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Novel adaptor protein is essential for insect cytokine signaling in hemocytes

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Abbreviation: GBP: growth-blocking peptide.

*Pseudaletia (Mythimna) separata* P77: AB521201, *Mamestra brassicae* P77: AB521202, *Spodoptera litura* P77: AB521203, *Pseudaletia (Mythimna) separata integrin β1*: AB548819.

## **Abstract**

Growth-blocking peptide (GBP) is an insect cytokine that stimulates a class of immune cells called plasmatocytes to adhere to each other and foreign surfaces. Although structure-activity studies have been extensively performed on the GBP and its mutants in Lepidoptera *Pseudaletia separata*, the signaling pathway of GBP-dependent activation of plasmatocytes remains unknown. We identified a novel adaptor protein (P77) with a molecular mass of 77 kDa containing SH2/SH3 domain binding motifs and an immunoreceptor tyrosine-based activation motif (ITAM)-like domain in the cytoplasmic region of the C-terminus. Although P77 did not show the capacity for direct binding with GBP, its cytoplasmic tyrosine residues were specifically phosphorylated within seconds after addition of GBP to a plasmatocyte suspension. Tyrosine phosphorylation of P77 was also observed when hemocytes were incubated with *Enterobacter cloacae* or *Micrococcus luteus*, but this phosphorylation was found to be induced by GBP released from hemocytes stimulated by the pathogens. We further detected tyrosine phosphorylation of the integrin  $\beta$  subunit in plasmatocytes stimulated by GBP. Double-stranded RNAs targeting *P77* not only decreased GBP-dependent tyrosine phosphorylation of the integrin  $\beta$  subunit, but also abolished GBP-induced spreading of plasmatocytes on foreign surfaces. *P77* RNAi larvae also showed significantly higher mortality than control larvae following infection with *Serratia marcescens*, thus indicating that P77 is essential for GBP to mediate a normal innate cellular immunity in insects. These results demonstrated that GBP signaling in plasmatocytes requires the adaptor protein P77 and that active P77-assisted tyrosine phosphorylation of integrins is critical for the activation of plasmatocytes.

## **\body**

### **Introduction**

The innate immune system of animals including insects is divided into humoral and cellular defense responses. The potent inducible antimicrobial defense system of *Drosophila melanogaster* has been intensively studied in the last fifteen years. The finding of the Toll signaling pathway in *Drosophila* allowed identification of human Toll-like receptors as the human homologues of the *Drosophila* Toll, which dramatically highlighted the study of the innate humoral immune system even in the medical sciences (1). In contrast to studies of the humoral defense system, the cellular defense

system in insects has not been vigorously analyzed, so that our knowledge of the factors mediating blood cell (hemocyte) activities is still quite limited. Some phagocytic receptors that are expressed in *Drosophila* hemocytes, such as Eater and Nimrod C1, were identified recently (2-4). Further, a possible link between the phagocytic activities of immune cells and the induction of antimicrobial peptides in the fat body has been suggested by several lines of evidence (3, 5, 6). Thus, the cellular defense system likely contributes to the clearance of pathogens not only by direct phagocytosis but also through activation of the humoral immune system. However, although the physiological importance of the cellular defense system has been increasingly recognized, the signaling pathway for hemocyte activation as well as the mechanism of cross-talk between cellular and humoral immune systems remains obscure (7).

Hemocytes in the armyworm *Pseudaletia separata*, like those of other Lepidoptera, consist of four subpopulations: granulocytes, plasmatocytes, spherule cells, and oenocytoids, which are distinguished from one another by their morphological, molecular, and functional characters (8, 9). The first two types, granulocytes and plasmatocytes, are active immune cells and contribute principally to performing the cellular defense mechanisms including phagocytosis, nodulation, and encapsulation (8). It is generally thought that the initiation of all these mechanisms requires a change in the nature of circulating hemocytes from non-adhesive to adhesive cells. Changes in the adhesive state of mammalian immunocytes are regulated by signaling molecules (cytokines), cell adhesion molecules, and their cognate receptors. For example, chemokine-triggered activation of leukocytes induces upregulation of the expression levels and activation states of the integrins, which enable leukocytes to adhere to the endothelial cells of the blood vessel walls before migrating into the tissues (10). We recently identified a chemokine-like peptide (hemocyte chemotactic peptide) in insects (11). This peptide and the other cytokine family referred to as the ENF peptide family, a name that was based on the consensus sequence of their N termini (Glu-Asn-Phe-), are known to increase hemocyte adhesion (12-16).

Growth-blocking peptide (GBP) was the first member of the ENF family discovered (12, 14). GBP exhibits multiple biological activities such as larval growth regulation, cell proliferation, paralysis induction, and activation of plasmatocytes, which have been reported as functions of the ENF peptide family (16, 17). Analysis of the hemolymph ENF peptides in several insects demonstrated that these peptides are present as

precursors that require precise processing by proteases to produce the active form of the ENF peptides (18, 19). Active GBP changes the plasmatocytes from a non-adhesive to an adhesive state, and the cells begin immediately to adhere to each other or to foreign surfaces. To characterize the intracellular signaling system of the GBP-induced changes in the adhesive state of plasmatocytes, membrane proteins of plasmatocytes were analyzed before and after treatment with GBP. The tyrosine residues of one single-pass plasmatocyte transmembrane protein (P77) with a molecular mass of 77 kDa was found to be phosphorylated within seconds after adding GBP to a hemocyte suspension. A binding assay using  $^{125}\text{I}$ -GBP showed no direct binding of the ligand to P77, indicating that P77 is not a GBP receptor itself. Sequence analysis showed that P77 contains ten tyrosine residues in the cytoplasmic region in which the SH2/SH3 domain-binding motifs and an ITAM-like motif, E-x<sub>2</sub>-Y-x<sub>2</sub>-L-x<sub>5</sub>-Y-x<sub>3</sub>-I, are present. The ITAM motif found in mammalian cells is located in the cytoplasmic domain of transmembrane adaptor molecules that are associated with and transmit signals from many mammalian immunoreceptors including Fc, T cell, and B cell receptors (20). In lymphocytes, ITAM-containing adaptors transmit antigen receptor signals that lead to cell activation and tolerance, depending on the intensity of the receptor stimulation and the presence of co-stimulatory signals. Recently, it has become clear that the Fc receptor  $\gamma$  and 12 kDa DNAX-activating protein (DAP12) associate with  $\beta_2$  and  $\beta_3$  integrins and contribute to signaling by these receptors (21). The fact that P77 contains an ITAM-like motif in the cytoplasmic region implies that the association of P77 with integrins controls the GBP-dependent activation of plasmatocytes. In order to confirm this hypothesis, we used RNA interference (RNAi) to reduce P77 gene expression, and measured the effects of this treatment on integrin and plasmatocyte activities. Treatment of larvae with dsRNA for P77 significantly reduced GBP-dependent activation of plasmatocytes as well as integrin activity.

## Results

**Identification of 77-kDa transmembrane protein.** When hemocytes suspended in Ex-Cell 420 medium in a plastic culture plate are activated by more than 1 nM GBP, plasmatocytes spread on the surface of the plate. We first verified that GBP bound directly to plasmatocytes by performing a receptor binding assay with  $^{125}\text{I}$ -GBP (Fig. S1). By Scatchard plot analysis of binding data, the number of GBP binding sites was

calculated to be 13,300/cell. Further, the dissociation constant for GBP was calculated to be 1.35 nM, which is close to the GBP concentrations with plasmatocyte-spreading activity (more than 1 nM) and also to those in the hemolymph of the last instar larvae of the armyworm (approximately 3-14 nM) (22). These results and facts led us to conclude that GBP-induced spreading of plasmatocytes occurs following the specific and direct binding of GBP to the cells.

To roughly characterize the GBP signaling pathway for plasmatocyte activation, we first examined whether tyrosine phosphorylation of plasmatocytes contributes to transmission of the GBP signal in this process. When plasmatocytes were preincubated with the tyrosine kinase inhibitor genistein before the addition of GBP, plasmatocytes did not change their morphology (Fig. 1A), thus indicating that GBP-dependent activation of plasmatocytes may be associated with intracellular tyrosine phosphorylation.

As a preliminary survey of phosphotyrosine-based signaling components stimulated by GBP, we used Western blot analysis with anti-phosphotyrosine antibody to compare tyrosine phosphorylated proteins in hemocytes treated with GBP or its deletion mutant peptides. Prior studies indicated that the wild type GBP (1-25GBP) and the C-terminal deletion analog (1-23GBP) activated plasmatocytes equally, whereas the N-terminal deletion analog (2-23GBP) did not have the activity (23). In accordance with these biological activities, the Western blots showed that hemocytes stimulated by 1-25GBP or 1-23GBP expressed the highly tyrosine phosphorylated protein with a molecular mass of 77 kDa, but that the same protein was only weakly tyrosine phosphorylated in hemocytes stimulated with BSA or 2-23GBP (Fig. 1B). To characterize the GBP-induced tyrosine phosphorylation of this protein, designated P77, the dose- and time-dependent effects of GBP on the tyrosine phosphorylation levels were analyzed. GBP showed a dose-dependent capacity to elevate the phosphorylation level of P77 (Fig. 1C). The tyrosine phosphorylation level increased soon after the addition of GBP and maintained a high level at least for 15 min (Fig. 1D).

