*Original Article*

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# Role of NPxY motif in Draper-mediated apoptotic cell clearance in *Drosophila*

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**ABSTRACT: Draper, a receptor responsible for the phagocytosis of apoptotic cells in *Drosophila*, possesses atypical epidermal growth factor (EGF)-like sequences in the extracellular region and the two phosphorylatable motifs NPxY and YxxL in the intracellular portion. We previously suggested that Pretaporter, a ligand for Draper, binds to the EGF-like repeat and augments the tyrosine phosphorylation of Draper. In this study, we first tested the binding of Pretaporter to various parts of the extracellular region of Draper and found that a single EGF-like sequence is sufficient for the binding. We next determined roles of the two intracellular motifs by forcedly expressing Draper proteins, in which tyrosine residues within the motifs had been substituted with phenylalanine, in hemocytes of Draper-lacking flies. We found that Draper proteins with Y-to-F substitution in either motif still underwent tyrosine phosphorylation, suggesting the occurrence of phosphorylation at both motifs. The Draper protein with substitution in the YxxL motif rescued a defect of phagocytosis, as did intact Draper, but the Draper protein with substitution in the NPxY motif did not, indicating a role of the motif NPxY, but not YxxL, in Draper-mediated phagocytosis. This coincides with our previous finding that Ced-6, an NPxY-binding signaling adaptor, is required for Draper's actions in apoptotic cell clearance. In summary, we demonstrated that Draper binds to its ligand Pretaporter using EGF-like sequences, and that the NPxY motif in the intracellular region of Draper plays an essential role in its actions as an engulfment receptor.**

***Keywords:*** Apoptosis, phagocytosis, tyrosine phosphorylation

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## 1. Introduction

The cells that constitute our body often become effete or harmful. Such altered own cells are induced to undergo apoptosis and become susceptible to phagocytosis (*1-4*). Most apoptotic cells expose substances that serve as ligands for receptors of phagocytes leading to engulfment (*1-4*). Genetic studies with *Caenorhabditis elegans* have shown the existence of two signaling pathways for the induction of phagocytosis (*5-8*). These pathways, namely, CED-6/CED-7/CED-10 and CED-2/CED-5/CED-12/CED-10, are most likely governed by the engulfment receptors CED-1 (*9*) and INA-1 (*10*), respectively. CED-1 is a single-path membrane protein containing atypical epidermal growth factor (EGF)-like sequences (*9*), and INA-1 is a α-subunit of *C. elegans* integrins (*10*). CED-1 (*11*), integrins (*12*), and the intracellular signaling molecules in the above two pathways (*8,13*) seem to be evolutionally conserved among species including humans. This suggests the phylogenetic conservation of the mode of apoptotic cell clearance.

We (*14*) and other investigators (*15*) have reported the participation of the CED-1 orthologue Draper in the phagocytic elimination of apoptotic cells by hemocytes and glia of *Drosophila melanogaster*. Draper is also responsible for the remodeling of neural circuits: removal of axons (*16,17*) and dendrites (*18*) of larval neurons during metamorphosis, axons in injury-induced Wallerian degeneration (*17,19*), and presynaptic membranes at neuromuscular junctions (*20*). The extracellular portion of Draper contains three cysteine-rich sequences that are shared with many other proteins, namely, the EMI, NIM, and EGF-like domains (*11,21*). We previously reported that a recombinant Draper protein corresponding to the entire EGF-like repeat binds Pretaporter, an endoplasmic reticulum protein that serves as a ligand for Draper, and that the binding of Pretaporter augments the tyrosine phosphorylation of Draper (*22*). There are two phosphorylatable tyrosine residues, which are contained in the motifs NPxY and YxxL, within the intracellular region of Draper. The NPxY motif serves as a binding site for proteins that possess the phosphotyrosine-binding (PTB) domain. In fact, the human orthologue of CED-6, which contains the PTB domain, interacts

with CED-1 through binding to the NPxY motif (*23*). In addition, we previously showed that Draper requires Ced-6 to induce the phagocytosis of apoptotic cells in embryos (*22*). The other motif YxxL is a part of the immunoreceptor tyrosine-based activation motif and serves as a site for binding of proteins containing the Src homology 2 domain (*24*). The importance of YxxL in Draper has been reported for the phagocytic elimination of injured axons by glia (*25*). It is thus likely that both motifs play roles in Draper-mediated phagocytosis. The present study was carried out to determine which region of the extracellular portion of Draper is responsible for the binding to Pretaporter as well as which of the two intracellular motifs is phosphorylated and required for Draper's actions.

