Integrin αPS3/βν-Mediated Phagocytosis of Apoptotic Cells and Bacteria in *Drosophila**

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Running title: Integrin αPS3/βν-Mediated Phagocytosis in *Drosophila*

Background: *Drosophila* integrin βν plays a role in the phagocytosis of apoptotic cells and bacteria, but its partner α-subunit remains to be identified.
Results: Of 5 α-subunits, αPS3 was physically and functionally associated with βν.
Conclusion: αPS3/βν serves as a receptor for phagocytosis in *Drosophila*.
Significance: The heterodimeric structure of *Drosophila* integrin has been genetically and biochemically solved.

SUMMARY

Integrins exert a variety of cellular functions as heterodimers of two transmembrane subunits named α and β. Integrin βν, a β-subunit of *Drosophila* integrin, is involved in the phagocytosis of apoptotic cells and bacteria. We here searched for a α-subunit that forms a complex and cooperates with βν. Examinations of RNAi-treated animals suggested that αPS3, but not any of four other α-subunits, is required for the effective phagocytosis of apoptotic cells in *Drosophila* embryos. The mutation of αPS3-encoding *scb*, deficiency, insertion of P-element, or alteration of nucleotide sequences, brought about a reduction in the level of phagocytosis. The defect in phagocytosis by deficiency was reverted by the forced expression of *scb*. Furthermore, flies in which the expression of both αPS3 and βν was inhibited by RNAi showed a level of phagocytosis almost equal to that observed in flies with RNAi for either subunit alone. A loss of αPS3 also decreased the activity of larval hemocytes in the phagocytosis of *Staphylococcus aureus*. Finally, a co-immunoprecipitation analysis using a *Drosophila* cell line treated with a chemical cross-linker suggested a physical association between αPS3 and βν. These results collectively indicated that integrin αPS3/βν serves as a receptor in the phagocytosis of apoptotic cells and bacteria by *Drosophila* phagocytes.

Phagocytosis plays an important role in the maintenance of homeostasis by eliminating materials foreign to host organisms (1, 2). Typical targets for phagocytic cells are invading microorganisms and altered own cells that have become unnecessary for or harmful to the host (1, 2). The elimination of pathogenic microorganisms helps host organisms avoid infectious diseases, and that of apoptotic cells is prerequisite to the morphogenesis in early development and the maintenance of tissue homeostasis (3, 4).

Most altered own cells are induced to undergo apoptosis and express substances, often called eat-me signals, at their surface, which are recognized by engulfment receptors of phagocytic cells (5–8). There are two partly overlapping signaling pathways for the induction of phagocytosis in *Caenorhabditis elegans* (9–12), namely, CED-6/CED-10 and CED-2/CED-5/CED-12/CED-10, which are most likely activated by the engulfment receptors CED-1 (13) and INA-1 (14), respectively. CED-1 is a single-path membrane protein containing atypical EGF-like repeats in its extracellular region (13), and INA-1 is a α-subunit of *C. elegans* integrins (14). CED-1 (15), integrins (16), and molecules that constitute the two signaling pathways (12, 17) seem to be evolutionally conserved among species including humans. This suggests the phylogenetic conservation of the mode of apoptotic cell clearance although the conservation of eat-me signals is yet to be determined.

Integrins are phylogenetically conserved transmembrane receptors consisting of heterodimers of two subunits called α and β (18, 19). Eighteen α-subunits and 8 β-subunits exist in mammals and form heterodimers giving rise to 24 different integrins (18, 19). Integrins play important roles in a variety of biological phenomena by mediating cell-cell adhesion. In addition, integrins connect the extracellular matrix with the cytoskeleton and activate intracellular signaling pathways (18–20). Integrins are capable of inducing phagocytosis probably due to their ability to remodel the cytoskeleton, and targets for integrin-mediated phagocytosis include apoptotic cells and microorganisms (21, 22). This

mechanism of action is sometimes exploited by microorganisms to gain entry into host cells (22). We recently identified integrin βν, a β-subunit of *Drosophila* integrins, as a receptor involved in the phagocytosis of apoptotic cells in *Drosophila* embryos (23). This subunit also induces the phagocytosis of *Staphylococcus aureus* by *Drosophila* hemocytes, recognizing peptidoglycan of this bacterium (24). There are five α-subunits, αPS1, 2, 3, 4 and 5, and two β-subunits, βPS and βν, for *Drosophila* integrins (16, 25). The present study was carried out aiming at the identification of a α-subunit that cooperates with βν in the phagocytosis of apoptotic cells and bacteria.

EXPERIMENTAL PROCEDURES

Fly Stocks, Bacterial Strains, and Cell Culture—The following lines of *Drosophila* were used: w^{1118}, *Oregon R* (Kyorin-Fly, Kyorin University Tokyo, Japan), $betaInt\text{-}nu^{2}$ (26), *UAS-betaInt-nu-IR* (National Institute of Genetics, Shizuoka, Japan), *UAS-mew-IR* (National Institute of Genetics), *UAS-if-IR* (National Institute of Genetics), *UAS-scb-IR* (National Institute of Genetics), *UAS-alphaPS4-IR* (National Institute of Genetics), *UAS-alphaPS5-IR* (National Institute of Genetics), *Df(2R)Exel7135* (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN), scb^{01288} (Bloomington Drosophila Stock Center), scb^{2} (27) (Drosophila Genetic Resource Center, Kyoto, Japan), *da-Gal4* (a gift from S. Hayashi), *srpHemoGAL4 UAS-srcEGFP* (28), *201Y-GAL4 UAS-GFP.S65T(T2)* (a gift from T. Awasaki), and *pxn-GAL4 8.1* (a gift from M. J. Galko). To establish a fly line for the expression of αPS3 isoforms A and B in a *scb* mutant, cDNA coding for αPS3A or αPS3B was prepared from RNA of w^{1118}, inserted into the vector pUAST (29), and used to generate a transgenic fly line with the background of w^{1118}. The resulting fly lines carrying the transgenes on the 3rd chromosome were intercrossed with the fly line *Df(2R)Exel7135* and used for the mating with *da-Gal4*. Other fly lines used in this study were generated through the mating of existing flies, and some lines were used after changing balancers. Genotypes of the fly lines analyzed are shown in the corresponding figure legends. The wild-type *S. aureus* strain RN4220 was cultured at 30 ˚C with Luria-Bertani medium. Bacteria were harvested at full growth, washed with PBS, and used in an assay for phagocytosis. The cell line l(2)mbn, established from larval hemocytes, was maintained at 25 ˚C with Schneider's Drosophila medium (Invitrogen), as described previously (30).