**Purification and characterization of P77.** Two affinity column chromatographies using  $\text{Fe}^{3+}$ -chelating Sepharose and anti-phosphotyrosine IgG columns, coupled with SDS-PAGE, isolated a purified P77 (Fig. S2). *De novo* sequencing of peptide fragments from P77 identified the amino acid sequences of two fragments (Fig. 2). These peptide sequences enabled us to clone a *P77* cDNA of 2,043 bp. The deduced protein encoded

by *P77* was 560 amino acids and contained a putative signal peptide sequence in the N-terminus and a single-pass transmembrane domain at position 168 to 190. The cytoplasmic tail of *P77* was found to be rich in proline and to contain SH2 and SH3 domain-binding motifs. Further, evidence that an immunoreceptor tyrosine-based activation motif (ITAM)-like sequence, E-x<sub>2</sub>-Y-x<sub>2</sub>-L-x<sub>5</sub>-Y-x<sub>3</sub>-I, was present near the C-terminus of *P77* implied its importance in the regulation of intracellular signaling of GBP. This interpretation was partially supported by demonstrating that this ITAM-like sequence together with the SH2/SH3 domain-binding motifs were completely conserved in the sequences of two *P77* orthologs found in two other lepidopteran insects, *Mamestra brassicae* and *Spodoptera litura* (Fig. S3). These two orthologous genes were identified by RT-PCR, and the complete cDNAs were cloned by RACE-PCR methods.

To test whether GBP directly binds with *P77*, binding of <sup>125</sup>I-GBP was measured using COS7 cells transformed with *P77* cDNA. Although we confirmed high levels of *P77* expression in the transformed COS7 cells, significant binding of <sup>125</sup>I-GBP to the cell membrane fraction was not detected (Fig. S4), thus suggesting that *P77* does not have the capacity for direct binding with GBP. However, these results cannot exclude the possibility that *P77* interacts directly with GBP *in vivo*, as part of a GBP receptor complex.

Hybridization of *P77* to a single mRNA of 2.1 kb on Northern blots revealed the full-length cloned cDNA (Fig. 3A), although the predicted molecular mass of the deduced *P77* protein (60,790 Da) remained about 16 kDa less than that estimated for purified *P77* by SDS-PAGE. Because the molecular mass was significantly decreased from 77 kDa to 70 kDa by treatment with N-glycosidase, *P77* is a glycoprotein with N-linked sugar chains (Fig. 3B). RT-PCR analysis indicated that expression of *P77* is restricted spatially to hemocytes and the nervous system (Fig. 3C). Spatially restricted expression of *P77* protein was also confirmed by Western blot analysis (Fig. S5A). Preferential expression of *P77* in plasmatocytes was demonstrated by immunocytochemistry and Western blotting (Figs. 3D, S5B). In plasmatocytes, *P77* was detected in the cytoplasm as well as the cell membrane by immunocytochemistry (Fig. 3E). Because *P77* is a transmembrane protein, immunoreaction signals in the cytoplasm were expected to be derived from *P77* in the membranes of organelles such as the endoplasmic reticulum. This expectation was confirmed by the result that immunoreactive proteins were present only in the insoluble fraction of the plasmatocyte

lysate (Fig. 3F). *P77* transcription was constantly maintained throughout the last larval stage (Fig. S5C).

**Tyrosine phosphorylation pattern of P77 at bacterial infection.** Tyrosine phosphorylation levels of *P77* were measured in hemocytes coincubated with bacteria. In contrast to the quick phosphorylation of *P77* by GBP, *E. cloacae*- or *M. luteus*-induced phosphorylation was detected 30 min after initiation of the coincubation (Fig. 4A), suggesting that the bacteria-induced tyrosine phosphorylation of *P77* is not caused directly by the bacteria themselves but by GBP secreted from hemocytes stimulated by the bacteria. This interpretation was supported by the results that a significant amount of the GBP precursor (pro-GBP) was secreted from hemocytes stimulated by bacteria (Fig. 4B). Further, the bacteria-induced elevation of phosphorylation levels of *P77* was significantly blocked by the addition of anti-GBP IgG or a serine protease inhibitor cocktail that inhibits the proGBP-processing enzyme, suggesting that bacterial infection activated the processing enzyme that elevated active GBP concentrations in the hemocyte incubation medium (Fig. S6). These data clearly demonstrated that the exposure of hemocytes to external pathogens such as bacteria enhanced the release of pro-GBP from hemocytes and induced its proteolytic processing to activate the GBP that triggered tyrosine phosphorylation of *P77*s in plasmatocytes.

**Functional role of P77 in insect immune system** Previous reports suggested that integrins are involved in plasmatocyte activation processes such as spreading, encapsulation, and phagocytosis (24-27). To examine the functional link between *P77* and integrins, we first measured tyrosine phosphorylation levels of integrin  $\beta$  chains in plasmatocytes stimulated by GBP because their tyrosine phosphorylation was previously reported to be associated with the activation of integrins (28). The results clearly showed that GBP enhanced tyrosine phosphorylation levels of the  $\beta$  chains whose primary structures were verified as the *P. separata* integrin (Fig. 4C, see Supporting information). We then performed RNA interference (RNAi) to reduce *P77* expression, and measured the effects of this treatment on the integrin  $\beta$  chain expression and its tyrosine phosphorylation level after GBP stimulation (Fig. 4D, S7). The reduction of *P77* expression by the RNAi significantly suppressed the integrin  $\beta$  chain expression level as well as its phosphorylation level, thus indicating a close functional link between *P77* and integrins (Fig. 4D, S7).

To test the biological function of *P77*, we used RNAi to reduce the *P77* expression



level and measured the effects of this treatment on cellular and individual levels. Control plasmatocytes prepared from larvae treated with dsRNA targeting *EGFP* were strongly spread by GBP, whereas plasmatocytes from larvae treated with *P77* dsRNA were not significantly activated by GBP (Fig. 4E). Further, injection of the pathogenic bacterium *Serratia marcescens* caused significantly higher mortality in *P77* dsRNA-treated larvae than in control *EGFP* RNAi larvae (Fig. 4F), strongly suggesting that *P77* is essential for GBP signaling to mediate normal innate cellular immunity in insects.

## Discussion

In the present study, we found the novel adaptor protein *P77*, which is involved in GBP signaling for activation of immune cells, plasmatocytes. Because the involvement of integrins in the adhesive and phagocytic responses of plasmatocytes has been demonstrated in some insect species (24-27), we examined whether the GBP-*P77* signaling contributes to activation (tyrosine phosphorylation) of integrins in plasmatocytes. Incubation of plasmatocytes with GBP clearly induced tyrosine phosphorylation of integrin  $\beta$  chains. However, plasmatocytes lacking *P77* due to the RNAi lacked sensitivity to GBP, and GBP-dependent tyrosine phosphorylation of integrin  $\beta$  chains did not occur in these plasmatocytes. The same plasmatocytes were also defective in GBP-mediated spreading or aggregation responses. Further, RNAi targeting *P77* significantly suppressed integrin expression, suggesting that GBP-signaling may control integrin transcription levels as well as its activity. These results strongly suggest that GBP-induced activation of plasmatocytes is directly catalyzed by integrins whose activities are mediated by the adaptor protein *P77*.

Although we do not have direct evidence that explains the mechanism underlying the regulation of integrin activities by *P77*, it is worth emphasizing that *P77* contains several notable motifs, such as SH2/SH3 domain-binding motifs and the ITAM-like motif E-x<sub>2</sub>-Y-x<sub>2</sub>-L-x<sub>5</sub>-Y-x<sub>3</sub>-I, in its cytoplasmic region. The fact that ITAM motifs are generally present in the cytoplasmic domain of transmembrane adaptor proteins that associate with and mediate cell activation by immunoreceptors enabled us to presume that *P77* transmits the GBP signal to integrins by using the ITAM-like motif. This assumption is consistent with recent findings that integrin signaling for the activation of cellular responses in mammalian immune cells requires ITAM sequences in FcR $\gamma$  and

DAP12 (29-31). The presumed function of the SH2/SH3 domain-binding motifs in P77 was also based on accumulated data on the mammalian adaptor protein, non-catalytic region of tyrosine kinase adaptor proteins (Nck and Nck2) containing SH2/SH3 domains (32). Through its SH2 domain, Nck binds to specific phosphotyrosine-containing sites on activated receptors and scaffolds, and through its SH3 domains, Nck binds to proline-rich motifs in downstream effectors. These latter targets include proteins involved in the control of cellular actin dynamics. Although *P. separata* Nck has not been identified, in *Drosophila* Nck/Dock SH2/SH3 adaptor protein has been found as a mammalian Nck homologue (33). Given that SH2/SH3 domain binding motifs in P77 interact with Nck-like adaptor protein, it is plausible that P77 is involved in organization of the actin cytoskeleton under the influence of GBP in immune cells. Further, it has been reported that Nck-2 associates with a number of growth factor receptors including EGF and PDGF receptors (34). Based on the facts that GBP functions as a cell growth factor and shares a striking similarity with the C-terminal loop subdomain of EGF (35-37), it is reasonable to assume that P77 may directly or indirectly interact with a GBP receptor, actin, and integrin molecules using its SH2/SH3 domain binding motifs and associate with integrin through the ITAM-like domain. Therefore, P77 may function as a focal point that transduces GBP signaling to integrin signaling.

It has been reported that GBP is produced as a precursor form (pro-GBP) mainly from the fat body, and a relatively high concentration of pro-GBP is normally present in the hemolymph (18, 19, 38). This observation has obscured the possible fluctuation of hemolymph pro-GBP levels depending on physiological conditions of the insects. However, the present *in vitro* experiments (Fig. 4B) showed that exposure of hemocytes to microbes enhanced the secretion of pro-GBP from hemocytes. Further, another *in vitro* experiments (Fig. 4A, S6) demonstrated that microbes stimulates hemocytes to induce proteolytic activation of pro-GBP to active GBP by a specific serine protease(s) that has been reported in some lepidopteran larvae (18, 19), implying that this microbe-induced elevation of active GBP in the medium (or in the hemolymph *in vivo*) must be very fast because the process of elevating pro-GBP levels is simple without necessity to pass through the transcriptional enhancement. Given that concentrations of active GBP are precisely regulated *in vivo* as demonstrated *in vitro*, it is reasonable to propose that GBP mediates cellular immune activities to defend insects against various

acute infections. Further functional studies of P77 should increase our understanding how the insect cytokine GBP coordinates cellular innate immune activities in infected insect bodies.