## 2. Materials and Methods

### 2.1. *Fly stocks and cell culture*

The following lines of *Drosophila* were used: $w^{1118}$, $drpr^{\Delta 5}$ (*15,26*), *srpHemoGAL4 UAS-srcEGFP* (*29*), *w*; +; *Dr/TM6B Dfd-GMR-nvYFP* (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN, USA), and *C(1)DX/FM7*; +; *Sb/TM3* (a gift from T. Awasaki). To establish fly lines expressing Draper proteins with amino acid alterations, the *Drosophila* EST clone GH03529 (Berkeley Drosophila Genome Project and National Institute of Genetics), in which nucleotide sequences had been modified so that tyrosine residues in the NPxY and YxxL motifs were changed to phenylalanine, was placed downstream of the *UAS* sequence and used to generate transgenic flies. The resulting flies together with one possessing intact *draper* were intercrossed with the *draper* null mutant $drpr^{\Delta 5}$ and used for mating with *srpHemoGAL4 UAS-srcEGFP*, a GAL4 driver for the hemocyte-specific expression of *UAS*-transgenes. Other flies used in this study were generated through the mating of existing flies. Genotypes of the fly lines analyzed are shown in the corresponding figure captions. The hemocyte-derived cell line l(2)mbn was maintained at 25°C with Schneider's Drosophila medium (Life Technologies Japan, Tokyo, Japan), as described previously (*14*). Sf9 insect cells were maintained at 29°C with Grace's Insect medium (Life Technologies Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum.

### 2.2. *Assay for protein-protein interaction*

Various regions within the extracellular portion of Draper fused to glutathione *S*-transferase (GST) at the N-terminus were prepared using the baculovirus-based vector system (Life Technologies Japan) and Sf9 cells, and affinity-purified by glutathione-Sepharose chromatography (GE Healthcare Japan, Tokyo, Japan), essentially as described previously (*22*). Pretaporter fused to maltose-binding protein (MBP) was prepared and purified as reported previously (*22*). The GST-fused Draper proteins were mixed with MBP-tagged Pretaporter (at an equal molar concentration) in a buffer consisting of 15 mM PIPES (pH 6.5), 0.1 M NaCl, 20 mM KCl, 20 mM $MgSO_4$, and 10 mM $CaCl_2$, precipitated with glutathione-sepharose, and analyzed by Western blotting for the co-precipitation of MBP-Pretaporter using the anti-MBP antibody.

### 2.3. *Assay for tyrosine phosphorylation*

The EST clone GH03529 and the plasmid pUAST (*28*) were used to prepare vectors to express HA-tagged Draper proteins with altered NPxY and YxxL motifs as well as with unaltered motifs. l(2)mbn cells were transfected with these vectors together with pAct5C-GAL4 (a gift from M. Miura), a GAL4 driver for the ubiquitous expression of *UAS*-transgenes, by lipofection (Cellfectin II; Life Technologies Japan) and cultured for 2 days. The cells were then lysed with a buffer consisting of 40 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 2% (w/v) CHAPS, protease inhibitors (Nakalai Tesque, Kyoto, Japan), and phosphatase inhibitors (Sigma-Aldrich Japan, Tokyo, Japan). The lysates were incubated with the anti-influenza virus hemagglutinin (HA) antibody, and HA-tagged Draper proteins were precipitated with protein G-sepharose (GE Healthcare Japan) and subjected to Western blotting with the anti-phosphotyrosine antibody. For the analysis of the tyrosine phosphorylation of Draper *in vivo*, pupae of the flies that expressed Draper proteins containing altered NPxY and YxxL motifs with the background of $drpr^{\Delta 5}$ were lysed with a buffer consisting of 20 mM Tris-HCl (pH 8.1), 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, and 5% (w/v) bovine serum albumin. The resulting lysates were subjected to immunoprecipitation with the anti-Draper antibody, and the precipitates were analyzed by Western blotting with the anti-phosphotyrosine antibody.

