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Article

Engulfment of Apoptotic Cells in *C. elegans* Is Mediated by Integrin α/SRC Signaling

Tsung-Yuan Hsu[1] and Yi-Chun Wu[1,2,*]
[1]Institute of Molecular and Cellular Biology
[2]Department of Life Science
National Taiwan University, Number 1, Section 4, Roosevelt Road, Taipei 10617, Taiwan

Summary

Background: Engulfment of apoptotic cells is important for cellular homeostasis and the development of multicellular organisms. Previous studies have shown that more than one engulfment receptors act upstream of the conserved signaling module CED-2/CrkII-CED-5/Dock180-CED-12/ELMO for cell corpse removal in *C. elegans*, but little is known about their identities, except for PSR-1.
Results: We show that in *C. elegans*, integrin functions as an engulfment receptor in the recognition and subsequent phagocytosis of apoptotic cells. Mutations in the integrin α gene *ina-1* result in inefficient engulfment of apoptotic cells. The INA-1 extracellular domain binds to the surface of apoptotic cells in vivo. This binding requires the phospholipid scramblase SCRM-1, which promotes the exposure of phosphatidylserine, a key "eat me" signal in apoptotic cells. Furthermore, we identify an essential role of the nonreceptor tyrosine kinase SRC-1 in INA-1-mediated cell corpse removal. INA-1 and SRC-1 both act in the engulfing cells during the engulfment process and are colocalized in the phagocytic cups extending around apoptotic cells. Finally, our genetic and biochemical data suggest that SRC-1 relays the *scrm-1*-dependent engulfment signal from INA-1 to the conserved motility-promoting signaling complex CED-2/CrkII-CED-5/Dock180-CED-12/ELMO for CED-10/Rac activation, probably by interactions with CED-2 and the INA-1 cytoplasmic domain, leading to the internalization of apoptotic cells.
Conclusions: Our findings provide evidence that integrin functions as an engulfment receptor at the whole-organism level and reveal a nonconventional signaling pathway in which SRC provides a FAK-independent linkage between integrin α and the common motility-promoting signaling module CED-2/CrkII-CED-5/Dock180-CED-12/ELMO to promote the internalization of apoptotic cells.

Introduction

When cells undergo apoptosis, their corpses are quickly recognized and phagocytosed by engulfing cells [1–3]. Engulfment of apoptotic cells needs to be tightly regulated, because too little engulfment has been shown to cause inflammation and autoimmune disease in mice and humans [1, 2], whereas too much engulfment in *C. elegans* leads to the loss of cells that normally survive [4, 5]. However, the regulation and mechanism of apoptotic cell engulfment are poorly understood.

Engulfment of apoptotic cells is a complex process that involves the recognition, internalization, and degradation of apoptotic cells. Apoptotic cells display "eat me" signals that are recognized by engulfment receptors either directly or indirectly through bridging molecules. The best-characterized "eat me" signal is the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane [6]. In living cells, PS is normally confined to the inner leaflet of the plasma membrane, but during apoptosis, it is externalized and acts as an "eat me" signal. Pioneering studies in mammalian cells have identified several potential engulfment receptors that recognize exposed PS on apoptotic cells. These include the PS receptor (PSR) [7], T cell immunoglobulin- and mucin-domain-containing molecule 4 (TIM-4) and TIM-1 [8, 9], stabilin-2 [10], brain-specific angiogenesis inhibitor 1 (BAI-1) [11], integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [12–14], and the receptor tyrosine kinase MER [15, 16]. Of these receptors, PSR, TIM-4, and BAI-1 have been shown to bind PS directly in vitro, whereas the integrins and MER respectively bind PS indirectly through the soluble bridging molecules milk fat globule-EGF factor 8 (MFG-E8) or growth-arrest-specific gene 6 (Gas-6) [12, 13, 15]. Only PSR and the integrins discussed above have obvious corresponding genes with similar sequences in worms and flies. PSR knockout experiments have shown that PSR may play a conserved role in eliminating apoptotic [17, 18] or non-apoptotic [19] cells in many organisms, including worms, flies, and zebrafish, but may not be the dominant engulfment receptor [6]. Thus, it is important to determine whether the function of the other receptor, the integrins, may be evolutionarily conserved.

During the development of a *C. elegans* hermaphrodite, 131 somatic cells undergo programmed cell death [3], and the cell corpses are removed by their neighboring cells within an hour. Previous studies have identified and characterized two conserved pathways that mediate the engulfment of apoptotic cells [3, 20]. In one, the engulfment receptor CED-1, together with CED-7 (an ATP-binding cassette transporter), acts upstream of the phosphotyrosine-binding domain-containing adaptor protein CED-6 and the dynamin DYN-1 to promote the engulfment and degradation of apoptotic cells [3, 20]. In the other, the engulfment receptor PSR-1 functions upstream of the intracellular signaling proteins CED-2, CED-5, and CED-12, which in turn activate the small GTPase CED-10/Rac, leading to actin-driven extension of pseudopods that ultimately enclose apoptotic cells [17, 21–25]. CED-2 is similar to the SH2/SH3 domain-containing adaptor protein CrkII, CED-5 is similar to Dock180, and CED-12 is similar to ELMO [21–25]. Dock180 and ELMO function together as a guanine nucleotide exchange factor for Rac [26]. CED-5/Dock180 and CED-12/ELMO may interact with CED-2/CrkII to form a trimeric complex [21, 24] but may also function as a submodule independent of CED-2/CrkII [27] to activate the GTPase CED-10/Rac. In addition, an UNC-73/TRIO-MIG-2/RhoG signaling module has been shown to control CED-10/Rac activation through its interaction with the armadillo repeat of CED-12/ELMO [28]. A genetic study has shown that CED-10 may also mediate certain activities in the CED-1 pathway [29]. Recently, negative regulators in the engulfment process have been revealed. The myotubularin MTM-1 inhibits cell corpse engulfment mediated by CED-5, CED-12, and CED-10, whereas the

*Correspondence: yichun@ntu.edu.tw

Table 1. *ina-1* Mutants Contain More Apoptotic Cells than Wild-Type during Embryogenesis

	Number of Cell Corpses				
Strain	Comma Stage	1.5-fold Stage	2-fold Stage	3-fold Stage	4-fold Stage
N2	10.1 ± 0.6	10.2 ± 0.6	8.3 ± 0.7	2.5 ± 0.4	0.3 ± 0.5
ina-1(RNAi)	13.1 ± 1.3**	14.1 ± 1.3**	10.9 ± 2.1*	2.2 ± 0.7	0.3 ± 0.5
ina-1(gm39)	11.3 ± 1.9*	11.1 ± 0.9*	8.7 ± 0.8	2.1 ± 0.8	0.4 ± 0.5
ina-1(gm144)	12.2 ± 1.6*	12.5 ± 1.9*	9.6 ± 2.1*	2.1 ± 0.6	0.5 ± 0.6
ina-1(gm88)	13.1 ± 0.8**	13.7 ± 0.9**	11.2 ± 2.1**	2.4 ± 0.8	0.4 ± 0.7
ina-1(gm86)	13.2 ± 1.1**	13.6 ± 0.8**	11.3 ± 1.1**	2.6 ± 0.8	0.5 ± 0.6
pat-3(RNAi)	15.1 ± 1.9**	15.2 ± 1.6**	ND	ND	ND
pat-3(st564)	15.0 ± 1.2**	14.8 ± 1.2**	ND	ND	ND
pat-3(RNAi) ina-1(gm144)	15.1 ± 1.4**	15.2 ± 1.3**	ND	ND	ND
ced-3(n717)	0.1 ± 0.3	0.4 ± 0.2	0.4 ± 0.2	0.1 ± 0.3	0.2 ± 0 .4
ina-1(RNAi); ced-3(n717)	0.1 ± 0.3	0.2 ± 0.4	0.4 ± 0.2	0.1 ± 0.2	0.2 ± 0.4
ced-4(n1162)	0.7 ± 0.7	0.2 ± 0.4	0.4 ± 0.5	0.2 ± 0.4	0.4 ± 0.8
ced-4(n1162); ina-1(RNAi)	0.7 ± 1.0	0.6 ± 0.7	0.4 ± 0.6	0.3 ± 0.5	0.4 ± 0.6
cep-1(gk138)	7.4 ± 2.1	9.2 ± 1.1	5.5 ± 1.1	1.2 ± 1.1	0.5 ± 0.5
cep-1(gk138); ina-1(RNAi)	12.8 ± 1.3	14.4 ± 1.0	10.9 ± 1.1	3.0 ± 1.3	0.6 ± 0.8