## **Materials and Methods**

**Animals.** *Pseudaletia separata* larvae were reared on an artificial diet at 25±1°C with a photoperiod of 16 h light: 8 h dark (39).

**Analysis of phosphorylated proteins in hemocytes.** Hemolymph was collected into an ice-cold microcentrifuge tube containing 1 ml of anticoagulant buffer (41 mM citric acid, 98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, pH 4.5) and immediately centrifuged at 500 g for 1 min at 4°C. Precipitated hemocytes were suspended in 1 ml anticoagulant buffer and, after leaving on ice for 1 h, cells were used for following assays. For isolation of plasmatocytes (over 90% purity), Percoll step-gradient centrifugation was used as described previously (40). Whole hemocytes or plasmatocytes were washed twice with anticoagulant buffer and once with Ex-Cell 420 medium (JHR Bioscience) by repeating suspending and centrifugation at 500 g. Washed cells were finally resuspended in Ex-Cell 420 medium (20 larvae equivalents/ml) and then stimulated with GBP at 25 . After stimulation, cells were solubilized by adding same volume of 2 x lysis buffer (100 mM HEPES-NaOH, 300 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, 20% glycerol, 2% TX-100 (w/v), 1 % sodiumdeoxycholate, 0.2 % SDS, pH 7.5) containing Protease inhibitor cocktail (Nacalai tesque), Phosphatase inhibitor cocktail set II (Calbiochem), and 0.2 % phenylthiourea. After centrifugation at 17,000 g for 15 min at 4°C, the supernatant was mixed with the same volume of sample buffer (125 mM Tris-HCl, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue) and separated by SDS-PAGE (8% or 10%), transferred onto an Immobilon-P PVDF membrane (Millipore). Following Western blot analysis with anti-phosphotyrosine mouse monoclonal antibody (PY-100; Cell Signaling) was carried out as described previously (39). For reprobing, membranes were washed for 30 min at 50°C in 62.5 mM Tris-HCl (pH 6.7) containing 100 mM 2-mercaptoethanol and 2 % SDS, and they were probed with anti-P77 rabbit IgG or anti-human integrin β3 IgG (Santa Cruz Biotechnology).

The protein band cross-reacted with the anti-integrin β3 IgG was demonstrated to be that of *P. separata* integrin β-chain as described in Supporting information.

**Northern blot analysis.** Twenty-five micrograms of total RNA was separated on a 1% formaldehyde-agarose gel and transferred onto a Hybond N<sup>+</sup> nylon membrane (GE Healthcare). Hybridization was performed at 42°C in 50% formamide containing 5xSSPE and 0.5% SDS. The P77 cDNA fragment (1-941 bp) labeled with [<sup>32</sup>P]dCTP was used as a probe. The membrane was washed with 2x NaCl/Cit containing 0.1% SDS at 42°C, according to the procedure described previously (41).

**Preparation of anti-P77 IgG.** The cDNA containing the ORF of *P77* (residue 200-379) was cloned into pGEX-5X-1 (GE Healthcare) and expressed in *E. coli* strain BL21(DE3)pLys. The recombinant GST-P77 fusion protein was purified by glutathione-Sepharose column (GE healthcare). The purified protein was emulsified by Titer Max Gold (CytRx Corporation) and injected to immunize a rabbit and mouse. Anti-P77 IgG was precipitated by adding ammonium sulfate to 40% saturation and further purified by an affinity column of protein G-Sepharose (GE healthcare).

**Immunoprecipitation.** For immunoprecipitation, Protein G-Sepharose conjugated with anti-P77 rabbit IgG (1 mg IgG/ml gel) was mixed with hemocyte lysate and left for 6 h at 4°C. After washing the immunoprecipitate three times with lysis buffer, protein samples were eluted by boiling in sample buffer and analyzed by Western blotting with anti-phosphotyrosine antibody described above. After stripping for 30 min at 50°C in a solution containing 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol and 2 % SDS, the membrane was reprobed with anti-p77 mouse IgG followed by incubation with HRP-conjugated secondary antibody (39).

**Glycosidase treatment.** *P77* immunoprecipitated from the hemocyte lysate as described above was incubated with 0.2 U of N-glycosidase F (Roche) in 50 mM phosphate buffer (pH 7.4) containing 1% Triton X-100 for 12 h at 37°C. Samples were eluted with sample buffer for SDS-PAGE, and separated by 10% SDS-PAGE and probed with anti-P77 mouse IgG after transferring it onto a PVDF membrane as described above.

**Immunocytochemical analysis.** Immunocytochemistry of hemocytes prepared from Day4 last instar larvae was performed as described previously (39).

**Purification of P77 and integrin β1, their cDNA cloning, RT-PCR, GBP binding assays, RNAi, bacterial treatment of hemocytes, bacterial infection.** All detailed procedures can be found in Supporting information text.

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regulate two separate groups of target genes. *FEBS J* 275:894-902.



## Figure legends

**Fig. 1.** GBP-induced tyrosine-phosphorylation is essential for plasmatocyte activation. (A) Plasmatocytes were stimulated by 10 nM GBP with or without 100  $\mu$ M of the tyrosine kinase inhibitor, Genistein, for 15 min. DMSO, solvent for Genistein, was used as a negative control. Note that GBP-induced spreading was completely blocked by Genistein. (B) GBP-induced tyrosine phosphorylation in P77. Hemocytes were stimulated by 100 nM of wild type 25 amino acid GBP (1-25GBP) or deletion mutant GBPs (1-23GBP and 2-23GBP) for 5 min. Arrow indicates tyrosine phosphorylated P77 protein. (C) Effect of various concentrations of GBP on tyrosine phosphorylation levels in P77. Hemocytes were stimulated with indicated concentrations of GBP for 5 min. (D) Effect of incubation time on GBP-induced tyrosine phosphorylation levels in P77. Hemocytes were incubated with 100 nM GBP for indicated periods.

**Fig. 2.** Nucleotide and deduced amino acid sequences of cloned cDNA for *P. separata* P77. The determined amino acid sequences of two peptides derived from purified P77 are dotted-underlined. The putative signal peptide and transmembrane domain are boxed. SH2 and SH3 domain binding motifs are underlined and double underlined, respectively. The ITAM-like sequence is marked by thick bar. #Potential phosphorylation sites; \*Potential N-glycosylation sites.

**Fig. 3.** Analysis of P77 expression. (A) Hybridization of P77 cDNA to a northern blot of total RNA from sixth *P. separata* larval hemocytes. Size makers (kb) are shown to the left. (B) Immunoblot of P77 treated with N-glycosidase. P77 (Control) purified by using anti-P77 IgG conjugated-beads was treated with N-glycosidase (Treated). (C) RT-PCR analysis of P77 expression in various tissues of *P. separata* sixth instar: hemocytes (HC), midgut (MG), fat body (FB), Malpighian tubule (MT), testes (TE), integument (IN), and brain (BR). Detection of actin expression served as the control. (D) Immunocytochemical analysis of P77 in hemocytes from sixth instar larvae. P77 was visualized with anti-P77 IgG and an Alexa 488-conjugated secondary antibody. PL: plasmatocyte, GR: granulocyte, OE: oenocytoid. Scale bar represents 50  $\mu$ m. Note that P77 signals are visible mainly in plasmatocytes. (E) Immunocytochemical visualization of P77 in plasmatocytes before and after GBP treatment. Blue signals indicate nuclei containing DNA conjugated with DAPI. Note that P77 signals are visible especially in plasma membranes of spread plasmatocytes. (F) Distribution of P77 in cell fractions extracted from plasmatocytes. T: crude extract, I: insoluble membrane fractions of cells,

S: soluble cytoplasmic fraction of cells.

**Fig. 4.** Biological functions of P77. (A) Induction of tyrosine phosphorylation in hemocytes by GBP and bacteria such as *Enterobacter cloacae* (Ec), *Micrococcus luteus* (Ml), and *Bacillus licheniformis* (Bl). Note that Ec and Ml induced tyrosine phosphorylation 30 min after mixing with hemocytes. (B) Release of pro-GBP into the incubation medium from hemocytes (Hc) or hemocytes coincubated with *E. cloacae* (Hc+Ec). Same number of hemocytes (approximately  $1 \times 10^5$  cells) were used for each assay. (C) Tyrosine phosphorylation of integrin  $\beta$  chain in plasmatocyte stimulated by GBP. Plasmatocytes were stimulated with GBP for 10 min, and tyrosine-phosphorylation levels in integrin  $\beta$ -chains were detected. For reprobing, anti-Integrin beta 3 mouse IgG (Santa Cruz Biotechnology) was used. This antibody was demonstrated to cross-react with *P. separata* integrin  $\beta 1$  band as shown in Supporting information. (D) Effect of RNAi targeting P77 on GBP-induced tyrosine phosphorylation levels in integrin  $\beta$  chain in plasmatocyte. Double strand (ds) RNA targeting P77 was injected as described in Supporting information. EGFP dsRNA was used as control. <upper RT-PCR analysis> Expression levels of P77 and integrin  $\beta 1$  were measured by real-time quantitative RT-PCR and were normalized by dividing by actin expression levels in each sample. <lower Western blot analysis> Plasmatocytes prepared from test larvae were stimulated by 100 nM GBP for 5 min, and tyrosine phosphorylation levels of P77 and integrin  $\beta 1$  were determined. Phosphotyrosine positive bands were quantified using IMAGEJ (NIH) and were normalized by dividing by actin band values in each sample. Data are given as means  $\pm$  SD for seven separate measurements using four test larvae for each time. Asterisks indicate significant differences from controls (t-test; \*P<0.05, \*\*P<0.01). (E) Effect of P77 RNAi on plasmatocyte behavior. Spreading was assayed by scoring 100 randomly selected cells after 20 min in culture with 100 nM GBP. Each bar represents the mean  $\pm$  S.D. for five independent measurements. Upper photographs were typical cases for each condition. Other explanations as in (D). (F) Effect of P77 RNAi on survival rate of last instar larvae infected with *Serratia marcescens*. Test larvae were injected with *S. marcescens* suspension 1 day after the last injection of P77 dsRNA. Data are given as means for four separate measurements. Eight larvae were used for each measurement. Significant difference of both slopes was determined using General Linear Models.