### 2.4. *Immunochemistry*

Cultured l(2)mbn cells were smeared on glass slides, incubated with phosphate-buffered saline (PBS) containing the anti-HA antibody and 1% (v/v) blocking reagent (Roche Diagnostics Japan, Tokyo, Japan), and washed with PBS. They were then successively reacted with biotin-conjugated anti-mouse IgG antibody (Life Technologies Japan) and Alexa488-labeled streptavidin (Life Technologies Japan), and examined by fluorescence microscopy. For the analysis of embryos, dispersed embryonic cells were smeared on glass slides, incubated with PBS containing the anti-Draper antibody and 3% bovine serum albumin, and washed with PBS. The samples were successively reacted with biotin-conjugated anti-rat IgG antibody (Life Technologies Japan) and Alexa546-labeled streptavidin (Life Technologies Japan) followed by microscopic examination.

2.5. *Other materials and methods*

Generation and use of the anti-Draper and anti-Croquemort rat antibodies were reported previously (*16*). The anti-MBP, anti-GST, anti-HA, and anti-phosphotyrosine (clone RC20) antibodies were purchased from New England Biolabs (Ipswich, MA, USA), Millipore (Billerica, MA, USA), Covance Japan (Tokyo, Japan), and BD Biosciences (San Jose, CA, USA), respectively. The level of phagocytosis of apoptotic cells was cytochemically determined with dispersed embryonic cells as described previously (*29*), and the ratio of hemocytes that had accomplished phagocytosis was exhibited as "phagocytosing hemocytes". Western blotting of lysates of cultured cells (*14*) and flies (*29*) was done essentially as reported previously.

2.6. *Data processing and statistical analysis*

Results from quantitative analyses were expressed as the mean ± SD of the data from at least three independent experiments. Other data were representative of at least three independent experiments that yielded similar results. Statistical analyses were performed using the two-tailed Student's *t*-test, and *p* values of less than 0.05 were considered significant and are indicated in the figures.

## 3. Results

3.1. *Identification of minimum region of Draper for binding to Pretaporter*

We first tried to determine the region of Draper required for the binding to its ligand Pretaporter. The EMI and NIM domains are located close to the N-terminus, and EGF-like sequences appear 15 times occupying the remaining part of the extracellular region (Figure 1A). We previously showed that a recombinant Draper protein lacking the EMI and NIM domains binds Pretaporter (*22*), and thus tested the binding of Pretaporter to Draper proteins with reduced numbers of the EGF-like sequences (Figure 1A). MBP-tagged Pretaporter (MBP-Prtp), portions of the extracellular region of Draper fused with GST (GST-DrprEx), and GST alone as a negative control were purified (Figure 1B) and mixed, and GST-proteins were recovered with glutathione-Sepharose followed by examination for the presence of MBP-Prtp by Western blotting using the anti-MBP antibody. We found that the reduction in the number of EGF-like sequences did not significantly influence the binding to Pretaporter, and that even only a single EGF-like sequence located the closest to the N-terminus effectively bound Pretaporter (Figure 1C, left panels). We next tested single EGF-like sequences located at other parts of Draper for the binding to

