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

PURIFICATION, CHARACTERIZATION, AND cDNA CLONING*

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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 foetida*. To evaluate the involvement of LGBP in the prophenoloxidase (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).

Invertebrate animals 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 in many insects (14, 15) 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 degranulation of blood cells (38) by the β-1,3-glucan-binding protein (β-GBP) 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

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ250128.

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[1] The abbreviations used are: LPS, lipopolysaccharide; PO, phenoloxidase; proPO system, prophenoloxidase activating system; β-GBP, β-1,3-glucan-binding protein; LGBP, lipopolysaccharide- and β-1,3-glucan-binding protein; DOPA, 3,4-dihydroxy phenylalanine; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HLS, hemocyte lysate supernatant; HPLC, high performance liquid chromatography.

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-GBP, the coelomic cytolytic factor-1, from the earthworm *Eisenia foetida* (27) and the peptidoglycan recognition protein from *B. mori* (30) appear to be involved in the activation of the proPO system.

In this paper, we describe the purification of 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 (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) (46). The hemocytes were spun down at 4 °C and 800 × *g* for 10 min, and then the hemocytes were homogenized with CAC buffer (10 mM sodium cacodylate, 0.1 M $CaCl_2$, 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 × 12 cm), previously equilibrated with CAC buffer. The flow-through containing LGBP was collected, and it was loaded to a phenyl-Sepharose CL-4B (1 × 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 erythrocyte carbonic anhydrase (30 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 treated with 5% skim milk in 20 mM Tris/HCl, pH 7.9, at 4 °C for 1 h and then incubated in 2-fold dilution anti-LGBP antiserum with rinse solution containing 10 mM Tris/HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-200, 1% NaN_3, and 0.5% skim milk at 4 °C for 12 h with gentle shaking. The strip was washed carefully with rinse solution and subsequently cut into pieces. The antibody bound to LGBP was eluted with 0.2 M 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 antiserum was loaded to protein A-Sepharose CL-4B equilibrated with 50 mM Tris/HCL, pH 7.0, and washed with the same buffer. Bound anti-LGBP antibody was eluted with 0.1 M 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 under reducing conditions and electrotransferred to nitrocellulose membranes in transfer buffer (25 mM Tris/HCl, 190 mM glycine, 20% MeOH) for 2 h at 280 mA on ice. All of the following steps were performed at room temperature. The membrane was subsequently blocked in TTBS (0.1% Tween 20 in 20 mM Tris/HCl, 150 mM NaCl, pH 7.4) containing 3% BSA for 1 h and incubated with antibody in TTBS containing 0.1% BSA for 1 h 30 min. A 2,000-fold dilution of affinity-purified antibody was used in immunoblotting. Then the membrane was washed with TTBS once for 15 min and three times for 5 min. The anti-rabbit IgG peroxidase-conjugated IgG diluted 1:10,000 with TTBS containing 0.1% BSA was incubated for 1 h, washed with TTBS once for 15 min and four times for 5 min. For detection, the enhanced chemiluminescence (ECL) Western blotting reagent kit (Amersham Pharmacia Biotech) was used.

Determination 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 lysyl endopeptidase 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 C_2/C_{18} 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 NH_2-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 Biosystem 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:B5 (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/HCl containing 50 mM NaCl, pH 8.5, was added for measuring the bound fluorescence using a fluorescence multiwell plate reader (Wallac 1420 multilabel counter) at emission/excitation 485 nm/530 nm. This experiment was repeated twice with similar results.

In another method, curdlan, 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 of *Staphylococcus aureus* (Fluka), or LPS of *E. coli* serotype 055:B5 (Sigma) was used for testing binding activity to LGBP. 4 μg of purified LGBP was incubated with 100 μg of curdlan, laminarin, LPS, or peptidoglycan in CAC buffer for 1 h at 4 °C. The supernatant was taken, and the pellets of curdlan, laminarin, LPS, or peptidoglycan were washed three times with CAC buffer. The bound protein was eluted with 30 μl of SDS-PAGE sample loading buffer (60 mM Tris/HCl, pH 6.8, containing 2% SDS, 1% glycerol, 0.01% bromphenol blue, and 0.1% dithiothreitol) and 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 supernatants 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-^{32}P]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, and the recombinant DNA was purified by using Wizard λ preparation DNA purification system (Promega). The insert was digested out by the restriction enzyme *Eco*RI (Amersham Pharmacia Biotech) and subcloned into *Eco*RI-digested pBluescript II (SK+) plasmid (Stratagene). It was subsequently sequenced in double strands by an Applied Biosystems PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). The cDNA sequence was analyzed with the MacVector 4.1.4