Antibodies—The anti-integrin αPS3 antibody was raised by immunizing rats with an extracellular region of integrin αPS3, corresponding to the amino acid positions 235–284 with the amino terminus numbered 1, that had been expressed in *E. coli* as a protein fused to GST and purified to homogeneity. Generation and use of the anti-integrin βν (23), anti-Croquemort (30), and anti-Ced-6 (31) rat antibodies were reported previously. The anti-αPS3 (32) and anti-βν (33) rabbit antibodies were provided by S. Hayashi and R. O. Hynes, respectively. Antigen specificity of the anti-αPS3 rabbit antibody (supplemental Fig. S1) and the anti-αPS3 rat antibody (supplemental Fig. S2) was confirmed in Western blotting.

Chemical Cross-linking and Co-immunoprecipitation—To examine the physical association of αPS3 and βν, l(2)mbn cells were transfected with cDNA coding for the isoform B of αPS3 and βν by lipofection (Cellfectin II; Invitrogen). The cells ($5\sim7 \times 10^{7}$) were then incubated with Sulfo-NHS-SS-Diazirine (Thermo Fisher Scientific Inc., Rockford, IL) (3 mM), an amine- and photo-reactive chemical cross-linker containing a disulfide bond for cleavage, for 10 min at room temperature, supplemented with Tris-HCl (pH 8.0) at 0.17 M, and centrifuged. The resulting cell pellets were washed 3 times with PBS, re-suspended with PBS, and exposed to UV using a fluorescent lamp for 15 min at 4 ˚C. The cells were collected by centrifugation, lysed with a buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton-X 100, and protease inhibitors (Nakalai Tesque, Kyoto, Japan), and immunoprecipitated with the rat antibody (anti-αPS3 or anti-βν). The precipitates were separated on a 6% SDS-polyacrylamide gel and subjected to Western blotting with the rabbit antibody (anti-αPS3 or anti-βν). The membrane containing the transferred proteins was first reacted with either the anti-αPS3 or anti-βν antibody followed by signal detection, washed with an alkaline solution to remove the bound antibody, and then re-probed with the other antibody.

Other Methods—The level of phagocytosis of apoptotic cells was cytochemically determined with dispersed embryonic cells, as described in our previous paper (23). The ratio of Croquemort-expressing hemocytes containing TUNEL-stained apoptotic cells was determined and exhibited as "phagocytosing hemocytes." An assay for the phagocytosis of *S. aureus in vitro* was carried out using hemocytes prepared from wandering larvae as phagocytes and the *S. aureus*

strain RN4220 surface-labeled with FITC as targets, as described previously (34). The ratio of hemocytes containing target bacteria and the number of bacteria contained in 100 hemocytes were determined and exhibited as "phagocytosing hemocytes" and "engulfed bacteria," respectively. Western blotting of lysates of cultured cells (30) and flies (23) was done essentially as described previously, except that: cultured cells were lysed by detergent without sonication as shown above; membranes containing separated proteins were incubated with antibodies in either a buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% (v/v) Tween-20 and 5% (w/v) dry skim milk or MaxBlot Solution (Medical & Biological Laboratories, Nagoya, Japan); and signals were visualized by a colorimetric reaction using Western Lightning (Perkin Elmer) or ECL Select Western Blotting Detection Reagent (GE Healthcare). Immunocytochemistry of dispersed embryonic cells was conducted as described before (23).

Data Processing and Statistical Analysis—Results from quantitative analyses are expressed as the mean ± S. D. of the data from at least three independent experiments, unless otherwise stated in the text. Other data are 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.

RESULTS

Identification of αPS3 as Integrin Subunit Involved in Phagocytosis of Apoptotic Cells—There are five α-subunits, namely, αPS1, 2, 3, 4 and 5, for *Drosophila* integrins (16). To determine which α-subunit is involved in the phagocytosis of apoptotic cells in *Drosophila*, we conducted RNAi-mediated knockdown of genes coding for the five α-subunits. Dispersed cells of embryos at stage 16, in which RNA with inverted repeats corresponding to mRNA of the target genes was specifically expressed in hemocytes, were analyzed to determine the level of phagocytosis. We found that RNAi of the αPS3-encoding *scb* and the αPS4-encoding *alphaPS4*, but not genes coding for the other three α-subunits, brought about a reduction of phagocytosis by embryonic hemocytes (Fig. 1*A*). We next examined the effect of the knockdown of these two α-subunit-encoding genes in cells other than hemocytes. For this purpose, we induced RNAi in γ neurons of the mushroom body and determined the level of phagocytosis by embryonic hemocytes. The results showed that RNAi of *alphaPS4* in neurons was also inhibitory to phagocytosis while this was not the case for *scb* (Fig. 1*B*), suggesting that inhibition of phagocytosis with RNAi of *alphaPS4* in either hemocytes or neurons is due to an artifact or a secondary effect of a loss of αPS4 in those cell types. In contrast, αPS3 seemed to be directly involved in the phagocytic action of hemocytes. We thus decided to further investigate the role of αPS3 in the phagocytosis of apoptotic cells.