The number of cell corpses in each genotype was scored at the indicated embryonic stage. *ina-1*, *pat-3*, and *pat-3ina-1* were compared to N2, and *cep-1 (gk138); ina-1(RNAi)* was compared to *ina-1(RNAi)* at each stage (*$p < 0.05$, **$p < 0.001$; $p < 0.05$ for *pat-3(RNAi)* compared to any of the *ina-1* mutant alleles). All comparisons were performed by unpaired t test. ND indicates not determined because of embryonic arrest at the 2-fold stage. Data are presented as mean ± standard deviation (SD) of $n > 20$ embryos per stage.

tyrosine kinase ABL-1 acts in a pathway that is distinct from the two known engulfment pathways [30, 31].

Of the two *C. elegans* engulfment receptors characterized thus far, PSR-1 binds to surface-exposed PS on apoptotic cells, whereas CED-1 may recognize additional "eat me" signals besides PS [17, 32]. Interestingly, distinct cell-surface proteins, SCRM-1 and CED-7, are involved in the externalization of PS on apoptotic cells for recognition of apoptotic cells by PSR-1 and CED-1, respectively [32, 33]. However, PSR-1 is not the sole receptor acting in the CED-2, CED-5, and CED-12 pathway, because the *psr-1* null mutant has a weaker engulfment defect than any of the *ced-2*, *ced-5*, or *ced-12* mutants [17]. The identity of the receptors other than PSR-1 in this pathway remains unknown.

In *C. elegans*, there are two integrin α subunits, INA-1 and PAT-2, and one β subunit, PAT-3. Whether these integrins play a role in the engulfment of apoptotic cells is unclear. In this study, we showed that INA-1 recognizes apoptotic cells and mediates signaling through CED-2, CED-5, and CED-12 in the engulfing cells to promote cell corpse removal. We also identified and characterized an additional component, SRC-1, which provides a missing link between INA-1 and the downstream signaling module CED-2-CED-5-CED-12 for CED-10/Rac activation as an engulfing cell extends pseudopods around apoptotic cells.

Results

ina-1 Is Important for the Efficient Engulfment of Apoptotic Cells during Embryogenesis

To understand the function of *ina-1* in apoptosis, we utilized *ina-1* mutant alleles and the RNA interference (RNAi) technique to specifically knock down *ina-1* gene activity. The *ina-1* weak alleles *gm39* and *gm144* are viable, whereas the strong alleles *gm86* and *gm88* result in first stage (L1) larval arrest [34]. A time-course analysis of cell corpses during embryogenesis showed that *ina-1* mutants contained more cell corpses than the wild-type at the comma, 1.5-fold, and 2-fold stages (Table 1). This increase in the number of cell corpses was statistically significant at these three stages in all four *ina-1* mutant alleles (except for *gm39* at the 2-fold stage) and in RNAi-treated embryos, indicating that this phenotype was caused by loss of *ina-1* function and was not allele specific. In addition, the extra cell corpses in the *ina-1* mutants displayed a refractile, raised button-like morphology characteristic of apoptotic cells, and their generation was blocked by strong mutations of the proapoptotic genes *ced-3* and *ced-4* [35] (Table 1), showing that the extra cell corpses were generated by programmed cell death.

Cell adhesion to the extracellular matrix is primarily mediated by integrins. An impaired cell-extracellular matrix interaction often causes apoptosis, a phenomenon known as anoikis [36]. The extra cell corpses in the *ina-1* mutants did not appear to result from anoikis. First, *ina-1* animals contained the same number of nuclei in their anterior pharynx as wild-type animals (data not shown). Second, in some specific cell lineages, cells that were programmed to die actually survived in *ina-1* animals (see Figure S1 available online). Finally, integrin-mediated anoikis requires *p53* in many types of mammalian cells [36], but the increase in cell corpses in *ina-1* mutants was independent of *cep-1*, the *p53* homolog in *C. elegans* [37], because the *cep-1(gk138)* mutation did not affect the number of apoptotic cells in *ina-1(RNAi)* embryos (Table 1).

We then examined whether the increase in embryonic apoptotic cells in the *ina-1* mutants might be caused by a defect in the removal of apoptotic corpses. To this end, we used four-dimensional differential interference contrast (DIC) microscopy analysis to measure and compare the duration of persistence of embryonic cell corpses in wild-type and *ina-1* embryos from the comma to the 2-fold stage, as described previously [17]. The majority (95%) of wild-type cell corpses disappeared within 40 min, and none lasted longer than 50 min (Figure 1). However, in *ina-1(gm86)* embryos, only 47% of the cell corpses disappeared within 40 min, and 19% persisted longer than 50 min (Figure 1). On average, cell corpses in *ina-1(gm86)* embryos persisted for 45 min, approximately 55% longer than in wild-type embryos (Figure 1). Similar results were observed in *ina-1(gm144)* and *ina-1(RNAi)* embryos (Figure 1). These data show that the *ina-1* mutants were defective in the engulfment of apoptotic cells. Because the apoptotic cells were ultimately engulfed in *ina-1* mutants during the recording period, *ina-1* is not absolutely

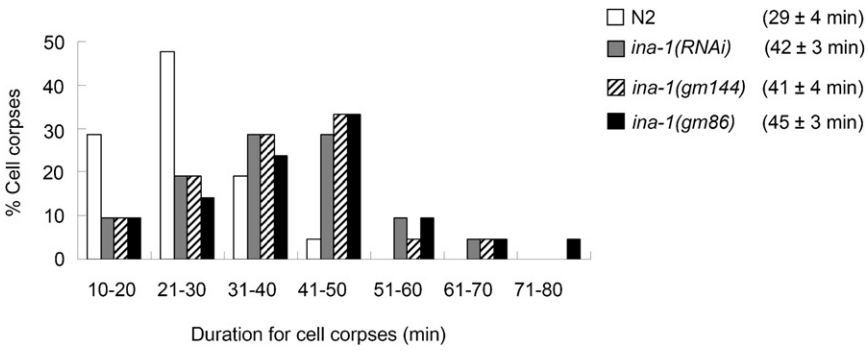


Figure 1. *ina-1* Is Important for Apoptotic Cell Engulfment during Embryogenesis