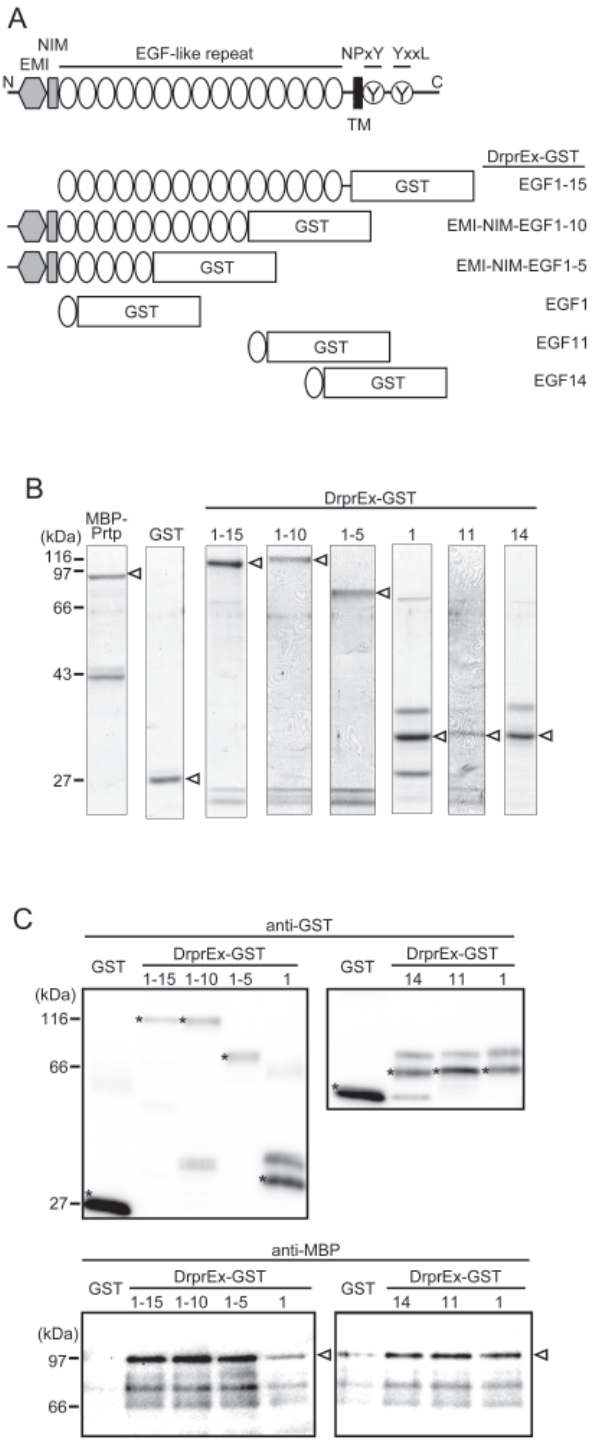


**Figure 1. Identification of minimum region of Draper necessary for binding to Pretaporter. Various parts of the extracellular region of Draper were examined for the binding to Pretaporter. (A)** Structures of Draper and the Draper proteins analyzed (GST-DrprEx) are schematically drawn not to scale. The positions of the EMI, NIM, EGF-like, NPxY, and YxxL domains together with the transmembrane region (TM) are shown. **(B)** Purified MBP-fused Pretaporter (MBP-Prtp), GST, and GST-DrprEx were analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue. The numbers above the slots correspond to those used to explain the structures of GST-DrprEx in **(A)**. The arrowheads point to the positions of the purified proteins. **(C)** GST-DrprEx and MBP-Prtp were incubated, pulled-down with glutathione-sepharose, and analyzed by Western blotting with anti-GST (top panels) and anti-MBP (bottom panels) antibodies. The asterisks and arrowheads indicate GST-DrprEx and MBP-Prtp, respectively.

Pretaporter, and found that all the sequences examined possessed the activity (Figure 1C, right panels). These results confirmed that EMI and NIM domains are dispensable for the binding of Draper to its ligand Pretaporter and suggested that any single EGF-like sequences of 15 repeats warrant the binding activity of Draper.

### 3.2. *Tyrosine phosphorylation of Draper with altered NPxY and YxxL motifs*

The cytoplasmic portion of Draper contains two short sequences susceptible to tyrosine phosphorylation, the NPxY and YxxL motifs, which have been presumed to be important for the actions of Draper. To delineate their roles, we first examined the effect of Y-to-F substitution in these motifs on the tyrosine phosphorylation of Draper. Nucleotide sequences of the cDNA of Draper, corresponding to Draper-I (*15*), were altered so that tyrosine residues located in the NPxY and YxxL motifs were changed to phenylalanine (Figure 2A). Draper proteins with Y-to-F substitution were expressed in l(2)mbn cells as proteins fused to HA at the N-terminus (HA-Drpr). When the cells were examined by immunocytochemistry for the surface localization of HA-Drpr using the anti-HA antibody, positive signals were obtained with cells expressing all the three Draper proteins, HA-Drpr-WT (no amino acid alteration), HA-Drpr-Y949F (Y-to-F substitution in YxxL), and HA-Drpr-Y858F (Y-to-F substitution in NPxY) (Figure 2B, left panel). The level of HA-Drpr-Y858F was somewhat higher than those of the other two proteins as examined by Western blotting of whole-cell lysates (Figure 2B, right panel). Under such conditions, the level of tyrosine-phosphorylated HA-Drpr was determined. For this purpose, l(2)mbn cells expressing HA-Drpr were lysed and subjected to immunoprecipitation with the anti-HA antibody followed by Western blotting with the anti-phosphotyrosine antibody (Figure 2C). As we reported previously for endogenous Draper (*22*), HA-Drpr-WT in l(2)mbn cells was already phosphorylated at tyrosine residues. We found that both HA-Drpr-Y858F and HA-Drpr-Y949F underwent tyrosine phosphorylation. These results suggested that Draper is phosphorylated at tyrosine residues in both NPxY and YxxL motifs.

