Signaling pathway for phagocyte priming upon encounter with apoptotic cells

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The phagocytic elimination of cells undergoing apoptosis is an evolutionarily conserved innate immune mechanism for eliminating unnecessary cells. Previous studies showed an increase in the level of engulfment receptors in phagocytes after the phagocytosis of apoptotic cells, which leads to the enhancement of their phagocytic activity. However, precise mechanisms underlying this phenomenon require further clarification. We found that the pre-incubation of a *Drosophila* phagocyte cell line with the fragments of apoptotic cells enhanced the subsequent phagocytosis of apoptotic cells, accompanied by an augmented expression of the engulfment receptors Draper and integrin αPS3. The DNA-binding activity of the transcription repressor Tailless was transiently raised in those phagocytes, depending on two partially overlapping signal-transduction pathways for the induction of phagocytosis as well as the occurrence of engulfment. The RNAi knockdown of *tailless* in phagocytes abrogated the enhancement of both phagocytosis and engulfment receptor expression. Furthermore, the hemocyte-specific RNAi of *tailless* reduced apoptotic cell clearance in *Drosophila* embryos. Taken together, we propose the following mechanism for the activation of *Drosophila* phagocytes after an encounter with apoptotic cells: two partially overlapping signal-transduction pathways for phagocytosis are initiated; transcription repressor Tailless is activated; expression of engulfment receptors is stimulated; and phagocytic activity is enhanced. This phenomenon most likely ensures the phagocytic elimination of apoptotic cells by stimulated phagocytes and is thus considered as a mechanism to prime phagocytes in innate immunity.

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This article contains supplemental Table S1 and Figs. S1–S2.

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Tens of billions of cells are lost every day in the human body, mostly by apoptosis. These cells need to be subjected to "silent" removal by phagocytosis; cells that have been induced to undergo apoptosis are engulfed and digested by phagocytic cells at an early stage of the apoptotic process before they lyse and damage surrounding healthy tissues (1, 2). Therefore, apoptosis is regarded as a biological phenomenon that earmarks unnecessary cells and makes them susceptible to phagocytic elimination (1–4). Cells undergoing apoptosis express molecules, often referred to as eat-me signals or phagocytosis markers, on their surfaces, and phagocytic cells bind, either directly or indirectly, with these molecules using engulfment receptors and then activate a signaling pathway for the induction of phagocytosis (3–10). There are partly overlapping two signal-transduction pathways in the nematode *Caenorhabditis elegans* that are composed of signal mediators encoded by cell death abnormal (*ced*) genes, namely CED-6/CED-10 and CED-2/CED-5/CED-12/CED-10 (11–14), which are located downstream of the engulfment receptors CED-1 and INA-1-PAT-3, respectively (15). CED-1 is a single-path membrane protein containing atypical EGF-like repeats in its extracellular region (16), whereas INA-1 and PAT-3 are the α- and β-subunits of *C. elegans* integrin (17), respectively. The phagocytosis of apoptotic cells not only serves as a mechanism to safely eliminate unnecessary cells but also plays an important role in morphogenesis during early development as well as in the maintenance of tissue homeostasis in adulthood (7, 18, 19). Malfunctions in this mechanism often result in the development of a number of diseases (20, 21).

Recently, Weavers *et al.* (22) demonstrated that hemocytes in the fruit fly *Drosophila melanogaster*, equivalent to mammalian macrophages, acquired greater migratory activity toward injured area and phagocytic activity against *Escherichia coli*, due to an elevated mRNA level of a gene coding for receptor named Draper, apparently after the engulfment of apoptotic cells. This finding has been interpreted as apoptotic cell engulfment playing two roles, to eliminate unnecessary cells and to enhance phagocyte ability through a change of gene expression, providing a concept of phagocyte priming by apoptotic cells. However, the following issues have yet to be clarified: whether phagocytic activity against apoptotic cells is also enhanced; what is the transcription factor(s) involved in the alteration of gene expression; and whether engulfment receptors and down-

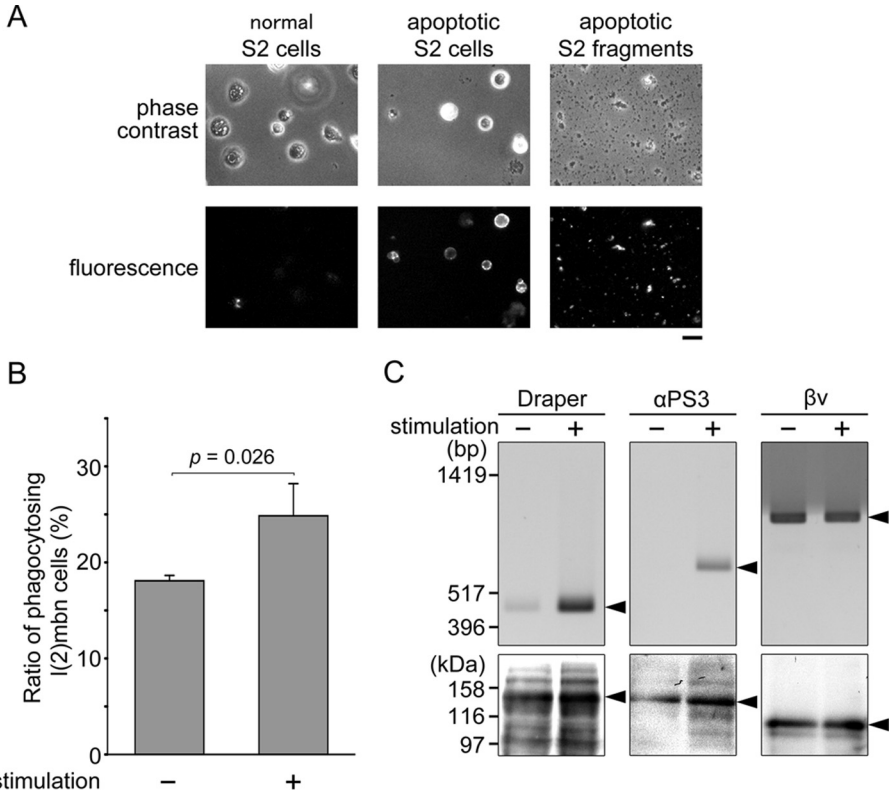


Figure 1. Enhancement of phagocytic activity and engulfment receptor expression in stimulated phagocytes. *A,* S2 cells were treated with cycloheximide for the induction of apoptosis, and total cell cultures (apoptotic S2 cells) and apoptotic cell fragments were prepared. Those materials, together with S2 cells not treated with cycloheximide (normal S2 cells), were incubated with FITC-conjugated annexin V and microscopically analyzed for the surface exposure of annexin V. Phase-contrast and fluorescence views of the same microscopic fields are shown. *Scale bar,* 10 μm. *B,* l(2)mbn cells were incubated with apoptotic cell fragments of S2 cells or left untreated for 30 min and subsequently used as phagocytes in an assay for phagocytosis with cycloheximide-treated S2 cells as targets. The means ± S.D. were obtained with the data from three independent experiments and analyzed by Student's *t* test. *C,* l(2)mbn cells were incubated with apoptotic cell fragments or left untreated for 30 min (for RT-PCR) or 1 h (for Western blotting), and their RNAs and whole-cell lysates were analyzed by RT-PCR (*top*) and Western blotting (*bottom*), respectively. The *arrowheads* point to the positive signals. Representative data from two (RT-PCR) and three (Western blotting) independent experiments that yielded similar results are shown.

stream signaling pathways are required for this priming mechanism. In this study, we investigated these issues using *Drosophila*. By conducting biochemical and genetic experiments, we found that an encounter with apoptotic cells enhances the phagocytosing activity against apoptotic cells in *Drosophila* phagocytes through an increase in the expression of genes coding for the engulfment receptors Draper and integrin αPS3. Furthermore, we identified the transcription factor Tailless responsible for the augmented expression of these engulfment receptors and the subsequent enhancement of phagocytic activity in primed phagocytes. Our results provide a mechanistic basis for the priming of phagocytes in cellular innate immunity.

Results

Increase in the levels of phagocytic activity and engulfment receptor expression in Drosophila phagocytes after incubation with apoptotic cell fragments

Drosophila possesses three types of blood cells or hemocytes: plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes, resembling mammalian macrophages, occupy a major population among hemocytes and are responsible for the phagocytic removal of apoptotic cells as well as invading microorganisms (23–25). A recent study demonstrated that the phagocytic activity of hemocytes in *Drosophila* embryos is enhanced after the engulfment of apoptotic cells through increased expression of Draper, an engulfment receptor of *Drosophila* (22). Although the phagocytic activity was examined only with *E. coli* as a target in that study, those hemocytes are likely to show an increased level of phagocytosis against apoptotic cells as well because we previously reported that Draper serves as an engulfment receptor in the elimination of apoptotic cells by embryonic hemocytes (26).

