A Lipopolysaccharide- and β-1,3-Glucan-binding Protein from Hemocytes of the Freshwater Crayfish *Pacifastacus leniusculus*

**PURIFICATION, CHARACTERIZATION, AND cDNA CLONING**

(Received for publication, August 18, 1999, and in revised form, September 30, 1999)

So Young Lee, Ruigong Wang, and Kenneth Söderhäll†‡

From the Department of Comparative Physiology, Evolutionary Biology Center, Uppsala University, Norbyvägen 18A, S-75236, Uppsala, Sweden

A lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP) was isolated and characterized from blood cells (hemocytes) of the freshwater crayfish *Pacifastacus leniusculus*. The LGBP was purified by chromatography on Blue-Sepharose and phenyl-Sepharose, followed by Sephacryl S-200. The LGBP has a molecular mass of 36 kDa and 40 kDa on 10% SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions, respectively. The calculated mass of LGBP is 39,492 Da, which corresponds to the native size of LGBP; the estimated pI of the mature LGBP is 5.80. LGBP has binding activity to lipopolysaccharides as well as to β-1,3-glucans such as laminarin and curdlan, but peptidoglycan could not bind to LGBP. Cloning and sequencing of LGBP showed significant homology with several putative Gram-negative bacteria-binding proteins and β-1,3-glucanases. Interestingly, LGBP also has a structure and functions similar to those of the coelomic cytolytic factor-1, a lipopolysaccharide- and glucan-binding protein from the earthworm *Eisenia fetida*. To evaluate the involvement of LGBP in the phenoloxidase (proPO) activating system, a polyclonal antibody against LGBP was made and used for the inhibition of phenoloxidase (PO) activity triggered by the β-1,3-glucan laminarin in the hemocyte lysate of crayfish. The PO activity was blocked completely by the anti-LGBP antibody. Moreover, the PO activity could be recovered by the addition of purified LGBP. These results suggest that the 36-kDa LGBP plays a role in the activation of the proPO activating system in crayfish and thus seems to play an important role in the innate immune system of crayfish.

Vertebrates and invertebrates are capable of initiating several kinds of defense mechanisms after recognition of bacterial and fungal cell wall molecules, such as lipopolysaccharides (LPS),1 peptidoglycans, and β-1,3-glucans (1–3). In the case of humans, monocytes and macrophages respond to LPS by inducing the expression of cytokines, cell adhesion proteins, and enzymes involved in the production of small proinflammatory mediators. Under pathophysiological conditions, LPS exposure can lead to an often fatal syndrome known as septic shock (4).

Invertebrates lack antibodies and hence an adaptive immune response, and instead they have efficient innate immune systems to defend themselves against invading foreign materials (5). The defense system of invertebrates is based on both cellular and humoral immune responses (6). The former includes encapsulation (7–9), phagocytosis (10), and nodule formation (11). The clotting system of arthropods (12, 13), the synthesis of a broad spectrum of potent antimicrobial proteins (14), and crustaceans (16–18), and the prophenoloxidase activating system (proPO system) (2) belong to the last immune response. Moreover, the humoral immune response is also triggered by LPS or β-1,3-glucans (2, 19) as in vertebrates. Therefore, the proteins involved in the recognition of LPS, peptidoglycans, and β-1,3-glucans have been named pattern recognition proteins (20), and they are involved in various ways in the biological defense mechanisms in both invertebrates and vertebrates. Recently, LPS- (21–24) and/or β-1,3-glucan-binding proteins (25–28), peptidoglycan recognition proteins (29–31), lectins (32–34), and hemolin (35–37) have been found in several different species of invertebrates, and their function in the immune response has been studied. For instance, in the horseshoe crab *Tachypleus tridentatus*, LPS or β-1,3-glucans both bind specifically to pattern recognition proteins, and as a result the coagulation cascade is activated (24, 28). In addition, the opsonic effect (26) and degradation of blood cells (38) by the β-1,3-glucan-binding protein (β-GGBP) in the crayfish *Pacifastacus leniusculus*, the opsonic effect of the LPS-binding protein in the cockroach *Periplaneta americana* (39), and the hemocyte nodule formation by the LPS-binding protein in the silkworm *Bombyx mori* (11) have already been reported as special biological properties of pattern recognition proteins.

