab5 is commonly used as a marker for the early endosome; owing to its localization within the cell, Rab5 is unlikely play a direct role in phagocytic signaling. However, ongoing studies have suggested a clear link between the ability to degrade an apoptotic cell and the ability to phagocytose additional apoptotic cells (see below).

Degradation of an apoptotic cell is an important feedback mechanism

Digestion of the apoptotic cell has long been the least considered stage in corpse removal, perhaps because it was believed to be little more than 'garbage-disposal'. However, new studies are challenging that view, suggesting that the process of phagocytosis can directly influence the capacity of the cell to phagocytose additional apoptotic cells (Schrijvers et al., 2005). Following closure of the plasma membrane around the apoptotic cell, the internalized corpse is contained within a phagolysosome (Fig. 1D). However, to be degraded properly, the apoptotic cell must enter the lysosomal network, a sequential series of endosomes leading to an acidic lysosome, where proteins are degraded and recycled. Studies in the field are now beginning to track this process and have provided several new insights.

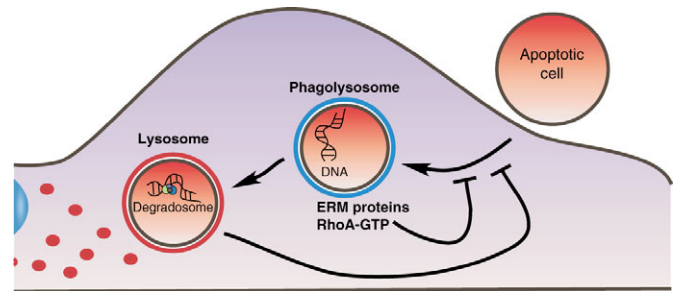


Fig. 3. Processing of the apoptotic cell corpse can act as a feedback mechanism to regulate further engulfment by the same phagocyte. Recent evidence suggests that, following engulfment, the apoptotic cell must progress through a series of endosomes before finally entering an acidic lysosome. The figure depicts two potential modes by which the processing of the engulfed corpse may regulate further phagocytosis. Activation of RhoA during later stages of engulfment, and subsequent RhoA-mediated signaling, could inhibit further uptake. Independently, proper degradation/disposal of the apoptotic-cell-derived DNA appears to regulate further engulfment by the same phagocyte. These feedback control points may link the early and late stages of engulfment to ensure proper disposal of phagocytosed apoptotic cells.

Addition of apoptotic cells to a culture of phagocytic cells results in transient Rac activation. Interestingly, RhoA-GTP levels also increase but at later time points (Erwig et al., 2006; Tosello-Trampont et al., 2003). The precise significance of this delayed RhoA activation has been the subject of some speculation. In migrating cells, activation of RhoA (and its effector, Rho kinase, ROCK) results in release of the 'lagging edge,' which is required for forward motion (Worthylake and Burridge, 2001). However, given the excess of apoptotic cells used in these experiments, it is unlikely that RhoA activation is due to induction of a migratory event. Henson and co-workers have suggested that the transient RhoA activation seen during phagocytosis is due to signaling as the apoptotic cell enters the phagolysosome (Erwig et al., 2006). The implications of these experiments are quite broad, suggesting that the timing of phagocytosis and lysosome entry is closely coordinated. When Rho activity is high because an apoptotic cell is entering an endosomal structure, the phagocyte may be prevented from engulfing an additional apoptotic cell. This could prevent problems related to unresolved phagocytic intermediates. RhoA signaling thus represents a potentially attractive therapeutic target for diseases such as lupus, where defects in phagocytosis may result in exposure of potential self-antigens. Interestingly, ERM proteins (a family of actin-binding proteins including ezrin, radixin and moesin) play a key role in RhoA-mediated inhibition at the phagosome. ELMO has been shown to interact with the ERM proteins; however, a biological role for this interaction could not be identified (Grimsley et al., 2006). The interaction of ERM proteins with both RhoA and ELMO could bring these two activities together, allowing RhoA-GTP to signal to inhibit ELMO (Fig. 3).

DNA degradation following phagocytosis

Reverse genetic screens in the nematode have identified a complex series of proteases and DNases (including DNase II) that interact together to form a large 'degradosome' responsible for degrading the DNA of the apoptotic cell (Parrish and Xue, 2003). Surprisingly, defects in DNase II

have been associated with decreased ability to engulf apoptotic cells both in a mouse model and in *C. elegans* (Krieser et al., 2002; Parrish and Xue, 2003; Wu et al., 2000). The evidence discussed above indicates that signaling downstream of phagocytosis can impact the ability of an apoptotic cell to remove additional targets (see above). Studies in the nematode support this hypothesis: mutations in NUC-1 mimic mutations in the engulfment machinery, resulting in increased cell survival (Wu et al., 2000). Interestingly, *Drosophila* lacking DNase II also show decreased ability to phagocytose bacteria, providing further support for the idea that signaling from the lysosome to the phagosome is important for efficient phagocytosis (Seong et al., 2006).

Conclusions and future directions

Although numerous studies have examined signaling of the apoptotic cell and events downstream of phagocytosis, we still know relatively little about intracellular signaling during phagocytosis. Numerous receptors have been implicated in the removal of an apoptotic cell; however, the role of many of these receptors is controversial, and the effect of loss-of-function mutations *in vivo* can be relatively mild. There are several possible explanations: removal of apoptotic cells in organisms could be quite redundant; signaling pathways for the removal of apoptotic cells by macrophages and nonprofessional phagocytes might be largely overlapping (nonprofessional phagocytes would thus substitute for deficient macrophages and vice-versa), or the majority of identified receptors might promote phagocytosis by enhancing binding to the apoptotic cell. It is crucial for future work to distinguish these possibilities.

Very few studies go the extra step and look at how each individual receptor takes part in cross-talk with other potential receptors to mediate corpse removal. Research in other contexts, such as signaling through the T-cell receptor, clearly shows that, although one key protein may be at the center of signaling, coordinated signaling from many accessory proteins is also required. The field must now begin the arduous task of teasing out and integrating the roles of each protein in corpse recognition and downstream signaling.

Finally, how anti-inflammatory signaling induced by apoptotic cells is overridden to produce an autoimmune state in mice that display deficiencies in corpse removal must be addressed. Perhaps anti-inflammatory signaling is limited to macrophages and other nonprofessional phagocytes – further work needs to ascertain what the response of the other cellular components of the immune system is to persistent cell corpses. The engulfment field is currently undergoing an expansion: more and more researchers are interested in solving the unique dilemma of how apoptotic cells, which represent a strong ‘self’ signal, are removed without generating an immune response. The groundwork has begun to be laid and portends an exciting new series of observations with insights drawn from multiple model systems.

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