An Insect Multiligand Recognition Protein Functions as an Opsonin for the Phagocytosis of Microorganisms*

Chong Han Kim‡, Yong Pyo Shin‡, Mi Young Noh‡, Yong Hun Jo‡, Yeon Soo Han‡, Yeon Sun Seong‡, and In Hee Lee‡1

From the ‡Department of Biotechnology, Hoseo University, Asan City, Chungnam 336-795, South Korea, the §Department of Agricultural Biology, College of Agriculture and Life Science, Chonnam National University, Gwangju 500-257, South Korea, and the ¶Department of Biochemistry, College of Medicine, Dankook University, Cheonan, Chungnam 330-714, South Korea

Received for publication, April 16, 2010, and in revised form, May 26, 2010. Published, JBC Papers in Press, June 2, 2010, DOI 10.1074/jbc.M110.134940

We characterize a novel pathogen recognition protein obtained from the lepidopteran Galleria mellonella. This protein recognizes Escherichia coli, Micrococcus luteus, and Candida albicans via specific binding to lipopolysaccharides, lipoteichoic acid, and β-1,3-glucan, respectively. As a multiligand receptor capable of coping with a broad variety of invading pathogens, it is constitutively produced in the fat body, midgut, and integument but not in the hemocytes and is secreted into the hemolymph. The protein was confirmed to be relevant to cellular immune response and to further function as an opsonin that promotes the uptake of invading microorganisms into hemocytes. Our data reveal that the mechanism by which a multiligand receptor recognizes microorganisms contributes substantially to their phagocytosis by hemocytes. A better understanding of an opsonin with the required repertoire for detecting diverse invaders might provide us with critical insights into the mechanisms underlying insect phagocytosis.

Over the past three decades, insect immunity has been studied extensively with a variety of insects, including flies, mosquitoes, and moths. The insect immune system consists of two arms that operate upon the invasion of microorganisms into the hemocoel: (i) a humoral system resulting in the production of soluble antimicrobial peptides (1), melanin formation, and clotting via the activation of the prophenoloxidase cascade (2) and (ii) a cellular system based on hemocytes that is involved in defense reactions, including phagocytosis, nodulation, and encapsulation (3–5). These immune responses are triggered by the specific recognition of microorganisms by host proteins referred to as pattern recognition receptors (PRRs),2 which are capable of binding to a variety of ligands, including lipopolysaccharides (LPS), lipoteichoic acid (LTA), peptidoglycan, and β-1,3-glucan (6–9), which are cell wall components of three major groups of microorganisms: Gram-negative and Gram-positive bacteria and fungi. These PRRs have principally been detected as soluble proteins in hemolymph and surface proteins on hemocytes.

The insect cellular responses primarily perform a function in the clearance of foreign invaders from hemolymph at the early phase of infection (10). Among three cellular immune reactions, phagocytosis is a highly conserved process that is known to occur in all metazoan phyla. This process is mediated by blood cells that either directly recognize the microorganisms or recognize targets that are coated in opsonic molecules. Thus far, insect phagocytosis has also generally been considered an essential immune reaction as it is in vertebrates (11). Nonetheless, phagocytosis is not currently as completely understood as other insect immune mechanisms. In particular, very little information has been discovered relevant to the phagocytic opsonins present in insect hemolymph, although several membrane receptors for phagocytosis have been identified.