In particular, the proPO system is an important non-self-recognition system in invertebrates which can be activated by LPS or peptidoglycan from bacteria and β-1,3-glucans from fungi (2). Non-self-molecules are recognized by endogenous pattern recognition proteins and their receptors, and then they cause activation of the proPO system (2). The active form of proPO, phenoloxidase (PO), is produced by a serine proteinase known as the proPO-activating enzyme. Subsequently, PO oxidizes DOPA to dopaquinone, which is converted to melanin through several non-enzymatic steps. The generated PO plays an important role as it can melanize pathogens (2), sclerotize the cuticle (40), and heal wounds (41) in invertebrates. Since...
proPO was first cloned from the crayfish *P. leniusculus* (42), a large number of invertebrate proPOs have been structurally determined, and recently, the primary structure of proPO-activating enzyme has been reported from three different insects (43–45). However, so far only two groups have reported that a LPS- and β-1,3-glucan-binding protein (LGBP) from crayfish blood cells, its cDNA cloning, and its role in the proPO system of the crayfish *P. leniusculus*.

**EXPERIMENTAL PROCEDURES**

**Animals**—Freshwater crayfish, *P. leniusculus*, were purchased from Berga Kräftodling, Södermanland, Sweden, and kept in an aquarium with tap water at 10 °C. Only intermoult male crayfish were used in these experiments.

**Protein Purification**—Hemocyte lysate supernatant (HLS) was prepared by collecting hemolymph from 200 crayfish in anticoagulant buffer (0.1 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 50 mM NaCl, pH 7.4) (46). The hemocytes were spun down at 1,500 × g and 800 × g for 10 min, and then the hemocytes were homogenized with CAC buffer (10 mM sodium cacodylate, 0.1 M CaCl₂, pH 6.5). After centrifugation at 4 °C and 25,000 × g for 30 min, the supernatant was applied to a Blue-Sepharose CL-6B column (1 cm × 4 cm) equilibrated with CAC buffer. The flow-through containing LGBP was collected, and it was loaded to a phenyl-Sepharose CL-4B (1 cm × 4 cm) equilibrated with CAC buffer and washed with the same buffer. Bound proteins were eluted with 65% ethylene glycol in CAC buffer. The eluted proteins were concentrated on a Centricon concentration filter (Amicon, Inc.) to a final volume of 0.5 ml. As a final purification step, Sephacryl S-200 gel filtration column (0.8 × 100 cm) equilibrated with CAC buffer was used.

**Electrophoresis**—10% SDS-PAGE was carried out by the method of Laemmli (47). Samples were denatured by heating them for 4 min at 95 °C in 2% (w/v) SDS and 0.1% dithiothreitol, and then the gels were stained according to the method of Fairbank et al. (48). A low molecular mass calibration kit for electrophoresis (Amersham Pharmacia Biotech) was used for size markers: rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.4 kDa).

**Antibody and Immunoblotting**—The purified LGBP was separated by 10% SDS-PAGE under reducing conditions. After Coomassie Blue staining, a band corresponding to LGBP was excised and homogenized in phosphate-buffered saline. Antibody against LGBP was raised by three injections of 20 μg of purified LGBP each time with Freund’s adjuvant (complete for the first injection, incomplete for the other two injections). To purify the antibody to be used for immunoblotting experiment, the purified LGBP was electrophoresed and transferred onto a nitrocellulose membrane. The region of LGBP on the filter was excised and homogenized with nitrocellulose solution containing 10 mM Tris/HC1, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-200, 1% NaN₃, and 0.5% skim milk at 4 °C for 12 h with gentle shaking. The strip was washed carefully with nitrocellulose solution and subsequently cut into pieces. The antibody bound to LGBP was eluted at 0.2 ml glycine HCl, pH 2.8, and then the eluted antibody solution was neutralized immediately with 1 M KOH, and bovine serum albumin (BSA) was added to a final concentration of 0.1%. To purify the antibody for PO activity test, the anti-LGBP antisera was loaded to protein A-Sepharose CL-4B equilibrated with 50 mM Tris/HC1, pH 7.0, and washed with the same buffer. Bound anti-LGBP antibody was eluted at 0.1 ml glycine HCl, pH 2.8. The eluted anti-LGBP antibody was concentrated on a Centricon concentration filter.