To validate this possibility, we determined the phagocytic activity of larval hemocyte-derived l(2)mbn cells using apoptotic *Drosophila* cells as targets with and without pre-incubation in the presence of the fragments of apoptotic cells. We used insoluble membranous particles derived from *Drosophila* S2 cells undergoing apoptosis, hereafter referred to as "apoptotic cell fragments," for stimulation to distinguish engulfed materials during pre-incubation from those in the subsequent phagocytosis. These particles were microscopically visible and mostly positive for the binding of annexin V as were apoptotic cells (Fig. 1*A*), indicative of the surface exposure of the membrane phospholipid phosphatidylserine. We found that treatment with apoptotic cell fragments made l(2)mbn cells more active in the phagocytosis of apoptotic S2 cells (Fig. 1*B*). *Drosophila* hemocytes possess at least two engulfment receptors for apo-

ptotic cell clearance, namely Draper (26) and integrin αPS3-βν (27, 28). We next tested a possible change of their expression in phagocytes during stimulation and found that the mRNA and protein level expression of Draper and integrin αPS3, but not integrin βν, increased in l(2)mbn cells after incubation with apoptotic cell fragments (Fig. 1*C*). These results suggested that the phagocytic activity of hemocytes against apoptotic cells is enhanced when they encounter apoptotic cells, which may be attributed to the elevated levels of the expression of genes coding for engulfment receptors. The level of integrin βν remained unchanged in phagocytes after the stimulation. However, we speculate that stimulated phagocytes acquire an increased level of a heterodimer of the integrin subunits αPS3 and βν because the surface expression of integrins appears to depend on the level of α-subunits, as exemplified by integrin α_1-β_1 in human osteosarcoma cells (29) and integrin αPS3-βPS in the epithelial follicle cells of *Drosophila* ovaries (30).

Identification of Tailless as a transcription factor activated in Drosophila phagocytes upon stimulation

We next searched for a transcription factor(s) activated in phagocytes during incubation with apoptotic cell fragments to gain a cue for the mechanism of gene regulation. The profile of mRNA was first determined to detect possible changes in gene expression pattern in *Drosophila* S2 cells, a cell line established from embryonic hemocytes, before and after incubation with fragments of the same S2 cells undergoing apoptosis. RNA was prepared from control and stimulated S2 cells and subjected to a DNA microarray analysis with GeneChip containing over 18,000 *Drosophila* transcripts. When we analyzed the data from triplicate experiments, a total of six samples, for hierarchical clustering, they were clearly separated into two clusters: one consisting of three samples with stimulated S2 cells and the other cluster with control cells (supplemental Fig. 1*A*). This result indicated that the mRNA profile of S2 cells significantly changed after incubation with apoptotic cell fragments. We noted that genes up-regulated in stimulated cells included those coding for proteins that participate in the development of *Drosophila* (supplemental Fig. 1*B*), whereas cell proliferation-related genes appeared to be down-regulated (supplemental Fig. 1*C*). This suggested that the overall pattern of gene expression in S2 cells drifts toward the cessation of cell division and the onset of differentiation after incubation with apoptotic cell fragments.

We identified ~340 up-regulated genes and bibliographically searched for transcription factors that had been reported to be responsible for the transcription of 50 higher-ranked genes among them (supplemental Table 1). The search identified 12 transcription factors (Table 1), and we analyzed their DNA-binding activities using EMSA. We found that 11 of 12 transcription factors gave "shift" bands in this assay (data not shown) and determined their DNA-binding activity with nuclear extracts of S2 cells before and after stimulation with apoptotic cell fragments. The results indicated that signal intensities of the shift bands obtained with probes for several transcription factors, including Max-like protein X (Mlx), Dorsal-related immunity factor (Dif), and CLOCK/CYCLE (CLK/CYC), were reduced and that only the shift band showing the slowest migration with the Tailless (Tll) probe increased its signal intensity after stimulation (Fig. 2*A*). Only this signal among four shift bands obtained with the Tailless probe was shown to be specific to the cognate Tailless-binding sequence (Fig. 2*B*) and disappeared when S2 cells were subjected to the RNAi knockdown of *tailless* (Fig. 2*C*), indicating that this shift band reflects the DNA-binding activity of Tailless. These results collectively suggested that Tailless is activated in phagocytes when they encounter apoptotic cells.

Table 1
Transcription factors analyzed in this study

Transcription factors that control the transcription of the top 50 up-regulated *Drosophila* genes in stimulated phagocytes were bibliographically searched, and 12 factors found are listed.

Gene symbols	Transcription factors (abbreviations)	References
Socs36E	Signal-transducer and activator of transcription protein at 92E (STAT92E)	68, 69
bab2	Rotund (Rn)	70
Chn	Achaete/Scute (Ac/Sc)	71
Cbt	Max-like protein X (Mlx)	72
Daw	Dorsal-related immunity factor (Dif)	73
dro2	Dorsal (Dl)	74
Ken	Tailless (Tll)	75
HLHm3, HLHmbeta, vri	CLOCK/CYCLE (CLK/CYC)	76
Ken	Hunchback (Hb)	75
Socs36E	Schnurri (Shn)	FlyBase
comm2, Psc	Bicoid (Bcd)	FlyBase
bab2	Distal-less (Dll)	70

Tailless-mediated enhancement of engulfment receptor expression and phagocytic activity in stimulated phagocytes

We next examined whether or not the enhanced expression of engulfment receptors in phagocytes after incubation with apoptotic cell fragments depends on the actions of Tailless. S2 cells expressing influenza virus HA-tagged Tailless (Tailless-HA) were incubated with the fragments for various periods of time, and the levels of Tailless activity and engulfment receptor mRNA were determined (Fig. 3*A*). We found that the DNA-binding activity of Tailless was raised at 15 min and returned to the original level at 60 min. In accordance with an increase in the activity of Tailless, the mRNA of both Draper and integrin αPS3 increased at 15 min. To more directly examine the involvement of Tailless, l(2)mbn cells were subjected to RNAi knockdown of *tailless* prior to the stimulation with apoptotic cell fragments and the subsequent determination of engulfment receptor expression. We found that an increase in the expression of Draper and integrin αPS3 in stimulated l(2)mbn cells was weakened upon the RNAi of *tailless* (Fig. 3*B*). Furthermore, the phagocytic activity of l(2)mbn cells remained the same regardless of the stimulation with apoptotic cell fragments after RNAi (Fig. 3*C*). These results indicated that Tailless is required for the augmented expression of Draper and integrin αPS3 and the subsequent enhancement of phagocytic activity in stimulated phagocytes. Tailless, as a transcription repressor (31), is likely to be involved, not directly but indirectly, in the transcription of Draper-encoding *drpr* and αPS3-encoding *scb* through inhibition of the transcription of a gene(s) coding for a protein(s) that negatively controls the expression of these genes.