In this study, we have purified a new protein with molecular mass of 9 kDa from the hemolymph of the Galleria mellonella larvae. In several respects, including the amino acid sequence homology, it was determined that the protein was quite similar to a cationic protein with mass of 9 kDa isolated from hemolymph of Manduca sexta (12). This Manduca protein was named cationic protein 8 (CP8) as its amino acid sequence was homologous to a small CP8 that had been found previously in Bombyx mori (GenBank accession number AY655143) and Bombyx mandarina (GenBank accession number EF126182). Therefore, our protein is referred to as GmCP8 (G. mellonella CP8) in this study. GmCP8 evidenced a marked binding activity to LPS, LTA, and β-1,3-glucan. As a multiligand receptor, the protein was shown to be capable of recognizing the three microorganisms selected in this study: Escherichia coli, Micrococcus luteus, and Candida albicans. Subsequently, we attempted to ascertain which immune responses were caused by the binding of the protein to microorganisms. According to the results of our in vitro and in vivo experiments, it was determined that GmCP8 had opsonin activity for the phagocytosis of the three microorganisms by hemocytes. A better understanding of this novel hemolymph protein, which was identified as both a multiligand receptor and a phagocytic opsonin, might provide us with crucial insights into a cellular defense system exploited by insects to deal with a broad variety of invaders.
New Insect Opsonin for Phagocytosis of Microorganisms

EXPERIMENTAL PROCEDURES

Insects and Preparation of Cell-free Hemolymph and Hemocytes—G. mellonella larvae were cultivated on an artificial diet at 30 °C in a dark incubator (13). Hemolymph was collected directly from the cut prolegs of the larvae into sterile tubes containing a few crystals of phenylthiourea. After the centrifugation of the sample at 10,000 × g for 10 min at 4 °C, the cell-free hemolymph, hereafter designated as plasma, was utilized immediately or stored at −70 °C. To prepare the hemocytes, hemolymph was collected in a chilled tube containing 1 ml of anticoagulant buffer (93 mM NaCl, 0.1M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, and a few crystals of phenylthiourea, pH 4.6). The hemolymph was subsequently centrifuged at 180 × g for 5 min at 4 °C. The sediments containing the hemocytes were then washed twice in Grace’s insect medium (GIM; Sigma). Isolated hemocytes were used immediately for experiments, although they were confirmed to be more than 90% viable at 4 h after breeding.

Purification of GmCP8 and Production of Anti-GmCP8 Antibody—In our previous study, GmCP8 was isolated as one of three fungus-binding proteins from the plasma of G. mellonella larvae (14). GmCP8, which was referred to as fungus-binding protein 1 in the previous study, was injected into New Zealand White rabbits to generate an anti-GmCP8 Ab in accordance with standard protocols. In this study, the anti-GmCP8 Ab was used to trace GmCP8 in the purification procedures. The purification process, which consisted of a three-stage procedure, permitted us to acquire a sizable quantity of GmCP8 from the G. mellonella plasma. In brief, plasma (10 ml) was mixed with an equal volume of 10% acetic acid and agitated overnight at 4 °C. After 30 min of centrifugation at 10,000 × g, the supernatant was removed and loaded onto a Sephadex G-50 gel filtration column equilibrated with 5% acetic acid. Fractions were eluted at a flow rate of 6 ml/h and collected at 20-min intervals. Every third fraction was analyzed via SDS-PAGE and immunoblotted with anti-GmCP8 Ab. The GmCP8-containing fractions were then pooled and applied to a C4 reversed-phase HPLC column (Vydac 214TP54). After washing the column with water containing 0.1% trifluoroacetic acid for 10 min, fractions were eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 0.5 ml/min. The purified GmCP8 was then subjected to Tricine SDS-PAGE and acid-urea-PAGE analyses to confirm purity. The molecular mass of the purified GmCP8 was determined via matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry at the Korea Basic Science Institute.