Figure 2

TCTAAACGGGACAGTTGTGATTTTAAATTTTATATAGAAAAAGAGGAGAAATAAAAATGTTACTAATAGTTTATTTTGTGTTTTGAGTTCGAACTTTTTGTTTATAAAAATCAGAAGATT 120  
M L L I V Y F V F L S S N F L F I K S E D S

CTGGTATTATAAACATAGATGTATATAGGACTGTAACCTGCATGATTAATCCAGCGGGTTTCAAGTTCAGTGAAGGACAAAAATGTGACGCTGATAGTTCACCCCGGAGAAGTGCATATAG 240  
G I I N I D V Y R T V N C M I N P A G F K F T E G Q N V T L I V H P G E V H I D  
\*  
ACCTAGGCTGGTGGCGCCTTTGAAAGCGCTGGTAGTAGTACCTGTATGATCAGTTAAAGGGAAAAATAAAGGAGCTTTTAATTACTCTCGAACCTGCACAAAGCTGTGGAGGGAT 360  
L G W W R L V K A S G S S D L Y D Q L K G K N K G A F N Y S R T V D K A V E G S  
\*  
CATGGCAGTTACATTTTTTAAATAAGATAATGCAATATTCAGGAATACCAGAGTGTACTCGACGGAACCTAACACTATGTCAGTATGCACAAAGTGCACAATAGTTC 480  
W Q F T F Y F N K D N A N I Q E L P R V L L D G T N Y T M S V C T T N V T I V P  
\* \*  
CACCATGCACAACTCCACCATCATCAACTGCAATGGTACCTTTACAAAAGATAAAGATAAGAGATGACATGTTGGTCATCATAGCGTCCGTCGTGGCCGAGTCTTTATAATCA 600  
P S T T I P P S S T A M V P L P K D K D K R Y D M L V I I A S V V A A V L I I I

TCATTATAGCTCAAGTGCCTACATCCTGTACTTGA AAAATAAGGCTCAAAAATTGATTACGCTCAACACACATAACAACTTATCGAAGAAGTCCGAATCTACATCAAACGATAGAGCTC 720  
I I A Q A A Y I L Y L K N K A Q K L I T L N T H N N L S K K S E S T S N D R A R

GCTACTGTTCAATCTGGTGAAGATTATGAAGAACCTATCCTAGTGAAGATGATAATCGGGACAGTGTATCTACGAGCACCGAATATTGAATGTTCTAGTAATACTCGGATGC 840  
Y C S N P G Q K I M K E P I L V E D D N R D S V I Y E A P N I E L S S N T R M P  
# #  
CAATGCAGCCACTCAGCCACGCGATTACTACAATGCCTCGAACCCCGGCTGACCACAACCTGAAGATTGGTCTCAAGCATTGCTGGCTTCTGCTCTCAACAGTCTGCTCGAA 960  
M Q P P Q P P A I T T M P R T P R P D H N L K I G P Q A L L A S A L N S P A R S

GCCCATGTGCGATACTTCTCTCTAATCCAGAGACCAATCAAGTGAACGTGAGATGAATCTGGCGAATCGGCCTCCGCTGCCATTGCGGAACCATCTCCCGAACCTATGTTTC 1080  
P M S D T L P P N F Q R P N Q A E R E M N L A N R P P L P L P N H P P E P M F Q

AAGAACTTTACGAAGAAATGCCAACTGAAGAAAACACCTATGAACCGCCACCGGTGCATCAACTATGCCACCTCGACTTCTGTGCTCTAAGAAAGTACTACTGGCAATCTTCTGG 1200  
E L Y E E M P T E E N T Y E P P P V I N T M P P R L P V P P K K V P T G N L L D  
# #

ACGTAAAGGAAAAACACTAAAGTCAAGACCTGGAAACAAGCCACCACTCACAGCAAAGCCACCGCAACGACCCAACATGATGACGGCCAATGAATTCGAACAGGCTCTCAGAAGGA 1320  
V K E N T L K S R P G N K P P L T A K P T P Q R P N M M T A N E F E Q A L R R R

GGAACGAAAACCTTACCAAGAGGCAATCCGGGCCACATGATGCACCAACAACAGCAGGAGCAGACTCATAGAAGAAGGCTCCGGGCGATGGAGCGACCAACGAACTAGAAGAAGCTT 1440  
N E N S L P R G N P G H M M H Q Q Q Q E Q T H R R R P P G D G A T N E L E E A L

TAAGAAGGAGAAATAAATGGGTCTTACCAAGTAACTGCGACCAACGAACTTGAAGGAGGCTTTAAAAAGAAATAAAGTTATGCAAGTGCCTATTGTCTCAATTCCTGAGCCCT 1560  
R R R N N N G S S P S N T A T N E L E E A L K R R N K V M Q V P I V S I P E P S  
- - - - -

CGAGTGCAAGAACTCACGAAGCCGAAACCCGCTGATTTAAACACCTGTA AAAACACCAACCCAGTACAAGAAGAAATCTATTACAATGAAGCATCATCAGATGAAGAAGAAAT 1680  
S A R N S P K P K P P P A F K P P V K T P H P V Q E E I Y Y N E A S S D E E E W

GGACTTACGAGCCTGAAACCTTGAACAGTTACGACCCAAATATTTACAACGTATAATTAACCTTATTTTAAATTTAATAGGGTTTAAATTTGAATGAAATGAGGATTGCTTGACT 1800  
T Y E P L K P L N S Y D P N I Y N V \*  
# #

GAGGCCTCGCCTGTAGCTGCACAATGCATTTATGTGTTGGCGATTTAAAGTAGTACATTTTTGCAAAGTCCAGGCTGAGATTATCAATGTGTTTCAAATAAGTAAGAAAGTAAGAAG 1920  
ACATTAATAATTATTATATTATGATTAGCATTAGCAGTCTGTTGAGATACAGTAAAGTGTGGTCTGTGGTCCACCATGCGGGTCCAGTGCATTTGGTGAATATCACACCTCTCAAAT 2040  
TTG 2043