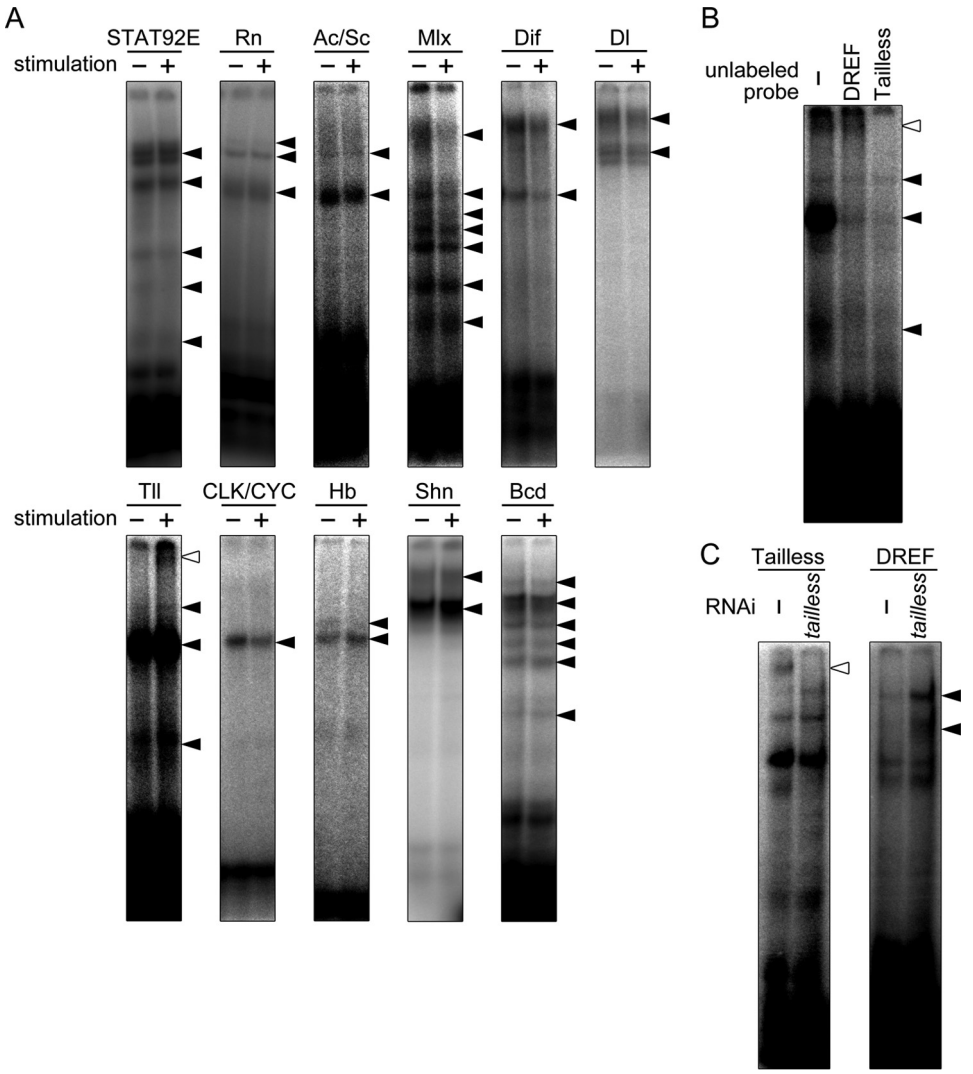


Figure 2. Identification of Tailless as the transcription factor activated in stimulated phagocytes. *A,* nuclear extracts of S2 cells, which had been incubated for 1 h in the presence (+) and absence (−) of apoptotic cell fragments, were analyzed by EMSA with radiolabeled oligonucleotide probes containing binding sequences for the indicated transcription factors (see Table 1 for abbreviations of the transcription factors and supplemental Fig. 2 for probe sequences). The *arrowheads* point to the signals with migration slower than that of free probes, and the *open arrowhead* denotes the shift band generated by Tailless. *B,* EMSA of Tailless was conducted with nuclear extracts of stimulated S2 cells in the presence and absence of unlabeled oligonucleotide probes in excess for Tailless and DNA replication-related element-binding factor (*DREF*), a transcription factor unrelated to the up-regulated genes analyzed as a negative control. *C,* EMSA of Tailless and DREF was performed with nuclear extracts of S2 cells, which had been subjected to *tailless* RNAi prior to the stimulation with apoptotic cell fragments.

Signaling pathway downstream of integrin αPS3-βν for the induction of phagocytosis

We next attempted to clarify how incubation with apoptotic cell fragments leads to the activation of Tailless in phagocytes. The eat-me signals, or phagocytosis markers, expressed at the surface of apoptotic cells are bound, either directly or indirectly, by engulfment receptors of phagocytes to initiate a signaling pathway(s) for the induction of phagocytosis (3–10). We reasoned that phagocytes could recognize eat-me signals, including phosphatidylserine (see Fig. 1*A*), contained in apoptotic cell fragments and activate the pathway during incubation. To examine the involvement of a phagocytosis-inducing signal-transduction pathway(s) in the stimulation of phagocytes, we first delineated the signaling pathway responsible for the induction of phagocytosis in *Drosophila* phagocytes. Previous studies on the mechanisms underlying and consequences of apoptosis (32–34) as well as the subsequent phagocytosis of apoptotic cells (35–37) in *Drosophila* revealed that there are many common and a few different points from those observed in studies using nematodes and mammals. We have suggested that, similar to the nematode, two partially overlapping signaling pathways exist in *Drosophila*, which are initiated by the engulfment receptors Draper (26) and integrin αPS3-βν (27, 28). Draper is a *Drosophila* counterpart of *C. elegans* CED-1 (16) and activates a pathway including Ced-6 as a signal mediator (38). In contrast, αPS3-βν is a member of the integrin family of proteins, similar to INA-1-PAT-3 of *C. elegans* (17), and thus may activate a signaling pathway resembling CED-2/CED-5/CED-12/CED-10 in *C. elegans*. Our previous finding showed that the depletion of Engulfment and cell motility, an orthologue of *C. elegans* CED-12, did not influence the phagocytosis of apoptotic cells in *Drosophila* embryos (39). There-

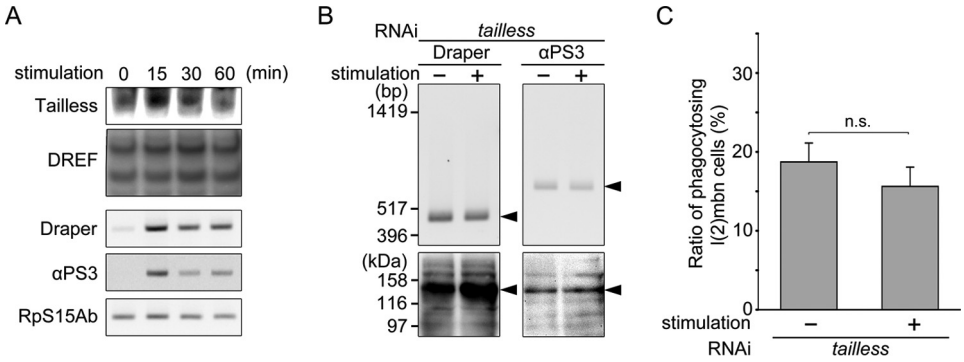


Figure 3. Requirement for Tailless in engulfment receptor expression and the enhancement of phagocytic activity in stimulated phagocytes. *A,* S2 cells expressing Tailless-HA were incubated with apoptotic cell fragments for the indicated periods of time and examined for the DNA-binding activity of Tailless and DREF by EMSA as well as the mRNA levels of the indicated proteins by RT-PCR. The mRNA of ribosomal protein S15Ab (*RpS15Ab*) was analyzed as an unchanged negative control. Representative data from two independent experiments that yielded similar results are shown. *B,* l(2)mbn cells, which had been subjected to the RNAi of *tailless*, were incubated with apoptotic cell fragments or left untreated for 30 min (for RT-PCR) or 1 h (for Western blotting), and their RNAs and whole-cell lysates were analyzed for the levels of Draper and integrin αPS3 by RT-PCR (*top*) and Western blotting (*bottom*), respectively. Representative data from two (RT-PCR) and three (Western blotting) independent experiments that yielded similar results are shown. *C,* l(2)mbn cells, which had been subjected to the RNAi of *tailless*, were incubated with apoptotic cell fragments for 30 min and subsequently used as phagocytes in an assay for phagocytosis with apoptotic S2 cells as targets. The means ± S.D. were obtained with the data from three independent experiments and analyzed by Student's *t* test. *n.s.,* not significant.

fore, we investigated the involvement of CT10 regulator of kinase (Crk)[4] and Myoblast city (Mbc), which are orthologues of *C. elegans* CED-2 and CED-5, respectively, in apoptotic cell clearance in *Drosophila* to delineate the pathway activated by integrin αPS3-βν.