The duration of cell corpses in wild-type (white bars), *ina-1(RNAi)* (gray bars), *ina-1(gm144)* (striped bars), and *ina-1(gm86)* (black bars) embryos was measured by four-dimensional microscopy analysis. Approximately 45 cell corpses from three embryos of each genotype were analyzed. The y axis represents the percentage of cell corpses in a specific duration range (shown on the x axis). Numbers in parentheses indicate the average duration time of cell corpses (mean ± standard deviation [SD]) for each genotype.

required for apoptotic cell engulfment but is important for the efficiency of this process. The increase in apoptotic cell number in *ina-1* mutants was observed at the comma to 2-fold stages, but not in late 3- or 4-fold embryos, suggesting either that the role of *ina-1* in cell corpse engulfment may be specific to midembryogenesis, when most embryonic cell deaths occur, or that *ina-1* acts redundantly with one or more other genes in late embryos.

ina-1 Acts in the *ced-2*, *ced-5*, and *ced-12* Signaling Pathway for the Clearance of Apoptotic Cells

A previous study showed that INA-1 associates with integrin β PAT-3 on the plasma membrane and that these two proteins function together in multiple cellular processes [34]. We found that *pat-3* mutants also had increased numbers of cell corpses from the comma to the 1.5-fold stage. In addition, the phenotype of the *pat-3(RNAi) ina-1(gm144)* double mutant was no stronger than that of the *pat-3(RNAi)* single mutant ($p = 0.7$ by unpaired t test; Table 1), in agreement with the notion that INA-1 may act together with PAT-3 in a protein complex to mediate cell corpse removal. We then sought to determine in which of the two partially redundant engulfment pathways *ina-1* acts. We generated and analyzed double mutants between *ina-1(gm144*, *gm86*, *gm88*, or *RNAi)* mutations and strong loss-of-function alleles of the engulfment *ced* genes for each pathway. The *ina-1* mutations significantly increased apoptotic cell numbers in *ced-1* and *ced-6* mutants (Table 2). In contrast, the *ina-1; ced-2*, *ina-1; ced-5*, and *ina-1; ced-12* double mutants contained a number of apoptotic cells similar to their respective *ced* single mutants (Table 2). These results indicate that *ina-1* acts in the genetic pathway mediated by *ced-2*, *ced-5*, and *ced-12* during the engulfment of apoptotic cells.

ina-1 Acts Genetically Upstream of *ced-2*, *ced-5*, and *ced-12* to Promote Engulfment of Apoptotic Cells

Previous genetic analyses have shown that *ced-2*, *ced-5*, and *ced-12* act upstream of *ced-10* to control cell corpse engulfment [21–25]. To determine whether *ina-1* acts upstream or downstream of *ced-2*, *ced-5*, *ced-10*, and *ced-12* to control cell corpse engulfment, we utilized the heat-shock promoter (P_{hsp}) to ectopically express *ced-2*, *ced-5*, *ced-10*, or *ced-12* cDNA in *ina-1(RNAi* and *gm144)* mutants. The phenotype of an increased number of cell corpses in *ina-1* mutants was significantly rescued by overexpression of *ced-2*, *ced-5*, *ced-10*, or *ced-12* (Table 3). In contrast, overexpression of *ced-1*, *ced-6*, or the control *gfp* failed to rescue the *ina-1* engulfment defect (Table 3). In the reciprocal experiments, *ina-1* cDNA was overexpressed by heat-shock promoter P_{hsp} in *ced-2*, *ced-5*, *ced-10*, and *ced-12* mutants. Overexpression of *ina-1* rescued the engulfment defect of the *ina-1* mutants but failed to rescue the defect in *ced-2*, *ced-5*, *ced-10*, or *ced-12* mutants (Table 3). Together, these observations indicate that *ina-1* likely acts upstream of *ced-2*, *ced-5*, *ced-10*, and *ced-12* in promoting engulfment.

A previous study has shown that the PS-binding receptor PSR-1 also acts upstream of CED-2, CED-5, and CED-12 in

Table 2. Number of Apoptotic Cells in *ina-1*, *src-1*, and Other Engulfment-Defective Mutants

Strain	Number of Cell Corpses	
	Comma Stage	1.5-fold Stage
N2	10.1 ± 0.6	10.2 ± 0.6
ina-1(RNAi)	13.1 ± 1.3	14.1 ± 1.3
ced-1(e1735)	19.9 ± 1.4	24.9 ± 2.4
ced-1(e1735); ina-1(RNAi)	25.1 ± 2.5**	28.1 ± 2.7**
ced-1(e1735); ina-1(gm144)	26.3 ± 1.5**	28.5 ± 2.1**
ced-1(e1735); ina-1(gm86)	26.3 ± 1.1**	28.2 ± 1.9**
ced-1(e1735); ina-1(gm88)	27.6 ± 2.0**	28.3 ± 2.4**
ced-1(n1995)	16.2 ± 1.1	19.0 ± 1.8
ced-1(n1995); ina-1(RNAi)	18.3 ± 1.6*	21.3 ± 1.1*
ced-6(n1813)	19.2 ± 2.1	20.3 ± 2.2
ced-6(n1813) ina-1(RNAi)	25.6 ± 1.8**	26.5 ± 2.3**
ced-2(n1994)	18.4 ± 2.1	22.8 ± 3.6
ina-1(RNAi); ced-2(n1994)	19.3 ± 1.9	23.4 ± 2.6
ina-1(gm144); ced-2(n1994)	18.5 ± 0.9	22.4 ± 1.1
ced-2(n1752)	15.8 ± 1.6	15.8 ± 1.7
ina-1(RNAi); ced-2(n1752)	15.6 ± 2.1	16.3 ± 2.3
ced-5(n1812)	32.4 ± 1.8	36.1 ± 2.8
ina-1(RNAi); ced-5(n1812)	31.4 ± 1.4	35.9 ± 2.1
ina-1(gm144); ced-5(n1812)	32.1 ± 3.5	33.9 ± 3.3
ina-1(gm86); ced-5(n1812)	32.5 ± 2.8	36.5 ± 2.1
ina-1(gm88); ced-5(n1812)	31.9 ± 2.8	37.1 ± 2.9
ced-5(n2002)	26.0 ± 2.3	29.6 ± 1.7
ina-1(RNAi); ced-5(n2002)	25.6 ± 1.6	29.3 ± 2.3
ced-12(n3261)	21.8 ± 2.4	25.6 ± 2.7
ced-12(n3261); ina-1(RNAi)	22.6 ± 1.3	25.1 ± 1.6
ced-12(tp2)	30.4 ± 2.8	33.9 ± 1.4
ced-12(tp2); ina-1(gm144)	30.9 ± 3.4	32.9 ± 2.0
ced-12(tp2); ina-1(gm88)	29.6 ± 2.1	31.5 ± 2.8
psr-1(tm469)	13.2 ± 2.7	13.1 ± 2.6
ina-1(RNAi); psr-1(tm469)	14.9 ± 2.2*	15.8 ± 1.2*
ina-1(gm144); psr-1(tm469)	15.1 ± 0.9*	16.1 ± 1.7**
src-1(cj293)	13.1 ± 0.8##	14.7 ± 1.2##
src-1(RNAi)	12.4 ± 1.0#	12.9 ± 0.8#
src-1(cj293); ina-1(RNAi)	13.5 ± 0.7	13.9 ± 0.9
src-1(RNAi); ced-2(n1994)	18.7 ± 1.6	22.4 ± 2.6
src-1(RNAi); ced-1(e1735)	24.6 ± 1.9**	30.7 ± 3.1**

Double mutants were compared to the corresponding single mutants (*$p < 0.05$, **$p < 0.001$). *src-1* single mutants were compared to N2 (#$p < 0.05$, ##$p < 0.001$). All comparisons were performed by unpaired t test. Data presented are mean ± SD of $n > 20$ embryos.