Quantitative Reverse Transcription-PCR and Tissue Culture—To evaluate the tissue specificity of GmCP8 expression, the non-immunized or immunized larvae were dissected in accordance with a predetermined time schedule. For the immunization of insects, 5 × 10⁵ cells of log phase E. coli were injected into each of the larvae. The fat body, midgut, and integument were collected and rinsed repeatedly with insect Ringer’s solution (IRS; 2.3 mM NaHCO₃, 128 mM NaCl, 1.3 mM KCl, and 1.8 mM CaCl₂, pH 6.2). In all cases, total RNA was isolated, and GmCP8 expression was analyzed immediately in the collected tissues. Total RNA samples were prepared with an RNA extraction kit (SV Total RNA Isolation System, Promega, Madison, WI). Similarly, the fat body and the hemocyte RNA samples were also isolated at predetermined times following immunization. First strand cDNA was synthesized with total RNA (1–2 μg), oligo(dT)₁₈, and Moloney murine leukemia virus reverse transcriptase (200 units; Bioneer, Daejon, Korea) at 42 °C for 1 h. G. mellonella actin transcripts were used as an internal standard for the normalization of the cDNA templates. Relative levels of GmCP8 cDNA in the samples were measured via semi-quantitative PCR using two primers corresponding to the reverse complement of nucleotides (dashed-underlined arrows 42 °C, 1 min; and 72 °C, 1 min. The cycle numbers were chosen to yield comparable band intensities while preventing saturation. For the tissue cultures, the fat body, midgut, and integument were prepared in accordance with the same procedure as described above. The dissected tissues were rinsed twice in GIM, and −250 mg of each tissue was transferred to separate wells of 12-well tissue culture plates (Nunclon), each of which contained 1 ml of the same medium supplemented with 100 units/ml penicillin G and 100 μg/ml streptomycin (Sigma). In the case of the hemocyte sample, ~2 × 10⁶ cells from 10 larvae were utilized for each culture. The cultures were incubated for 16 h at 30 °C with shaking at 100 rpm. The supernatants of each sample were collected via centrifugation and then dialyzed with a 1,000 molecular weight cutoff dialysis bag (Spectrum) in 50 mM Tris-Cl buffer, pH 7.0 for 16 h. Additionally, the protein extracts of tissues were prepared in SDS-PAGE sample buffer (1:1 Tris, 10% SDS, and 21% glycerol, pH 8.8) and homogenized with a pestle or by pipetting. Following a brief centrifugation, all protein samples were analyzed via immunoblot assays using anti-GmCP8 Ab.

Assays for Binding of GmCP8 to Microorganisms and Microrganismal Surface Molecules—1 × 10⁷ formaldehyde-fixed bacteria (E. coli and M. luteus) or C. albicans were resuspended in plasma diluted 3-fold with IRS. The mixture was incubated for 2 h with gentle shaking at 30 °C and then centrifuged for 10 min at 2,000 × g at 4 °C. The supernatant was removed, and cells in the sediment were washed three times in phosphate-buffered saline (PBS; 1.47 mM KH₂PO₄, 10 mM Na₂HPO₄, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) containing 0.02% Tween 20. The bound proteins were subsequently eluted with 100 μl of SDS-PAGE sample buffer. After 10 min of centrifugation at 2,000 × g at 4 °C, the supernatants were subjected to Tricine-SDS-PAGE analysis. Duplicate gels were used for immunoblot assays using anti-GmCP8 Ab. In an effort to assess binding activity to polysaccharides, 2 μg of GmCP8 was incubated for 2 h with 2 mg of peptidoglycan (Fluka, 53243), curdlan (insoluble polymer of β-1,3-glucan; Sigma, C7821), or cellulose (insoluble polymer of β-1,4-glucan; Sigma, S5504) in 100 μl of IRS containing 1% bovine serum albumin at 30 °C. Each sample was then centrifuged for 10 min at 10,000 × g at 4 °C. The sediment was washed three times with PBS containing 0.02% Tween 20 and then diluted 3-fold with Tricine-SDS-PAGE sample buffer. After a brief centrifugation, the supernatants were analyzed via immunoblot assay. Additionally, the specific binding activity of GmCP8 to LPS (Sigma, L2630) or LTA (Sigma, L2515) was determined via a dansyl polymyxin (DPX) displacement assay (15). In this test, polymyxin B (Sigma, P0972) and apoli-
New Insect Opsonin for Phagocytosis of Microorganisms