We previously determined the role for phagocytosis-related molecules in apoptotic cell clearance by examining their loss-of-function effect on hemocyte phagocytosis of apoptotic cells in *Drosophila* embryos (27). In this assay, dispersed embryonic cells are simultaneously analyzed by immunocytochemistry and TUNEL to identify cells containing the hemocyte marker Croquemort and fragmented DNA, respectively, and those positive for both signals are regarded as embryonic hemocytes that have phagocytosed apoptotic cells. A similar strategy was taken to find the involvement of Crk and Mbc in a phagocytosis-inducing pathway. The lysates of stage 16 embryos of the fly line $Crk^{KG00336}$ with a mutation on *Crk* were first analyzed for the level of Crk protein, and we confirmed a lower Crk level than that in the lysates of control embryos (y^1 w^{1118}) (Fig. 4*A*, *top panel*). We then conducted an assay for phagocytosis with those embryos and found that a decrease in the level of Crk reduced the hemocyte phagocytosis of apoptotic cells by one-third (Fig. 4*A*, *bottom panel*). The embryos of the *mbc* mutant mbc^{C1} used in this study did not seem to normally develop into stage 16, showing abnormal morphology (data not shown). Therefore, we analyzed embryos at an earlier stage of this mutant fly line for the level of phagocytosis. We first determined the amount of Mbc protein in embryos at stage 13 by Western blotting and confirmed that signal intensity with the lysates of mutant embryos was weaker than that obtained using control flies (Fig. 4*B*, *top panel*). Cells collected from stage 13 embryos of control y^1 w^{1118} flies were then subjected to an assay for phagocytosis, comparing with those from embryos at stage 16. We observed Croquemort-positive hemocytes containing TUNEL-stained

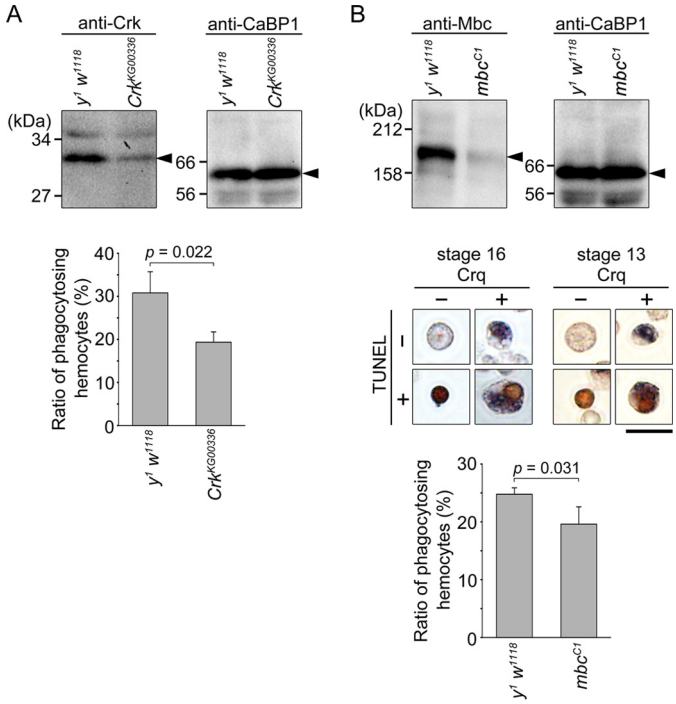


Figure 4. Involvement of Crk and Mbc in apoptotic cell clearance by *Drosophila* hemocytes. *A,* stage 16 embryos of the indicated fly lines were analyzed by Western blotting with anti-Crk and anti-CaBP1 antibodies (*top*) and an assay for phagocytosis *in vivo* (*bottom*). In the analysis of phagocytosis, the means ± S.D. were obtained with the data from three independent experiments and analyzed by Student's *t* test. *B,* in the *top panel*, lysates of stage 13 embryos of the indicated fly lines were subjected to Western blotting with anti-Mbc and anti-CaBP1 antibodies. In the *middle panel*, dispersed cells from embryos at stages 13 and 16 of control flies (y^1 w^{1118}) were analyzed by immunohistochemistry with an anti-Croquemort (*Crq*) antibody, stained in *purple*, for hemocytes and TUNEL, and stained in *brown*, for apoptotic cells. *Scale bar,* 10 μm. In the *bottom panel*, stage 13 embryos of the indicated fly lines were subjected to an assay for phagocytosis *in vivo*, and the means ± S.D. were obtained with the data from at least three independent experiments and analyzed by Student's *t* test.

nuclei besides Croquemort-positive hemocytes with no engulfed materials as well as unengulfed TUNEL-positive cells in either preparation of embryonic cells (Fig. 4*B*, *middle panel*), indicating a successful analysis of phagocytosis using embryos

[4] The abbreviations used are: Crk, CT10 regulator of kinase; Elmo, Engulfment and cell motility; FDR, False Discovery Rate; GO, Gene Ontology; Mbc, Myoblast city; DREF, DNA replication-related element-binding factor.

at stage 13. We then compared stage 13 embryos of the *mbc* mutant with those of $y^1\ w^{1118}$ flies in an assay for phagocytosis *in vivo*. The level of phagocytosis in embryos of the *mbc* mutant was lower than that of control flies (Fig. 4*B*, *bottom panel*). The above-described results collectively indicate that Crk and Mbc are both required for the maximum level of apoptotic cell clearance by hemocytes in *Drosophila* embryos.

To establish whether Crk and Mbc function in the same signaling pathway, we performed a genetic interaction experiment. The level of phagocytosis was measured with embryos of a heterozygote of the *Crk* or *mbc* mutant as well as those of a "double heterozygous" mutant, $mbc^{C1}/+;Crk^{KG00336}/+$. We found that phagocytosis in a heterozygote of either mutant was similar to that in control $y^1\ w^{1118}$ flies but was weaker in the double heterozygote than in the control (Fig. 5*A*, *left panel*). This indicates the occurrence of genetic interaction between *Crk* and *mbc* in the double heterozygote, suggesting that these two genes function in the same pathway. We then investigated whether Crk and Mbc are located downstream of the engulfment receptor integrin αPS3-βν. To achieve this, the level of phagocytosis was determined in the embryos of flies, in which the RNAi of only integrin βν-encoding *Itgbn*, both *Crk* and *mbc*, and all of *Itgbn*, *Crk*, and *mbc* were specifically induced in hemocytes using the GAL4-UAS system (40). The level of apoptotic cell clearance was similar among these three flies (Fig. 5*A*, *right panel*), suggesting that *Crk* and *mbc* are required in the pathway involving *Itgbn*. When a similar experiment was performed applying the RNAi of *drpr* instead of *Itgbn*, phagocytosis was weaker after the knockdown of three genes, *Crk*, *mbc*, and *drpr*, than in flies with the knockdown of *drpr* alone or both *Crk* and *mbc* (Fig. 5*B*, *left panel*), suggesting that *drpr* does not function in the pathway in which *Crk* and *mbc* play a role. Similarly, *Itgbn* and Ced-6-encoding *ced-6*, the latter of which was previously shown to function downstream of *drpr* (37), appeared to be located in different signaling pathways (Fig. 5*B*, *right panel*). These results collectively showed that Crk and Mbc function in the pathway initiated by the engulfment receptor integrin αPS3-βν but not Draper. In addition, the results of an assay for genetic interaction indicated that Rac1 and Rac2, which are orthologues of *C. elegans* CED-10, act in the pathways downstream of Draper (Fig. 5*C*, *left panel*) as well as integrin βν (Fig. 5*C*, *right panel*). In conclusion, two partly overlapping signal transduction pathways exist for the induction of apoptotic cell clearance in *Drosophila*, namely Draper/Ced-6/Rac1, Rac2, and αPS3-βν/Crk,Mbc/Rac1,Rac2; however, some signal mediators contained in both pathways have yet to be identified.

Requirement for phagocytosis-inducing signaling pathways and engulfment in the activation of Tailless

We next investigated whether the engulfment receptors and signal mediators that constitute the pathways for the induction of phagocytosis are required for the activation of Tailless. S2 cells were subjected to the RNAi of genes coding for these molecules before the incubation with apoptotic cell fragments. S2 cell cultures were supplemented first with dsRNA containing sequences of the mRNAs of Draper and integrin βν to simultaneously inhibit the expression of these two receptors. This treatment reduced the expression of Draper and integrin βν (Fig. 6*A*, *left panel*); the expression of integrin βν was examined by RT-PCR because its level in S2 cells was below the detection limit of Western blotting. These cells were incubated with apoptotic cell fragments, and their nuclear extracts were examined for the DNA-binding activity of Tailless. We found that the activity of Tailless did not significantly increase in stimulated S2 cells after the RNAi of *drpr* and *Itgbn* (Fig. 6*A*, *right panels*), indicating a requirement for the actions of Draper and/or integrin βν in the activation of Tailless. We then attempted to identify which receptor is important and found that the RNAi of either *drpr* or *Itgbn* reduced the level of Tailless activation to a certain extent (Fig. 6*B*), suggesting the involvement of both receptors. Possible participation of Draper in the recognition of phosphatidylserine-exposing apoptotic cell fragments by phagocytes is reasonable because this engulfment receptor directly recognizes phosphatidylserine (41). Next, the requirement for signal mediators in the Draper- and integrin αPS3-βν-initiated pathways was examined, and we found that the extent of an increase in Tailless activity became small after the RNAi of *ced-6*, *Crk*, or *mbc* (Fig. 6*C*). This result, which coincided with the data for the involvement of Draper and integrin βν, indicated that Ced-6, Crk, and Mbc are all necessary for the full activation of Tailless. We then tested Rac1 and Rac2 in a similar manner and again demonstrated the importance of these molecules in the activation of Tailless (Fig. 6*D*). Collectively, we concluded that two partly overlapping signal transduction pathways for the phagocytosis of apoptotic cells, Draper/Ced-6/Rac1,Rac2 and αPS3-βν/Crk,Mbc/Rac1,Rac2, participate in the activation of Tailless in phagocytes during incubation with apoptotic cell fragments.