Table 3. Expression of *ina-1* and Other Engulfment Genes in Engulfment-Defective Mutants

Genotype	Transgene	Heat Shock	Number of Cell Corpses: Comma Stage	Number of Cell Corpses: 1.5-fold Stage
N2	–	–	10.1 ± 0.6	10.2 ± 0.6
N2	P_{hsp} *gfp*	+	10.1 ± 0.8	10.0 ± 0.7
ina-1(RNAi)	–	–	13.1 ± 1.4	14.1 ± 1.3
ina-1(RNAi)	P_{hsp} *gfp*	+	13.1 ± 1.4	13.5 ± 1.3
ina-1(RNAi)	P_{hsp} *ced-2*	–	13.2 ± 1.4	13.8 ± 1.2
ina-1(RNAi)	P_{hsp} *ced-2*	+	9.2 ± 1.1**	9.9 ± 0.8**
ina-1(RNAi)	P_{hsp} *ced-5*	–	13.4 ± 1.1	13.7 ± 1.3
ina-1(RNAi)	P_{hsp} *ced-5*	+	10.2 ± 1.2*	9.4 ± 0.7**
ina-1(RNAi)	P_{hsp} *ced-10*	–	13.3 ± 1.1	13.2 ± 1.1
ina-1(RNAi)	P_{hsp} *ced-10*	+	9.7 ± 1.2**	9.7 ± 1.1**
ina-1(RNAi)	P_{hsp} *ced-12*	–	13.2 ± 1.2	13.8 ± 1.5
ina-1(RNAi)	P_{hsp} *ced-12*	+	9.7 ± 0.8**	9.4 ± 1.0**
ina-1(gm144)	–	–	12.2 ± 1.6	12.5 ± 1.9
ina-1(gm144)	P_{hsp} *gfp*	+	12.3 ± 1.3	12.4 ± 1.2
ina-1(gm144)	P_{hsp} *ced-2*	–	12.3 ± 1.4	12.6 ± 1.3
ina-1(gm144)	P_{hsp} *ced-2*	+	10.2 ± 1.1*	9.9 ± 0.6*
ina-1(gm144)	P_{hsp} *ced-5*	–	12.1 ± 1.2	12.3 ± 1.4
ina-1(gm144)	P_{hsp} *ced-5*	+	10.1 ± 1.0*	10.1 ± 0.8*
ina-1(gm144)	P_{hsp} *ced-1*	–	12.5 ± 1.3	12.6 ± 1.1
ina-1(gm144)	P_{hsp} *ced-1*	+	12.2 ± 1.3	12.6 ± 1.1
ina-1(gm144)	P_{hsp} *ced-6*	–	12.6 ± 1.4	12.7 ± 1.4
ina-1(gm144)	P_{hsp} *ced-6*	+	12.5 ± 1.2	12.4 ± 1.3
ina-1(gm144)	P_{hsp} *ina-1*	–	12.5 ± 1.3	12.5 ± 1.9
ina-1(gm144)	P_{hsp} *ina-1*	+	9.9 ± 0.7**	9.9 ± 0.5**
ced-2(n1994)	P_{hsp} *ina-1*	–	18.8 ± 2.0	22.8 ± 3.6
ced-2(n1994)	P_{hsp} *ina-1*	+	19.3 ± 0.5	20.7 ± 1.1
ced-5(n1812)	P_{hsp} *ina-1*	–	32.0 ± 1.4	36.1 ± 2.8
ced-5(n1812)	P_{hsp} *ina-1*	+	31.2 ± 1.2	35.3 ± 2.1
ced-10(n3246)	P_{hsp} *ina-1*	–	26.1 ± 1.6	30.1 ± 2.0
ced-10(n3246)	P_{hsp} *ina-1*	+	27.2 ± 1.6	29.5 ± 2.2
ced-12(tp2)	P_{hsp} *ina-1*	–	31.3 ± 1.6	33.9 ± 1.4
ced-12(tp2)	P_{hsp} *ina-1*	+	31.1 ± 1.8	33.2 ± 1.4
ina-1(gm144)[a]	P_{ina-1} *ina-1::gfp*	–	10.2 ± 0.4#	10.1 ± 0.5#
ina-1(gm144)[a]	P_{egl-1} *ina-1::gfp*	–	12.3 ± 0.5	12.4 ± 0.6
ina-1(gm144)[a]	P_{ced-1}*ina-1::gfp*	–	10.6 ± 0.5#	10.1 ± 0.8#
ina-1(gm144)[a]	P_{ced-1} *ina-1ΔC::gfp*	–	12.3 ± 0.6	13.1 ± 0.5

For heat-shock treatments, transgenic animals were subjected to heat shock (+) at 33°C for 30 min or left at 20°C (–). Comparison of heat-shocked transgenic worms with the corresponding non-heat-shocked transgenic worms at the same stage was performed by unpaired t test (*$p < 0.05$, **$p < 0.001$). For P_{hsp} *ina-1* mutants, apoptotic cells were counted at the 1.5-fold stage. Data are presented the mean ± SD of $n > 20$ embryos.
[a] Comparison of *ina-1(gm144)* transgenic embryos with nontransgenic *ina-1(gm144)* embryos at the same stage was performed by unpaired t test (#$p < 0.05$).

mediating cell corpse removal [17]. We therefore generated and analyzed *ina-1; psr-1* double mutants, and we found that the double mutants had slightly, but significantly, more cell corpses than the *ina-1* or *psr-1* single mutants (Table 2). This result shows that *ina-1* and *psr-1* probably act in a partially redundant manner in promoting the engulfment of apoptotic cells. Interestingly, the number of cell corpses in the *ina-1; psr-1* double mutant was still less than that in any of the *ced-2*, *ced-5*, or *ced-12* mutants, suggesting that additional engulfment receptors other than PSR-1 and INA-1 act upstream of the CED-2-CED-5-CED-12 signaling module. Similarly, a number of engulfment receptors have been shown to function in cell corpse removal in mammals [1, 2, 6]. Thus, it appears to be evolutionarily conserved in worms and mammals that one or two engulfment receptors may not suffice to activate the engulfment machinery to the level needed to trigger efficient engulfment of apoptotic cells and that multiple receptors need to be engaged.

ina-1 Functions in the Engulfing Cells, but Not in the Dying Cells, to Promote Cell Corpse Removal

INA-1 is widely expressed during embryogenesis [34]. We confirmed this observation by using the transgene P_{ina-1}*ina-1::gfp*, in which the translational fusion INA-1::GFP is expressed under the control of the endogenous *ina-1* promoter P_{ina-1} [34]. INA-1::GFP was localized to the plasma membrane in many cells, including apoptotic cells and their neighboring engulfing cells, and rescued the Ina-1 engulfment defect (Figure S2; Table 3).

