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Structure and Biological Activity of a 1,3-β-D-Glucan-binding Protein in Crustacean Blood*

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The prophenoloxidase activating system, an enzyme cascade present in arthropod blood, has been shown to be involved in defense and recognition reactions. This system is converted to its active form by fungal 1,3-β-D-glucans through binding to a plasma protein, a 1,3-β-D-glucan-binding protein. Here the molecular cloning and carbohydrate composition of the 1,3-β-D-glucan-binding protein from the freshwater crayfish *Pacifastacus leniusculus* are reported. It is also demonstrated that this protein can act as an opsonin, stimulating phagocytic uptake of yeast particles by isolated blood cells. The deduced amino acid sequence of 1,339 residues shows no significant similarity to proteins with similar functions in other animals such as the mannan-binding and lipopolysaccharide-binding proteins present in mammals. However, a short sequence motif with similarity to the active site of microbial 1,3–1,4-β-D-glucan 4-glucanohydrolases was found to occur twice in the 1,3-β-D-glucan-binding protein.

Defense reactions in vertebrates, invertebrates, and plants can be triggered by 1,3-β-D-glucans, polysaccharides generally present in the cell walls of fungi (1). In invertebrates, the horseshoe crab coagulation cascade and the prophenoloxidase activating system (proPO-system)[1] can be induced into their active forms by these polysaccharides (2, 3). The minimal glucan entity capable of eliciting proPO-system activation in crustaceans is a linear pentasaccharide consisting of five 1,3-β-D-linked glucopyranosyl residues (4). In horseshoe crabs coagulation is initiated in the presence of glucans by the conversion of the glucan-binding zymogenic factor G to a catalytically active proteinase (2, 5). In crustaceans and insects 1,3-β-D-glucan-binding proteins (βGBPs) have been purified from plasma (6–9). These proteins enhance the glucan-mediated activation of the proPO-system (6–9). A membrane receptor protein for a glucan-binding protein was recently purified from crayfish blood cells and was shown to bind the βGBP only after the βGBP had been treated with glucan (10). The binding of the βGBP-glucan complex to the membrane receptor triggers the blood cell to partially degranulate and spread (11). During degranulation the proPO-system and associated factors, such as a 76-kDa cell adhesion protein, are released in a biologically inactive form (12, 13) and are activated in the presence of microbial polysaccharides such as 1,3-β-D-glucans or lipopolysaccharides (LPS) (12, 13). As a consequence of this activation proPO-components will gain their biological activity and assist in mounting an effective defense toward microorganisms such as promotion of phagocytosis and encapsulation by the blood cells (13, 14). It has also been demonstrated in *Drosophila melanogaster* that 1,3-β-D-glucans can induce the expression of genes for the antimicrobial cecropin peptides (15), although a glucan-binding factor has not yet been characterized in this system.

In light of the widespread occurrence of 1,3-β-D-glucan-mediated defense reactions in different types of organisms, we decided to characterize further the βGBP from freshwater crayfish. Here, we report for the first time the cDNA cloning and properties of a βGBP involved in the activation of the proPO-system of arthropods.

MATERIALS AND METHODS

Tissue Preparation and Blood Cell Isolation—Freshwater crayfish, *Pacifastacus leniusculus*, from lake Halmsjön, Sweden, were kept in aquaria at 10–12 °C. Only intermolt animals were used in the experiments. Different tissues were excised and incubated in anticoagulant (16) for primary tissue culture. Blood cell (hemocyte) isolation, primary tissue culture, and metabolic labeling with [^{35}S]methionine were performed as described earlier (17).

RNA Isolation and cDNA Library Construction—Total RNA was isolated from the hepatopancreas by extraction in acid guanidine thiocyanate as described by Chomczynski and Sacchi (18). For library construction a total of approximately 5 mg of total RNA were isolated from the hepatopancreas of eight animals. A cDNA library was constructed in λgt11 (CLONTECH, Palo Alto, CA).