The results described above suggested the involvement of phagocytosis in the activation of Tailless in stimulated phagocytes. To directly validate this possibility, we treated phagocytes with cytochalasin B, which inhibits the polymerization of actin and thus serves as an inhibitor of phagocytosis, before stimulation with apoptotic cell fragments. To confirm the phagocytosis-inhibiting action of this drug, we analyzed the phagocytosis of apoptotic cells by l(2)mbn cells that had been treated with cytochalasin B and found a lowered activity of phagocytes after the treatment (Fig. 6*E*, *top panel*). We then similarly treated S2 cells with cytochalasin B, further incubated them in the presence of apoptotic cell fragments, and determined the DNA-binding activity of Tailless in their nuclear extracts. The treatment with the drug led to the activation of Tailless in stimulated S2 cells being undetectable (Fig. 6*E*, *bottom panels*). Taken together, the occurrence of engulfment is required for the activation of Tailless and, most probably, the subsequent enhancement of phagocytic activity in phagocytes during incubation with apoptotic cell fragments.

Involvement of Tailless in apoptotic cell clearance in flies

To gain insight into the physiological significance of Tailless-mediated enhancement of phagocytosis, we examined the effect of the RNAi of *tailless* on apoptotic cell clearance *in vivo*. Flies that harbor a *UAS* transgene for the generation of dsRNA containing the Tailless mRNA sequence (*UAS-tailless-IR*) were crossed with flies expressing GAL4 in a cell type-nonspecific

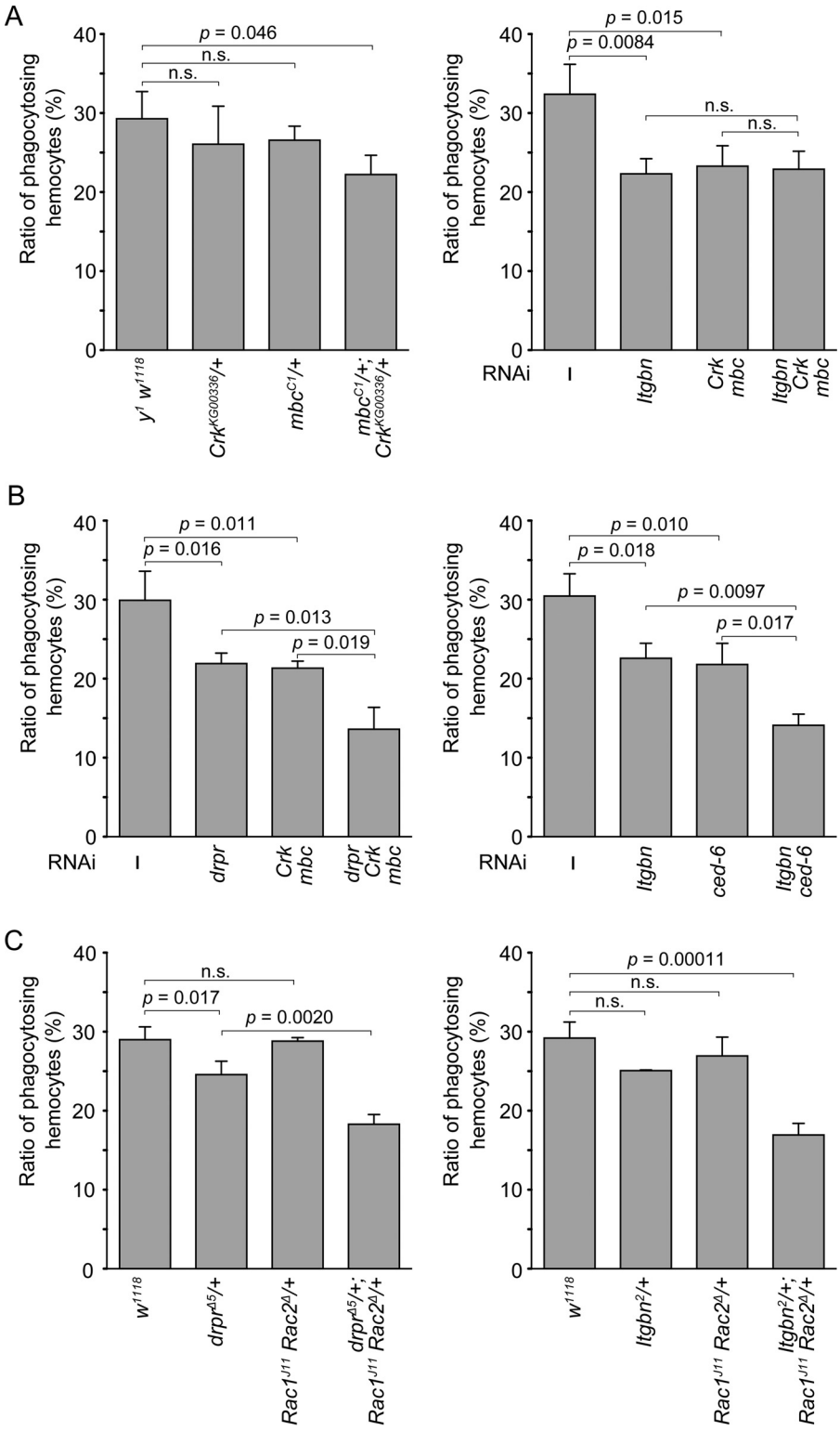
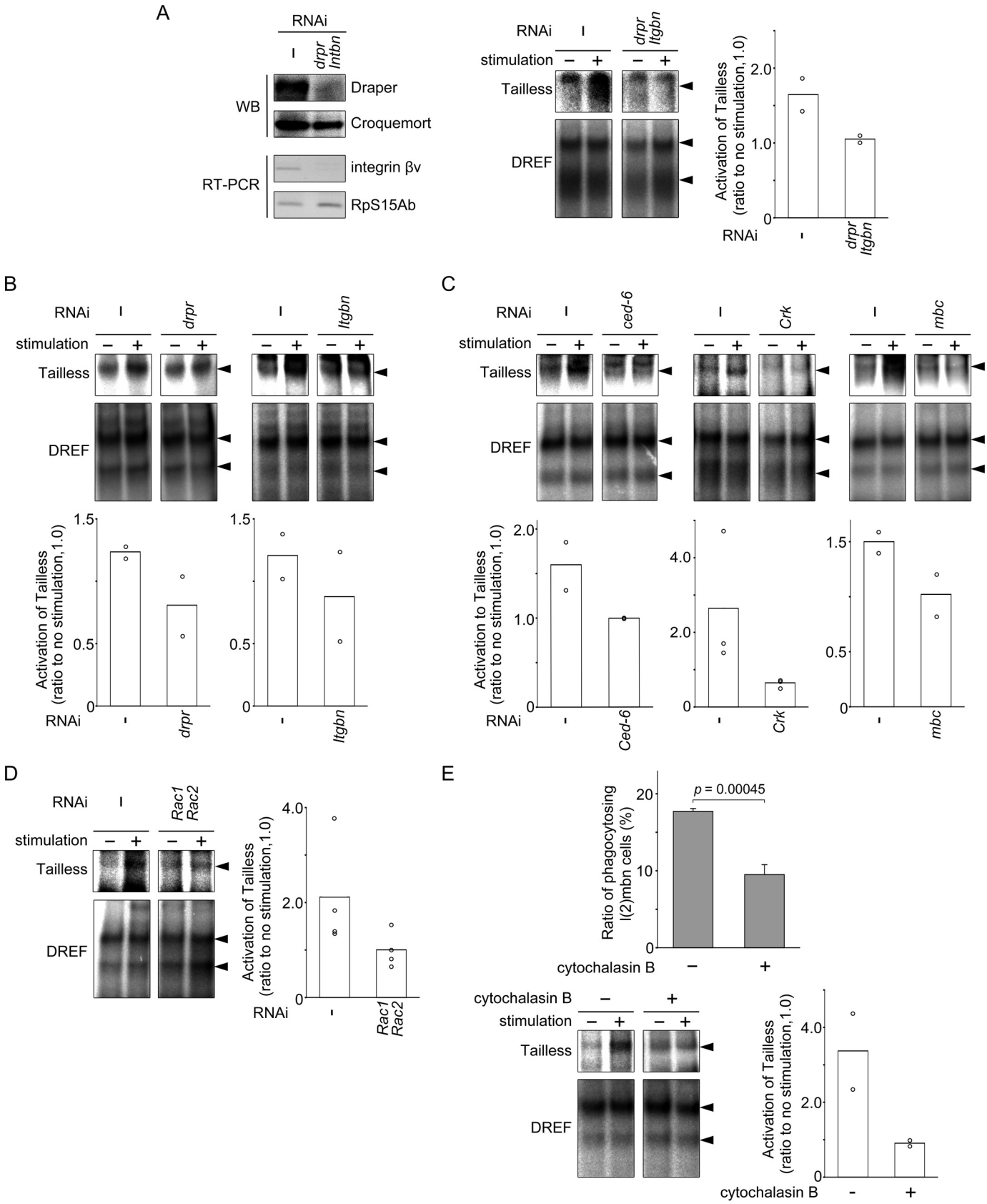