For immunoblotting, the proteins were subjected to 10% SDS-PAGE and subsequently developed for partial amino acid sequencing by the following procedures.

**Determinations of Partial Amino Acid Sequences**—To determine the internal amino acid sequence of peptide fragments of LGBP, the protein was subjected to 10% SDS-PAGE under reducing conditions, stained with 0.2% Coomassie Blue in 50% methanol, and destained with 30% methanol. The band corresponding to LGBP was excised and treated with lysoendopeptidase according to Wilm et al. (49). The resulting digest was subjected to reverse phase high performance liquid chromatography (HPLC, Pharmacia Smart chromatography system) using a phase μRPC C₁₈/a column (MIC-15-03-MRP, Amersham Pharmacia Biotech). The HPLC was performed with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid for 75 min at a flow rate of 30 μl/min, and the most prominent peaks were sequenced using an Applied Biosystem 476A sequencer.

For determination of the NH₂-terminal amino acid sequence, the protein was electrotransferred onto a polyvinylidene difluoride membrane. The membrane was stained with Coomassie Blue, destained, washed with distilled water, and dried. The LGBP band was cut into small pieces and subjected to an Applied Biosysytem 476A automated protein sequencer for amino acid sequencing.

**Assay of LPS, Peptidoglycan, or β-1,3-Glucan Binding Activity of LGBP**—Fluorescein isothiocyanate-labeled LPS (smooth types) from *Salmonella abortus* (Sigma) and smooth types of LPS from *Escherichia coli* serotype 055:BS (Sigma) were used for LPS binding activity of LGBP. Microtiter 96-well plates were coated with purified LGBP (200 μl/well of 10 μg/ml in CAC buffer, pH 6.5) overnight at 4 °C. Excess binding sites were blocked with 1% BSA in CAC buffer at 37 °C for 2 h. The same concentration (10 μg) of BSA was used as control. After washing three times with CAC buffer, different doses of fluorescein isothiocyanate-labeled LPS were added in 100 μl of CAC buffer containing 0.1% BSA, incubated for 3 h at 37 °C, and then the plates were washed three times with CAC buffer. 100 μl/well 50 mM Tris/HC1 containing 50 mM NaCl, pH 8.5, was added for measuring the bound fluorescence using a fluorescence microplate reader (Wallac 1420 multilabel counter) at emission/excitation 485 nm/530 nm. This experiment was repeated twice with similar results.

In another method, curdian, a linear polymer of glucose units linked with β-1,3-linkages (Wako), laminarin, which consists of β-1,3-glucan chain with occasional β-1,6-linked glucose units (Calbiochem), peptidoglycan from *Staphylococcus aureus* (Fluka), or LPS of *E. coli* serotype 055:BS (Sigma) was used for testing binding activity to LGBP. 4 μg of purified LGBP was incubated with 100 μg of curdian, laminarin, LPS, or peptidoglycan in CAC buffer for 1 h at 4 °C. The supernatant was taken, and the pellets of curdian, laminarin, LPS, or peptidoglycan were washed three times with CAC buffer. The bound protein was eluted with 50 μl of SDS-PAGE sample loading buffer (60 mM Tris/HC1, pH 6.8, containing 2% SDS, 1% β-mercaptoethanol, 0.1% dithiothreitol, and 0.1% Triton X-100) and the supernatant was treated by heating at 95 °C for 4 min. The supernatant was treated by trichloroacetic acid for protein precipitation. The precipitated proteins were dissolved with 30 μl of SDS-PAGE sample loading buffer. To investigate LGBP binding activity to LPS, β-1,3-glucans, or peptidoglycan, the eluted proteins and the supernatant were applied to 10% SDS-PAGE and subsequently developed for immunoblotting using anti-LGBP antibody as a probe.