Figure 3

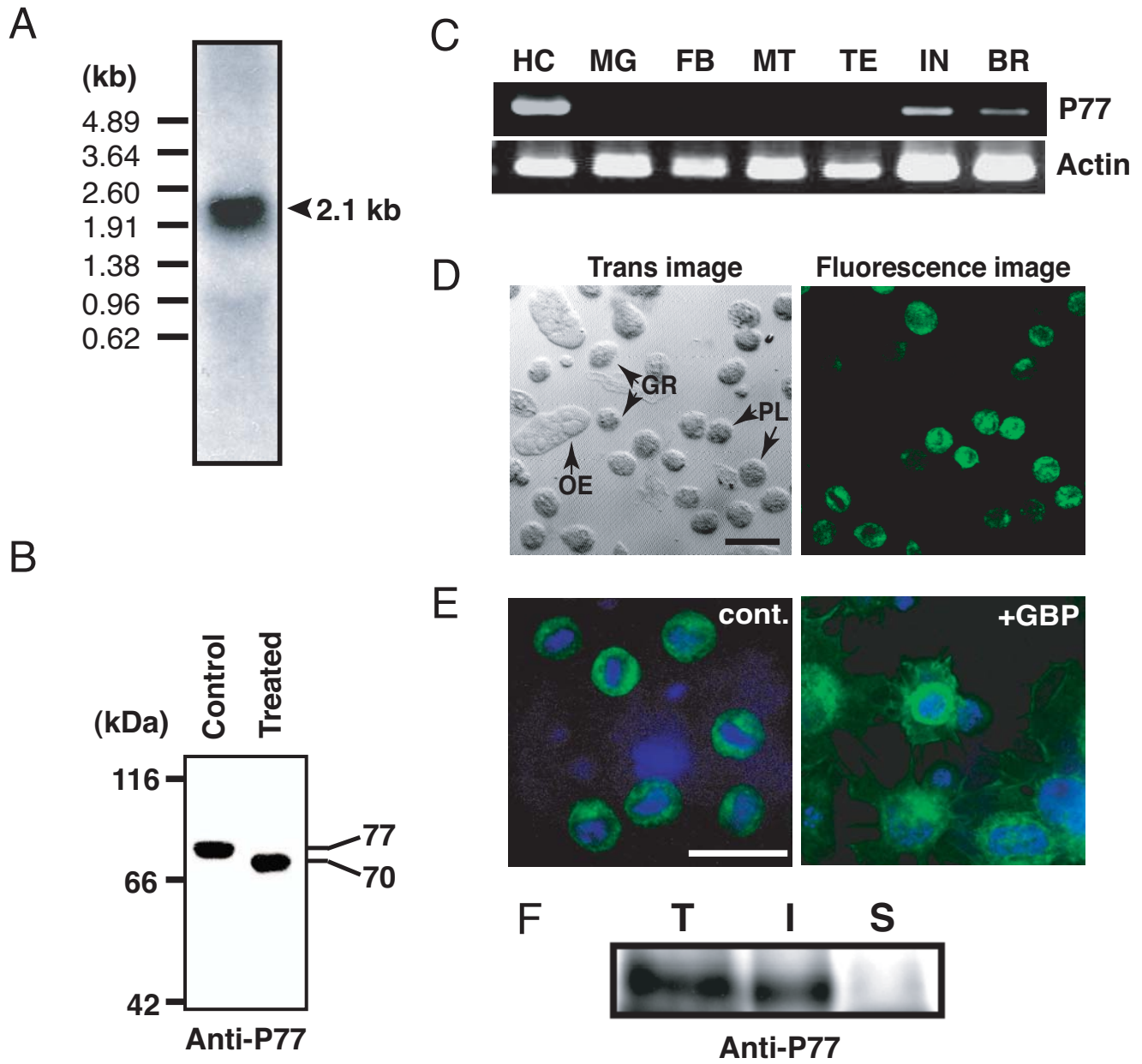
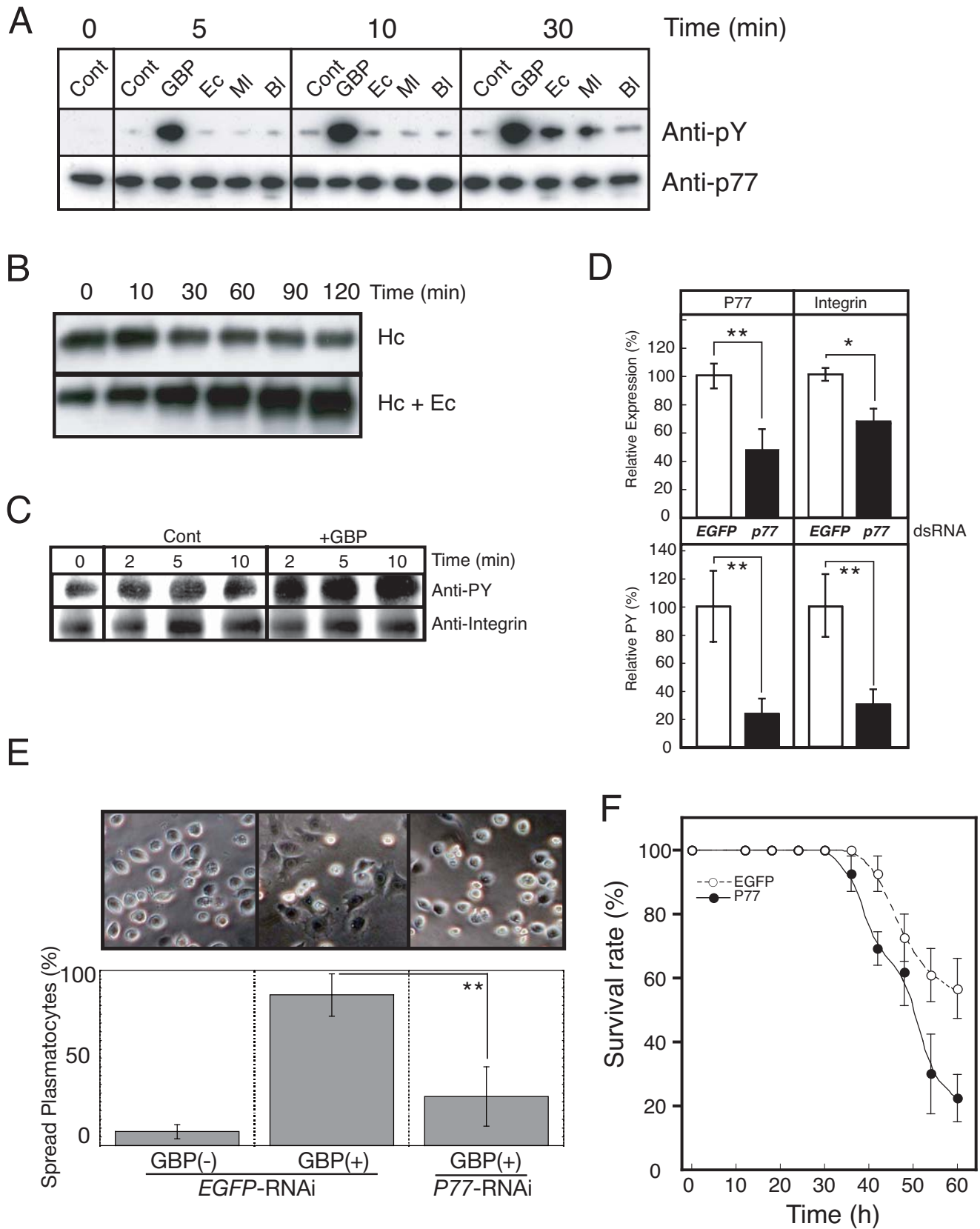


Figure 4



## Supporting information

### Materials and Methods

**Purification of P77** To purify phosphorylated P77 from hemocytes, we first prepared hemocyte extracts after stimulation of hemocytes with GBP as follows. The hemocyte suspension was prepared from last instar larvae (Day 4) as described in Materials and Methods and then stimulated with 100 nM GBP at 25 °C for 5 min. After GBP stimulation, the hemocyte suspension was mixed with 1/9 volume of 10% SDS to solubilize membrane proteins and stored at –20 °C until the next purification step.

The hemocyte extract was dialyzed against MES (2-morpholinoethanesulfonic acid) buffer (50 mM MES-NaOH, 150 mM NaCl, pH 6.0) containing 1% SDS through a dialysis membrane with an exclusion limit of 50 kDa nominal molecular weight (Spectra/Por Float-A-Lyzer, Spectrum Labs). The dialyzed fraction was then loaded onto a Fe<sup>3+</sup>-chelating Sepharose Fast Flow (GE Healthcare) column (15 x 250 mm) equilibrated with MES buffer. After washing with five column volumes of MES buffer containing 0.1% SDS, phosphorylated proteins were eluted with 0.1 M Tris-HCl (pH 9.5) containing 0.1% SDS and immediately dialyzed against 0.1 M Tris-HCl (pH 7.5) containing 0.1% SDS. After mixing with 1/9 volume of 10% NP-40, the phosphorylated proteins fraction was subjected to a column (10 × 20 mm) of Protein G-Sepharose (GE Healthcare) conjugated with anti-phosphotyrosine IgG (Cell Signaling) and washed with five column volumes of 0.1 M Tris-HCl (pH 7.5) containing 1% NP-40. Tyrosine-phosphorylated proteins were eluted with 1x sample buffer (0.5 M Tris-HCl (pH 6.8) containing 10% SDS, 5% bromophenol blue, and 7% glycerol) for SDS-PAGE and concentrated in a stacking gel as a single band according to the method of Staudenmann et al. (1). After being equilibrated with sample buffer for SDS-PAGE, the isolated gel containing tyrosine-phosphorylated proteins was separated by SDS-PAGE (8%), and the tyrosine phosphorylated protein with 77 kDa was isolated from the gel for *de novo* peptide sequence analysis.

**cDNA cloning and sequence analysis of P77** Total RNA was isolated from hemocytes of last instar larvae using Trizol reagent (Invitrogen). First-strand cDNA was synthesized with 5 µg of total RNA and oligo(dT) primer using ReverTra Ace (Toyobo Co., Ltd.). The cDNA was amplified with degenerate primers designed based on the peptide sequences (forward primer: 5'-GCIWSIGCIHTNAAYWSNCC-3', reverse

primer: 5'-CKIGCISWNSWNSSYTCNNGG-3') using a following profile: 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 1 min at 72 °C. The cDNA fragment with the predicted size was subcloned into a TA vector (pGEM-T Easy, Promega), and sequenced by a 310 DNA sequencer (ABI). Full-length cDNA was isolated using the 3' and 5' rapid amplification of cDNA ends (RACE) technique with the following five primers based upon the partial DNA sequence obtained above: P77F-1, (5'-GCGACCAACGAACTAGAAGAAGC-3') and P77F-2 (5'-GGTCTTCACCAAGTAACACTGCG-3') for 3' RACE, and P77R-1 (5'-TGGGTCGTTGCGGTGTTGGCTTTGC-3'), P77R-2 (5'-GTAGGTACTTTCTTAGGAGGCACAG-3'), and P77R-3 (5'-TCGAGGTGGCATAGTGTGATGACC-3') for 5' RACE. Computer-assisted sequence analyses were performed by Genetyx-Mac ver. 13.0.9 (Software Development Co.). The signal peptide sequence and transmembrane region were predicted using SignalP ver. 3.0 ([www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) and TMHMM ver. 2.0 ([www.cbs.dtu.dk/services/TMHMM/](http://www.cbs.dtu.dk/services/TMHMM/)), respectively (2).

**cDNA cloning and sequence analysis of *Mamestra brassicae* and *Spodoptera litura***

**homologues of P77** First-strand cDNA was prepared from *M. brassicae* or *S. litura* hemocyte total RNA as described above. For cloning of the *M. brassicae* homologue, the cDNA was amplified with primers (forward primer: 5'-TGGCTTCTGCTCTCAACAGTCCTG-3', reverse primer: 5'-CTTAAAGCTTCTTCTAGTTCGTTGG-3', which correspond to 931-954 and 1420-1444 of the *P. separata* P77 cDNA sequence, respectively) using a following profile: 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C, and 40 sec at 72 °C. The approximately 600 bp PCR fragment was cloned into the TA vector (pGEM-T Easy, Promega), and sequenced by a 310 DNA sequencer (ABI). Full-length cDNA was isolated using the 3' and 5' RACE-PCR methods with the following six kinds of primers based upon the partial cDNA sequence obtained above: MbP77F-1 (5'-GGAATAATGTAGAGCGTGAGAGCAC-3'), MbP77F-2 (5'-CAAGAGGTCCTTCTCCAGGACAC-3') and MbP77F-3 (5'-GCCACAGCATCAAGAGCAGAACC-3') for 3' RACE, and MbP77R-1 (5'-TTCTCGTCGGTATACGCTTCTTCG-3'), MbP77R-2 (5'-GATGATTCTGAGGCGGCAAAGGC-3') and MbP77R-3 (5'-CGTTCAATATGCTGCAGCCTCTG-3') for 5' RACE.