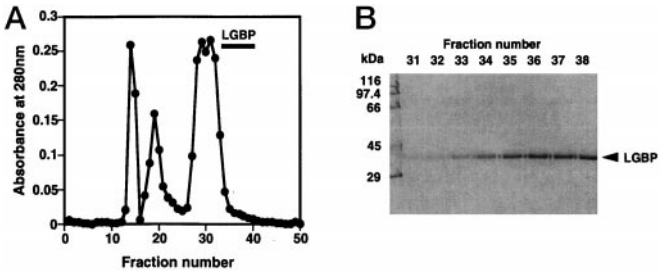

FIG. 1. **Elution profiles of LGBP from a Sephacryl S-200 column.** *Panel A*, Sephacryl S-200 chromatography pattern; *panel B*, 10% SDS-PAGE of the different fractions from Sephacryl S-200 under reducing conditions. Molecular mass markers are indicated on the *left*; 116, *E. coli* β-galactosidase; 97.4, rabbit muscle phosphorylase *b*; 66, bovine albumin; 45, egg albumin; 29, bovine erythrocyte carbonic anhydrase.

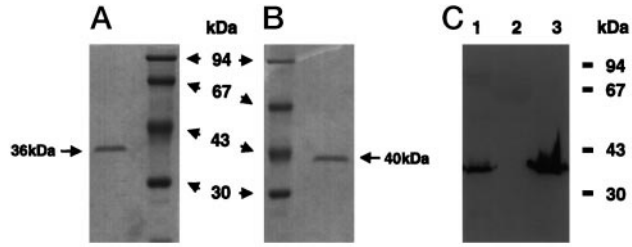

FIG. 2. **SDS-PAGE of purified LGBP and immunoblotting analysis.** 2 μg of LGBP was precipitated with trichloroacetic acid and then subjected to SDS-PAGE. *Panel A*, 10% SDS-PAGE of purified LGBP under reducing conditions; *panel B*, 10% SDS-PAGE of purified LGBP under nonreducing conditions; *panel C*, 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.

software (Kodak). The nucleotide sequence, and the deduced amino acid sequence was compared with the BLAST program (National Center Biotechnology International, Bethesda, MD).

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, spanning the coding region and 3′-untranslated region, respectively, were labeled with [α-^{32}P]dCTP using the Megaprime labeling kit (Amersham Pharmacia Biotech). Northern blot hybridization was performed overnight at 65 °C in a solution composed of 5 × SSC, 5 × Denhardt's solution, 0.5% (w/v) SDS, and 100 μg/ml denatured salmon sperm DNA. The membrane was subsequently washed twice with 2 × SSC and 0.1% SDS at room temperature for 10 min, once with 1 × SSPE and 0.1% SDS at 65 °C for 15 min, and finally twice with 0.5 × SSC and 0.1% SDS at 65 °C for 10 min. The washed membrane was used for autoradiography.

Effects of Anti-LGBP Antiserum on PO Activity—To confirm the involvement of LGBP in the crayfish proPO system, a polyclonal antibody against LGBP was used, and PO activity was assayed according to Aspán and Söderhäll. (50). Briefly, 30 μg of HLS was incubated for 1 h at 4 °C with different concentrations of anti-LGBP antibody purified by protein A column chromatography and 10 μg of L-DOPA (3-(3,4-dihydroxylphenyl)-L-alanine). These mixtures were incubated at room temperature for 5 h in a final volume of 30 μl of 30 mM sodium cacodylate, pH 6.5, with or without 10 μg of laminarin, curdlan, LPS, or peptidoglycan. The oxidation of L-DOPA was measured at 490 nm. For reconstitution of PO activity, 3 μg or 15 μg of purified LGBP, previously incubated with laminarin for 20 min at room temperature, was incubated with HLS, in which PO activity had been previously inhibited by addition of anti-LGBP antibody. The recovered PO activity was measured after addition of L-DOPA by spectrophotometry at 490 nm.

Determination of Protein—The concentration of protein was measured with the Bradford method (51), using BSA as a standard protein.