Assays for Microbicidal Activity of Hemocytes—Each sample of two log phase bacteria (1 × 10⁸ cells) or C. albicans (1 × 10⁷ cells) was incubated for 2 h in 100 µl of IRS containing GmCP8 (100 µg) or plasma (10 µl) as a positive control at 30 °C in a shaking incubator. After two washes with IRS, 1 µl of each sample was mixed with 99 µl of GIM, and 10-µl portions were then incubated with GmCP8 or C. albicans for 1.5 h at 30 °C. The slides were washed three times in PBS and stained with TO-PRO-3 iodide (Invitrogen) as a control sample. The slides were then incubated with anti-GmCP8 Ab diluted to 1:500 in 1% bovine serum albumin in PBS for 1.5 h at 30 °C. The samples were washed three times in PBS and incubated for 1 h with a fluorescein rhodamine-conjugated goat anti-rabbit IgG Ab (Alexa Flour 546 goat anti-rabbit, Invitrogen) diluted 1:300 in 1% bovine serum albumin in PBS. After treatment with the secondary Ab, the samples were again washed three times in PBS and stained with TO-PRO-3 iodide (Invitrogen) to visualize the nuclei of the hemocytes. For each of the samples, 10 sequential optical sections of 1 µm each were collected, and only one section is shown.

RESULTS

Purification of GmCP8 and Its Amino Acid Sequence—Via a three-step procedure consisting of acid extraction, gel permeation chromatography, and reverse phase HPLC, GmCP8 was purified from the plasma of G. mellonella larvae. As shown in Fig. 2A, GmCP8 was eluted in a symmetric HPLC peak. Fig. 1, B and C, show the purified GmCP8 on the Tricine-SDS-PAGE and acid-urea-PAGE gels, respectively. As the protein appeared in two discrete bands on the acid-urea-PAGE gel, it was assumed that GmCP8 might occur in two isoforms, namely an amidated and an acidic C-terminal form. The results of MALDI analysis showed two mass peaks with a difference of 58 Da, which corresponds to the mass of glycine (Fig. 1D). The full-length cDNA encoding GmCP8 was cloned from the larval fat body using a combination of reverse transcription-PCR and 5′-rapid amplification of cDNA ends-PCR (supplemental Fig. S1). The observed cDNA structure indicated that it comprised 87 amino acids with a C-terminal glycine, which has been observed frequently in the cDNA structures of other proteins harboring an amidated C terminus (18). It was also noted that GmCP8 harbored six intradisulfide bonds because it contained 12 cysteine residues (Fig. 1E). Overall, the calculated
New Insect Opsonin for Phagocytosis of Microorganisms

**FIGURE 1.** Purification and characterization of GmCP8 from plasma of *G. mellonella* larvae. *A*, GmCP8-enriched fractions after gel permeation chromatography were pooled and subjected to C4 reverse phase HPLC. The arrow indicates the peak containing GmCP8. *B*, Tricine-SDS-PAGE and immunoblot analysis. *Left panel*, a Tricine-SDS-PAGE gel stained with Coomassie Blue; *right panel*, immunoblot analysis with anti-GmCP8 Ab. Lane 1, plasma; lane 2, purified GmCP8. *C*, acid-urea-PAGE and immunoblot analysis. *Left panel*, an acid-urea PAGE gel stained with Coomassie Blue; *right panel*, immunoblot analysis with anti-GmCP8 Ab. Lane 1, acid extract of plasma; lane 2, purified GmCP8. *D*, MALDI mass analysis for GmCP8. Two major peaks appeared at m/z values of 9,170.86 and 9,228.73 Da, respectively. *E*, the amino acid sequences of mature GmCP8. Asterisks (*) indicate the 12 Cys residues. The C-terminal glycine residue is indicated in parentheses as it participated in the formation of C-terminal amide in one of two isoforms.