We next examined the effect of mutations in *scb* on apoptotic cell clearance. Lysates were prepared from embryos of fly lines having a deletion of a chromosomal region including *scb* (*Df(2R)Exel7135*), an insertion of P-element within *scb* (scb^{01288}), or an alteration of nucleotide sequences within a coding region of αPS3 (scb^{2}), which was caused by treatment with ethylmethanesulfonate, and analyzed by Western blotting for the level of αPS3 using the anti-αPS3 rabbit antibody. The lysates of flies with the deficiency or P-element insertion showed a reduced level of αPS3 while αPS3 with altered amino acid sequences seemed to be produced much more than the canonical protein (Fig. 1*C*, *left panel* and supplemental Fig. S3). When embryos of these mutant flies were analyzed, they all showed a reduction in the level of phagocytosis compared to those of *Oregon R*, a wild-type fly line (Fig. 1*C*, *right panel*). There are two subtypes for αPS3, called isoforms A and B, which differ in the N-terminal 63 amino-acid residues of 1,115 residues in total (35, 36). They are derived from two different mRNA transcribed with two distinct start sites on the same *scb* gene (36). These two isoforms cannot be distinguished from each other in SDS-PAGE and are recognized by the anti-αPS3 antibodies used in this study. To determine which of the two isoforms is required for phagocytosis, we forcedly expressed each protein in the *scb* mutant with deficiency. We found that the expression of either isoform sufficiently restored phagocytosis in embryos of the mutant fly line (Fig. 1*D*). These results also confirmed that αPS4 is not involved in the phagocytosis of apoptotic cells by hemocytes because the chromosomal region lost in this mutant line, *Df(2R)Exel7135*, includes *alphaPS4* as well. The results described above collectively indicated that αPS3 is required for hemocytes to achieve effective phagocytosis of apoptotic cells, and suggested that the αPS3 isoforms A and B produced by two different transcripts from the same gene are redundant for this function of hemocytes.

We next determined the expression pattern of αPS3 during the development of *Drosophila*.

Lysates of wild-type flies at various developmental stages were prepared and analyzed by Western blotting using the anti-αPS3 rabbit antibody and the anti-βν rat antibody. We found that the level of αPS3 varied depending on developmental stage, being highest in pupae and lowest in larvae (Fig. 2*A*, *top panel*), whereas βν was most abundant in embryos and least abundant in pupae (Fig. 2*A*, *bottom panel*), as reported previously (23). The surface expression of αPS3 was then examined with embryonic hemocytes. Dispersed cells of embryos were subjected to immunocytochemistry under conditions without membrane permeabilization using the anti-αPS3 rat antibody, and cells containing GFP, indicative of hemocytes, were analyzed for the signal derived from αPS3. Punctate signals were detected in GFP-positive cells while no such signals were seen with control antibody (Fig. 2*B*). The pattern of distribution of αPS3 was quite similar to that of βν (23). These results indicated that αPS3 as wells as βν is present at the surface of embryonic hemocytes.

Functional Association of αPS3 and βν in Phagocytosis of Apoptotic Cells and Bacteria—We next examined the functional interaction between αPS3 and βν in the phagocytosis of apoptotic cells. The level of phagocytosis with flies that had been subjected to RNAi of both *scb* and *betaInt-nu*, which codes for βν, was determined and compared with that in flies subjected to RNAi of either one of the two genes. We found that phagocytosis of apoptotic cells occurred almost equally in embryos of the three fly lines analyzed (Fig. 3*A*), suggesting that αPS3 and βν function in the same pathway for the induction of phagocytosis.

We previously reported that βν is involved in the phagocytosis of *S. aureus* by hemocytes (24). We therefore examined the participation of αPS3 in the βν-mediated phagocytosis of this bacterium. Flies were subjected to RNAi of *scb*, and hemocytes prepared from third-instar larvae were tested for phagocytic activity in an assay *in vitro*. The data indicated that a loss of αPS3 brought about a reduction of phagocytosis (Fig. 3*B*). We next examined the relationship between αPS3 and βν in the phagocytosis of *S. aureus*, as done for the phagocytosis of apoptotic cells. We found that hemocytes prepared from flies, which had been subjected to RNAi of both *scb* and *betaInt-nu*, possessed an activity almost equal to that of hemocytes with knockdown of either subunit alone (Fig. 3*C*). These results indicated that αPS3 is also required for hemocytes to effectively engulf *S. aureus*, and suggested cooperation between αPS3 and βν.

Physical Association of αPS3 and βν—We next tried to observe a physical association between αPS3 and βν in l(2)mbn cells, a cell line established from larval hemocytes. The cells were treated with a membrane-impermeable cross-linker to covalently combine substances existing at the cell surface in close proximity to each other. Whole-cell lysates were prepared, treated with a buffer for SDS-PAGE in the presence and absence of 2-mercaptoethanol, and analyzed by Western blotting. The treatment with the cross-linker did not affect the migration of αPS3 when the lysates were separated after incubation with 2-mercaptoethanol (Fig. 4*A*, *left panel*). However, the same lysates showed a smear-like additional signal with a slower migration when incubated in the absence of 2-mercaptoethanol (Fig. 4*A*, *left panel*). This was almost the same for βν except that incubation with 2-mercaptoethanol seemed to cause a reduction of migration (Fig. 4*A*, *middle panel*), suggesting the presence of an intramolecular disulfide bond. In contrast, no additional signal was observed after cross-linking for Ced-6, a signaling molecule located inside cells, examined as a negative control (Fig. 4*A*, *right panel*). These results suggested the occurrence of a structural change for αPS3 and βν, but not for Ced-6, after the treatment of cells with the cross-linker. Next, l(2)mbn cells were transfected with plasmid vectors for the overexpression of both αPS3 and βν, treated with the cross-linker, and lysed. The lysates were then immunoprecipitated with the anti-αPS3 rat antibody or control rat serum, and the resulting precipitates were separated by SDS-PAGE after incubation with a buffer containing 2-mercaptoethanol followed by Western blotting with the anti-αPS3 and anti-βν rabbit antibodies. We found signals corresponding to αPS3 and βν in the immunoprecipitates obtained with the anti-αPS3 antibody but not with the control serum (Fig. 4*B*, *left two panels*). Similar results were observed in the reverse experiment, that is, immunoprecipitation with the anti-βν antibody and Western blotting with the anti-αPS3 antibody (Fig. 4*B*, *right two panels*). When immunoprecipitates with the anti-αPS3 antibody were analyzed for the presence of βν in Western blotting without treatment for reduction, the signal observed in the experiment with reduction disappeared, and instead signals of different migration that resembled the pattern without immunoprecipitation (see Fig. 4*A*, *middle panel*) became detectable (Fig. 4*C*). These results indicated that βν and αPS3 were co-immunoprecipitated depending on the treatment of cells with the cross-linker. The above results collectively suggested that αPS3 and βν are

physically associated with each other at the surface of l(2)mbn cells.

Taken together, it is most likely that the two integrin subunits αPS3 and βν form a complex to serve as a receptor for the phagocytosis of apoptotic cells and bacteria by *Drosophila* hemocytes.