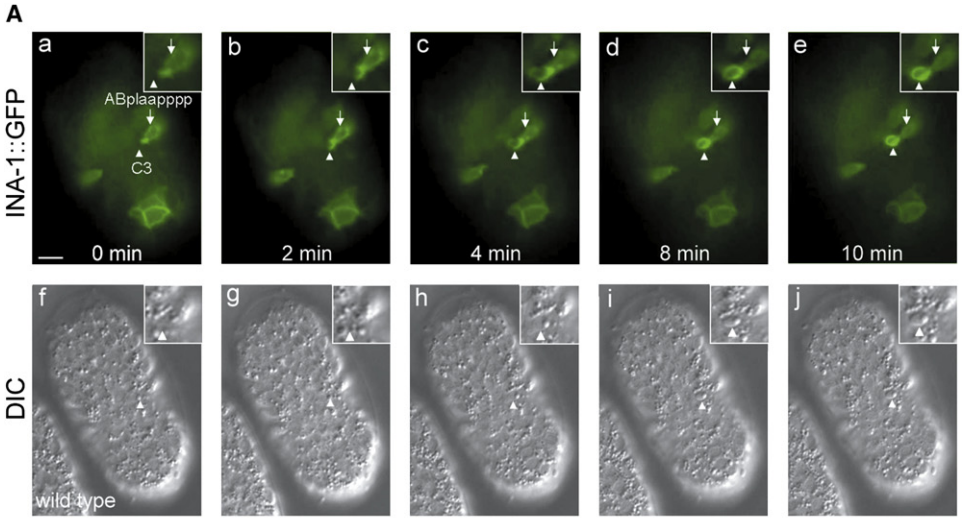
To determine whether *ina-1* acts in the dying cell and/or the engulfing cell in cell corpse engulfment, we expressed the INA-1::GFP fusion protein under the control of the cell-type-specific promoters P_{ced-1} and P_{egl-1} in *ina-1* mutants. P_{ced-1} is active in cell types that act as engulfing cells for cell corpses [38] but is not expressed in dying cells, whereas P_{egl-1} is active predominantly in dying cells [39]. We found that the P_{ced-1}*ina-1::gfp* transgene significantly rescued the engulfment defect of *ina-1(gm144)* mutants (Table 3). However, no significant rescue was observed in *ina-1(gm144)* embryos transgenic for P_{egl-1}*ina-1::gfp* (Table 3). These results indicate that *ina-1* activity in the engulfing cells, but not in the dying cells, is sufficient for the rescue of the engulfment defect of *ina-1* mutants. Thus, *ina-1* likely functions in the engulfing cells to promote the removal of apoptotic cells.

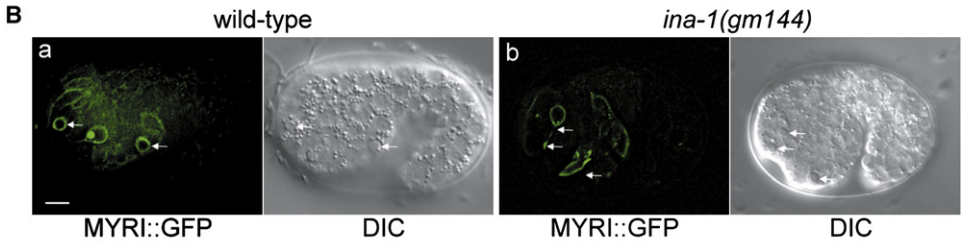
INA-1 Localizes to Pseudopods Extending around Apoptotic Cells

We further examined the INA-1::GFP localization pattern during the engulfment process in transgenic embryos expressing P_{ced-1}*ina-1::gfp* via time-lapse microscopy analysis. Specifically, we monitored the INA-1::GFP signal of the hypodermal cell ABplaapppp, which engulfs an apoptotic cell (hereafter called C3, as in [20]) between the comma and 1.5-fold stages [20]. The INA-1::GFP protein was localized to the cell surface of the ABplaapppp cell (indicated by arrows in Figure 2A). When the engulfment process was initiated, INA-1::GFP accumulated at the contact site with C3 (Figure 2Aa), even before C3 displayed a characteristic apoptotic morphology under DIC optics (Figure 2Af). This observation is consistent with a previous finding that an engulfing cell recognizes a dying cell before it shows any overt morphological changes [40] and supports the notion that the engulfment-promoting signal is generated at an early stage of apoptosis. INA-1::GFP then localized to the budding pseudopods, which extended around and eventually enclosed C3 (Figures 2Ab–2Ad). C3 became refractile and adopted an apoptotic appearance when it was almost fully enclosed by the INA-1::GFP circle (Figures 2Ad and 2Ai). The whole internalization process took approximately 6 min. Endosomes were subsequently recruited to the INA-1::GFP-labeled phagosome (Figure S3), as previously observed during the maturation of the apoptotic cell-containing phagosome [20].

Mutations in *ina-1* Affect the Internalization of Apoptotic Cells

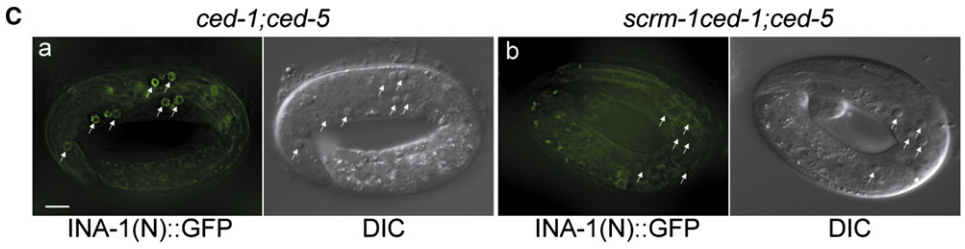
Because INA-1 was localized to the growing pseudopods around apoptotic cells, we further investigated whether mutations in *ina-1* affected the internalization of cell corpses. To this end, we utilized the transgene P_{ced-1}*myri::gfp*, in which GFP was tagged with a myristoylation signal at the N terminus





c

Genotype	% of apoptotic cells with the MYRI::GFP circle	
	comma	1.5-fold
N2	46.1 ± 2.8% (n = 60)	50.1 ± 2.8% (n = 60)
ina-1(RNAi)	18.6 ± 3.1% (n = 130)	20.5 ± 3.6% (n = 136)
ina-1(gm144)	22.2 ± 2.7% (n = 140)	20.2 ± 2.6% (n = 151)



c

Genotype	No. of total cell corpses	No. of GFP$^+$ cell corpses	% of GFP$^+$ cell corpses
ced-1(e1735);ced-5 (n1812)	33.1 ± 5.5	13.1 ± 4.0	39.3 ± 7.0
scrm-1(RNAi)ced-1(e1735); ced-5(n1812)	32.7 ± 3.0	2.3 ± 2.0	7.0 ± 4.0

Figure 2. INA-1 Recognizes Apoptotic Cells and Is Required for Their Internalization by Engulfing Cells

(Aa–Aj) INA-1::GFP is enriched in the pseudopods extending around apoptotic cells. Time-lapse images of INA-1::GFP (Aa–Ae) and differential interference contrast (DIC; Af–Aj) during cell corpse internalization in an embryo expressing $P_{ced\text{-}1}$*ina-1::gfp* are shown. 0 min indicates the time point at which INA-1::GFP was first seen to accumulate at the contact site with C3. Arrowheads indicate the apoptotic cell (C3); arrows indicate the engulfing cell (ABplaapppp). Scale bar in (Aa) represents 5 μm.

(Ba–Bc) *ina-1* mutations reduce the efficiency of cell corpse internalization in engulfing cells.

(Ba and Bb) MYRI::GFP and DIC images of wild-type (Ba) and *ina-1(gm144)* (Bb) embryos expressing $P_{ced\text{-}1}$*myri::gfp*. Arrows indicate apoptotic cells. Scale bar in (Ba) represents 5 μm.