Isolation and Sequencing of βGBP Clones—The cDNA library was screened with an anti-βGBP antiserum (8) using a primary antibody dilution of 1:500 and biotinylated goat secondary antibodies conjugated to avidin and horseradish peroxidase. An initial screening of 100,000 recombinants resulted in the isolation of two clones, one with a size of 1,950 base pairs (bp) (BG-1) and the other 1,030 bp (BG-2). For the isolation of additional clones BG-2 was labeled with ^{32}P by random priming according to the manufacturer's protocol using the Megaprime labeling kit (Amersham Corp.) and used to screen the cDNA library. Plaque lifting onto Amersham Hybond N membranes and hybridizations were performed according to the instructions supplied by the manufacturer. The clones were subcloned into the *Eco*RI site of pBluescript II KS(+) phagemid (Stratagene). Nucleotide sequence analysis was performed by the dideoxy chain-termination method using Sequenase 2.0 from U. S. Biochemical Corp. Nested deletions in both directions were made using Pharmacia Biotech. Inc.'s Nested Deletion Kit

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X80687.

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[1] The abbreviations used are: proPO-system, prophenoloxidase activating system; βGBP, 1,3-β-D-glucan-binding protein; LPS, lipopolysaccharide; CFS crayfish saline; HPLC high performance liquid chromatography; bp, base pair(s).

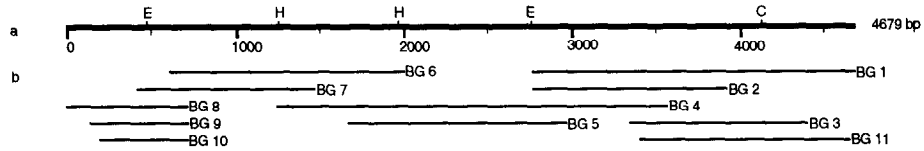

FIG. 1. **Restriction enzyme map (*a*) and sequencing strategy (*b*) of the βGBP cDNA.** The *BG-1* and *BG-2* were isolated by immunoscreening. *BG-3*, *BG-4*, *BG-5*, and *BG-11* were isolated using *BG-2* as a probe. *BG-6* was isolated by screening with *BG-5* and *BG-7* by the use of *BG-6*. *BG-7* finally was used to isolate clones *BG-8–10*. *E*, *Eco*RI; *H*, *Hin*dIII; and *C*, *Cla*I denote restriction enzyme sites.

according to the manufacturer's protocol. All sequences were determined in both directions and are based on several overlapping clones as shown in Fig. 1.

Determination of Partial Amino Acid Sequences of βGBP—βGBP was purified from *P. leniusculus* plasma by immunoaffinity chromatography as described previously (8). The purified protein was reduced and *S*-alkylated as follows: 0.5 nmol of dried protein was dissolved in 100 µl of 0.4 M ammonium carbonate, pH 8.0, containing 8 M urea or in 100 µl of 150 mM Tris-HCl, pH 7.5, containing 6 M urea, depending on the enzyme used for digestion. Reduction was achieved by addition of 5 µl of 45 mM dithiothreitol and an incubation at 50 °C for 15 min. After cooling down to room temperature, 5 µl of 100 mM iodoacetamide were added to the reduced protein, and the mixture was kept at room temperature for 15 min. The reduced and *S*-alkylated protein was, after dilution with distilled water, digested with either lysyl endopeptidase (enzyme:substrate ratio = 1:100, w/w) overnight at 37 °C in 100 mM ammonium carbonate, pH 8.0, containing 2 M urea or with asparaginyl endopeptidase (enzyme: substrate ratio = 1:100, w/w) at 37 °C for 20 h in 50 mM Tris-HCl, pH 7.5, containing 3 M urea. The resulting digests were separately subjected to reverse-phase high performance liquid chromatography (HPLC) using a Chemcosorb 5-ODS-H column (2.1 × 150 mm). The HPLC was performed with an isocratic elution of 0.06% (v/v) trifluoroacetic acid for 5 min, followed by a linear gradient of 0–80% (v/v) acetonitrile in 0.06% trifluoroacetic acid for 120 min at a flow rate of 0.2 ml/min. The isolated peptides were further purified by HPLC using a µBondasphere S5–300 column (2.0 × 150 mm). The conditions for elution were the same as described above for the Chemcosorb run. The effluents were monitored at 210 nm. In separate experiments the βGBP was digested with *Achromobacter lyticus* protease I in 4 M urea, 0.1 M Tris-HCl, pH 9.0, overnight at 37 °C. These digests were separated on a Brownlee Aquapure column, 2.1 × 30 mm. The purified peptides were subjected to N-terminal sequence analysis on an Applied Biosystems Protein Sequencer 477A and the phenylthiohydantoin-derivatives were identified with an on-line Applied Biosystems 120A phenylthiohydantoin analyzer.