**cDNA Cloning of Crayfish LGBP**—Six pairs of nested degenerate primers were synthesized according to amino acid sequences of four lysyl endopeptidase-derived peptide fragments of LGBP. Two cDNA fragments, both coding for LGBP, were amplified by polymerase chain reaction from the crayfish hemocyte cDNA library using each combination of 12 degenerate primers, one of which was labeled with [α-32P]dCTP by random priming using the Megaprime labeling kit (Amersham Pharmacia Biotech) and was used as a probe to screen more LGBP-specific clones. From an initial screening of approximately 120,000 recombinants of the crayfish hemocyte cDNA library λ-phage resulted in more than 300 positive clones. The largest one that was identified by restriction enzyme digestion was cultured and amplified. After double-strand sequencing, the recombinant DNA was purified using the following DNA purification system (Promega). The insert was digested out by the restriction enzyme EcoRI (Amersham Pharmacia Biotech) and subcloned into EcoRI-digested plBluescript II (SK+) plasmid (Strategene). It was subsequently sequenced in double strands by an Applied Biosytems PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). The cDNA sequence was analyzed with the MacVector 1.4.1
Northern Blot Analysis—Total RNA was extracted from crayfish hemocytes by using Trizol LS reagent (Life Technologies) according to the manufacturer’s instructions. Approximately 20 μg of total RNA was fractionated on a 1% agarose gel in the presence of formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) by capillary blotting following standard procedures. The 0.24–9.5-kilobase RNA ladder (Life Technologies) was electrophoresed simultaneously and stained with ethidium bromide. The cDNA probes, spanned through fraction, containing LGBP, was then purified by hydrophobic chromatography on phenyl-Sepharose followed by gel filtration on Sephacryl S-200 (Fig. 1A). The purity of LGBP in the different fractions was ascertained by SDS-PAGE (Fig. 1B), immunoblotting used for localization of LGBP. The samples were prepared from crayfish hemocyte lysate supernatant, and plasma and was then analyzed by immunoblotting using an affinity-purified antibody against LGBP. Lane 1, 20 μg of hemocyte lysate supernatant; lane 2, 30 μg of plasma; lane 3, 1 μg of purified LGBP. The arrows indicate the position of the size marker proteins.

RESULTS

Purification of LGBP—LGBP was purified from crayfish hemocytes. The hemocyte lysate of crayfish was first subjected to a Blue-Sepharose column chromatography, and the flow-through fraction, containing LGBP, was then purified by hydrophobic chromatography on phenyl-Sepharose followed by gel filtration on Sephacryl S-200 (Fig. 1A). The purity of LGBP in the different fractions was ascertained by SDS-PAGE (Fig. 1B), immunoblotting used for localization of LGBP. The samples were prepared from crayfish hemocyte lysate supernatant, and plasma and was then analyzed by immunoblotting using an affinity-purified antibody against LGBP. Lane 1, 20 μg of hemocyte lysate supernatant; lane 2, 30 μg of plasma; lane 3, 1 μg of purified LGBP. The arrows indicate the position of the size marker proteins.

Characterization of LGBP—Anti-LGBP antibody was used to confirm the localization of LGBP in crayfish hemolymph using immunoblotting. Fig. 2C shows that the anti-LGBP antibody could recognize LGBP in hemocytes but not in plasma. This result suggests that LGBP exists only in the hemocytes.

Two different methods were used for testing the binding activity of purified LGBP to LPS, β-1,3-glucans, or peptidoglycan. First, to examine the LPS binding activity fluorescein isothiocyanate-labeled LPS was used, and 10 μg of purified LGBP was immobilized to microtiter plates. When the fluorescence intensity of LGBP was compared with that of control BSA fluorescence intensity, the binding activity of LGBP to LPS was gradually increased in a dose-dependent manner (Fig. 3). This result shows that LGBP exhibits LPS binding activity. In a second approach, the purified LGBP was incubated with curdian, laminarin, LPS, or peptidoglycan to demonstrate the binding activity of LGBP. Fig. 4 shows the result of an immunoblot of curdian, laminarin, LPS, and peptidoglycan binding activity of LGBP. However, the purified LGBP could not be found in the supernatants that were incubated with LPS, laminarin, or curdian, whereas the supernatant solution of peptidoglycan showed a 36-kDa LGBP band (lane 9). These results clearly show that LGBP has β-1,3-glucan binding activity as well as LPS binding activity, but it does not exhibit any peptidoglycan binding activity.