Figure 5. Identification of signal mediators located downstream of Draper and integrin αPS3-βν. An assay for phagocytosis *in vivo* was conducted with stage 16 embryos of genetically manipulated flies. The means ± S.D. were obtained with the data from at least three independent experiments and analyzed by Tukey's test, except for the data shown in the *left panel* in *A* that were analyzed by Dunnett's test. *A,* occurrence of genetic interaction using the indicated fly lines (*left panel*) and consequences of hemocyte-specific RNAi of the indicated genes in combinations (*right panel*) were examined. *n.s.*, difference not significant. *B,* consequences of hemocyte-specific RNAi of the indicated genes in combinations were examined. *C,* occurrence of genetic interaction was examined using the indicated fly lines. *n.s.,* difference not significant.

manner (*Act-GAL4*), and Tailless mRNA levels in embryos were determined by RT-PCR. We observed a decreased level of Tailless mRNA, whereas the mRNA of RpS15Ab, tested as a negative control, remained almost unchanged (Fig. 7*A*), indicating the successful knockdown of *tailless* in flies using this *UAS* transgene. We then examined the hemocyte phagocytosis



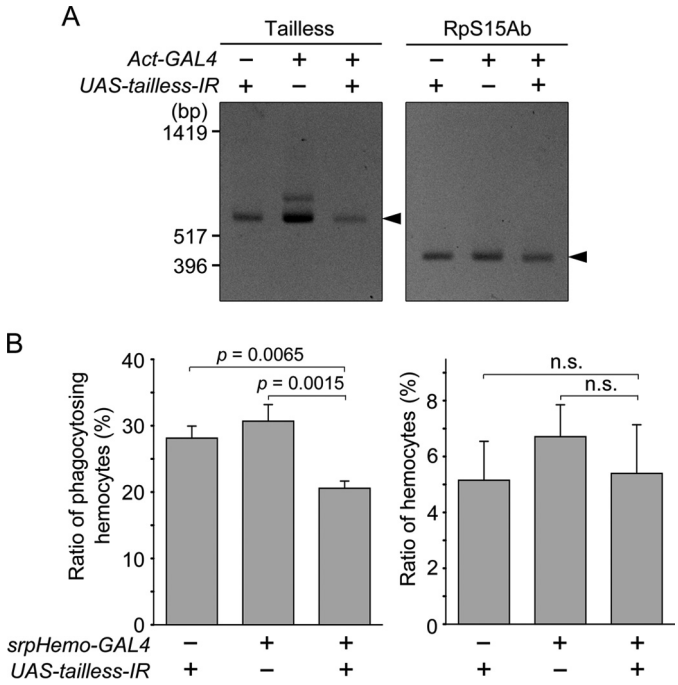


Figure 7. Requirement for Tailless in maximal level of apoptotic cell clearance in *Drosophila* embryos. *A,* flies were subjected to the cell type-nonspecific RNAi of *tailless*, and the RNA of embryos at stages 13–16 was analyzed by RT-PCR for the mRNAs of Tailless and RpS15Ab. Data from one of two independent experiments that yielded similar results are presented. *B,* flies were subjected to the hemocyte-specific RNAi of *tailless*, and dispersed cells of stage 16 embryos were immunocytochemically analyzed for the phagocytosis of apoptotic cells (*left panel*) and the number of Croquemort-positive hemocytes (*right panel*). The level of phagocytosis is shown as the ratio of hemocytes with TUNEL signals to all hemocytes, and the number of hemocytes is as the ratio of hemocytes to total dispersed cells. The means ± S.D. deviations were obtained with the data from three independent experiments and analyzed by Tukey's test. *n.s.,* not significant.

of apoptotic cells in embryos of flies, in which *tailless* was knocked down using a hemocyte-specific GAL4 driver (*srpHemo-GAL4*). We found that a decrease in the level of Tailless in hemocytes reduced apoptotic cell clearance in embryos to approximately two-thirds of its normal level (Fig. 7*B*, *left panel*). This is not due to a reduction in the number of hemocytes (Fig. 7*B*, *right panel*), indicating that Tailless is necessary for embryonic hemocytes to exert the maximal level of phagocytosis. This suggests that the phagocytic activity of hemocytes is enhanced in a manner mediated by Tailless when they first encounter and engulf apoptotic cells *in vivo*.

Discussion

Accumulating evidence has suggested that phagocytes increase their phagocytic activity after the engulfment of cells undergoing apoptosis. This phenomenon most likely ensures the phagocytic removal of apoptotic cells that phagocytes meet thereafter and is thus considered to be a priming mechanism of phagocytes in innate immunity. Previous studies reported that an increase in the level of engulfment receptors is a cause, at least in part, for the enhancement of phagocytic activity; the expression of the engulfment receptor Mer is raised in peritoneal (42) and bone marrow-derived (43) macrophages of the mouse after the phagocytosis of apoptotic thymocytes. A more recent study showed that the embryonic hemocytes of *Drosophila* acquired phagocytic activity against bacteria after the engulfment of apoptotic cells, accompanied by an elevated level of the engulfment receptor Draper (22). In this study, we demonstrated that *Drosophila* phagocytes produce a higher level of Draper and integrin αPS3, another engulfment receptor, after incubation with apoptotic cell fragments and become more active in the phagocytosis of apoptotic cells. Therefore, phagocytes that have accomplished phagocytosis seem to increase the amount of engulfment receptors and acquire enhanced activity for the phagocytosis of multiple targets, and this phenomenon is likely to be conserved among mammals and insects.

We here investigated the mechanisms underlying the engulfment-mediated priming of phagocytes. The data obtained in a series of experiments collectively indicate that the transcription repressor Tailless is responsible for the elevated expression of Draper and integrin αPS3 and thus for the enhanced phagocytic activity in *Drosophila* phagocytes that have recognized apoptotic cells (Fig. 8). As a transcription repressor, activated Tailless is likely to inhibit the expression of a gene(s) that negatively controls the transcription of Draper-encoding *drpr* and integrin αPS3-encoding *scb*. To identify such a target gene(s) of Tailless is one of the important issues in future investigation. *Drosophila* Tailless (44) is an orphan nuclear receptor belonging to the nuclear receptor subfamily 2 group E (45, 46). The orthologue of *tailless* is known in mammals and nematodes, *Tlx* of the mouse (47) and *nhr-67* of the *C. elegans* (48), and Tailless and its counterparts have been shown to function in the organogenesis during early development. We found that Tailless is required for the maximal level of apoptotic cell clearance in *Drosophila* embryos, but whether or not Tlx and NHR-67