Production of GmCP8 in Insect Tissues—The sites of GmCP8 synthesis were identified via immunoblotting and quantitative reverse transcription-PCR analyses. Fig. 2A shows an SDS-PAGE analysis for proteins extracted from the hemocytes, fat body, midgut, and integument (left panel). In the immunoblotting assay, the GmCP8 band was detected only in the fat body extract (right panel). However, GmCP8 was detected in the culture supernatants of three tissues: fat body, midgut, and integument (Fig. 2B). Therefore, it was determined that GmCP8 was synthesized in these three tissues and secreted into the hemolymph. It is worth noting that GmCP8 was not generated in the hemocytes. Consistent with these results, reverse transcription-PCR analysis showed that the GmCP8-encoded gene was expressed in three tissues but not in the hemocytes (Fig. 2C).

**FIGURE 2.** Production of GmCP8 in *G. mellonella*. The same amounts of proteins (60 μg) extracted from each tissue (A) and proteins (60 μg) of tissue culture supernatant (B) were subjected to Tricine-SDS-PAGE analysis (left panel), and each duplicate gel was utilized for immunoblot assays (right panel) using anti-GmCP8 Ab. *CP8, 1 μg of GmCP8; Pl, plasma; Hc, hemocyte; Fb, fat body; Mg, midgut; It, integument.* C, tissue-specific expression of GmCP8 mRNA. The GmCP8 mRNA was detected in the three tissues but not in the hemocytes. *D*, time course analysis of GmCP8 mRNA expression after *E. coli* injection. The GmCP8 mRNA was detected at constant levels in the fat body but not in the hemocytes. Corresponding actin bands for these samples are shown in the lower panel.
New Insect Opsonin for Phagocytosis of Microorganisms

binding of DPX to LPS or LTA was inhibited by GmCP8 in a dose-dependent fashion (Fig. 3B). The inhibitory effects of GmCP8 on the binding of DPX to LPS and LTA were similar to or stronger than those of apoLp-III, which was shown previously to bind to LPS and LTA (20, 21). It was therefore concluded that GmCP8 could recognize E. coli and M. luteus via specific binding to LPS or LTA. Additionally, the specific binding of GmCP8 to fungal cell wall components was assayed using insoluble polymers of different polysaccharides (Fig. 3C).

As a result, GmCP8 was shown to bind to curdlan (polymer of \( \beta-1,3\)-glucan) but not to peptidoglycan or cellulose (polymer of \( \beta-1,4\)-glucan). It was also concluded that the recognition of C. albicans by GmCP8 was mediated by its specific binding to \( \beta-1,3\)-glucan. Additionally, the specific microbial recognition of GmCP8 was confirmed by determining whether or not GmCP8 was capable of binding to the microbial surface after its incubation with each of three cell wall components. As a result, it was determined that each ligand substantially inhibited the binding of GmCP8 to the microbial surface (Fig. 3D).

Antimicrobial Effect of G. mellonella Hemocytes Mediated by GmCP8—Fig. 4A shows the microbicidal effects of hemocytes against three microorganisms under different conditions. When hemocytes were incubated directly with three microorganisms for 1.5 h, no antimicrobial activity of hemocytes was detected. In contrast, the hemocytes evidenced marked microbicidal activity against three microorganisms that had been pretreated with plasma. These results showed that G. mellonella hemocytes could not mount their defense reactions against invading microorganisms without the aid of plasma components. It was also determined that hemocytes exerted their antimicrobial effects against three microorganisms coated with purified GmCP8, thereby indicating that GmCP8 performed a critical function in the cellular defense reactions of G. mellonella. In the following experiment, we attempted to determine whether or not GmCP8 was involved in nodule formation via an in vitro hemocyte aggregation assay.

Our results showed that, in contrast to the two control samples prepared with plasma and apoLp-III, hemocyte aggregation was not observed when the hemocytes were incubated with microorganisms pre-treated with GmCP8 (Fig. 4B).