Determination of the C-terminal Amino Acid Residue of βGBP—The C-terminal residue was determined by the vapor phase hydrazonolysis method as described previously (19, 20). Briefly, highly dried βGBP was treated with vaporized hydrazine at 90 °C for 3 h, and the sample was then treated with benzaldehyde. The amino acid released from the C terminus of the protein was identified with a Hitachi model L-8500 amino acid analyzer. Three independent determinations were performed each with 1 nmol of protein. The average yield was about 900 pmol of histidine.

Component Sugar Analysis—The sugar composition of βGBP was determined by the method of Takemoto *et al.* (21). Briefly, the sample (500 pmol) was hydrolyzed in 4 N trifluoroacetic acid at 100 °C for 3 h in an evacuated tube. The acid hydrolyzates were *N*-acetylated and then pyridylaminated with 2-aminopyridine using a pyridylamination instrument, Palstation model 4000 (Takura Shuzo Co., Ltd., Kyoto, Japan). Finally, the sugar derivatives were analyzed by HPLC on a Palpak type A column (4.6 × 150 mm). The HPLC was performed with an isocratic elution of 0.7 M potassium borate, pH 9.0, containing 10% acetonitrile (v/v) at a flow rate of 0.3 ml/min.

Assay for 1,3-β-D-Glucanase Activity—To produce 1,3-β-D-[^{14}C]glucans a scaled up reaction mixture of isolated 1,3-β-D-glucan synthase from the fungus *Aphanomyces astaci* was used as described previously (22) using UDP-[^{14}C]glucose as substrate. To assay for glucanase activity 20,000 cpm ^{14}C-labeled glucan was incubated with various amounts of βGBP for up to 6 h at 30 °C and the release of low M_r sugars was followed (22).

Northern Blot Analysis—Different tissues (hepatopancreas, gills, abdominal muscle, epidermis, and green gland) were ground in liquid nitrogen immediately upon dissection, and total RNA was isolated as above, whereas hemocyte RNA was extracted from hemocytes which had been immediately suspended in homogenization buffer (10 mM sodium cacodylate, 0.25 M sucrose, and 100 mM $CaCl_2$, pH 7.0) without prior freezing. The RNA was run on 1% agarose gels in the presence of formaldehyde (23) and transferred to nylon filters (Hybond N, Amersham) by capillary blotting overnight. Twenty µg of total RNA were loaded to each lane. For hybridization 10 µCi of the ^{32}P-labeled BG-5 clone was used in a hybridization solution containing 5 × SSPE (20 × SSPE is 3.6 M NaCl, 0.2 M sodium phosphate, and 0.02 M EDTA, pH 7.7), 5 × Denhardt's solution (100 × Denhardt's solution is 2% (w/v) bovine serum albumin, 2% (w/v) Ficoll (Pharmacia) and 2% polyvinylpyrrolidone (Sigma)), 0.1% SDS, and 100 µg/ml salmon sperm DNA. The samples were hybridized overnight at 65 °C, the filters were washed three times for 20 min with 0.2 × SSPE and 0.1% SDS at 65 °C, and then they were subjected to autoradiography.

Binding of the β-1,3-Glucan-binding Protein to Isolated Crayfish Blood Cells—Binding of βGBP to fixed cells was visualized by indirect immunofluorescence as described by Barracco *et al.* (11) with the exception that the concentration of laminarin-treated βGBP was 40 µg/ml instead of 200 µg/ml. To analyze the influence of an Arg-Gly-Asp containing peptide, the fixed, isolated cells were pre-incubated with 0.2 mM Arg-Gly-Asp-Ser or as a control with 0.2 mM Arg-Gly-Glu-Ser (both from Sigma) in phosphate-buffered saline, pH 7.4, for 4 h at 20 °C. After this preincubation, laminarin-treated βGBP was added to the cells for a 30-min incubation (11), and after washing, the cell-bound protein was visualized by indirect immunofluorescence as described earlier (11).