Nucleotide Sequence of LGBP cDNA and the Deduced Amino Acid Sequence—The NH₂-terminal and four internal amino acid sequences of LGBP were determined. The NH₂-terminal sequence commences at Val-16, and the internal amino acid sequences is underlined (Fig. 5). The internal amino acid sequence data of LGBP was used to design and synthesize degenerate primers. Two cDNA fragments, which were amplified by polymerase chain reaction from the crayfish hemocyte cDNA library, were sequenced and identified as LGBP-specific clones. More clones were isolated from the library using one of the LGBP-specific cDNA fragments as a probe. The largest clone was shown to code for the complete amino acid sequence of the crayfish LGBP. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 5. The cDNA has an open reading frame of 1083 nucleotides corresponding to 361 amino acid residues. The termination codon is followed by a long 3'-untranslated flanking region in the mRNA of LGBP (Fig. 5). It contains more than 17 tandem repeats of a 32-nucleotide sequence.
amino acid residues form a typical signal sequence. Therefore, the mature LGBP consists of 346 amino acid residues with a calculated molecular mass of the protein portion of 39,492 Da and a predicted isoelectric point of 5.80 for the mature protein. Two putative glycosylation sites (Asn-Xaa-Ser/Thr) for and a predicted isoelectric point of 5.80 for the mature protein. One short-linked carbohydrate chains are present in the mature protein.

Northern Blot Analysis—Northern blot analysis shows that the mRNA of LGBP was expressed constitutively as a single expression codon. Putative recognition motifs for known as a putative recognition site for 

Comparison of the Amino Acid Sequence of LGBP with Other Proteins—The amino acid sequence of the mature LGBP was compared with the sequences in the BLAST protein sequence search program (National Center Biotechnology International).

The cloning and sequence analysis revealed that LGBP shows significant amino acid homology with the Gram-negative bacteria-binding proteins and with bacterial glucanases. Alignment of the part of the LGBP sequence, from Ile-31 to Gln-361, reveals closest homology with 

The activation of proPO system is initiated by the recognition of microbial cell wall components such as LPS, peptidoglycans, or β-1,3-glucans. It was thus interesting to identify whether the LGBP participates in the proPO system. LGBP, BSA was incubated with LPS, curdlan, laminarin, and peptidoglycan was analyzed by immunoblotting using affinity-purified (SDS-PAGE sample loading buffer and subjected to 10% SDS-PAGE with trichloroacetic acid. The precipitated proteins were dissolved with 10% SDS-PAGE. The amino acid sequence of the mature LGBP was incubated with LPS, curdlan, laminarin, and peptidoglycan were eluted with purified LGBP was incubated with LPS, curdlan, laminarin, and peptidoglycan.

Involvement of LGBP in the Prophenoloxidase Activating System—The activation of proPO system is initiated by the recognition of microbial cell wall components such as LPS, peptidoglycans, or β-1,3-glucans. It was thus interesting to identify whether the LGBP participates in the proPO system. Hemocyte lysate of crayfish P. leniusculus by itself causes only low level of PO activity. However, as shown in Fig. 7, the PO activity in hemocyte lysate can be increased by the presence of triggering stimuli such as curdlan, laminarin, or LPS but not

Peptidoglycans, or β-1,3-glucans. It was thus interesting to identify whether the LGBP participates in the proPO system. Hemocyte lysate of crayfish P. leniusculus by itself causes only low level of PO activity. However, as shown in Fig. 7, the PO activity in hemocyte lysate can be increased by the presence of triggering stimuli such as curdlan, laminarin, or LPS but not

Involvement of LGBP in the Prophenoloxidase Activating System—The activation of proPO system is initiated by the recognition of microbial cell wall components such as LPS, peptidoglycans, or β-1,3-glucans. It was thus interesting to identify whether the LGBP participates in the proPO system. Hemocyte lysate of crayfish P. leniusculus by itself causes only low level of PO activity. However, as shown in Fig. 7, the PO activity in hemocyte lysate can be increased by the presence of triggering stimuli such as curdlan, laminarin, or LPS but not.
by peptidoglycan.