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. 1*A*). The purity of LGBP in the different fractions was ascertained by SDS-PAGE (Fig. 1*B*). Hemocyte lysate supernatants from 200 crayfish with a protein content of about 35 mg of protein gave 210 μg of purified LGBP. The purified LGBP ran as a single band of approximately 36 kDa and 40 kDa in 10% SDS-PAGE under reducing and nonreducing conditions, respectively (Fig. 2, *A* and *B*).

Characterization of LGBP—Anti-LGBP antibody was used to confirm the localization of LGBP in crayfish hemolymph using immunoblotting. Fig. 2*C* 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 curdlan, laminarin, LPS, or peptidoglycan to demonstrate the binding activity of LGBP. Fig. 4 shows the result of an immunoblot of the corresponding LGBP binding activity to curdlan, laminarin, LPS, or peptidoglycan. In the pellets, LGBP is shown to bind strongly to LPS and curdlan, less to laminarin (*lanes 2*, *3*, and *4* in Fig. 4), and not at all to peptidoglycan (*lane 5*). LGBP could not be found in the supernatants that were incubated with LPS, laminarin, or curdlan, (*lanes 6*, *7*, and *8* in Fig. 4), 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 NH_2-terminal and four internal amino acid sequences of LGBP were determined. The NH_2-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, CGCCTCACCCAGCCCATCCAGCAGTAGACCCA. Underlined amino acid sequences of the cDNA are partial amino acid sequences determined from the LGBP, suggesting that the cDNA clone of the LGBP is authentic. The first 15

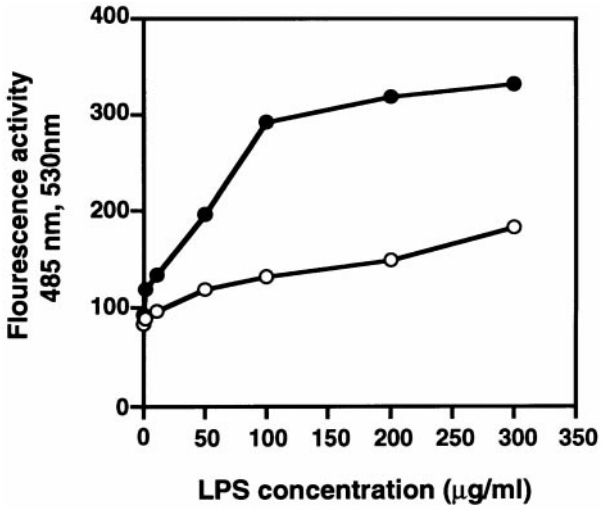

FIG. 3. **LPS binding activity of LGBP.** Purified LGBP or BSA was immobilized on microtiter plates and incubated with different doses of fluorescein isothiocyanate-labeled smooth type LPS from *S. abortus*. LPS binding activity was determined using a fluorescence spectrophotometer. The *closed circles* represent 10 μg of purified LGBP, and the *open circles* represent 10 μg of BSA.

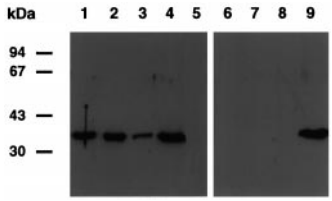

FIG. 4. **β-1,3-Glucans and LPS binding activity of LGBP.** 3 μg of purified LGBP was incubated with LPS, curdlan, laminarin, and peptidoglycan in CAC buffer. The supernatants were taken, and the pellets of the LPS, curdlan, laminarin, or peptidoglycan were eluted with SDS-PAGE sample loading buffer. The eluted proteins were subjected to 10% SDS-PAGE (*lanes 2–5*). The supernatants were precipitated with trichloroacetic acid. The precipitated proteins were dissolved with SDS-PAGE sample loading buffer and subjected to 10% SDS-PAGE (*lanes 6–9*). The binding activity of LGBP to LPS, curdlan, laminarin, or peptidoglycan was analyzed by immunoblotting using affinity-purified anti-LGBP antibody. *Lane 1*, purified LGBP; *lanes 2* and *6*, LPS; *lanes 3* and *7*, laminarin; *lanes 4* and *8*, curdlan; *lanes 5* and *9*, peptidoglycan.