DISCUSSION

In the present study, we adopted both genetic and biochemical approaches aiming at the identification of a α-subunit that cooperates with the β-subunit βν in the phagocytosis by *Drosophila* hemocytes. We successfully identified αPS3 as a partner of βν and showed that αPS3/βν serves as an engulfment receptor responsible for the phagocytic elimination of apoptotic cells and *S. aureus* in *Drosophila*. Cooperation between αPS3 and βν was previously suggested for midgut migration in embryos (26) as well as for synaptic morphogenesis at neuromuscular junctions in third instar larvae (37). This indicates that integrin αPS3/βν plays roles in various biological events in *Drosophila*.

There are two isoforms for αPS3, namely, αPS3A and αPS3B, which are produced through alternative transcription initiation on the same gene (35, 36). The expression of the two αPS3 mRNA is seemingly under both spatial and temporal regulation: αPS3A mRNA is predominantly present in the head while αPS3B mRNA is in both head and body tissues (36); and αPS3A mRNA is abundant in embryos, pupae, and adults while αPS3B mRNA is effectively produced in larvae, pupae, and adults (35). Such a spatiotemporal control of *scb* expression makes us assume that the two isoforms function differently, but no evidence for this has been provided so far. Our data indicated that αPS3A and αPS3B are equivalent in hemocytes to rescue a defect of phagocytosis caused by a mutation in *scb*, but the predominant expression of αPS3A mRNA at the embryonic stage suggests that this isoform of αPS3 plays the role of a partner for βν to serve as a receptor for the phagocytosis of apoptotic cells in embryonic hemocytes. On the other hand, αPS3B/βν is likely responsible for the phagocytosis of *S. aureus* by larval hemocytes.

In *C. elegans*, a α-subunit named INA-1 is required for the phagocytosis of apoptotic cells by embryonic epithelial cells (14). There exists only one β-subunit, PAT-3, for *C. elegans* integrins. INA-1 appears to form a complex with PAT-3 in embryos (38), and functional cooperation between these two integrin subunits has been suggested (14). It is thus likely that INA-1/PAT-3 plays a role as an engulfment receptor to remove apoptotic cells during embryogenesis. More recently, the other α-subunit of *C. elegans* integrins, PAT-2, was shown to be responsible for the phagocytic removal of apoptotic cells by muscle cells in embryos (39). PAT-2 too likely forms a heterodimer with PAT-3 to act as a receptor for phagocytosis.

It is most probable that CED-1 (and its orthologue) and integrins are the receptors that govern two partly overlapping signaling pathways for the induction of phagocytosis of apoptotic cells in *C. elegans* and *Drosophila* although a redundancy of receptors for each pathway cannot be excluded. CED-1 (40, 41) and Draper (42, 43), a *Drosophila* counterpart of CED-1, appear to bind proteins in the recognition of apoptotic cells by phagocytes. Draper also recognizes lipoteichoic acid, a cell wall component, as a ligand in the phagocytosis of *S. aureus* by hemocytes (34), suggesting a multiplicity of ligands for this receptor. In contrast, ligands for integrins in the recognition of apoptotic cells by phagocytes of *C. elegans* and *Drosophila* are yet to be identified. In mammals, integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are known to act as engulfment receptors in apoptotic cell clearance (21). Both integrins recognize the amino acid sequence Arg-Gly-Asp or the RGD motif, most probably owing to the action of α_V. In fact, integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ use a RGD motif-containing protein called milk fat globule EGF-factor 8 as a ligand, which at the same time binds phosphatidylserine to connect apoptotic cells and phagocytic cells (44). The alignment of amino acid sequences, however, reveals that αPS3 of *Drosophila* is not similar to human α_V (16), and that *Drosophila* βν does not resemble human β_3 or β_5 (16). This suggests that the *Drosophila* integrin αPS3/βν does not require the RGD motif for the recognition of ligand molecules. In fact, we found that βν binds peptidoglycan in the phagocytosis of *S. aureus* (24). Similarly, *C. elegans* INA-1 does not share much structural similarity with RGD motif-binding human integrins. In addition, there found no appreciable similarity in the primary structure between αPS3 and INA-1 / PAT-2, and βν and PAT-3. A molecular basis for the recognition of apoptotic cells by αPS3/βν, INA-1/PAT-3, and PAT-2/PAT-3 remains to be clarified. Despite an evolutionally conserved role of integrins as an engulfment receptor in the elimination of cells unnecessary for host organisms, their manner of action appears to differ between vertebrates and invertebrates.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. **Identification of αPS3-endocing *scb* required for phagocytosis of apoptotic cells in *Drosophila* embryos.** Dispersed cells of embryos of various fly lines were analyzed for the level of phagocytosis of apoptotic cells by hemocytes. *A*, embryos of flies that had been subjected to hemocyte-specific RNAi, using a GAL4 driver with the promoter of *srp*, of genes coding for five α-subunits, *mew* (αPS1), *if* (αPS2), *scb* (αPS3), *alphaPS4* (αPS4), and *alphaPS5* (αPS5), were analyzed. *B*, embryos of flies that had been subjected to RNAi of *scb* and *alphaPS4* specifically in mushroom body γ neurons (using the enhancer-trap line *201Y-GAL4*) or hemocytes were analyzed. *n.s.*, not significant. *C*, embryos of flies having a deletion of a chromosomal region including *scb* (*Df(2R)Exel7135*), an insertion of P-element within *scb* (scb^{01288}), and an alteration of amino acid sequences of αPS3 (scb^{2}) were analyzed for the level of phagocytosis (*right*) as well as for the level of αPS3 and Croquemort (*left*), an engulfment receptor of hemocytes (45, 46) analyzed as a positive control. In the *left* panel, lysates of embryos were separated on a SDS-polyacrylamide gel (0.15 mg of protein on a 5.5% gel for the analysis of αPS3, and 0.02 mg of protein on a 10% gel for Croquemort) followed by Western blotting using the anti-αPS3 rabbit antibody or anti-Croquemort rat antibody. Portions of the data containing signals corresponding to αPS3 and Croquemort are exhibited (full-length blots are shown as supplemental Fig. S2). *D*, embryos of *Df(2R)Exel7135* flies that forcedly express the isoform A (*scbA*) or B (*scbB*) of αPS3 in whole bodies (using a GAL4 driver with *da* promoter) were analyzed. Genotypes of the fly lines analyzed are: *srpHemoGAL4 UAS-srcEGFP/+* (*UAS-IR* – in *A*), *srpHemoGAL4 UAS-srcEGFP/UAS-mew-IR* (*UAS-IR mew* in *A*), *srpHemoGAL4 UAS-srcEGFP/+; UAS-if-IR/+* (*UAS-IR if* in *A*), *srpHemoGAL4 UAS-srcEGFP/UAS-scb-IR* (*UAS-IR scb* in *A*, *UAS-IR scb* with *GAL4 srp* in *B*), *srpHemoGAL4 UAS-srcEGFP/+; UAS-alphaPS4-IR/+* (*UAS-IR alphaPS4* in *A*, *UAS-IR alphaPS4* with *GAL4 srp* in *B*), *srpHemoGAL4 UAS-srcEGFP/+; UAS-alphaPS5-IR/+* (*UAS-IR alphaPS5* in *A*), *201Y-GAL4 UAS-GFP.S65T(T2)/UAS-scb-IR* (*UAS-IR scb* with *GAL4 201Y* in *B*), *201Y-GAL4 UAS-GFP.S65T(T2)/+; UAS-alphaPS4-IR/+* (*UAS-IR alphaPS4* with *GAL4 201Y* in *B*), cn^{1} scb^{01288} (scb^{01288} in *C*), cn^{1} scb^{2} bw^{1} sp^{1} (scb^{2} in *C*), *Df(2R)Exel7135; da-Gal4/+* (*Df(2R)Exel7135* with *UAS* – and *GAL4* + in *D*), *Df(2R)Exel7135; da-Gal4/UAS-scbA* (*Df(2R)Exel7135* with *UAS scbA* and *GAL4* + in *D*), and *Df(2R)Exel7135; da-Gal4/UAS-scbB* (*Df(2R)Exel7135* with *UAS scbB* and *GAL4* + in *D*).