Phagocytosis of Yeast Cells by Isolated Blood Cells—Heat-killed bakers' yeast was prepared for phagocytosis and labeled with fluorescein isothiocyanate as described in Hed (24). Before the phagocytosis assay the fluorescein isothiocyanate-labeled yeast particles were incubated either with 200 µg/ml purified βGBP in crayfish saline (CFS) (16), with 200 µg/ml bovine serum albumin in CFS, with crayfish plasma depleted of βGBP by treatment with anti-βGBP (11) or with CFS alone. The yeast was incubated for 2 h at room temperature, then washed three times with CFS by centrifugation at 1,200 × *g* for 10 min, and finally resuspended in CFS for immediate use in the phagocytosis assay.

Hyaline hemocytes were isolated on preformed Percoll gradients as described by Smith and Söderhäll (16). Monolayers were prepared by allowing 180 µl of a hemocyte suspension to attach on glass coverslips (12). The monolayers were washed with CFS and incubated for 1 h at 20 °C with 40 µl fluorescein isothiocyanate-conjugated yeast particles pretreated as above to a final concentration of 5 × 10^6 yeast particles/ml. To quench external fluorescence, a small volume of trypan blue was added (24). Ingested yeast particles are fluorescent and could therefore be easily detected in the epifluorescence microscope. The percentage of phagocytosing cells was determined by counting at least 200 hemocytes on each coverslip.

Computer-aided Analysis—The cDNA sequence was analyzed with the MacVector 4.1.1 software (Kodak). The deduced protein sequence was compared with all entries in the Entrez data base, release 10.0.

RESULTS

cDNA Cloning of the 1,3-β-D-Glucan-binding Protein—Using a monospecific antiserum raised against the *P. leniusculus* βGBP (8) and metabolic labeling of a primary tissue culture of hepatopancreas, a polypeptide, which was specifically immunoprecipitated with this antiserum, was found. Therefore, a cDNA expression library was constructed from this tissue and screened for recombinants reacting with the anti-βGBP antiserum. Two clones, BG-1 and BG-2, were isolated, subcloned and sequenced and also used as probes to isolate further overlapping clones (Fig. 1). Nine more clones were isolated and sequenced and the composite nucleotide sequence obtained from these clones spanned 4,679 bp. The first ATG codon at nucleotide 154, which is preceded by an in-frame termination codon, begins an open reading frame which encompasses 4,017 bp followed by a TAG stop codon and a 506-bp 3′-noncoding sequence. The deduced 1,339 amino acid residues of this open