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 isoelectic point of 5.80 for the mature protein. Two putative glycosylation sites (Asn-Xaa-Ser/Thr) for *N*-linked carbohydrate chains are present in the mature protein sequence at Asn-64 and Asn-313. However, the calculated molecular mass is similar to that of native LGBP, suggesting that LGBP cDNA contains no carbohydrate moiety. One short putative cell adhesion site and integrin binding site (52), Arg-Gly-Asp (RGD), is present in the sequence of the mature protein from Arg-149 to Asp-151.

Northern Blot Analysis—Northern blot analysis shows that the mRNA of LGBP was expressed constitutively as a single band of 5.3 kilobases (data not shown) in the hemocytes of crayfish. Two probes, spanning the coding region and the 3′-untranslated region with 17 repeated sequences, gave the same result, which indicates that the 17 repeated sequences of the 3′-untranslated flanking region in LGBP mRNA are not an artifact (data not shown).

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).

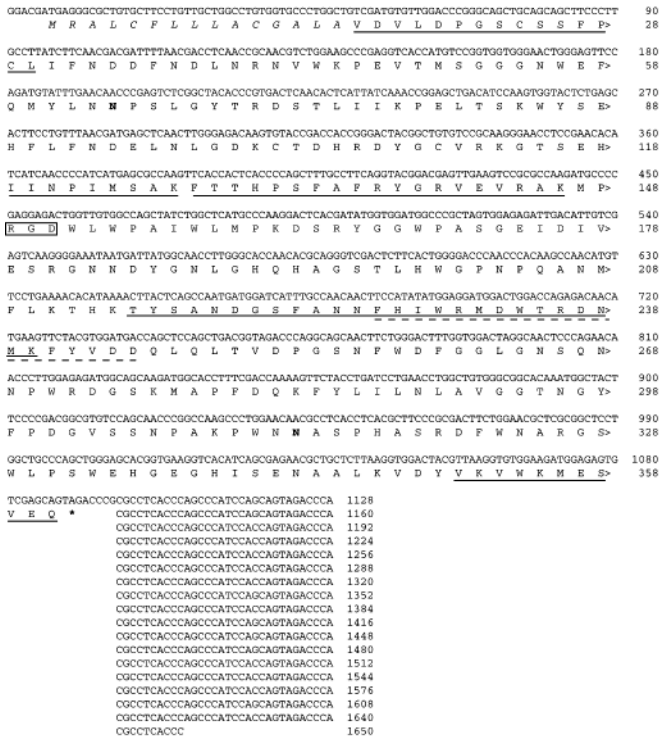

FIG. 5. **Nucleotide and deduced amino acid sequences of the crayfish *P. leniusculus* LGBP.** Nucleotides (*upper*) are numbered on the *right*. 17 repeat motifs of 32 nucleotide sequences were found in the 3′-untranslated region of mRNA. Amino acids (*lower*) are also numbered on the *right* and counted from the first methionine. The putative signal peptide is in *italics*. The *underlined* amino acid residues were confirmed by amino acid sequencing of peptide fragments. The NH_2-terminal sequence starts at Val-16. An *asterisk* represents the termination codon. Putative recognition motifs for β-(1->3) linkages of polysaccharides are *dot-lined*. The *bold N* represents the potential glycosylation sites. The Arg-Gly-Asp sequence for a putative cell adhesive site is *bold-boxed*.

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 β-1,3-glucanase of the sea urchin *Strongylocentrotus purpuratus* (53), 47% identity and 60% similarity (Fig. 6). It also shows 54% similarity with coelomic cytolytic factor-1 of the earthworm *E. foetida* (27), which was reported as a LPS- and β-glucan-binding protein exhibiting opsonizing properties as well as being able to cause activation of the proPO system of this earthworm. Moreover, LGBP shows sequence similarity with the putative Gram-negative bacteria-binding proteins of the mosquito *Anopheles gambiae* (54), the silkworm *B. mori* p50 (22), and the webworm *Hyphantria cunea* (55). Interestingly, LGBP also contained a region that is known as a putative recognition site for β-(1->3) linkages of polysaccharides (56) (Fig. 5). This region of LGBP has homology with the glucanase A1 domain of β-1,3-D-glucan-sensitive coagulation factor G α-subunit of horseshoe crab *T. tridentatus* (57).