FIGURE 2. **Expression profile of αPS3.** *A*, lysates (0.1~0.14 mg of protein) of scb^{+} flies at the indicated developmental stages were analyzed by Western blotting using the anti-αPS3 rabbit antibody or the anti-βν rat antibody. The arrowheads indicate the positions of αPS3 and βν. The positions of markers with molecular masses in kDa are shown on the left. *B*, dispersed cells of stage-16 embryos of s*rpHemoGAL4 UAS-srcEGFP* flies were immunocytochemically analyzed under membrane-unpermeabilized conditions for the presence of αPS3 using the anti-αPS3 rat antibody. As a negative control, the cells were similarly analyzed with an antibody that recognizes an intracellular region of βν (*control antibody*). Phase contrast and fluorescence views as well as overlays of the same microscopic fields that contain GFP-expressing hemocytes are shown as vertically aligned panels. The arrowheads denote positive signals. *Scale bar*, 10 μm.

FIGURE 3. **Functional interaction of αPS3 with βν in phagocytosis of apoptotic cells and bacteria.** *A*, embryos of flies that had been subjected to hemocyte-specific RNAi, using a GAL4 driver with *srp* promoter, of genes coding for αPS3 (*scb*), βν (*betaInt-nu*), or both αPS3 and βν were analyzed for the level of phagocytosis of apoptotic cells. *B*, hemocytes prepared from 3rd-instar larvae of flies that had been subjected to hemocyte-specific RNAi, using a GAL4 driver with *pxn* promoter, of the gene coding for αPS3 were used in an assay for phagocytosis *in vitro* with FITC-labeled *S. aureus* as targets. *C*, the phagocytosis of *S. aureus* was analyzed as in *B* using flies that had been subjected to hemocyte-specific RNAi (with *pxn-GAL4* driver) of genes coding for αPS3, βν, or both αPS3 and βν. *n.s.*, not significant. Genotypes of the fly lines analyzed are: *srpHemoGAL4 UAS-srcEGFP/+* (*UAS-IR –*), *srpHemoGAL4 UAS-srcEGFP/UAS-scb-IR* (*UAS-IR scb*), *srpHemoGAL4 UAS-srcEGFP/+; UAS-betaInt-nu-IR/+* (*UAS-IR betaInt-nu*), and *srpHemoGAL4 UAS-srcEGFP/UAS-scb-IR; UAS-betaInt-nu-IR/+* (*UAS-IR betaInt-nu scb*).

FIGURE 4. **Physical association between αPS3 and βν.** *A*, l(2)mbn cells were transfected with cDNA coding for αPS3B (for the analysis of αPS3) or left untransfected (for the analysis of βν and Ced-6). The cells were reacted or not reacted with a membrane unpermeable, SH reagent-cleaved chemical cross-linker (*CL*), and whole-cell lysates were prepared. The lysates (0.08 mg of protein) were treated with a buffer containing or not containing 2-mercaptoethanol (*2-ME*), separated by SDS-PAGE (5.5% gel for the analysis of αPS3, 6% gel for βν, and 10% gel for Ced-6), and subjected to Western blotting with the anti-αPS3, anti-βν, and anti-Ced-6 rat antibodies. The closed arrowheads indicate the positions of canonical proteins, the arrows show the positions of αPS3 and βν that were retarded on the gel after cross-linking, and the open arrowhead points to βν that migrated differently without 2-mercaptoethanol treatment. The positions of markers with molecular masses in kDa are shown on the left. *B*, l(2)mbn cells were transfected with cDNA coding for αPS3B and βν, reacted with the cross-linker, and lysed. Whole-cell lysates (2~2.4 mg of protein) were immunoprecipitated (*IP*) with the anti-αPS3 rat antibody (*left two panels*), the anti-βν rat antibody (*right panel*), or control normal rat serum, and the precipitated materials were treated with 2-mercaptoethanol, separated on a 6% SDS-polyacrylamide gel, and analyzed by Western blotting (*WB*) using the anti-αPS3 and anti-βν rabbit antibodies. The arrowheads point to the positive signals. *C*, immunoprecipitates of l(2)mbn cell lysates with the anti-αPS3 rat antibody were prepared as in *B* and analyzed by Western blotting using the anti-βν rabbit antibody with and without 2-mercaptoethanol treatment. The symbols are the same as those used in *A* (*middle panel*).