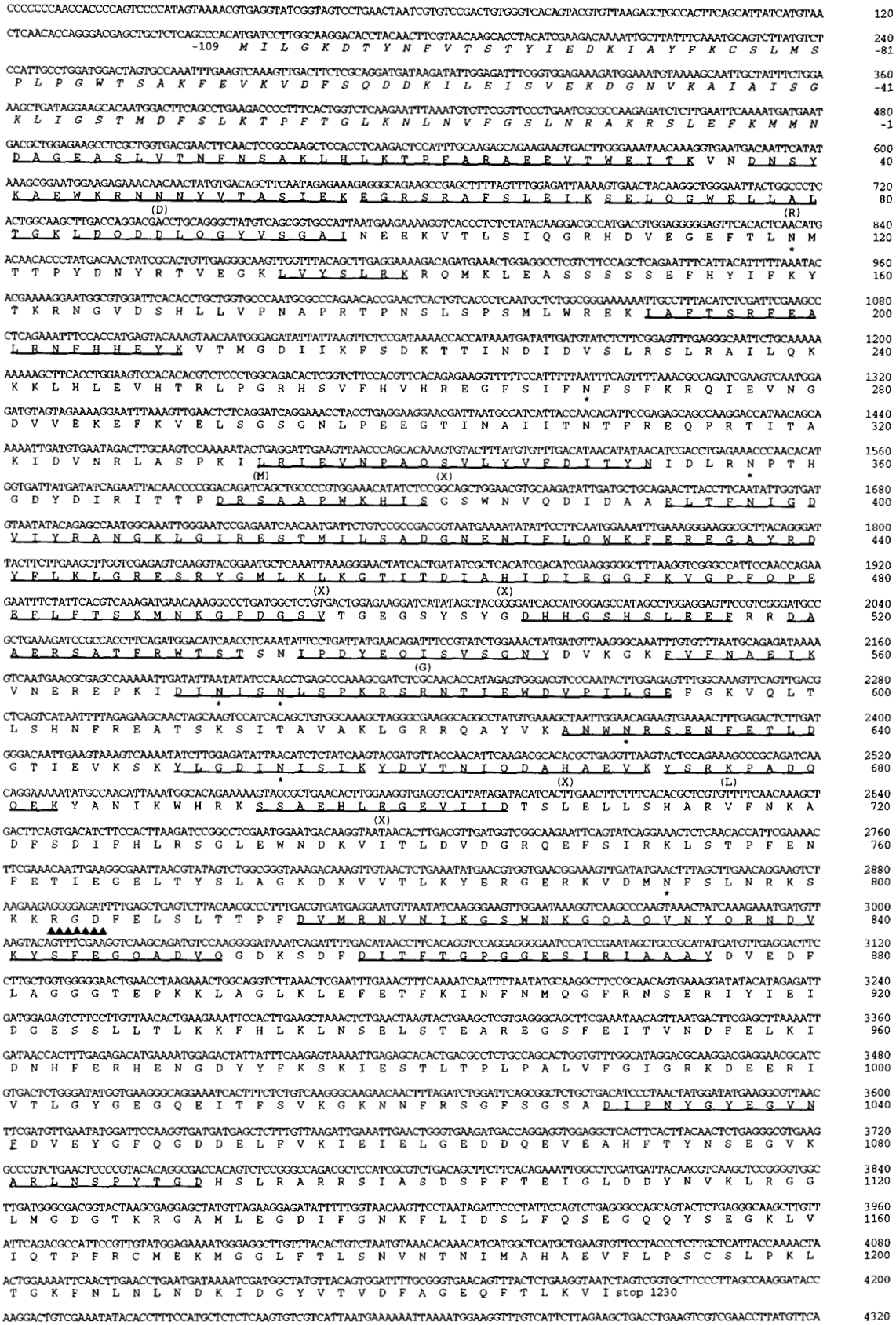

FIG. 2. **Nucleotide sequence and deduced amino acid sequence of the βGBP cDNA.** The sequences of the N terminus of the mature protein and of the peptide fragments obtained after partial proteolysis of the βGBP are *underlined*. Amino acid residues which were ambiguous

TABLE I
Carbohydrate composition of βGBP

Sugar	βGBP
	mol/mol
N-Acetylgalactosamine	0.2
N-Acetylglucosamine	1
Xylose	2
Glucose	2
Mannose	6
Fucose	1–2
Galactose	1–2

reading frame would give rise to a polypeptide with a calculated mass of 151,834 Da (Fig. 2). The apparent mass of the plasma βGBP as judged by SDS-polyacrylamide gel electrophoresis is approximately 100 kDa (8), and it is thus likely that the protein undergoes relatively extensive post-translational processing.

Determination of the Partial Amino Acid Sequence and C-terminal Amino Acid of βGBP—The authenticity of the cDNA sequence is corroborated by the determination of partial amino acid sequences from proteolytic digests of homogeneous *P. leniusculus* βGBP as well as from the determination of the N-terminal amino acid sequence of the mature protein. In total, sequences covering 464 amino acid residues were determined, and from partially overlapping fragments, 25 different peptide fragments ranging in length from 97 to 7 amino acid residues were obtained (Fig. 2). Judging from the cDNA sequence the N terminus is preceded by a prosequence comprising maximally 109 amino acid residues if counting the first ATG codon. There appears to be no typical signal peptide sequence located upstream of the N terminus of the mature protein. The C-terminal amino acid of the mature protein was determined after hydrazonolysis to be a histidine. The yield of histidine after hydrazonolysis was about 90%. The C-terminal amino acid residue of the deduced cDNA sequence is an isoleucine and thus it is likely that the protein is undergoing C-terminal processing. This processing may leave His^{1187} or His^{1091} as the terminal amino acid residue. It is not likely that this cleavage would take place immediately after His^{1071}, because amino acids 1081–1090 were identified from amino acid sequencing of the purified protein (Fig. 2).

βGBP Is a Glycoprotein and Exhibits No 1,3-β-D-Glucanase Activity—The deduced amino acid sequence contains eight putative *N*-glycosylation sites (Fig. 2). However, the Asn^{571} and Asn^{574} residues were clearly identified by amino acid sequencing, which suggests that these residues are probably not glycosylated. For a further characterization of the protein a carbohydrate analysis was performed. This analysis confirmed that the crayfish βGBP is a glycoprotein and showed that the major sugar constituent is mannose (Table I). The protein also contains glucose, xylose, fucose, galactose, and *N*-acetylglucosamine. No sialic acid was found. No glucanase activity was detected when βGBP was incubated with ^{14}C-labeled 1,3-β-D-glucans.