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

FIG. 6. **Amino acid sequence alignment of LGBP and other proteins.** *PL*, crayfish *P. leniusculus* LGBP; *SP*, sea urchin *S. purpuratus* β-1,3-glucanase (53); *AG*, mosquito *A. gambiae* putative Gram-negative bacteria-binding protein (54); *CCF-1*, earthworm *E. foetida* LPS- and glucan-binding protein, coelomic cytolytic factor-1 (27); *BM*, silkworm *B. mori* Gram-negative bacteria-binding protein (22); *HC*, webworm *H. cunea* Gram-negative bacteria-binding protein (55). The *asterisks* indicate that the residues are identical. The *dots* indicate that the amino acids have similar properties. Similar amino acids were defined as follows: AG; ST; ED; RKH; QN; VILM; YF; W; P; C. Gaps were introduced to obtain maximal sequence alignment. The number is based on the sequence of LGBP.

```
         .  . *        *               *   *                *  *    *                        *
PL    31 IFNDDFNDLN RNVWKPEVTM SGGG-NWEFQ MYLNNP--SL GYTRDSTLII KPELTSKWYS ------EHFL FNDELNLG-- 99
SP       IFQEEFDSFN LDIWEHEMTA GGGG-NWEFE YYTNNR--SN SYVRDGKLFI KPTLTTDKLG ------EGSL SSGTLDLWGS
AG       IFEDNFDFFD FEKWEHVNTL AGGG-NWEFQ WYTNNR--SN SFVEDGALNI RPTLTADQFG ------LDFM TSGTLSLQGS
CCF1     VWQDEFDYFD GAKWQHEVTA TGGG-NSEFQ LYTQDG--AN SFVRDGKLFI KPTLLADNIN PQTGAPFGTD FMYNGVLDVW
BM       IFEEQFDSLD ENVWQIEQYI PIYHPEYPFV SYQRNN--LT VSTADGNLHI NAKLQQHMPG ------FLDD SIYSGTLN--
HC       IFEENFNTFR EDVWQIEQYI PVYSTEFPFV SYQHLSQDPT VAVTGGNLRI TPKLQQRMPG ------FTDS SIYSGSLN--
              **       *          . *       *    . *   *   . ** .* ***. *  * . *    *     ** .
PL   100 ---DKCTDHR DYGCVRKGTS EHIINPIMSA KFTTHPSFAF RYGRVEVRAK MPRGDWLWPA IWLMPKDSRY G-GWPASGEI 175
SP       SPANLCTGNA WYGCSRTGSN DNLLNPIQSA RLRTVESFSF KYGRLEVEAK LPTGDWLWPA IWLLPKHNGY G-EWPASGEI
AG       YPTDHCTNDA FYGCVRVGNR QHIVNPVKSA RIRTISSFNF KYGRAEVRAK LPTGDWLWPA IWLLPKRNAY G-TWPASGEM
CCF1     AMYGACTNTD NNGCYRTGAA G-DIPPAMSA RVRTFQKYSF THGRVVVHAK MPVGDWLWPA IWMLPEDWVY G-GWPRSGEI
BM       ---LFSGCTS SAEACIKQAS GADILPPIVS GRITSIGFAF TYGTVEIRAK LPQGDWLYPE ILLEPFLKKY GSMNYASGVV
HC       ---IFSGCTA PAEACMKDAW GASILPPVVS GRITSKAFAF TYGTVFVKAK LPQGDWIYPE ILLEPFLKKY GSTHYSSGVI
          .    **                .    .  .   **                            .     .     .   .
PL   176 DIVESRGN-- -----NDYGN LGHQHAGSTL HWGPNPQANM FLKTHKTYSA NDG-SFANNF HIWRMDWTRD NMKFYVDDQL 247
SP       DLVESRGN-- ADIKDADGLS AGVDQMGSTM HWGPFWPLNG YPKTHATK-- ---------- ---------- ---FYVDDEL
AG       DLMESRGN-- -ENLYLDGVQ IGTRQVGQTL HFGPNPSYNG YPTATLTKNA LPEQEFSKSF STFGFVWTPD NITVSINGED
CCF1     DIIETIGNR- -DFKNTGGEF LGIQKMGSTM HWGPGWDDNR YWLTSLPKHS DDWN-YGDNF HTFWFDWSPN GLRFFVDDEN
BM       KIACARGNAE LYSGPNDYSN T-------VL YGGPIMDLEC RENFLSTKRR RDGTSWGDSF HTYSVQWTPD FIALSVDGEE
HC       KIASARGNRE LTSGYTDYSN K-------ML FGGPVMNLQC YDTLLESKAS SNGRQWGDDF HEYVLRWAPE RITLSVDGVE
            .                                          . ***  *    . .* *        *.            *
PL   248 QLTVDPGSNF WDFG---GLG NSQNNPWRDG S--------- -KMAPFDQKF YLILNLAVGG TNGYFPDG-- VSSNP---AK 309
SP       LLNVDPATGF WDLGEFENDA PGIDNPWAYN PN-------- -KLTPFDQEF YLILNVAVGG VN-YFGDG-- LTYTP---AK
AG       LATIGGDFWT RGG----FDK HNLENPWRHG T--------- -RMAPFDQEF HFIINLAVGG VA-FFPDAAT NPGG-----K
CCF1     QALLDVPYPL IDANPWWVDF WEWGKPWLPQ YENDNPWAGG TNLAPFDQNF HFILNVAVGG TNGFIPDGCI NRGGDPALQK
BM       WARVEAPRDA LP----AVCA HAPRHLLQAG S--------- -QMAPFDDHF YITLGVAAGG IT-EFRDGSI TSGGV---TK
HC       WARVEPTASG LSGRFPQTCS KLPRTFLAAG T--------- -KMAPFDDHF YLTLGVAAGS IT-EFPDGVQ TSGSR---PK
         **         *   *       *   *             .  * ...*                    % identity  % similarity
PL   310 PWNNASPH-- ASRDFWNARG SWLPSWEHGE GHISENAALK VDYVKVWKME SVEQ 361       100         100
SP       PWSNDSPT-- ASKDFWSDFN TWYPTWNGEE ------AAMQ VNYVRVYXEP GQTTYXLRDR       47          60
AG       PWKNNSPQ-- AATDFWNGRA QWLPTWNLER DGGKS-ASLL VDYVKVWAL                    45          57
CCF1     PWSNGDWYND AMRKFFDARG NWKWTWDDEG D----NNAMQ VDYIRVYKRN                   41          54
BM       PWRDSARK-- ASVHFWRHMS DWFPRWSQPS --------LI VDFVKVIAL                    31          44
HC       PWTNTGSK-- AMLHFWEDMD SWFATWNQPQ --------LL VDYVKVVAL                    28          42
```