To evaluate the role of LGBP in the proPO system of crayfish blood cells, anti-LGBP antibody that had been purified by protein A column chromatography was incubated with hemocyte lysate of crayfish in a dose-dependent manner. The anti-LGBP antibody could inhibit the PO activity of hemocyte lysate significantly even in the presence of laminarin, but preimmune serum could not affect inhibition of PO activity at the same concentration as the anti-LGBP antibody (Fig. 8). The PO activity of hemocyte lysate that had been inhibited by anti-LGBP antibody could be recovered by the addition of purified LGBP in the presence of laminarin. However, laminarin itself and BSA could not recover the PO activity in this system (Fig. 9). These results indicate that LGBP is directly involved in the proPO system and plays a role as an initiator of the proPO system of the freshwater crayfish *P. leniusculus*.

**DISCUSSION**

One candidate for an immediate noninducible system in invertebrates is the proPO system, which has been shown to have a role in both recognition and defense. The specific activation of this system by LPS, peptidoglycans, or β-1,3-glucans but not by other carbohydrates has been described in several invertebrate animals. Several proteins, the so-called pattern recognition proteins (20), are capable of binding specifically to these carbohydrates, and subsequently they can induce activation of the proPO system (2). However, so far only coelomic cytolytic factor-1 (27) and the peptidoglycan-binding protein (30) of *B. mori* have been shown to be involved in the activation of the proPO system in their respective animals.

In the present investigation, we have purified a protein that has strong affinity to LPS and β-1,3-glucans, i.e., LGBP, from hemocytes, and which is involved in the proPO system of the freshwater crayfish *P. leniusculus*. LGBP has instead a primary structure that is different from that of factor G. LGBP has instead a structure that is different from that of factor G. LGBP has instead a structure that is different from that of factor G.
primary structure similar to that of coelomic cytolytic factor-1, the LPS- and glucan-binding protein involved in the activation of the proPO system of the earthworm *E. foetida*. Another invertebrate defense protein, the β-GBP from the crayfish *P. leniusculus* was found to contain a short sequence motif with similarity to the active site of bacterial β-1,3-1,4 glucanases (26). However, glucanase activity was not found in this protein, the β-GBP of the earthworm *E. foetida* (27), or the Gram-negative bacteria-binding protein of the silkworm *B. mori* (22). From these results, it is reasonable to suggest that these invertebrate proteins, containing the bacterial glucanase motif, have evolved from a common ancestral glucanase that has lost its glucanase activity, whereas the β-GBP of the cockroach *E. foetida* is present in vesicles in the hemocytes, and the plasma β-GBP after reaction with β-1,3-glucans binds to a specific hemocyte membrane receptor (58) which will cause an exocytotic release of the proPO system and LGBP. However, β-GBP itself or β-1,3-glucans do not affect the crayfish granular cells. Once the components of proPO system as well as LGBP have been released from the hemocytes, the LGBP reacted with β-1,3-glucans will cause activation of the released proPO system, and as a result several factors associated with the crayfish proPO system will gain their biological function such as for example peroxinectin (59) which will participate in the defense toward an invading pathogen.