We then conducted similar experiments *in vivo*. Draper with Y-to-F substitution in either NPxY or YxxL were forcedly expressed in hemocytes of $drpr^{\Delta 5}$, a null mutant for *draper*, and lysates of pupae were subjected to immunoprecipitation with the anti-Draper antibody followed by Western blotting with the anti-phosphotyrosine antibody (Figure 2D). We found that either Draper protein with the substitution of the tyrosine residue was phosphorylated, suggesting again the occurrence of tyrosine phosphorylation in both NPxY and YxxL motifs.

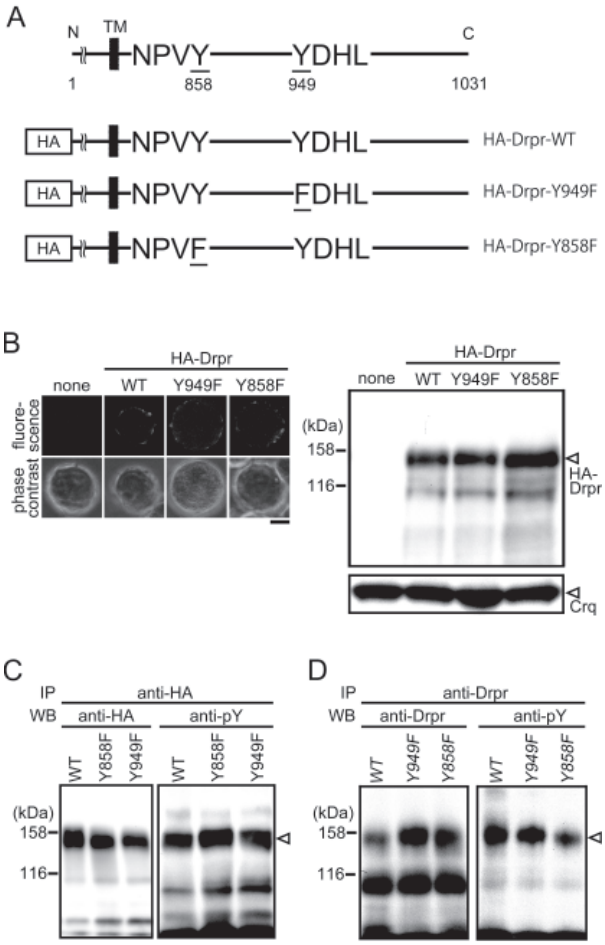


**Figure 2. Tyrosine phosphorylation of Draper with altered NPxY and YxxL motifs.** The levels of tyrosine-phosphorylated Draper proteins containing the sequences NPxY-YxxL (WT), NPxY-FxxL (Y949F), and NPxF-YxxL (Y858F) were determined. **(A)** Amino acid sequences of the three Draper proteins around NPxY and YxxL motifs are shown. The numbers indicate amino acid positions with the N-terminus as 1. **(B)** Expression of the HA-fused Draper proteins (HA-Drpr) in l(2)mbn cells was determined by immunocytochemistry (left) and Western blotting (right) using anti-HA antibody. In immunocytochemistry, phase contrast and fluorescence views of the same microscopic fields are shown. Scale bar = 5 μm. In Western blotting, the level of hemocyte-specific Croquemort (Crq) was determined for equal loading of the lysates. **(C)** Lysates of l(2)mbn cells expressing HA-Drpr were immunoprecipitated (IP) with anti-HA antibody (anti-HA) followed by Western blotting (WB) with anti-HA and anti-phosphotyrosine antibody (anti-pY). The arrowhead points to the position of HA-Drpr. **(D)** Three Draper proteins with no tags were forcedly expressed in hemocytes of $drpr^{\Delta 5}$, and lysates of pupae were immunoprecipitated with anti-Draper antibody (anti-Drpr) followed by Western blotting with anti-Drpr and anti-pY. The arrowhead points to the position of Draper proteins. Note that the intense signals below those of Draper seen in the left panel are derived from immunoglobulin. Genotypes of the fly lines analyzed are: *yw/w* (or *Y*); *UAS-drpr-WT/ srpHemoGAL4 UAS-srcEGFP*; $drpr^{\Delta 5}$ (*WT*), *UAS-drpr-Y949F/w* (or *Y*); *srpHemoGAL4 UAS-srcEGFP/+*; $drpr^{\Delta 5}$ (*Y949F*), and *UAS-drpr-Y858F/w* (or *Y*); *srpHemoGAL4 UAS-srcEGFP/+*; $drpr^{\Delta 5}$ (*Y858F*).