(Bc) Percentage of apoptotic cells with a MYRI::GFP circle in the indicated genotype. Data (mean ± SD) are the average for two independent transgenic lines. Numbers in parentheses indicate the number of cell corpses scored.

(Ca–Cc) INA-1(N) binds to the surface of apoptotic cells in a *scrm-1*-dependent manner.

(Ca and Cb) INA-1(N)::GFP and DIC images of *ced-1(e1735); ced-5(n1812)* (Ca) and *scrm-1(RNAi)ced-1(e1735); ced-5(n1812)* (Cb) embryos expressing P_{hsp}*ina-1(N)::gfp*. Arrows indicate apoptotic cells. Scale bar in (Ca) represents 5 μm.

(Cc) Percentage of INA-1(N)::GFP$^+$ cell corpses in the indicated genotype. Cell corpses fully enclosed by the INA-1(N)::GFP circle were scored as INA-1(N)::GFP$^+$. Each sample point represents the data from 12 embryos. Data are shown as mean ± SD.

(see Supplemental Experimental Procedures); the expressed protein was localized to the surface of the engulfing cells and was used to monitor the pseudopod extension of these cells in wild-type and *ina-1* embryos. In the wild-type, MYRI::GFP was localized to the growing pseudopods and was observed to fully enclose apoptotic cells within 8 min (Figure S4), similar to the dynamics of cell corpse internalization observed with INA-1::GFP (Figure 2A). We then calculated the percentage of apoptotic cells with a complete MYRI::GFP circle in wild-type and *ina-1(RNAi* or *gm144)* embryos. At the 1.5-fold stage, about 50% of wild-type apoptotic cells were enclosed by the MYRI::GFP circle (Figures 2Ba and 2Bc), whereas the corresponding figure in *ina-1(RNAi* or *gm144)* embryos was only approximately 20% (Figures 2Bb and 2Bc). A similar result was observed at the comma stage (Figure 2Bc). These data show that internalization of apoptotic cells is defective in the *ina-1* mutants and that *ina-1* is important for the initiation and/or execution of that process.

INA-1 Recognizes Apoptotic Cells

We next investigated whether *ina-1* mediates the recognition of apoptotic cells. We fused the INA-1 extracellular region [termed INA-1(N)] to GFP and overexpressed the fusion protein INA-1(N)::GFP under the control of the heat-shock promoter P_{hsp} in *ced-1(e1735); ced-5(n1812)* double-mutant embryos, which contain nonengulfed apoptotic cells and thus provided a sensitized background for our assay. INA-1(N)::GFP was found to cluster on the surface of apoptotic cells in *ced-1; ced-5* mutants (Figure 2Ca). In control experiments, secreted GFP tagged with the INA-1 signal sequence (termed ssGFP) was expressed under the control of the P_{hsp} promoter under the same conditions, and no specific binding of ssGFP to apoptotic cells was observed (data not shown). In addition, we also used the P_{ced-1} promoter to express INA-1(N)::GFP and observed INA-1(N)::GFP clustering around apoptotic cells in *ced-1(e1735); ced-5(n1812)* double-mutant embryos, despite the fact that the INA-1(N)::GFP signal was not as strong as that expressed by the P_{hsp} promoter (Figure S5). These results provide evidence that INA-1(N) specifically recognizes, and binds to, the surface of apoptotic cells.

PS, which is normally restricted to the inner leaflet of the plasma membrane, is exposed on the surface of apoptotic cells and acts as an "eat me" signal to induce phagocytosis [6]. SCRM-1 and CED-7 are necessary for the efficient presentation of PS on the surface of apoptotic somatic cells [32, 33]. Because *scrm-1*, but not *ced-7*, acts with *ced-2*, *ced-5*, and *ced-12* in the same genetic pathway as *ina-1* [33], we examined whether the binding of INA-1(N)::GFP to apoptotic cells requires *scrm-1*. In *ced-1; ced-5* double mutants, approximately 39% of apoptotic cells displayed the INA-1(N)::GFP circle (Figure 2Cc). However, in *scrm-1ced-1; ced-5* triple mutants, which had the same number of cell corpses as the *ced-1; ced-5* mutants, only 7% of cell corpses exhibited the INA-1(N)::GFP circle (Figures 2Cb and 2Cc), indicating that the binding of INA-1(N)::GFP to apoptotic cells requires *scrm-1* activity.

src-1 Likely Acts Downstream of *ina-1* and Upstream of *ced-2* in the Genetic Pathway that Controls Cell Corpse Engulfment

Two types of engulfment receptors have been implicated in the recognition of cell corpses. Tethering receptors, including TIM-4 and *Drosophila* SIMU, in which the intracellular domain is not needed for their engulfment function, do not appear to mediate signaling [41, 42], while docking receptors, including BAI-1 and *C. elegans* PSR-1 and CED-1, mediate signaling through their intracellular domains [11, 17, 38]. We therefore tested whether the cytoplasmic domain of INA-1 is required for engulfment. We generated the mutant construct P_{ced-1}*ina-1*Δ*C* in which the INA-1 cytoplasmic domain was deleted, expressed the truncated protein under the control of the P_{ced-1} promoter in engulfing cells, and tested its ability to rescue the Ina-1 engulfment defect. We found that deletion of the cytoplasmic domain completely abolished INA-1 rescuing activity (Table 3). This result supports the idea that the INA-1 cytoplasmic domain mediates signaling for cell corpse removal.

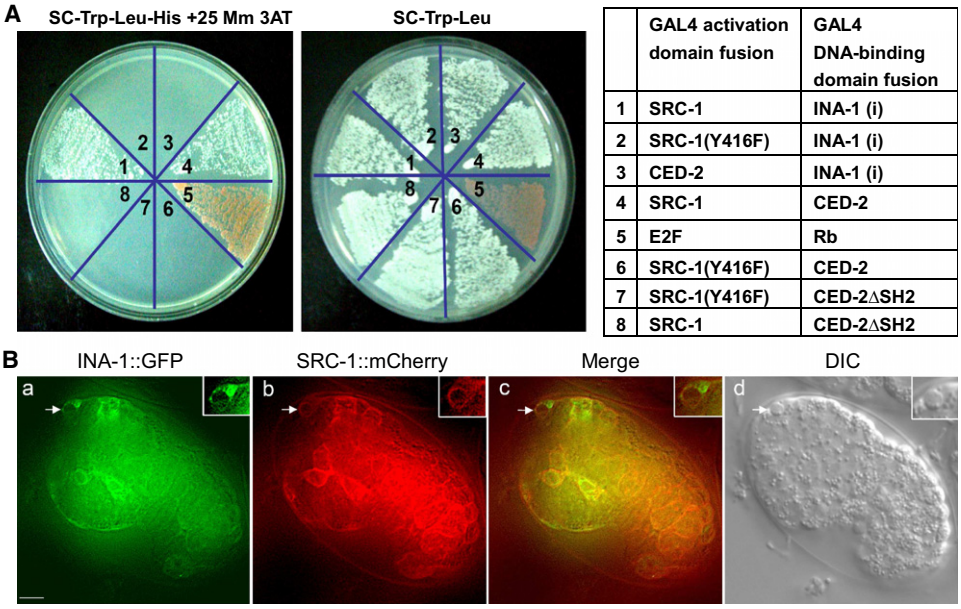
To understand how INA-1 may transduce the engulfment signal, we tested whether its intracellular domain interacts directly with CED-2, CED-5, or CED-12 in the yeast two-hybrid assay. No direct interaction was detected (Figure 3A and data not shown), suggesting that one or more additional components are needed to link INA-1 to the CED-2-CED-5-CED-12 signaling module. The adaptor protein CED-2 contains an SH2 domain [22], which typically interacts with a phosphotyrosine residue. We therefore screened for nonreceptor tyrosine kinases, inactivation of which by a mutation or RNAi phenocopied the engulfment defect of *ina-1* mutants, and identified *src-1*. The *src-1(cj293)* and *src-1(RNAi)* embryos contained an increased number of cell corpses at the comma and 1.5-fold stages (Table 2) similar to the level seen in *ina-1* mutants (Table 1). In addition, *src-1(cj293* or *RNAi)* did not increase the cell corpse number in *ina-1(RNAi)* or *ced-2* mutants but markedly increased the number in *ced-1* mutants (Table 2), suggesting that *src-1* functions in the same genetic pathway as *ina-1* and *ced-2*. Moreover, when *src-1* was fused to the *mcherry* reporter and expressed under the control of the P_{ced-1} promoter, it fully rescued the phenotype of cell corpse increase in *src-1(cj293)* mutants (Table 4), supporting the idea that *src-1*, like *ina-1* and *ced-2*, functions in an engulfing cell to promote the engulfment of apoptotic cells.