Figure 6. Requirement for engulfment receptors and signal mediators in Tailless activation. *A,* S2 cells were subjected to the RNAi of *drpr* and *Itgbn* prior to the incubation with apoptotic cell fragments. In the *left panel*, S2 cells were analyzed for the indicated proteins and mRNA by Western blotting (*WB*) and RT-PCR, respectively. In the *right panel*, nuclear extracts of S2 cells stimulated or not stimulated with apoptotic cell fragments for 1 h were analyzed by EMSA for Tailless and DREF. Data from a single experiment (*left panel*) and from two independent experiments with similar results (*right panel*) are shown. *B,* EMSA of Tailless and DREF was performed with the nuclear extracts of S2 cells expressing Tailless-HA, which had been subjected to the RNAi of the indicated genes before the stimulation with apoptotic cell fragments for 15 min (*left panel*) or 1 h (*right panel*). Representative data from one of two independent experiments that yielded similar results are shown. *C,* EMSA of Tailless and DREF was performed with the nuclear extracts of Tailless-HA-expressing S2 cells, with and without the RNAi of the indicated genes before a 15-min stimulation with apoptotic cell fragments. Data from one of two (*left panel*), three (*middle panel*), and two (*right panel*) independent experiments with similar results are shown. *D,* nuclear extracts of Tailless-HA-expressing S2 cells, which had been subjected to the RNAi of *Rac1* and *Rac2* prior to a 15-min incubation with apoptotic cell fragments, were subjected to EMSA of Tailless and DREF. Data from one of four independent experiments with similar results are shown. *E, top panel*, l(2)mbn cells were treated with cytochalasin B before they were used as phagocytes in an assay for phagocytosis *in vitro* with cycloheximide-treated S2 cells as targets. The means ± S.D. were obtained with the data from three independent experiments and analyzed by Student's *t* test. In the *bottom panel*, cultures of S2 cells expressing Tailless-HA were supplemented with cytochalasin B prior to the stimulation with apoptotic cell fragments for 15 min, and their nuclear extracts were subjected to EMSA of Tailless and DREF. Representative data from two independent experiments that yielded similar results are shown. The *bar graphs* in EMSA indicate values for signal intensities obtained in independent experiments shown with the mean values.

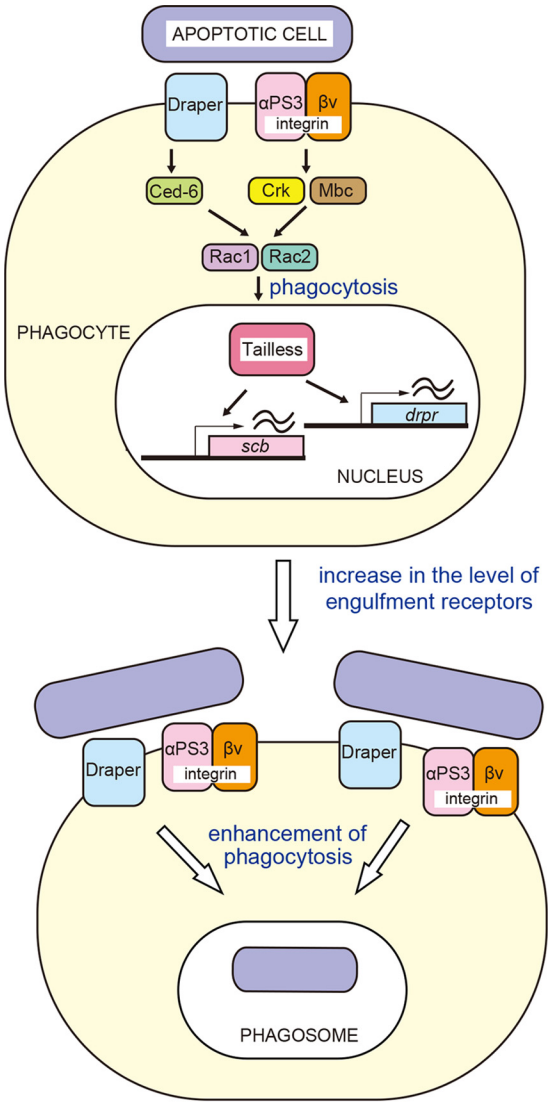


Figure 8. Model depicting the mechanism of phagocyte priming by apoptotic cells. Refer to the text for description.

play a similar role remains to be known. A loss of Tailless makes *Drosophila* embryonic lethal (49), suggesting its role in the development of embryos. Hemocyte-specific reduction in the expression of *tailless* brought about a decreased level of apoptotic cell clearance but not of hemocyte number. This suggests that Tailless primarily supports the phagocytic activity rather than the development of hemocytes in embryos.

The DNA-binding activity of Tailless was raised in stimulated phagocytes, although the data from a microarray analysis indicated no increase in the level of Tailless mRNA after stimulation, suggesting a post-transcriptional control of its expression and function. An increase in the DNA-binding activity of Tailless was dependent on two partly overlapping signaling pathways for the induction of phagocytosis, namely Draper/Ced-6/Rac1,Rac2 and αPS3-βν/Crk,Mbc/Rac1,Rac2 (Fig. 8) as well as the occurrence of phagocytosis. Therefore, either a morphological change in phagocytes after the reorganization of the cytoskeleton or an apoptotic cell-derived material(s) incorporated into phagocytes is responsible for the activation of Tailless. As a possible mechanism for the latter, apoptotic cell fragments engulfed by phagocytes could supply Tailless with a ligand for activation. Tailless is known to function forming a complex with transcriptional co-repressors and other proteins (45). Another possibility to explain the activation of Tailless is that the quantity and/or quality of those associated proteins are altered in stimulated phagocytes.

Experimental procedures

Cell culture

S2 cells, a cell line established from *Drosophila* embryonic hemocytes, were maintained at 25 °C in Schneider's *Drosophila* medium (Life Technologies, Inc., Japan, Tokyo, Japan) containing 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The l(2)mbn cells, a cell line derived from the larval hemocytes of a tumorous *Drosophila* mutant, were cultured similarly to S2 cells. Prior to use as phagocytes in an assay for phagocytosis *in vitro*, l(2)mbn cells at ~70–80% confluence were incubated with 20-hydroxyecdysone (Sigma Japan, Tokyo, Japan) (1 μM) for 48–96 h (26). To generate an S2 cell line that expresses Tailless-HA, a DNA fragment containing a sequence corresponding to entire Tailless fused to the HA tag at the C terminus was inserted into the vector pMT/V5-HisA (Thermo Fisher Scientific K.K., Yokohama, Japan), and the resulting plasmid was used, together with a plasmid for the expression of a blasticidin-resistant gene, to transfect S2 cells using the *Drosophila* Expression System (Thermo Fisher Scientific). Cells were maintained in the presence of blasticidin for 2 weeks, and subclones were established and tested for the expression of Tailless-HA after incubation with 0.5 mM $CuSO_4$ for 12–24 h. A subclone that expressed Tailless-HA at a higher level than others was selected, maintained, and used in the subsequent experiments. To inhibit phagocytosis, l(2)mbn cells and S2 cells expressing Tailless-HA were incubated for 1 h in the presence of cytochalasin B (Sigma Japan) at 50 μM prior to an assay for phagocytosis *in vitro* and the stimulation with apoptotic cell fragments, respectively.

Fly maintenance

All flies were maintained with standard cornmeal/agar medium at 25 °C. The following lines of *Drosophila* were used after changing balancers when necessary: w^{1118}, y^{1} w^{1118}, $drpr^{\Delta 5}$ lacking Draper (50); $Itgbn^{2}$ lacking integrin βν (51); the *P*-element insertion mutant $Crk^{KG00336}$ (52) (Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN; stock no. 13652); the deficiency mutant mbc^{C1} (53) (Bloomington *Drosophila* Stock Center; stock no. 1671); $Rac1^{J11}$ $Rac2^{\Delta}$ lacking both Rac1 and Rac2 (Bloomington *Drosophila* Stock Center; stock no. 6677); *Act-GAL4* (*Drosophila* Genomics and Genetic Resources, Kyoto Stock Center, Kyoto Institute of Technology, Kyoto, Japan; DGRC number 107727) used as a cell type-nonspecific GAL4 driver after making it possess *Act-GFP*; *srpHemo-GAL4 UAS-srcEGFP* (54) used as a hemocyte-specific GAL4 driver; *UAS-drpr-IR* (Vienna *Drosophila* RNAi Center, Vienna, Austria: VDRC ID 4833); *UAS-Itgbn-IR* (National Institute of Genetics, Mishima, Japan: stock ID 1762R-1); *UAS-Crk-IR* (Vienna *Drosophila* RNAi Center; VDRC ID 106498); *UAS-mbc-IR* (Vienna *Drosophila* RNAi Center; VDRC ID 16044); *UAS-ced-6-IR* (Vienna *Drosophila*

RNAi Center; VDRC ID 108101); and *UAS-tailless-IR* (Vienna *Drosophila* RNAi Center; VDRC ID 6236).