3.3. *Identification of tyrosine residue required for Draper-mediated phagocytosis*

We next determined the importance of the NPxY and YxxL motifs for the actions of Draper in the phagocytosis of apoptotic cells. To do so, a defect of phagocytosis due to a loss of Draper expression in *drpr*$^{\Delta 5}$ was rescued by forced expression of Draper with Y-to-F substitution at the NPxY and YxxL motifs (see Figure 2A) using a hemocyte-specific promoter. To examine the presence of Draper proteins in hemocytes of the transgenic flies, dispersed embryonic cells were immunocytochemically analyzed with the anti-Draper antibody. Most hemocytes, which were identified by the presence of green fluorescent protein (GFP), were positive for the binding of the anti-Draper antibody (Figure 3A, left panel). When lysates of those embryos were examined by Western blotting, the level of intact Draper (*WT*) was lower than those of the other two Draper proteins (Figure 3A, right panel). We then examined the extent of apoptotic cell clearance in the embryos of those flies together with various control flies. The expression of intact Draper, even the least among the three exogenous Draper proteins, sufficiently recovered the level of phagocytosis in *drpr*$^{\Delta 5}$ (Figure 3B). This was the first to genetically confirm the involvement of Draper in the phagocytosis of apoptotic cells by hemocytes. A similar result was obtained for the embryos that expressed the Draper protein with Y-to-F substitution in YxxL (*Y949F*) (Figure 3C). However, the expression of the Draper protein with Y-to-F substitution in NPxY (*Y858F*) failed to rescue the defective phagocytosis caused by a loss of endogenous Draper (Figure 3C). These results collectively indicated that the NPxY motif, but not the YxxL motif, is necessary for Draper to exert actions as a receptor for phagocytosis in hemocytes.

## 4. Discussion

Draper contains atypical EGF-like sequences and two phosphorylatable motifs, NPxY and YxxL, in the extracellular and intracellular regions, respectively. This study was undertaken to examine the importance of the EGF-like repeat for the binding to the ligand Pretaporter as well as of the NPxY and YxxL motifs for the induction of phagocytosis. The results revealed that a single EGF-like sequence is sufficient for Draper to bind Pretaporter, and that the motif NPxY, but not YxxL, is required for Draper to induce phagocytosis. In general, the NPxY motif, upon tyrosine phosphorylation, binds proteins that contain the PTB domain. In fact, we previously reported that Ced-6, a PTB domain-containing adaptor, is located downstream of Draper (*22*). Our data provide a molecular basis for the idea that Draper activates the pathway CED-6/CED-7/CED-10. The following mechanism is now presumed for Draper-mediated phagocytosis: Pretaporter binds to Draper through an EGF-like sequence; tyrosine phosphorylation of the NPxY motif is augmented; Ced-6 becomes associated with the phosphorylated NPxY motif; and signals are further transmitted to *Drosophila* orthologues of CED-7 and CED-10 leading to the induction of phagocytosis. On the other hand, Freeman and co-workers reported that the other motif YxxL (*25,30*) together with *Drosophila* components constituting the pathways CED-6/CED-7/CED-10 and CED-2/CED-5/CED-12/CED-10 (*31,32*) are all required for the Draper-mediated phagocytosis of injured axons by glia. Therefore, Draper appears to differentially use the two intracellular motifs for transmitting signals to induce the phagocytosis of apoptotic cells and injured axons. This difference in the mode of Draper's actions could be due to a difference in ligands for Draper and/