**Acknowledgments**—We thank Ragnar Ajaxon and Anbar Khodabandeh for technical assistance.

**REFERENCES**

1. Rietschel, E., and Brade, H. (1992) *Sci. Am.* 267, 54–61
2. Soderhijl, K., and Cerevis, L. (1998) *Curr. Opin. Immunol.* 10, 23–28
3. Yang, R.-B., Mark, M. R., Gray, A., Huang, A., Xia, M. Z., Goddard, A., Wood, C. F., I., Gurney, A. L., and Godowski, P. J. (1998) *Nature* 395, 284–289
4. Parillo, J. E. (1993) *N Engl. J. Med.* 328, 1471–1477
5. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., Jr., and Ezekowitz, R. A. B. (1999) *Science* 284, 1313–1318
6. Smith, V. J., and Soderhijl, K. (1986) in *Immune Mechanisms in Invertebrate Vectors* (Luckie, A. M., ed) pp. 59–79, Oxford University Press, Oxford, U. K.
7. Kobayashi, M., Johannsson, M. W., and Soderhijl, K. (1990) *Cell Tissue Res.* 260, 13–18
8. Cho, M. Y., Lee, H. S., Lee, K. M., Homma, K.-I., Natori, S., and Lee, B. K. (1999) *Eur. J. Biochem.* 262, 737–744
9. Agsari, S., Theopold, U., Welfly, C., and Schmidt, O. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 3690–3695
10. Foukas, L. C., Katsoulas, H. L., Parakkasoupolu, N., Methenitii, A., Lambropoulou, M., and Marmara, V. J. (1998) *J. BioI. Chem.* 273, 14813–14818
11. Koizumi, N., Imamura, M., Kodotani, T., Yaii, K., Iwashana, H., and Sato, R. (1999) *FEBS Lett.* 443, 139–143
12. Iwagana, S., Kawabata, S.-A., and Muta, T. (1998) *Biochem. Tokyo* (123), 1–15
13. Hall, M., Wang, R., Antwerpens, R. V., Sottrup-Jensen, L., and Soderhijl, K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 1085–1079
14. Hoffmann, J. A., Reichhart, J.-M., and Hetru, C. (1996) *Curr. Opin. Immunol.* 8, 8–13
15. Bulet, P., Petru, C., Dimaroj, J.-L., and Hoffmann, D. (1999) *Dev. Comp. Immunol.* 23, 329–344
16. Destoumieux, D., Bulet, P., Loew, D., Van Dorselaer, A., Rodriguez, J., and Bachere, E. (1997) *J. BioI. Chem.* 272, 28398–28406
17. Schopp, D., Kemm, G. D., and Smith, V. J. (1999) *Eur. J. Biochem.* 259, 532–539
18. Kawano, K., Yoneya, T., Miyata, T., Yoshikawa, K., Tokunaga, F., Terada, Y., and Iwagana, S. (1990) *J. BioI. Chem.* 265, 15365–15367
19. Fearon, D. T., and Locksley, R. M. (1996) *Science* 272, 50–53
20. Medzhitov, R., and Janeway, C. A., Jr. (1997) *Cell* 91, 295–298
21. Komori, T., and Natori, S. (1991) *J. BioI. Chem.* 266, 13318–13323
22. Lee, W.-J., Lee, J. D., Krawchekno, V. V., Ulevitch, R. J., and Brys, P. T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 7888–7893
23. Koizumi, N., Morozumi, A., Imamura, M., Tanaka, E., Iwashana, H., and Sato, R. (1997) *Eur. J. Biochem.* 248, 211–224
24. Muta, T., Miyata, T., Misumi, Y., Tokunaga, F., Nakamur, T., Toh, Y., Ikebarya, Y., and Iwagana, S. (1991) *J. BioI. Chem.* 266, 6554–6561
25. Duvie, B., and Soderhijl, K. (1990) *J. BioI. Chem.* 265, 9327–9332
26. Ceninici, N., Li, D., Davidson, R. E., Helman, U., and Paladino, E. T., Iwagana, S., and Soderhijl, K. (1994) *J. BioI. Chem.* 269, 29462–29467
27. Beschin, A., Bilej, M., Hanssens, F., Raymarkers, J., Van Dyck, E., Revets, H., Breys, L., Gonzalez, J., De Baetselier, P., and Timmermans, M. (1998) *J. BioI. Chem.* 273, 24948–24954