For cloning of the *S. litura* homologue, the cDNA was amplified with degenerate primers designed based on conserved amino acid sequences of *P. separata* P77 and *M. brassicae* P77 homologues (forward primer: 5'-CNCCNGTNATHAAYACNATGCC-3', reverse primer: 5'-YTCRTRTARTADATYTCYTCYTG-3', which correspond to PPVINTMP and QEEIYYNE amino acid sequences, respectively) using a following profile: 35 cycles of 30 sec at 94 , 30 sec at 50 , and 40 sec at 72 . The approximately 600 bp PCR fragment was cloned and sequenced as described above. Full-length cDNA was isolated using the 3' and 5' RACE-PCR methods with the following five kinds of primers based upon the partial cDNA sequence obtained above: SIP77F-1 (5'-GGAGACAAGCGGGAATTGAAACTG-3') and SIP77F-2 (5'-CAAGTCCAGCAAAGACCAGACCTG-3') for 3' RACE, and SIP77R-1 (5'-GTCTGTTCGCCATATCACGTTCTG-3'), SIP77R-2 (5'-GAATGGGTATGGTCCATCATTGCG-3') and SIP77R-3 (5'-TTTAGGGTCGCTCCATGCATCTGC-3') for 5' RACE. All identified cDNA sequences were subjected to computer-assisted sequence analysis as described above.

**Analysis of *P. separata* integrin  $\beta 1$  cDNA and protein** *P. separata integrin  $\beta 1$*  cDNA was amplified with degenerate primers designed based on conserved region of *Manduca sexta* and *Spodoptera frugiperda integrin  $\beta 1$*  genes (forward primer: 5'-GGNWSNTTYGTNGANAARCC-3', reverse primer: 5'-GGNTCYTGRTANARNGRIT-3') using the following profile: 35 cycles of 30 sec at 94°C, 30 sec at 53°C, and 2 min at 72°C. The cDNA fragment (1599bp) was subcloned into a pGEM-T Easy vector (Promega) and sequenced using a 310 DNA sequencer (ABI) (AB548819).

*P. separata* protein cross-reacted with anti-human integrin  $\beta$ -3 IgG was isolated as follows. Hemocyte extract was mixed with anti- human integrin  $\beta$ -3 IgG, The mixture was put at 4°C for 24 h and was incubated with Protein G-Sepharose beads (GE Healthcare) at 25°C for 1 h. The beads were isolated by filtration, and proteins cross-reacted with the beads were solubilized by adding 1x sample buffer for SDS-PAGE. Following separation of solubilized proteins by SDS-PAGE, the protein band in the same position as the protein cross-reacting with anti-human integrin  $\beta$ -3 IgG was isolated, trypsinized and sequenced by *de novo* peptide sequence analysis. Four peptide fragments were sequenced as follows: LTIQLQVDCK, CHGAGFMQCGICK, AFCDKENQDATSGK, and NQTYDVIVSPVGLPDK. The fact that these peptides are

coded on the *P. separata integrin β1* cDNA indicated that the protein band cross-reacted with the mouse integrin antibody is that of the armyworm integrin β1 chain.

**Reverse transcription PCR (RT-PCR)** Total RNA was isolated from hemocytes, midgut, fat body, Malpighian tubule, testis, integument, and brain using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed with oligo (dT) primer using ReverTra Ace (Toyobo). The cDNA was amplified with a *P77*-specific primer pair (forward primer: 5'-GATAATGCAAATATTCAGGAATTACC-3' and reverse primer: 5'-TCGAGGTGGCATAGTGTGATGACC-3') and an actin primer pair (forward primer: 5'-TTCGAGCAGGAGATGGCCACC-3' and reverse primer: 5'-GAGATCCACATCTGYTGGAAGGT-3'). PCR was conducted under the following condition, 30 cycles at 94°C for 30 sec, 53°C for 30 sec, and 72°C for 60 sec.

**Quantitative real-time PCR** Total RNA prepared from hemocytes of last instar larvae was reverse transcribed as described above. Real-time quantitative PCR was carried out with 2.5% of the reverse transcription product in a 20 μl reaction volume of LightCycler Fast DNA Master SYBR Green I (Roche Diagnostics) using the following primer sets: *p77* forward primer 5'-CCAACAACAGCAGGAGCAGA-3', reverse primer 5'-GGCTTCGGTGAGTTTCTTGC-3'; *integrin β1* forward primer 5'-ACTGTGTCTGTGGCGTGTGC-3', reverse primer 5'-CCTGATGTTGCGTCCTGGTT-3'; actin forward primer 5'-TTCGAGCAGGAGATGGCCACC-3', reverse primer 5'-GAGATCCACATCTGYTGGAAGGT-3'. Amplification was performed using the Light-Cycler 1.2 instrument and software with the following profile: 45 cycles of 10 sec at 95°C, 10 sec at 55°C, and 10 sec at 72°C. The gene expression values of *P77* and *integrin β1* were standardized against those of actin gene expression in each sample.

**GBP binding assay using plasmatocytes** Plasmatocytes were isolated as described in Materials and Methods. GBP-binding assay was performed by adding 50 μl of various concentrations (0.02-7.5 nM) of <sup>125</sup>I-labeled GBP (1.27 x 10<sup>5</sup> cpm/pmol) in Ex-Cell 420 medium with or without an excess amount of non-labeled GBP (100 μM) to 50 μl plasmatocyte suspension (5 x 10<sup>5</sup> cells). After 2 h incubation at 4°C, plasmatocytes were washed with PBS by repeating centrifugation and suspending, and the radio activity was measured by a liquid scintillation counter (Aloka LSC-5100). Specific binding was calculated by subtracting the non-specific binding count (with excess non-labeled GBP)

from the total binding count (without non-labeled GBP).

**GBP binding assay using COS7 cells** P77 cDNA containing its full length ORF was cloned into a pEGFP-N3 expression vector (Clontech) previously digested by PstI and BamHI. The expression plasmid for P77-EGFP fusion protein (pEGFP-P77) or pEGFP (negative control) was transfected into COS7 cells using TransFast transfection reagent (Promega). Two days later, transfected cells were harvested, homogenized in Ex-Cell medium, and centrifuged at 17,000 g for 10 min at 4°C. The pellet was washed twice with Ex-Cell medium, suspended in the same medium, and used as the COS7 cell membrane fraction. GBP-binding assay was initiated by adding 50 µl of 200 nM <sup>125</sup>I-labeled GBP (1.27 x 10<sup>5</sup> cpm/pmol) in Ex-Cell 420 medium with or without excess non-labeled GBP (100 µM) to 50 µl COS7 cell membrane fraction. After 2 h incubation at 4°C, the membrane fraction was washed 3 times with PBS on a glass fiber filter (Whatman) and its radioactivity was measured by a liquid scintillation counter (Aloka LSC-5100). Specific binding was calculated by subtracting the non-specific binding count (with excess cold GBP) from the total binding count (without cold GBP).

**RNA interference** Double stranded RNA (dsRNA) was prepared by synthesizing two complementary RNA strands using a MEGAscript RNAi kit (Ambion) and annealing them. For *P77* dsRNA, two *pBluescript II-P77* plasmids containing *P77* cDNA (740 bp) in opposite directions were used as templates after BamHI digestion to linearize plasmid DNA. For *EGFP* dsRNA (as a control), the PCR product (701 bp), which was amplified from a *pEGFP* vector with two primers containing a T7 RNA polymerase promoter sequence at their 5' ends (forward primer: 5'-GCGTAATACGACTCACTATAGGGAGAGTGAGCAAGGGCGAGGAG-3', reverse primer: 5'-GCGTAATACGACTCACTATAGGGAGAGATCGCGCTTCTCGTTGG-3') was used as a template for synthesis of two complementary RNAs.

Prior to injection of *P77* dsRNA, approximately 50 µl hemolymph was removed from test penultimate (5th) instar larvae through a small puncture in one of the forelegs. At 6 h after bleeding, five microgram of *P77* dsRNA (or *EGFP* dsRNA) in 5 µl FuGENE HD transfection reagent (Roche) was injected into the test larvae. Three days after the injection, hemolymph was collected from each larva for hemocyte analyses. For investigating the effects of *P77* RNAi on survival rates after a bacterial challenge, 8 larvae were injected with bacterial suspension 3 days after dsRNA injection.

**Bacterial treatment of hemocytes** *Enterobacter cloacae*, *Micrococcus luteus*, or *Bacillus licheniformis* was cultured in LB medium at 37°C for 15 h and collected by centrifugation. Bacteria were washed with Grace's medium by repeating suspension and centrifugation three times, and suspended in Excell 420 medium to optical density (OD) =1. Hemocytes were suspended in Ex-Cell 420 medium and incubated with equal volume of the bacteria suspension. After incubation for indicated periods, hemocytes were solubilized by adding the lysis buffer, mixed with anti-P77 IgG beads. Immunoprecipitated proteins were separated by SDS-PAGE and P77 activation (tyrosine phosphorylation) was analyzed by Western blotting with anti-phosphotyrosine monoclonal antibody and anti-P77 antibody as described in Materials and Methods. For detection of pro-GBP, hemocytes were incubated with or without *E. cloacae* for indicated periods, and the supernatant after centrifugation at 3,000 rpm for 5 min was used for Western blot analysis with anti-GBP IgG.

In Fig. S6 experiments, the incubation medium of only hemocytes, hemocytes and *E. cloacae*, or only *E. cloacae* with or without anti-GBP IgG or serine protease inhibitor was again incubated with a plasmatocyte suspension for 5 min to measure the tyrosine phosphorylation levels of plasmatocyte P77s as described above.