Fig.1

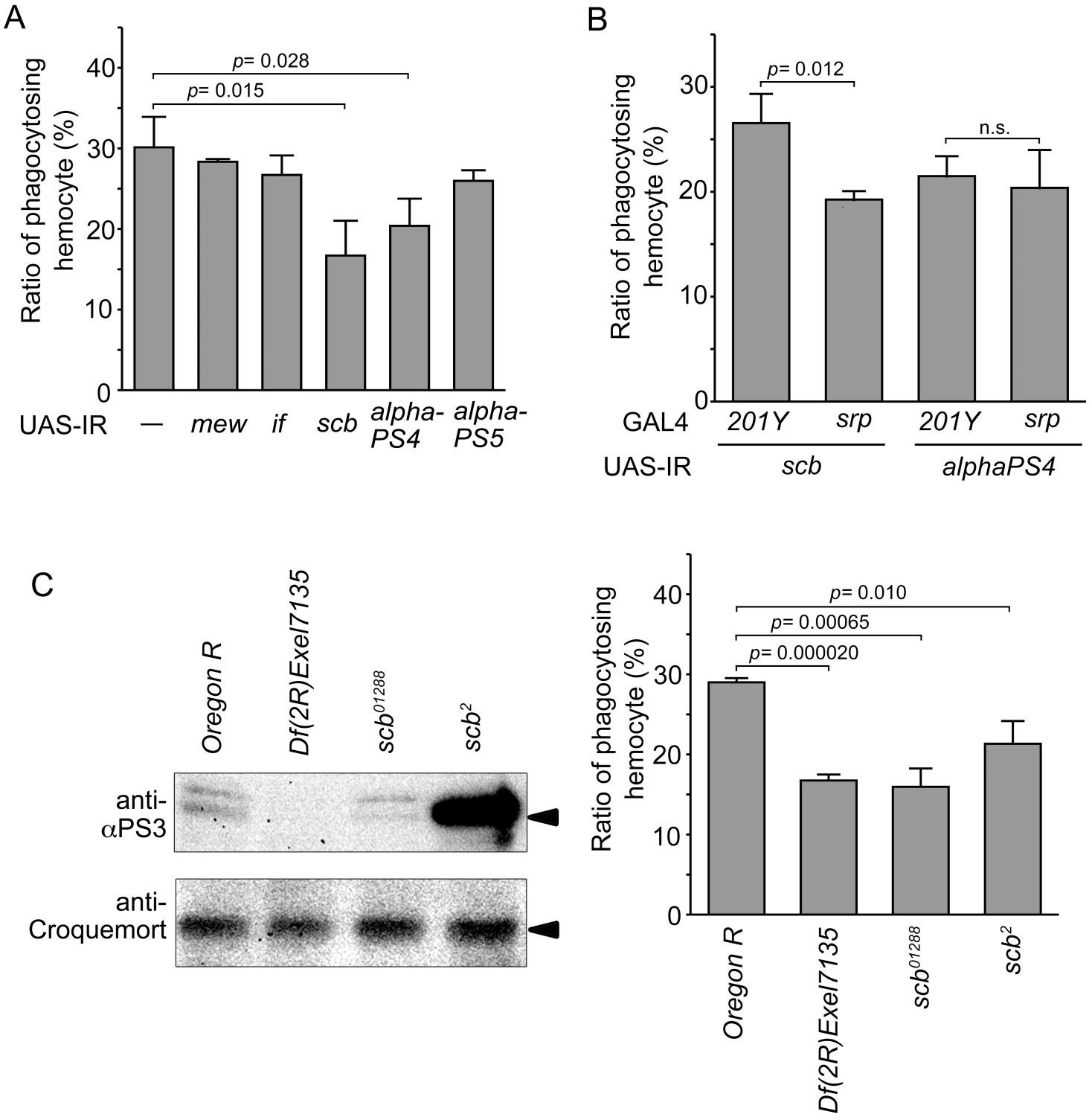
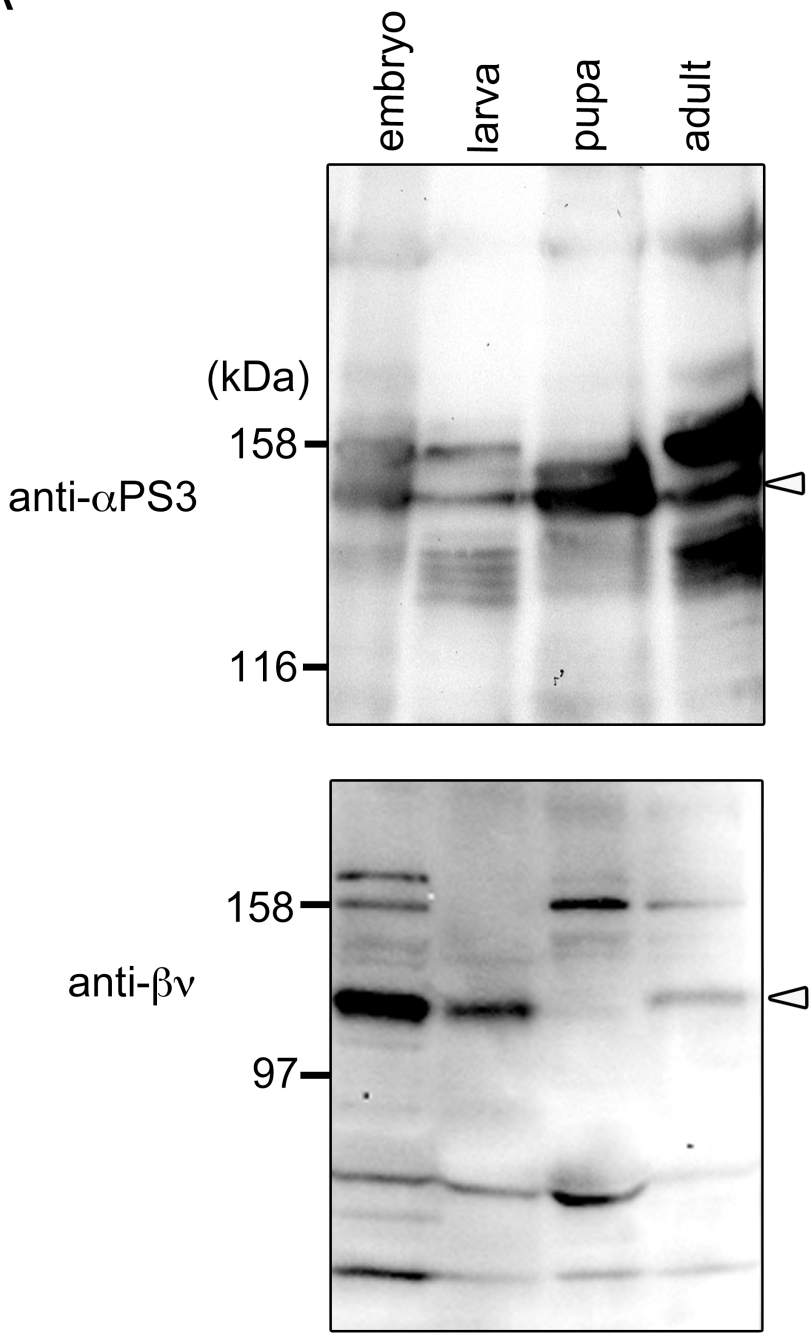