We then positioned *src-1* relative to *ina-1* and *ced-2* in the engulfment pathway via the genetic bypass assay. We utilized the P_{hsp}*src-1* transgene to examine whether overexpression of *src-1* by the P_{hsp} promoter could bypass the functional requirement for *ina-1* or *ced-2* in cell corpse engulfment. Heat-shock-induced overexpression of *src-1* from the transgene P_{hsp}*src-1* rescued the engulfment defect of *ina-1* mutants, but not *ced-2* mutants (Table 4). In the converse experiment, overexpression of *ced-2* under the control of the P_{hsp} promoter rescued the engulfment defect of *src-1(RNAi)* mutants (Table 4). Together, these results suggest that *src-1* genetically acts downstream of (or in parallel with) *ina-1* and upstream of (or in parallel with) *ced-2* in the genetic pathway for cell corpse removal.

SRC-1 Autophosphorylates and Interacts with INA-1 and CED-2 In Vitro

We next examined whether SRC-1 links CED-2 to INA-1 by a direct interaction with CED-2 and the INA-1 intracellular domain [INA-1(i)] via the yeast two-hybrid assay. SRC-1 was found to bind to CED-2 and INA-1(i), whereas no interaction between INA-1(i) and CED-2 was detected (Figure 3A). These results are consistent with the notion that SRC-1 acts as a bridging molecule linking CED-2 and INA-1(i).

Autophosphorylation of c-Src at Tyr416 in the kinase domain increases its in vitro kinase activity [43], and mutation of Tyr416 to phenylalanine (Phe) eliminates c-Src in vitro kinase



	GAL4 activation domain fusion	GAL4 DNA-binding domain fusion
1	SRC-1	INA-1 (i)
2	SRC-1(Y416F)	INA-1 (i)
3	CED-2	INA-1 (i)
4	SRC-1	CED-2
5	E2F	Rb
6	SRC-1(Y416F)	CED-2
7	SRC-1(Y416F)	CED-2ΔSH2
8	SRC-1	CED-2ΔSH2

Figure 3. SRC-1 Physically Interacts with INA-1(i) and CED-2 In Vitro and Is Colocalized with INA-1 in Phagocytes to the Pseudopods Extending around Apoptotic Cells

(A) SRC-1 interacts with INA-1(i) and CED-2 in the yeast two-hybrid assay. Pairs of constructs expressing the indicated fusion proteins were transformed into the yeast strain MaV203. The resulting transformants were streaked on SC-Trp-Leu-His or SC-Trp-Leu plates containing 25 mM 3AT. Growth on the SC-Trp-Leu-His + 25 mM 3AT plate indicates interaction of fusion proteins.

(Ba–Bd) INA-1::GFP and SRC-1::mCherry are colocalized to the pseudopods extending around an apoptotic cell. INA-1::GFP (Ba), SRC-1::mCherry (Bb), merged INA-1::GFP and SRC-1::mCherry (Bc), and DIC (Bd) images of the same embryo coexpressing P_{ced-1}*ina-1::gfp* and P_{ced-1}*src-1::mcherry* are shown. Apoptotic cells are indicated by arrows and are magnified in the insets. Scale bar in (Ba) represents 5 μm.

activity and its ability to induce foci when overexpressed in NIH 3T3 cells [43, 44]. We mutated Tyr416 to Phe in SRC-1 and found that the resulting mutant SRC-1(Y416F) failed to autophosphorylate in vitro (Figure S6) or interact with INA-1(i) or CED-2 in the yeast two-hybrid assay (Figure 3A). In addition, SRC-1(Y416F) expressed under the control of the P_{ced-1} promoter failed to rescue the engulfment defect of the *src-1*(*cj293*) mutant, whereas the wild-type SRC-1 was successful (Table 4). The expression of the mutant protein was validated by the mCherry tag (data not shown). Thus, the conserved Tyr416 and/or the kinase activity of SRC-1 may be important for its interaction with CED-2 and INA-1(i) and for its in vivo function in the engulfment of apoptotic cells.

We next investigated the potential involvement of the SH2 domain of CED-2 in its interaction with SRC-1 and the functional significance of such an interaction for CED-2 activity in cell corpse engulfment by generating the mutant CED-2ΔSH2, in which the SH2 domain was deleted. This deletion abolished the interaction of CED-2 with SRC-1 (Figure 3A) and significantly reduced the ability of *ced-2* to rescue the engulfment defect of *ced-2* mutants (Table 4). These results support that CED-2 may bind to SRC-1, possibly through the SH2 domain of CED-2, and that this binding may be important for CED-2 activity in mediating cell corpse engulfment.

SRC-1 Colocalizes with INA-1 to the Pseudopods Extending around Apoptotic Cells

We then explored the localization of SRC-1 with respect to INA-1 during the engulfment process. In embryos coexpressing P_{ced-1}*src-1::mcherry* and P_{ced-1}*ina-1::gfp*, SRC-1::mCherry colocalized with INA-1::GFP to the phagocytic cups around apoptotic cells (Figure 3B), consistent with our yeast two-hybrid finding that SRC-1 physically interacts with INA-1(i). These data and the aforementioned genetic and yeast two-hybrid studies support a model wherein SRC-1 is recruited by INA-1(i) to growing phagocytic cups to mediate downstream signaling by interacting with the CED-2-CED-5-CED-12 complex for GTPase CED-10/Rac activation, which then leads to actin-based cytoskeleton rearrangement and the phagocytosis of apoptotic cells.

Discussion

The recognition of apoptotic cells by the engulfing cells is a complex process and involves multiple engulfment receptors [1, 2, 6]. A previous genetic study in *C. elegans* indicated that additional receptors besides *psr-1* act upstream of *ced-2*, *ced-5*, and *ced-12* to mediate the engulfment of apoptotic cells [17]; however, the identities of these receptors remain unknown. Here, we have provided evidence that integrin is an engulfment receptor in the *ced-2*, *ced-5*, and *ced-12* signaling pathway. We have shown that integrin α INA-1 recognizes apoptotic cells through its extracellular domain and mediates signaling via its intracellular domain to promote apoptotic cell internalization. In addition, we have characterized the INA-1 signaling pathway and identified a novel role of SRC-1 in cell corpse removal. We propose that INA-1, upon recognizing and binding to apoptotic cells, recruits the conserved signaling module CED-2-CED-5-CED-12 to the phagocytic cups through SRC-1, which acts as a bridging molecule linking CED-2 to the INA-1 cytoplasmic domain to trigger GTPase CED-10 activation, which in turn leads to the internalization of apoptotic cells.