28. Seki, N., Muta, T., Oda, T., Iwaki, D., Kuma, T., Miyata, T., and Iwagana, S. (1994) *J. BioI. Chem.* 269, 1370–1374
29. Kang, D., Lui, G., Lindstrom, I., Gelius, E., and Steiner, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 11854–11858
30. Yoshida, H., Kinoshita, K., and Ashida, M. (1996) *J. BioI. Chem.* 271, 13854–13860
31. Ochiinai, M., and Ashida, M. (1999) *J. BioI. Chem.* 274, 11854–11858
32. Inamori, K., Iwaki, D., Nagira, T., Iwagana, S., Arisaka, F., and Kawabata, S. (1999) *J. BioI. Chem.* 274, 3273–3278
33. Kawabata, S., and Iwagana, S. (1999) *Dev. Comp. Immunol.* 23, 391–400
34. Vasta, G. R., and O’Leary, N. (1999) *Biochem. Tokyo* (123), 1–15
35. Sun, S. C., Lindstrom, I., Boman, H. G., Faye, I., and Schmidt, O. (1990)
36. Su, X.-D., Gastinel, L. N., Vaughan, D. E., Faye, I., Poon, P., and Bjorkman, P. J. (1998) Science 281, 991–995
37. Mendez, H. L., and Faye, I. (1999) Dev. Comp. Immunol. 23, 359–374
38. Barracco, M. A., Duvic, B., and Soderhall, K. (1991) Cell Tiss. Res. 266, 491–497
39. Jomori, T., and Natori, S. (1992) FEBS Lett. 296, 283–286
40. Sugumaran, M. (1993) FEBS Lett. 295, 233–239
41. Lai-Fook, J. (1996) J. Insect Physiol. 12, 195–226
42. Aspán, A., Huang, T.-s., Cerenius, L., and Soderhall, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 939–943
43. Lee, S. Y., Cho, M. Y., Hyun, J. H., Lee, K. M., Homma, K., Natori, S., Kawahata, S. I., Iwanaga, S., and Lee, B. L. (1998) Eur. J. Biochem. 257, 615–621
44. Jiang, H., Wang, Y., and Kanost, M. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12220–12225
45. Sato, D., Horii, A., Ochiai, M., and Ashida, M. (1999) J. Biol. Chem. 274, 7441–7453
46. Leonard, C., Soderhall, K., and Ratcliffe, N. A. (1985) Insect Biochem. Mol. Biol. 15, 803–810
47. Laemmli, U. K. (1970) Nature 227, 680–685
48. Fairbank, G., Steck, T. L., and Wallach, D. L. H. (1971) Biochemistry 10, 2606–2617
49. Wilm, M., Shcherbunka, A., Houthaere, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996) Nature 380, 466–469
50. Aspán, A., and Soderhall, K. (1991) Insect Biochem. 21, 363–373
51. Bradford, M. (1976) Anal. Biochem. 2, 248–254
52. Ruoslahti, E. (1991) J. Clin. Invest. 87, 1–5
53. Bachman, E. S., and McClay, D. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6808–6813
54. Dimopoulos, G., Richman, A., Muller, H.-M., and Kafatos, F. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11508–11513
55. Shin, S. W., Park, S. S., Park, D. S., Kim, M. G., Kim, S. C., Brey, P. T., and Park, H. Y. (1998) Insect Biochem. Mol. Biol. 28, 827–837
56. Yahata, M., Watanabe, T., Nakamura, Y., Yamamota, Y., Kamimiy, S., and Tanaka, H. (1990) Gene (Amst.) 86, 113–117
57. Muta, T., Seki, N., Takaki, Y., Hashimoto, R., Oda, T., Iwanaga, A., Tokunaga, F., and Iwanaga, S. (1995) J. Biol. Chem. 270, 892–897
58. Duvic, B., and Soderhall, K. (1992) Eur. J. Biochem. 207, 223–228
59. Johansson, M. W., Holmlad, T., Thoreqvist, P.-O., Cammarata, M., Parinello, N., and Soderhall, K. (1999) J. Cell Sci. 112, 917–925