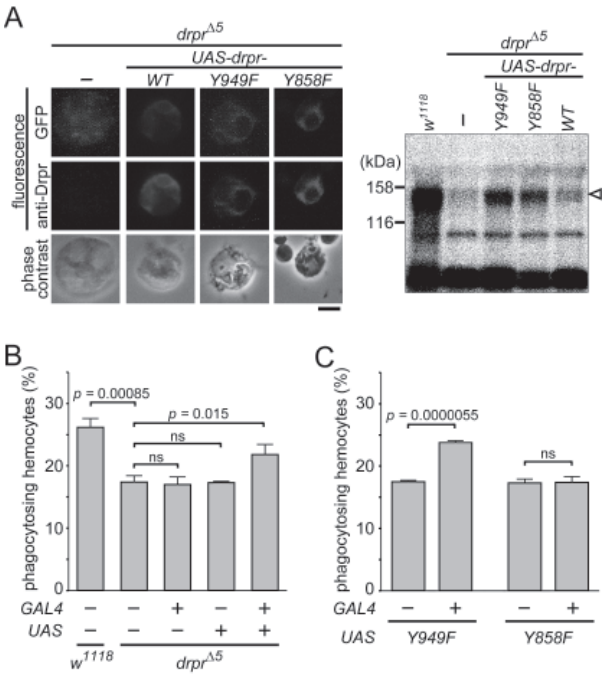


**Figure 3. Rescue of defect in apoptotic cell clearance in Draper-lacking flies by expression of Draper with altered NPxY and YxxL motifs.** Three Draper proteins containing the sequences NPxY-YxxL (WT), NPxY-FxxL (Y949F), and NPxF-YxxL (Y858F) were expressed in hemocytes of *drpr*$^{\Delta 5}$, and the level of phagocytosis was determined. **(A)** Expression of the Draper proteins was determined by immunocytochemistry (left) and Western blotting (right) using anti-Draper antibody. In immunocytochemistry, phase contrast and fluorescence views of the same microscopic fields that contain GFP-expressing hemocytes are shown. Scale bar = 5 μm. In Western blotting, lysates of whole embryos were analyzed. **(B)** Dispersed embryonic cells obtained from the indicated flies were subjected to an assay for phagocytosis. The fly line *w*$^{1118}$ was used as a *draper*$^{+}$ control. ns, not significant. Genotype of the fly line (*GAL4+* and *UAS+* with *drpr*$^{\Delta 5}$) is *yw/w* (or *Y*); *UAS-drpr-WT/srpHemoGAL4 UAS-srcEGFP*; *drpr*$^{\Delta 5}$. **(C)** The level of phagocytosis was determined as in **(B)**. Genotypes of the fly lines analyzed are: *UAS-drpr-Y949F/w* (or *Y*); *srpHemoGAL4 UAS-srcEGFP/+*; *drpr*$^{\Delta 5}$ (*GAL4+* with *Y949F*) and *UAS-drpr-Y858F/w* (or *Y*); *srpHemoGAL4 UAS-srcEGFP/+*; *drpr*$^{\Delta 5}$ (*GAL4+* with *Y858F*).

or types of phagocytes. It is necessary to further clarify the mechanism by which Draper transmits signals to downstream molecules, particularly focusing on the roles of the two phosphorylatable motifs located in the cytoplasmic portion of this receptor.

Integrin β-subunits contain the motif NPxY in their cytoplasmic region, which serves as a binding site for various adaptor molecules possessing the PTB domain (*33*). Mammalian $\alpha_V\beta_3$ and $\alpha_V\beta_5$ (*34*), *Drosophila* αPS3βυ ((*29*) and our unpublished observation), and *C. elegans* INA-1-PAT-3 (*10*) act as receptors in the phagocytosis of apoptotic cells. However, $\alpha_V\beta_5$ (*35*) and INA-1-PAT-3 (*10*) seem to reside at the point furthest upstream in the pathway CED-2/CED-5/CED-12/CED-10, not CED-6/CED-7/CED-10. In addition, the NPxY motif contained in $\beta_5$ integrin was shown to be dispensable for $\alpha_V\beta_5$-mediated phagocytosis (*36*). In contrast, mammalian stabilin-2, a phosphatidylserine-binding engulfment receptor, uses its NPxY motif to recruit the mammalian orthologue of CED-6 (*37*). Interestingly, stabilin-2 may also activate the CED-2/CED-5/CED-12/CED-10 pathway through physical association with $\alpha_V\beta_5$ (*38*). Therefore, which of the two conserved signaling pathways is used by NPxY motif-containing engulfment receptors seemingly depends on the ligand-receptor combination and/or the receptor repertoire in phagocytes.

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