**Bacterial infection experiment** *Serratia marcescens* was cultured in LB medium and collected by centrifugation. Bacteria were washed with Grace's medium and suspended in the medium to optical density (OD) =1. The bacteria solution was diluted to 100,000-fold, and then 5 µl of the solution (approximately  $6 \times 10^2$  cfu/ml) was injected into the larval abdominal hemocoel with a glass capillary needle.

## References

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### Figure legends

Fig. S1. Binding of  $^{125}\text{I}$ -GBP to plasmatocytes. The saturation curve at equilibrium was established from 2 h incubation at  $4^{\circ}\text{C}$ , with non-specific binding being evaluated in the presence of an excess amount of non-labeled GBP. Each plot represents the mean of binding (bound GBP/cell) for 3 separate measurements. (Inset) Scatchard plot analysis of specific binding. Note that 13,300 binding sites/cell and 1.35 nM of dissociation constant were calculated by Scatchard plot analysis. (●), specific binding; (□), total binding; (○), non-specific binding.

Fig. S2. Purification of P77. The protein band of P77 was followed by Western blotting with anti-phosphotyrosine IgG. Positive fractions (Nos. 2–5) from a  $\text{Fe}^{3+}$ -chelate affinity column were collected and applied to an anti-phosphotyrosine antibody-conjugated column. The positive fraction was applied to a preparatory SDS-PAGE, and the positive protein band of 77 kDa was isolated from the acrylamide gel. Purified P77 was proteolyzed by trypsin, and digested peptide fragments were analyzed by *de novo* peptide sequencing. To obtain complete amino acid sequences of P77 peptide fragments, hemocytes collected from over 7,000 last instar larvae were used.

Fig. S3. Comparison of deduced amino acid sequences of the three lepidopteran P77s. The putative signal peptide sequences and transmembrane domains are boxed. The sequences of SH2 and SH3 domain-binding motifs and ITAM-like domains are indicated by yellow, blue, and black colored backgrounds, respectively. \*Identical residues. #Putative phosphorylation sites (<http://www.cbs.dtu.dk/services/NetPhos/>). Ps, *Pseudaletia separata* P77 (AB521201); Mb, *Mamestra brassicae* P77 (AB521202); Sl, *Spodoptera litura* P77 (AB521203).

Fig. S4. Binding of  $^{125}\text{I}$ -GBP to COS7 cell membrane fractions prepared from cells expressing EGFP protein (pEGFP) or P77-EGFP fusion protein (pEGFP-P77).  $^{125}\text{I}$ -GBP bound to the COS7 cell membrane fraction was measured after 2 h incubation at  $4^{\circ}\text{C}$ . Specific binding was calculated by subtracting the non-specific binding count (with an excess amount of non-labeled GBP) from the total binding count (without non-labeled GBP). Data are given as means  $\pm$  SD (n = 6).

Fig. S5. Expression of P77 and its transcript. (A) P77 expression was analyzed by Western blot using anti-P77 IgG in indicated tissues: brain (Br), nerve cord (Nc), hemocytes (Hc), epidermis (Ep), inter-segment muscle (Im), fat body (Fb), Malpighian tubule (Mt), midgut (Mg), and testis (Te). (B) Western blot analysis of P77 expression in specific hemocytes isolated by Percoll density gradient centrifugation as previously described (3). Gr: granulocytes, Pl: plasmatocytes, Oe: oenocytoids. (C) P77 transcriptional levels in hemocytes of *P. separata* during the last larval instar.

Fig. S6. Effects of anti-GBP IgG or serine protease inhibitor on GBP released from hemocytes during pre-incubation. After a 30-min pre-incubation of indicated components (only hemocytes (Hc), hemocytes with *E. cloacae* (Hc+Ec), or only *E. cloacae* (Ec)) with or without anti-GBP IgG or serine protease inhibitor, the medium of each pre-incubation was collected and used to measure its ability of P77 activation (phosphorylation of P77 tyrosine residues) after removing anti-GBP IgG by absorbing with Protein G-Sepharose beads. The tyrosine phosphorylation level in plasmatocyte P77 was assayed at 5 min after adding the pre-incubation medium to a plasmatocyte suspension. Note that adding anti-GBP IgG or serine protease inhibitor during pre-incubation blocked bacteria-induced P77 activation in plasmatocytes, suggesting that bacteria-induced activation of P77 was caused mainly by GBP generated from pro-GBP by serine protease. Both pro-GBP and serine protease are thought to be secreted into the pre-incubation medium from hemocytes. See above Materials and Methods.

Fig. S7. Western blot analyses of P77 and integrin  $\beta 1$  expression levels in hemocytes of P77 RNAi larvae. Three days after injection of P77 dsRNA, hemocytes were collected from test larvae. EGFP dsRNA was used as a control. Hemolymph proteins were analyzed by Western blotting after separating them by SDS-PAGE.

Fig. S8. Model of GBP signaling via P77 after bacterial infection. Bacterial infection may trigger a proteolytic activation of GBP precursor (pro-GBP) that is normally present in hemolymph and is released from hemocytes stimulated by bacteria. Active GBP may stimulate GBP receptor (GBPR), which activates P77. Active P77 may assist activation of integrins and also recruit actins, which changes the adhesiveness of

plasmacytes, followed by morphological changes.