Stimulation of phagocytes with fragments of apoptotic cells

S2 cells were incubated in the presence of cycloheximide (1.5 μg/ml) for 24 h to induce apoptosis (26). Cells were then centrifuged at 300 × *g* at room temperature for 3 min, and the resulting supernatants were collected. They were re-centrifuged at 1500 × *g* at 4 °C for 3 min, and the supernatants were further centrifuged at 3500 × *g* at 4 °C for 3 min. The resulting precipitates were recovered, suspended in Schneider's *Drosophila* medium with 1% heat-inactivated FBS, microscopically evaluated for the number of membranous particles using a hemocytometer, and used as the fragments of apoptotic S2 cells, referred to as "apoptotic cell fragments" in this study. Semi-confluent S2 cells, expressing or not expressing Tailless-HA, or hormone-treated l(2)mbn cells maintained in Schneider's *Drosophila* medium with 1% heat-inactivated FBS were supplemented with apoptotic cell fragments at a responder/effector ratio of 1:25. The mixtures, together with phagocyte cultures with no fragments added as a negative control, were incubated at 25 °C for various periods of time as indicated in the figure legends, and cells were detached from culture containers by treatment with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA. The recovered cells were collected by centrifugation at 300 × *g* at room temperature for 3 min, washed twice with PBS, and used as phagocytes stimulated by apoptotic cell fragments or an untreated control in the preparation of RNA for a microarray analysis and the preparation of nuclear extracts for EMSA. In some experiments, cells after stimulation with apoptotic cell fragments were washed twice with PBS, supplemented with a lysis buffer, and detached from culture containers using a cell scraper. The resulting cell lysates were used in the RNA extraction for RT-PCR, Western blotting, and the preparation of nuclear extracts for EMSA. In an assay for phagocytosis *in vitro*, hormone-treated l(2)mbn cells were pre-incubated with and without apoptotic cell fragments, culture media including the stimulant were removed, and l(2)mbn cells remaining attached to culture containers were used as phagocytes.

DNA microarray analysis

Total RNA was prepared from S2 cells, which had been stimulated or left untreated with apoptotic cell fragments, using TRIzol reagent (Thermo Fisher Scientific K.K.) and subsequently purified with RNeasy micro kit (Qiagen K.K., Tokyo, Japan). The quality and quantity of the purified RNA were confirmed by agarose gel electrophoresis and spectrophotometry, respectively. One hundred and fifty nanograms of purified RNA was subjected to a reaction for the synthesis of biotinylated cRNA using GeneChip 3′ IVT express kit (Affymetrix), and the resulting cRNA was fragmented and subsequently used in hybridization with a DNA microarray (GeneChip *Drosophila* Genome 2.0 Array, Affymetrix). The hybridized cRNA was labeled with streptavidin/phycoerythrin using GeneChip Hybridization, Wash, and Stain Kit and Fluidics Station 450 System (Affymetrix), and fluorescence signals derived from cRNA were measured using GeneChip Scanner 3000 7G (Affymetrix). All experimental procedures were performed according to the manufacturer's instructions. Affymetrix GeneChip Command Console software was used to reduce array images to the intensity of each probe (CEL files). All microarray data are Minimum Information About a Microarray Experiment-compliant and have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov, GEO Series accession number GSE85429), as detailed on the website of the Functional Genomics Data Society. The original Affymetrix CEL files were quantified using the Distribution Free Weighted method (55) with statistical language R (56) and Bioconductor (57). Hierarchical clustering was performed using the pvclust() function (58) in R. To identify differentially expressed genes, the Rank Products method (59) was applied to data that had been quantified using the Distribution Free Weighted method, with the number of permutations being set at 500. Probe sets presenting false discovery rate (FDR) of <0.05 were regarded as having significantly different expression levels between the two groups. A gene-annotation enrichment analysis of differentially expressed genes was performed using the Database for Annotation, Visualization, and Integrated Discovery 6.8 beta (60) (david-d.ncifcrf.gov) and QuickGO (61). Expression Analysis Systematic Explorer scores, which are a modified version of Fisher's exact test *p* values (62), were used to statistically evaluate over-represented Gene Ontology (GO) terms from differentially expressed genes. Benjamini and Hochberg FDR corrections for multiple testing (63) were used to correct the results. GO terms with *p* values of <0.05, after corrections using FDR, were regarded as significantly enriched, unless otherwise stated in the text.

Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared from S2 cells expressing or not expressing Tailless-HA, according to the previously described procedure (64). In brief, S2 cells, which had been treated or not treated with apoptotic cell fragments, were suspended in a buffer containing 0.6% (w/v) Nonidet P-40, vigorously vortexed, and centrifuged. The resulting precipitates, a crude nuclear fraction, were suspended in a buffer containing 0.4 M NaCl, incubated on ice for 15 min, and centrifuged. The supernatants were collected as nuclear extracts, and the aliquots were stored frozen at −80 °C until use. In EMSA, nuclear extracts, 2–4 μg of proteins, were incubated with oligonucleotides labeled with ^{32}P at the 5′-end, which contained sequences corresponding to the binding sites for the transcription factors analyzed (refer to supplemental Fig. 2 for the sequences), in the presence of poly(dI-dC) (Sigma Japan) on ice for 10 min. The reaction mixtures were separated by 6% (w/v) PAGE, and the radioactive signals were visualized using an imaging plate and BAS-1800II (GE Healthcare Japan, Tokyo, Japan). In some experiments, the images were analyzed using Adobe Photoshop for determining signal intensities.

Other materials and methods

An assay for phosphatidylserine exposure was carried out as described previously (26) with some modifications using FITC-conjugated annexin V (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Western blotting was performed accord-

ing to a standard procedure. The generation and use of anti-integrin $\beta\nu$ (27), anti-DmCaBP1 (65) for determining the level of DmCaBP1 as an internal control, and anti-Croquemort (26) rat antisera were described elsewhere. The anti-Draper mouse monoclonal antibody 8A1, which had been deposited to the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) by Mary A. Logan, was used in this study, except for the experiment shown as Fig. 6*A* (*left panel*) in which anti-Draper rat antiserum (26) was used. An anti-integrin αPS3 rabbit antibody (66) was a gift from Shigeo Hayashi. Anti-Crk antiserum was generated by immunizing rats with recombinant *Drosophila* Crk that had been expressed in *E. coli* as a protein fused to GST and purified to homogeneity. Anti-Mbc rat antiserum, which had been raised against recombinant *Drosophila* Mbc corresponding to the amino acid positions 1717–1970 at the C terminus (67), was a gift from Susan Abmayr. Primary antibodies were located by a chemiluminescence reaction using an anti-rat IgG antibody conjugated with alkaline phosphatase and the Immun-Star system (Bio-Rad, Tokyo, Japan), anti-rabbit IgG antibody conjugated with HRP and Western Lightning (PerkinElmer Life Sciences, Japan Co., Ltd., Yokohama, Japan), or an anti-mouse IgG antibody conjugated with HRP and Western Lightning. RNAi with culture cell lines was performed by incubating cells in the presence of dsRNA that contained a part of the sequence of target mRNAs, as described previously (26). In RT-PCR, total RNA was prepared from cells and used as a template for reverse transcription with oligo(dT) as a primer, and the resulting complementary DNA was subjected to semi-quantitative PCR. The DNA oligomers used as the primers in PCR for the synthesis of dsRNA as well as in RT-PCR are shown in supplemental Fig. 1. An assay for phagocytosis *in vitro* was conducted using 20-hydroxyecdysone-treated l(2)mbn cells as phagocytes and S2 cells undergoing cycloheximide-induced apoptosis as target cells, as described previously (26) with modifications. Phagocytes and target cells were incubated and stained with hematoxylin, and the number of phagocytes containing or not containing target cells was microscopically determined. An assay for phagocytosis *in vivo* using cells dispersed from embryos was performed according to the established procedure (27). Dispersed embryonic cells were subjected to immunocytochemistry using an anti-Croquemort antibody for hemocytes and TUNEL for nuclei with fragmented DNA, and the number of cells positive for only Croquemort (hemocytes without phagocytosis) and those positive for both Croquemort and fragmented DNA (hemocytes after phagocytosis) was determined.

Data processing and statistical analysis

Results from quantitative analyses are expressed as the means ± S.D. of data from three independent experiments, unless otherwise stated in the figure legends. Other data are representative of at least two independent experiments that yielded similar results. Statistical analyses were performed using the two-tailed Student's *t* test, Tukey's test, or Dunnett's test, as indicated in the figure legends. *p* values are shown in the corresponding figures or figure legends; any p values <0.05 were considered significant.

Author contributions—S. N., Y. A., K. N., and Y. Nakanishi conceived the research. S. N., Y. A., T. K., C. H., and Y. Nakai conducted the experiments. S. N., Y. A., Y. Nakai, K. N., F. N., A. S., and Y. Nakanishi analyzed the data. S. N. and Y. Nakanishi wrote the paper.

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