Role of GmCP8 in Phagocytosis of Microorganisms by G. mellonella Hemocytes—As GmCP8 was determined not to be involved in nodule formation, which was one of two cellular immune responses that might occur in response to the invasion of microorganisms into the
hemocytes, we evaluated its possible role in the phagocytosis of microorganisms by hemocytes. First, we attempted to determine whether or not the binding of GmCP8 to three types of microorganisms could opsonize them for phagocytosis. Fig. 5 shows the fluorescence microscopic and flow cytometric analyses for the phagocytosis of the three microorganisms by G. mellonella hemocytes. The results showed that the GmCP8-treated microorganisms were internalized into the hemocytes to a substantially greater extent than were the untreated microorganisms. To further assess the function of GmCP8 as a molecular bridge between microorganisms and hemocytes, we evaluated the inhibitory effects of three soluble ligands (LPS, LTA, and laminarin) on the opsonophagocytic activity of GmCP8. As a consequence, the treatment of microorganisms with GmCP8 preincubated with each ligand did not augment the uptake of the corresponding microorganism by hemocytes. As shown in all FACS data in Fig. 5, peak c in the three graphs overlapped almost completely with peak a for the control samples, which were not treated with GmCP8. These results clearly revealed that the opsonin activity of GmCP8 was attributable to its specific binding to the surfaces of microorganisms. Furthermore, the GmCP8-mediated phagocytosis noted in the hemocoel was examined in greater detail under in vivo conditions. Each of three FITC-labeled microorganisms was injected into the hemocoel of live G. mellonella larvae, and then the hemocytes collected from the insects were subjected directly to confocal microscopic analysis. As shown in Fig. 6, each microorganism was observed around the nucleus of hemocytes, and its FITC fluorescence was shown to overlap with the rhodamine fluorescence of the secondary Ab attached to anti-GmCP8 Ab in the case of E. coli or enclosed by rhodamine fluorescence in the cases of M. luteus and C. albicans. Considering that GmCP8 was not generated in the hemocytes, it became apparent that GmCP8 was internalized into the hemocytes from the hemolymph while bound to the surfaces of microorganisms. Collectively, our results demonstrated that invading microorganisms were specifically bound to GmCP8 occurring in the hemolymph and were then subjected to GmCP8-mediated phagocytosis by the hemocytes.

**DISCUSSION**

Insects have become a preferred system for the study of certain aspects of innate immunity as insects can defend themselves against microbial pathogens without an adaptive immune system. Whereas the fruit fly and the mosquito are useful and genetically tractable models that permit the rapid identification of novel immune factors, lepidopteran insects are frequently utilized because their large size makes them suitable for biochemical experiments for the analysis of immune mechanisms. Because insects lack the specificity of microbial recognition achievable with an adaptive system, they generate a variety of PRRs to cope with a very diverse range of microbial pathogens and also utilize a combination of several PRRs, thereby expanding their ability to detect invaders (22, 23). In this study, we have purified a new multiligand receptor from the plasma of G. mellonella larvae and have also demonstrated its immunological role in host-pathogen interactions using the purified protein. GmCP8 evidenced potent microbial binding and phagocytosis-promoting activities, clearly indicating this protein as an opsonin for the phagocytosis of the three microorganisms selected in this study.

As is the case in higher vertebrates, phagocytosis is believed to be an essential cellular process in insect immune responses...
New Insect Opsonin for Phagocytosis of Microorganisms

against invading microorganisms. Insect phagocytosis is also mediated by the binding of PRRs to the surfaces of microorganisms (10, 11). Thus far, four main classes of PRRs associated with insect phagocytosis have been detected in the form of soluble proteins secreted from immune cells and membrane proteins attached to hemocytes, which interact with their cognate ligands expressed on the surfaces of Gram-negative and Gram-positive bacteria as well as fungi (11, 24). (i) A group of thioester-containing proteins (TEPs) are the best characterized phagocytic opsonins that have been detected in Drosophila melanogaster and Anopheles gambiae (25, 26). It has also been noted that a variety of TEPs appear to be involved in the recognition and phagocytosis of different microorganisms: TEPII for E. coli, TEPIII for Staphylococcus aureus, and TEPVI for C. albicans (27). (ii) The scavenger receptor families comprise structurally unrelated transmembrane proteins that participate in the recognition of polyanionic ligands. They have been recognized as PRRs crucial for phagocytosis in many species, including mammals and insects (28). The insect scavenger receptor families are expanded to cope with a broad variety of microorganisms as each member has only a limited repertoire for microbial recognition (29, 30). (iii) Down syndrome cell adhesion molecule (Dscam) is the immunoglobulin superfamily receptor previously identified as a potential phagocytic membrane protein and opsonin in D. melanogaster and A. gambiae this is reminiscent of mammalian immunoglobulin (31, 32). Therefore, it seems plausible that Dscam is an insect phagocytic PRR with the required repertoire to cope with a broad range of pathogens. (iv) Two novel phagocytosis-mediated transmembrane receptors, Eater and Nimrod C1 (33, 34), have been previously identified in D. melanogaster. Whereas Eater was well defined as a phagocytic receptor that promotes bacteria binding and uptake, it remains to be clarified whether Nimrod C1 can recognize bacteria directly, although it has been demonstrated that the protein was capable of contributing to the phagocytosis of E. coli (34).