A
Ratio of phagocytosing hemocyte (%)
p= 0.028
p= 0.015
UAS-IR
—
mew
if
scb
alpha-PS4
alpha-PS5
B
Ratio of phagocytosing hemocyte (%)
p= 0.012
n.s.
GAL4
201Y
srp
201Y
srp
UAS-IR
scb
alphaPS4
C
Oregon R
Df(2R)Exel7135
scb01288
scb2
anti-αPS3
anti-Croquemort
Ratio of phagocytosing hemocyte (%)
p= 0.010
p= 0.00065
p= 0.000020
Oregon R
Df(2R)Exel7135
scb01288
scb2
D
Ratio of phagocytosing hemocyte (%)
p= 0.037
p= 0.0047
p= 0.0095
p= 0.00079
GAL4
UAS
scbA
scbB
Oregon R
Df(2R)Exel7135

Fig.2

A


embryo
larva
pupa
adult
(kDa)
158
anti-αPS3
116
158
anti-βν
97

B

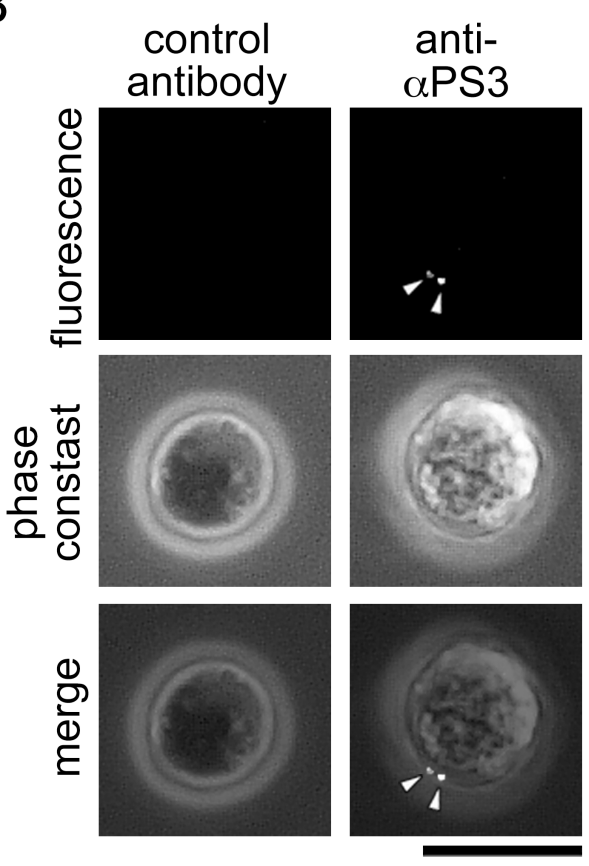

control antibody
anti-αPS3
fluorescence
phase constast
merge

Fig.3