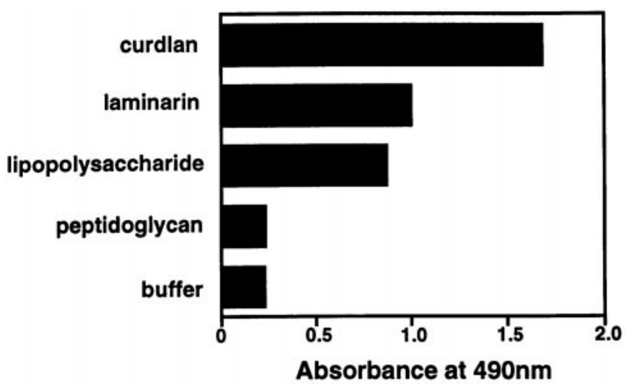

FIG. 7. **Activation of the proPO activating system in HLS of crayfish by curdlan, laminarin, LPS, or peptidoglycan.** The PO activity was determined by measurement of the absorbance at 490 nm. This experiment was repeated three times with similar results.

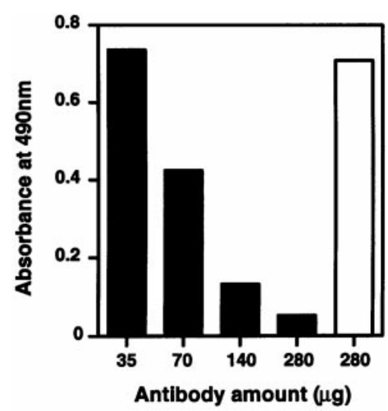

FIG. 8. **Effects of anti-LGBP antibody on β-1,3-glucan-triggered activation of the proPO system.** Different doses of anti-LGBP antibody purified by protein A column chromatography were preincubated with 30 μg of HLS for 1 h at 4 °C. L-DOPA was added to these fractions as substrate in the presence of laminarin. PO activity was measured by reading the absorbance at 490 nm. The *black bars* represent the PO activity in the presence of different amount of the anti-LGBP antibody, and the *white bar* represents the PO activity in the presence of the preimmune serum as control.