As described under “Results,” the amino acid sequence of GmCP8 was shown to be homologous with those of ISPI-1 from G. mellonella, CP8 from M. sexta, and AmFPI-1 from A. mylitta (supplemental Fig. 52). Regarding their physiological functions, it has been reported that ISPI-1 and AmFPI-1 are involved in an insect defense mechanism as an inhibitor to regulate the proteolytic activity of a variety of proteases, including fungal protease (13, 19), and Manduca CP8 with no inhibitor activity contributed to the activation of prophenoloxidase cascade in the hemolymph (12). However, it remains to be determined whether those three proteins also exhibit the same microbial binding and/or phagocytic activity as GmCP8. On the other hand, it was postulated that GmCP8 might be functionally similar to the CP8 from M. sexta rather than the other two proteins (ISPI-1 and AmFPI-1) in that it evidenced no activity as a protease inhibitor (data not shown), and its production was not inducible upon microbial infection as shown in Fig. 2D. Additionally, as in the case of Manduca CP8, GmCP8 was not generated in the hemocytes, which allowed us to confirm its opsonin activity via the direct injection of microorganisms into the hemocoels of live insects.

Thus far, there has been only minimal information regarding insect phagocytic opsonin as compared with other immune fac-

REFERENCES

1. Boman, H. G. (1998) Scand. J. Immunol. 48, 15–25
2. Nagai, T., and Kawabata, S. (2000) J. Biol. Chem. 275, 29264–29267
3. Watanabe, A., Miyazawa, S., Kitami, M., Tabunoki, H., Ueda, K., and Sato, R. (2006) J. Immunol. 177, 4594–4604
4. Whitten, M. M., Tew, I. F., Lee, B. L., and Ratcliffe, N. A. (2004) J. Immunol. 172, 2177–2185
5. Lemaitre, B., and Hoffmann, J. (2007) Annu. Rev. Immunol. 25, 697–743
6. Yu, X. Q., and Kanost, M. R. (2000) J. Biol. Chem. 275, 37373–37381
7. Yu, X. Q., and Kanost, M. R. (2002) Eur. J. Biochem. 269, 1827–1834
8. Yoshida, H., Kinoshiba, K., and Ashida, M. (1996) J. Biol. Chem. 271, 13854–13860
9. Wang, X., Rocheleau, T. A., Fuchs, J. F., and Christensen, B. M. (2006) Cell. Microbiol. 8, 1581–1590
10. Hoffmann, J. A., Kaufatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999) Science 284, 1313–1318
11. Stuart, L. M., and Ezekowitz, R. A. (2008) Nat. Rev. Immunol. 8, 131–141
12. Ling, E., Rao, X. J., Ao, J. Q., and Yu, X. Q. (2009) Insect Biochem. Mol. Biol. 39, 263–271
13. Fröbius, A. C., Kanost, M. R., Götz, P., and Vilcinskas, A. (2000) Eur. J. Biochem. 267, 2046–2053
14. Lee, Y. S., Yun, E. K., Jang, W. S., Kim, I., Lee, J. H., Park, S. Y., Ryu, K. S., Seo, S. J., Kim, C. H., and Lee, I. H. (2004) Insect Mol. Biol. 13, 65–72