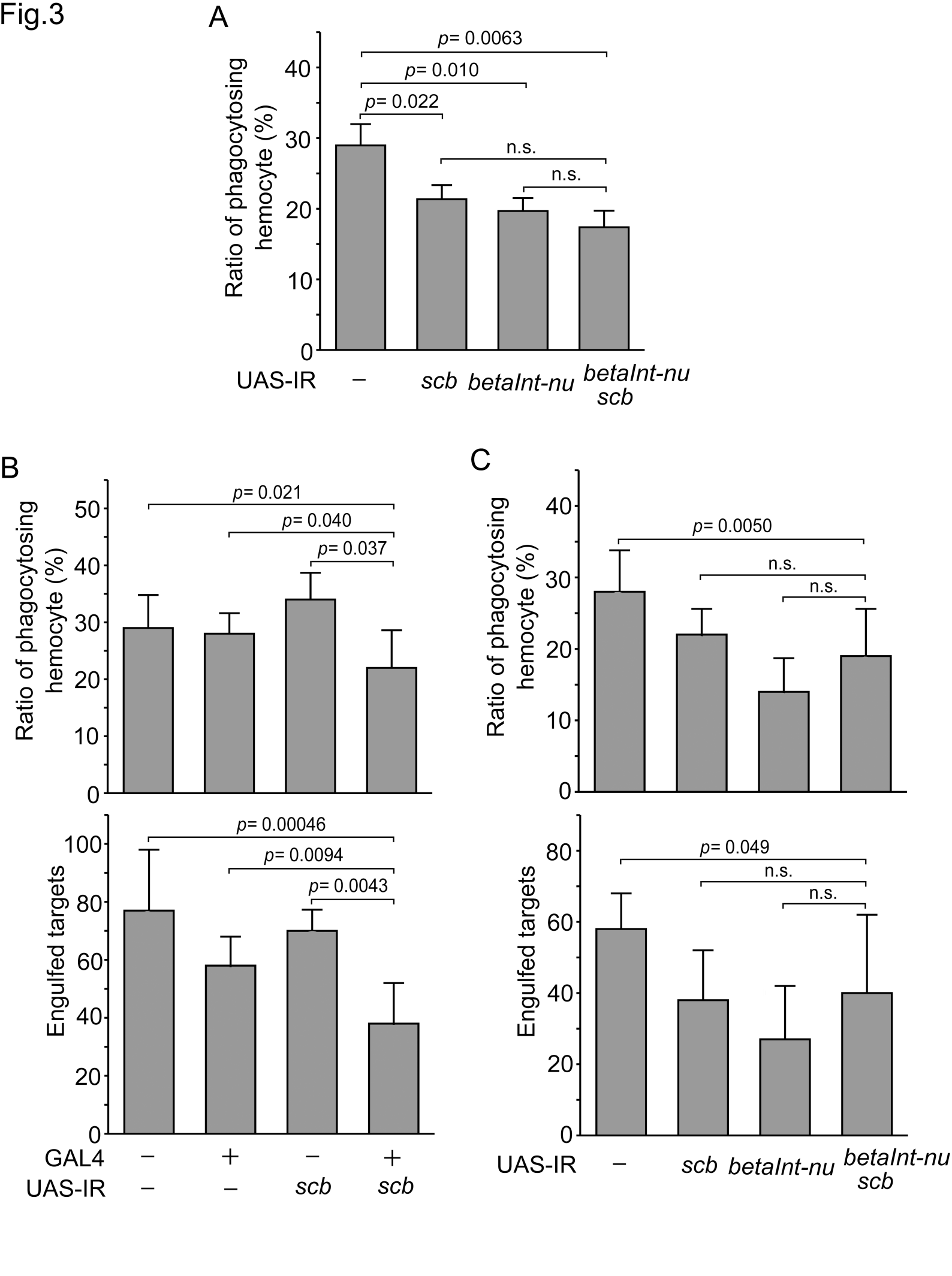
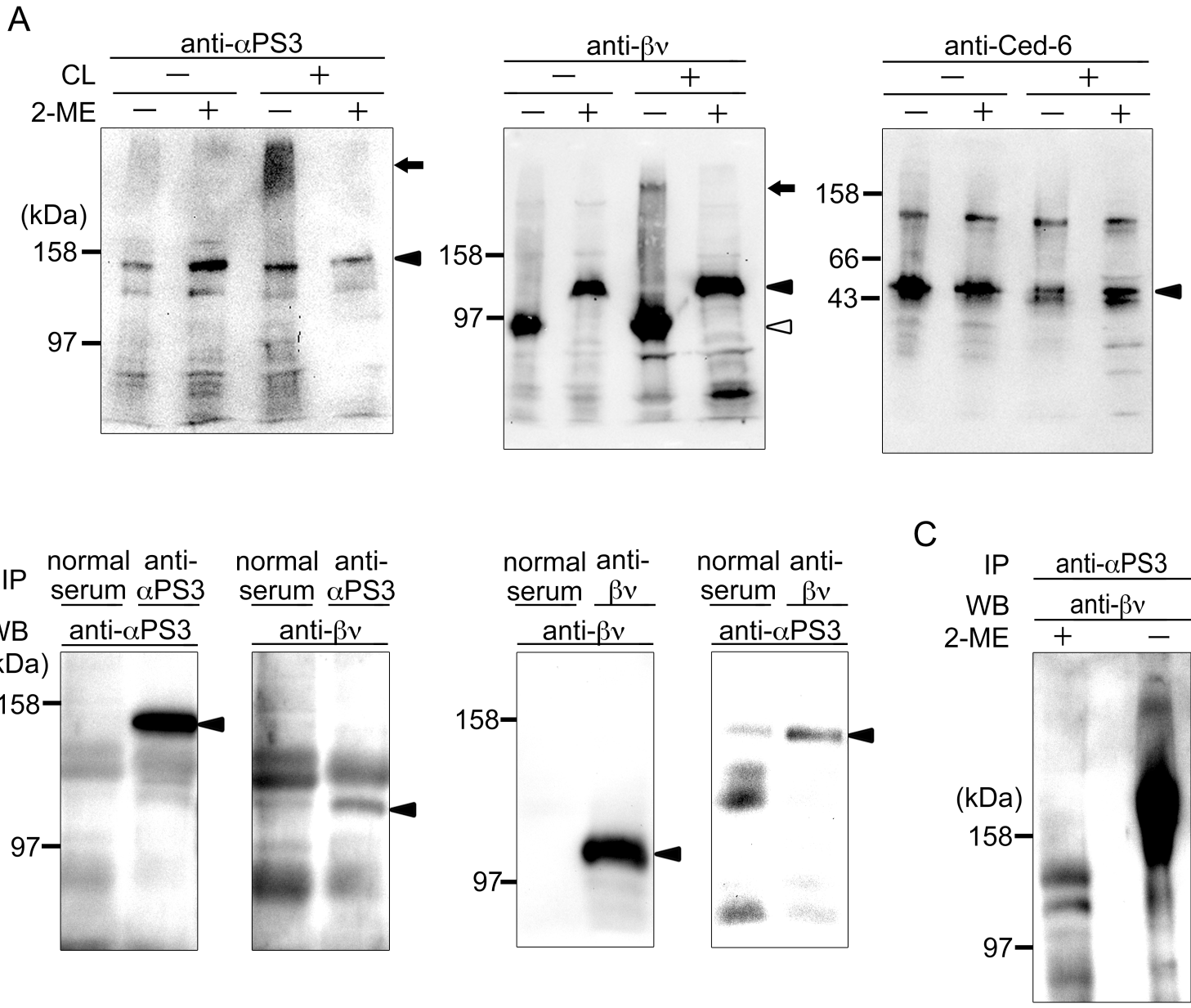

A
p= 0.0063
p= 0.010
p= 0.022
n.s.
n.s.
Ratio of phagocytosing hemocyte (%)
0
10
20
30
40
UAS-IR
–
scb
betaInt-nu
betaInt-nu scb
B
p= 0.021
p= 0.040
p= 0.037
Ratio of phagocytosing hemocyte (%)
0
10
20
30
40
50
p= 0.00046
p= 0.0094
p= 0.0043
Engulfed targets
0
20
40
60
80
100
GAL4
–
+
–
+
UAS-IR
–
–
scb
scb
C
p= 0.0050
n.s.
n.s.
Ratio of phagocytosing hemocyte (%)
0
10
20
30
40
p= 0.049
n.s.
n.s.
Engulfed targets
0
20
40
60
80
UAS-IR
–
scb
betaInt-nu
betaInt-nu scb

Fig.4


A
anti-αPS3
anti-βν
anti-Ced-6
CL
2-ME
(kDa)
158
97
66
43
B
IP
normal serum
anti-αPS3
WB
anti-αPS3
anti-βν
C
IP
anti-αPS3
WB
anti-βν
2-ME