We have shown that INA-1(N) specifically binds to the surface of apoptotic cells when it is fused to the GFP reporter

Table 4. Overexpression of *src-1* and *ced-2* in Engulfment-Defective Mutants

Genotype	Transgene	Heat Shock	Number of Cell Corpses: Comma Stage	Number of Cell Corpses: 1.5-fold Stage
N2	–	–	10.1 ± 0.6	10.2 ± 0.6
ina-1(gm144)	–	–	12.2 ± 1.6	12.5 ± 1.9
ina-1(gm144)	P_{hsp} *src-1*	–	12.2 ± 1.4	12.6 ± 1.0
ina-1(gm144)	P_{hsp} *src-1*	+	9.9 ± 1.2**	10.3 ± 0.7**
src-1(RNAi)	–	–	12.4 ± 1.0	12.9 ± 0.8
src-1(RNAi)	P_{hsp} *ced-2*	–	12.5 ± 1.2	12.8 ± 1.1
src-1(RNAi)	P_{hsp} *ced-2*	+	9.9 ± 1.1**	10.4 ± 0.9**
ced-2(n1994)	–	–	18.4 ± 2.1	22.8 ± 3.6
ced-2(n1994)	P_{hsp} *src-1*	–	18.2 ± 2.1	22.1 ± 2.7
ced-2(n1994)	P_{hsp} *src-1*	+	19.7 ± 1.4	21.6 ± 1.1
src-1 (cj293)	–	–	13.1 ± 0.8	14.7 ± 1.2
src-1(cj293)	P_{ced-1} *src-1::mcherry*	–	10.8 ± 1.1##	11.1 ± 0.7##
src-1(cj293)	P_{ced-1} *src-1(Y416F)::mcherry*	–	13.2 ± 0.7	13.9 ± 1.3
ced-2(n1994)	P_{hsp} *ced-2*	–	18.3 ± 1.8	22.1 ± 2.8
ced-2(n1994)	P_{hsp} *ced-2*	+	9.8 ± 0.5**	10.1 ± 0.4**
ced-2(n1994)	P_{hsp} *ced-2ΔSH2*	–	18.6 ± 1.6	22.3 ± 2.2
ced-2(n1994)	P_{hsp} *ced-2ΔSH2*	+	15.1 ± 1.1**	16.3 ± 1.7**

For heat-shock treatments, transgenic animals were subjected to heat shock (+) at 33°C for 30 min or left at 20°C (–). Comparison of heat-shocked transgenic worms with the corresponding non-heat-shocked transgenic worms at the same stage was performed by unpaired t test (*$p < 0.05$, **$p < 0.001$). For each transgene, two (B and C) or three (A) independent stably transmitting lines were analyzed, except for those of *ced-2(n1994); P_{hsp} ced-2* embryos, which are from only one line as described previously [24], and the data were similar. Data of one representative line are shown. Transgenic embryos were compared to nontransgenic embryos in (B) (##$p < 0.001$). All comparisons were performed by unpaired t test. Data are presented as mean ± SD of $n > 20$ embryos.

and ectopically expressed in embryos. Interestingly, this binding depends on SCRM-1, suggesting a potential involvement of PS externalization on the surface of apoptotic cells in the recognition by INA-1. Mammalian integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have been shown to bind indirectly to externalized PS on apoptotic cells via the bridging molecule MFG-E8 secreted locally by phagocytes [12, 13]. MFG-E8 has an RGD domain, which is essential for its interaction with integrin [12]. However, *C. elegans* does not appear to have an MFG-E8 homolog, and INA-1 is closer to the laminin-binding integrins than to the RGD-binding integrins on the basis of amino acid sequence. In addition, integrin $\alpha_v\beta_3$ also binds synergistically with the cell-surface protein CD36 to apoptotic cells through the bridging molecule thrombospondin (TSP), an extracellular matrix glycoprotein [45]. The molecular nature of the TSP-binding site on apoptotic cells is poorly understood. It remains to be determined whether the "eat me" signal recognized by INA-1 is externalized PS or as yet unidentified *scrm-1*-dependent moieties on the surface of apoptotic cells and whether INA-1 binds to the "eat me" signal directly or indirectly via a bridging molecule. In addition, *C. elegans* has CD36-like proteins [46]. Given that the *Drosophila* CD36-related receptor Croquemort is required for the phagocytosis of apoptotic cells [47], a role of *C. elegans* CD36-like proteins in engulfment is possible. This possibility and the potential interaction of INA-1-PAT-3 with CD36-like proteins in promoting cell corpse removal need to be tested.

On binding to the apoptotic cell opsonin MGF-E8, integrin $\alpha_v\beta_5$ recruits the intracellular signaling complex $p130^{Cas}$-CrkII-DOCK180 for Rac1 activation, probably through focal adhesion kinase (FAK), and promotes the engulfment of apoptotic cells [12, 48]. The cytoplasmic domain of β_5 is important for this signaling [48]. We found that *C. elegans* integrin also acts upstream of CED-2/CrkII and CED-5/Dock180 but does not require *kin-32*, the *C. elegans* homolog of FAK, to promote engulfment. The deletion allele *ok166* of *kin-32*, which results in the loss of more than three-quarters of the conserved FERM (*f*our-point-one-ezrin-*r*adixin-*m*oesin) domain [49], did not cause a detectable defect in cell corpse engulfment during embryogenesis, even when the *ok166* mutant was treated with *kin-32(RNAi)*. For example, the *kin-32(ok166)* mutants treated with *kin-32* RNAi showed 10.6 ± 1.4 and 10.3 ± 1.2 cell corpses at the comma and 1.5-fold stages, respectively (data not shown). Thus, the FERM domain of KIN-32, and possibly KIN-32 activity, is not needed for the engulfment of embryonic cell corpses. Our yeast two-hybrid system showed that SRC-1 physically interacts with INA-1(i) and CED-2. However, it is not clear whether SRC-1 may directly interact with INA-1(i) or CED-2 in *C. elegans*. Nevertheless, our genetic data strongly indicate that *ina-1*, *src-1*, and *ced-2* function in the same genetic pathway in the order that *ina-1* acts upstream and *ced-2* downstream to control the engulfment of apoptotic cells. Notably, this SRC-dependent, but FAK-independent, integrin α/SRC signaling is not unique to *C. elegans*. Mammalian integrin $\alpha_4\beta_1$ also does not require FAK for its function in promoting cell motility, and, more importantly, $\alpha_4\beta_1$ acts through the α_4 cytoplasmic domain for downstream Src activation [50], which in turn recruits the $p130^{Cas}$-CrkII-Dock180 complex and leads to GTPase Rac activation to promote cell motility [51]. These results, in combination with our findings, define a nonconventional but conserved integrin signaling pathway in which activation of SRC by integrin α provides a FAK-independent linkage to a conserved motility-promoting signaling complex containing CED-2/CrkII and CED-5/Dock180 for CED-10/Rac activation during engulfment and cell migration in *C. elegans* and mammals.

Supplemental Information

Supplemental Information includes Supplemental Results, six figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.01.062.

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