Fig. S1

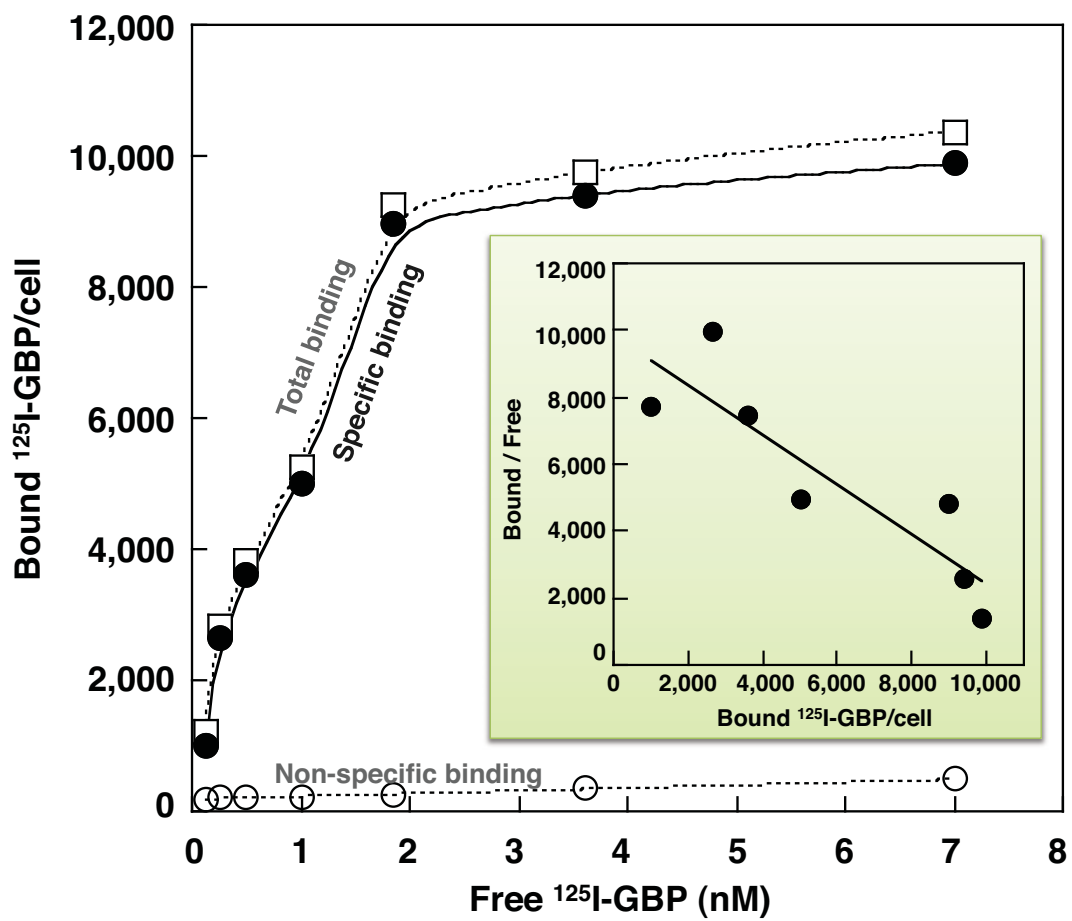




Fig. S2

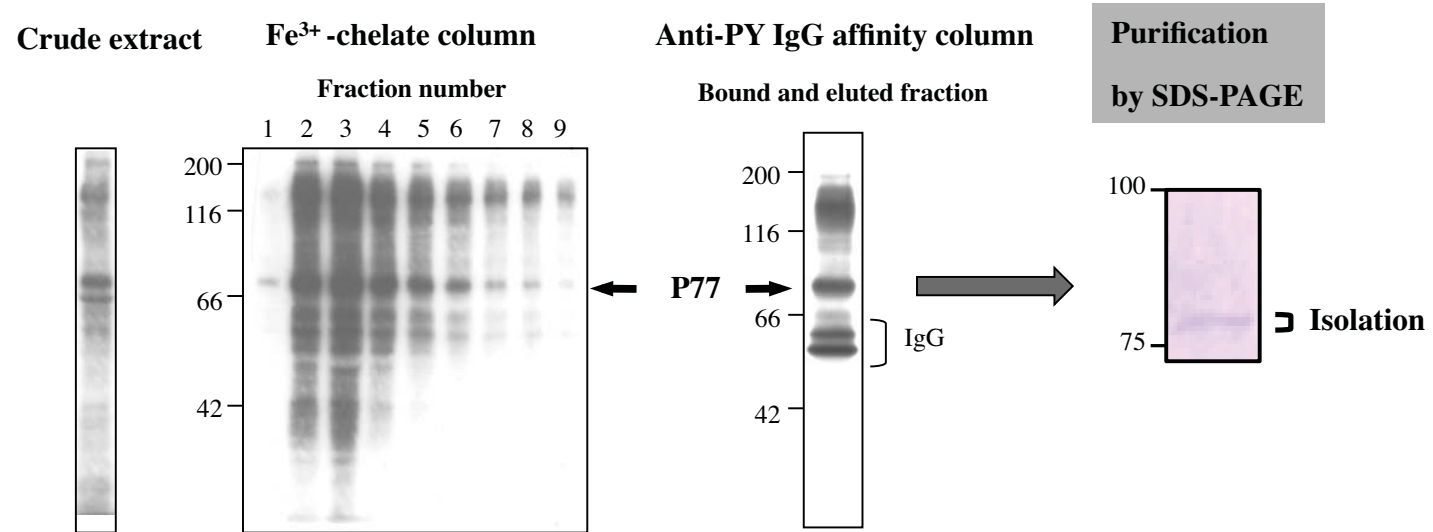




Fig. S4

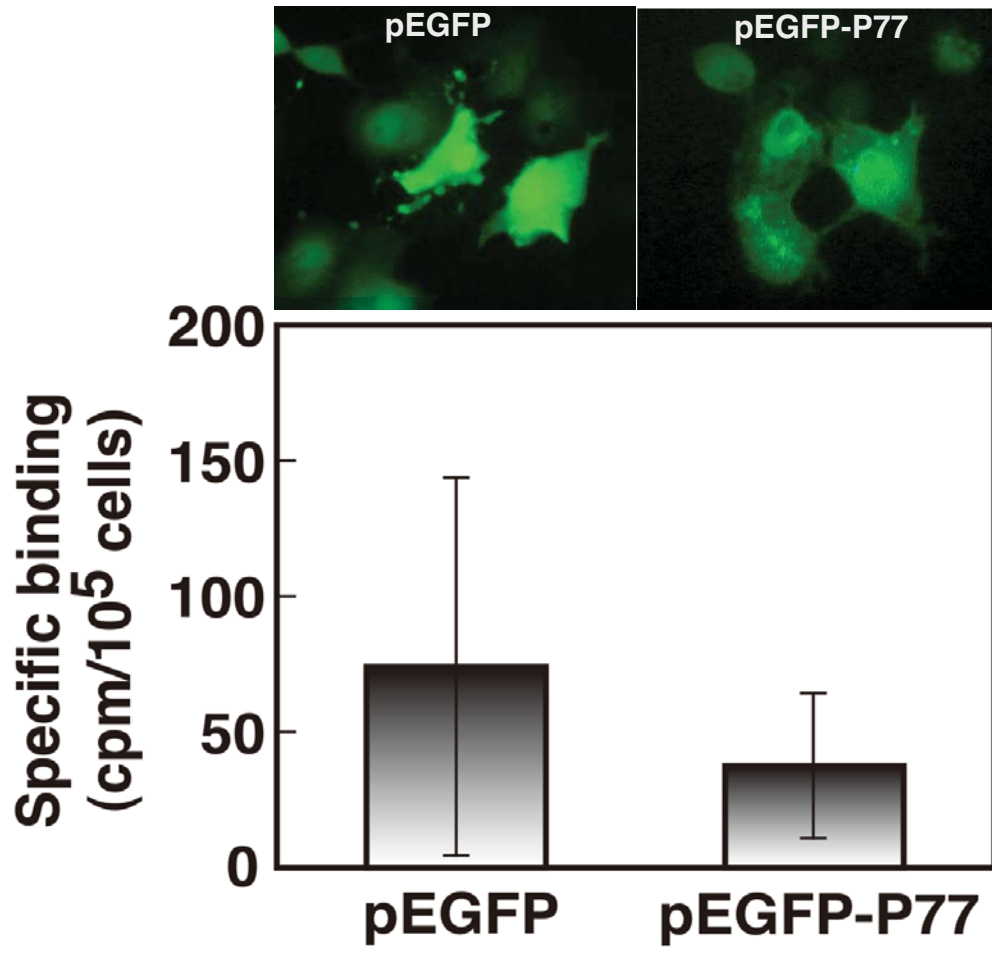
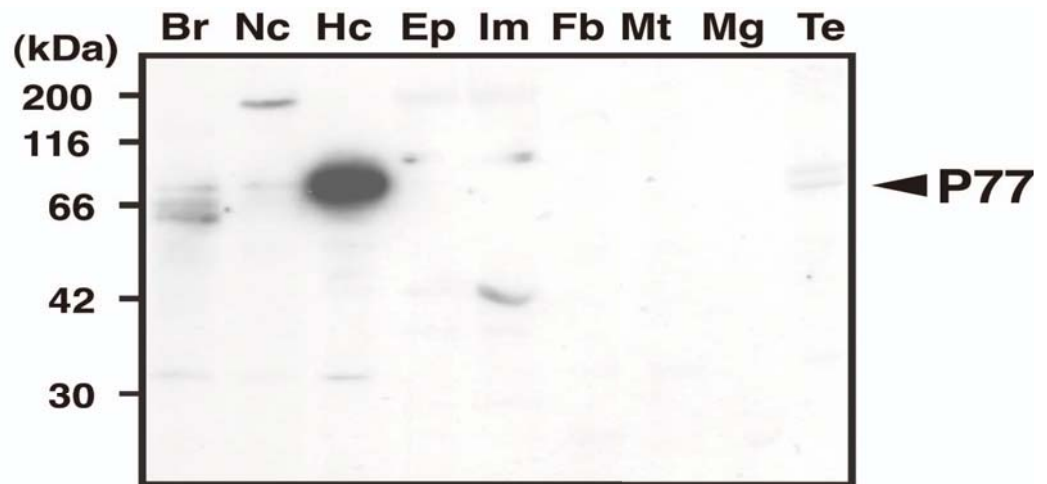
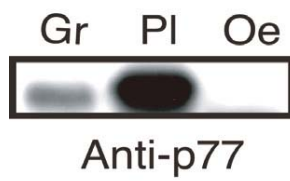


Fig. S5

**A**



**B**



**C**

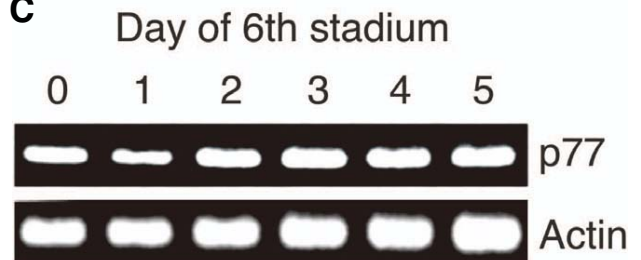


Fig. S6

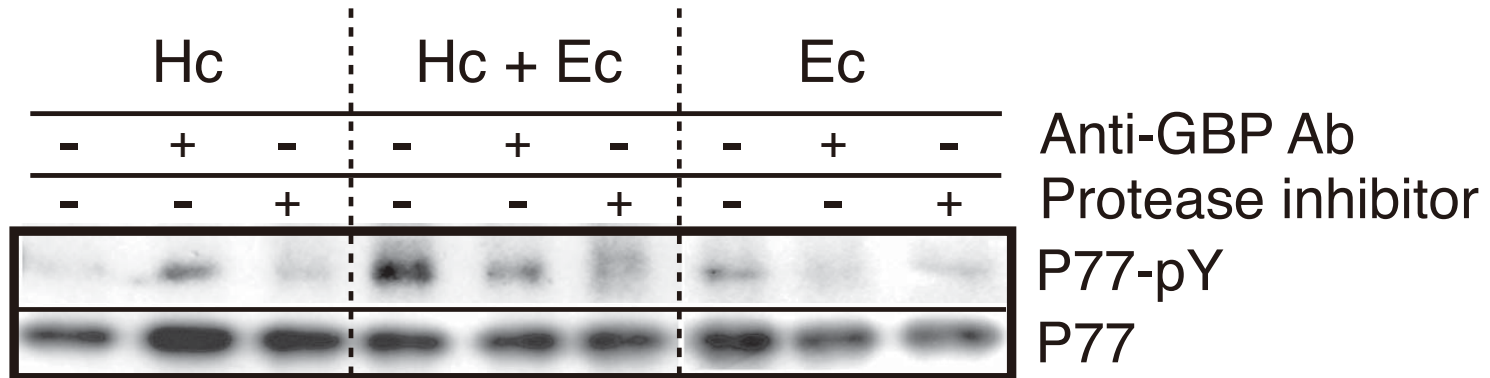


Fig. S7

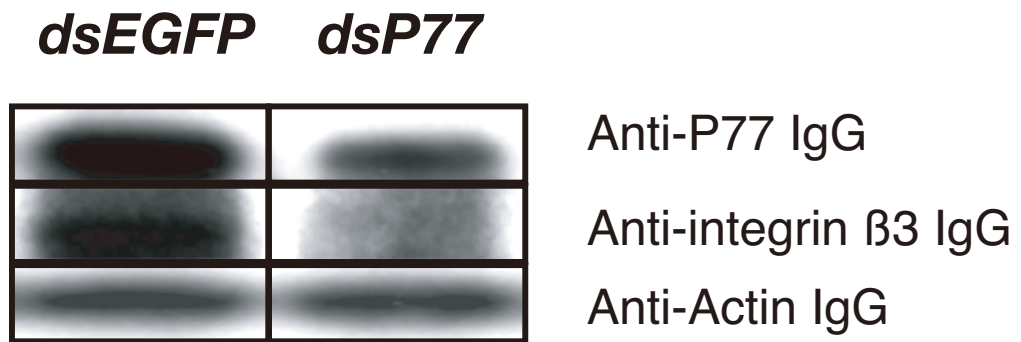


Fig. S8