15. Scott, M. G., Gold, M. R., and Hancock, R. E. (1999) Infect. Immun. 67, 6445–6453
16. Park, S. Y., Kim, C. H., Jeong, W. H., Lee, J. H., Seo, S. J., Han, Y. S., and Lee, I. H. (2005) Dev. Comp. Immunol. 29, 43–51
17. Costa, S. C., Ribeiro, C., Girard, P. A., Zumbihl, R., and Brehe´lin, M. (2005) J. Insect Physiol. 51, 39–46
18. Kim, C. H., Lee, J. H., Kim, I., Seo, S. J., Son, S. M., Lee, K. Y., and Lee, I. H. (2004) Mol. Cells 17, 262–266
19. Shrivastava, B., and Ghosh, A. K. (2003) Insect Biochem. Mol. Biol. 33, 1025–1033
20. Imura, Y., Ishikawa, H., Yamamoto, K., and Sehnal, F. (1998) Arch. Insect Biochem. Physiol. 38, 119–125
21. Halwani, A. E., Niven, D. F., and Dunphy, G. B. (2000) J. Invertebr. Pathol. 76, 233–241
22. Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., and Kurata, S. (2004) EMBO J. 23, 4690–4700
23. Ferrandon, D., Imler, J. L., Hetru, C., and Hoffmann, J. A. (2007) Nat. Rev. Immunol. 7, 862–874
24. Schmidt, O., Söderhäll, K., Theopold, U., and Faye, I. (2010) Annu. Rev. Entomol. 55, 485–504
New Insect Opsonin for Phagocytosis of Microorganisms

25. Lagueux, M., Perrodou, E., Levashina, E. A., Capovilla, M., and Hoffmann, J. A. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 11427–11432
26. Moita, L. F., Wang-Sattler, R., Michel, K., Zimmermann, T., Blandin, S., Levashina, E. A., and Kafatos, F. C. (2005) *Immunity* 23, 65–73
27. Stroschein-Stevenson, S. L., Foley, E., O’Farrell, P. H., and Johnson, A. D. (2006) *PLoS Biol.* 4, e4
28. Janeway, C. A., Jr. (1989) *Cold Spring Harb. Symp. Quant. Biol.* 54, 1–13
29. Franc, N. C., Dimarcq, J. L., Lagueux, M., Hoffmann, J., and Ezekowitz, R. A. (1996) *Immunity* 4, 431–443
30. Stuart, L. M., Deng, J., Silver, J. M., Takahashi, K., Tseng, A. A., Hennessy, E. J., Ezekowitz, R. A., and Moore, K. J. (2005) *J. Cell Biol.* 170, 477–485
31. Schnucker, D., Clemens, J. C., Shu, H., Worby, C. A., Xiao, J., Muda, M., Dixon, J. E., and Zipursky, S. L. (2000) *Cell* 101, 671–684
32. Watson, F. L., Pu¨ttmann-Holgado, R., Thomas, F., Lamar, D. L., Hughes, M., Kondo, M., Rebel, V. I., and Schmucker, D. (2005) *Science* 309, 1874–1878
33. Kocks, C., Cho, J. H., Nehme, N., Ulvila, J., Pearson, A. M., Meister, M., Strom, C., Conto, S. L., Hetrui, C., Huet, L. M., Stehle, T., Hoffmann, J. A., Reichhart, J. M., Ferrandon, D., Räm, M., and Ezekowitz, R. A. (2005) *Cell* 123, 335–346
34. Kurucz, E., Márkus, R., Zsámboki, J., Folkl-Medzihradszky, K., Darula, Z., Vilmor, P., Udvardy, A., Krausz, I., Lukacsovich, T., Gateff, E., Zettervall, C. J., Hultmark, D., and Andó, I. (2007) *Curr. Biol.* 17, 649–654