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 crayfish *P. leniusculus*. The purified LGBP is a 36- or 40-kDa single polypeptide under reducing and nonreducing conditions, respectively. Amino acid sequence analysis shows that LGBP has a sequence motif similar to the β-1,3-glucanase A1 of *Bacillus circulans* WL-12 (56), and it has homology with several other β-1,3-glucanases as well as to Gram-negative bacteria-binding proteins described earlier from different species of invertebrates (31, 53–56). Interestingly, the amino acid sequence of LGBP is similar to the glucanase A1 domain of the α-subunit of factor G, a β-GBP of horseshoe crab *T. tridentatus* (57), but the LGBP clearly has an overall primary structure that is different from that of factor G. LGBP has instead a

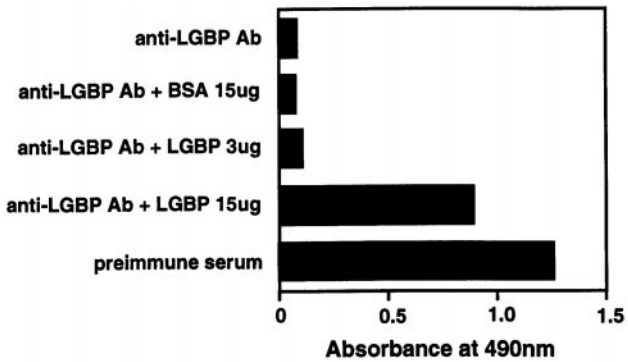

FIG. 9. **Involvement of LGBP in the proPO system.** 30 μg of crayfish HLS was preincubated with 280 μg of anti-LGBP antibody to block completely the L GBP activity in the crayfish HLS for 1 h at 4 °C; 3 μg or 10 μg of purified LGBP was also preincubated with the laminarin for 20 min at room temperature in the other tube. These two fractions were mixed and then incubated with L-DOPA as substrate. The recovered PO activity was measured by reading the absorbance at 490 nm. 10 μg of BSA was used as control.

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 β-1,3-glucanase gene, which later during evolution has lost its glucanase activity, whereas the glucan binding activity has been retained, and hence the protein can be involved in immune reactions (26). We can also speculate that the putative β-1,3-glucanase catalytic site of LGBP is the region in which the LPS and β-1,3-glucans are binding, but this remains to be shown for LGBP and for all other LPS- or β-GBPs described so far.

In the nucleotide sequence of LGBP, 17 repeated sequence motifs in the 3′-untranslated region were found. These repeated sequences are not homologous with any known sequence. Although the poly(A) tail part could not be found, the result of Northern blotting data showed that a long untranslated region is present between the termination codon and the poly(A) addition site (data not shown). A similar phenomenon was also found in the LPS-binding protein of the cockroach *P. americana*, which has 15 tandem repeats of a 28-nucleotide sequence in the mRNA 3′-untranslated region (21). However, the biological significance of these repeated sequences is unknown, and the role if any of this sequence region of crayfish LGBP is unknown.

LGBP has binding activity to both LPS and β-1,3-glucans, whereas peptidoglycans of Gram-positive bacteria have no binding activity to LGBP (Fig. 4). The proPO system of crayfish can be activated by both LPS and β-1,3-glucans, but in our experimental conditions peptidoglycans could not induce proPO activation (Fig. 7). Anti-LGBP antibody could completely inhibit β-1,3-glucan-triggered activation of the proPO system in the crayfish hemocyte lysate (Fig. 8). The results of the LPS and β-1,3-glucan binding affinity to LGBP and its participation in proPO system strongly suggest that LGBP is an important pattern recognition protein, and it has been shown to be clearly involved as an initiator of the proPO system in crayfish.

We have reported previously the purification and characterization of the β-GBP from the plasma of the crayfish *P. leniusculus* (25, 26). The β-GBP is also a component of the proPO system because it can induce activation of proPO in a crude source of proPO system. As a consequence, two glucan binding pattern recognition proteins seem to be required for complete activation of the proPO system in crayfish. The proPO system is contained 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.

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A Lipopolysaccharide- and β-1,3-Glucan-binding Protein from Hemocytes of the Freshwater Crayfish *Pacifastacus leniusculus* : PURIFICATION, CHARACTERIZATION, AND cDNA CLONING

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