Expression of βGBP mRNA—To determine if tissues other than the hepatopancreas could synthesize βGBP in crayfish, Northern analysis was performed on RNA isolated from different organs. In contrast to the hepatopancreas, no signal was found after hybridizing a βGBP cDNA probe to RNA from hemocytes, gills, epidermis, green gland or abdominal muscle (Fig. 3). Thus, the hepatopancreas appears to be the major if not the only organ responsible for the biosynthesis of βGBP in

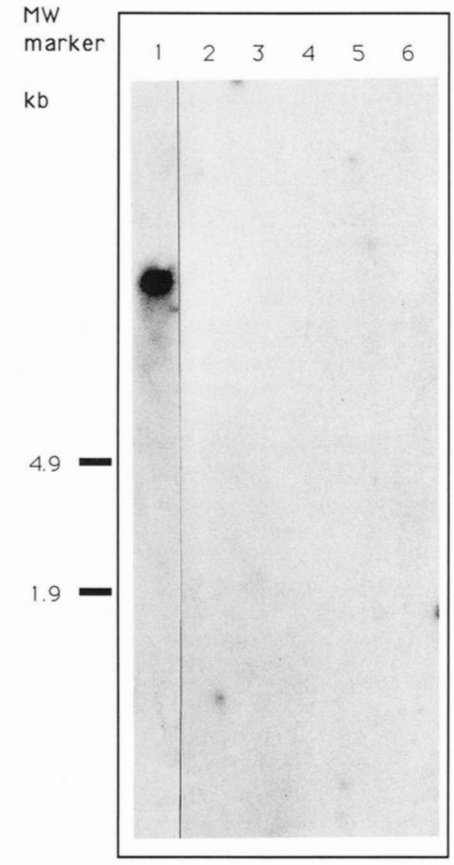

FIG. 3. **Tissue specific expression of βGBP.** The lanes contain total RNA isolated from *P. leniusculus*; from the *left* to *right*: *1*, hepatopancreas; *2*, gills; *3*, hemocytes; *4*, abdominal muscles; *5*, epidermis; *6*, green gland. The blot was hybridized with a ^{32}P-labeled βGBP cDNA probe. Twenty μg of total RNA were loaded on each lane.

crayfish. In some experiments we tried to inject laminarin, a 1,3-β-D-glucan, into the animals (25), but no increase in βGBP expression could be detected (not shown). Thus it appears that βGBP is constitutively expressed in *P. leniusculus*.

Binding of βGBP to Its Blood Cell Receptor Protein—The deduced sequence contains an Arg-Gly-Asp sequence and, furthermore, it was earlier demonstrated (26) that a synthetic peptide, Arg-Gly-Asp-Ser, having this sequence motif had the same biological effects on isolated blood cells as did purified βGBP, *i.e.* to induce degranulation and cell spreading. We therefore tested whether this peptide had any effect on βGBP binding to fixed blood cells. The binding of βGBP as visualized by immunofluorescence was not influenced by this peptide. Thus, the Arg-Gly-Asp sequence in βGBP may not be involved in βGBP-binding to the blood cell receptor protein. Alternatively, the Arg-Gly-Asp triplet alone may not be sufficient for binding and additional domains of the βGBP are also required.

βGBP Functions as an Opsonin—To compare the βGBP with the mammalian mannan-binding protein and LPS-binding protein, which can act as opsonins, we investigated whether the crayfish βGBP, at a physiologically relevant concentration (the concentration in plasma is 200–400 μg of βGBP/ml), could enhance phagocytosis of yeast cells by isolated crustacean hemocytes. If killed *S. cerevisiae* cells were pre-incubated with 200 μg/ml βGBP before assay, the percentage of hemocytes that ingested yeast particles increased to 20% compared to 6.7% in a control with buffer-treated yeast (Fig. 4). Also, preincubation

in the amino acid sequencing are indicated with (*X*) below the deduced sequence. Five residues, where peptide sequencing and DNA sequencing resulted in different amino acid assignments, are indicated *below* the deduced amino acid sequence. Putative *N*-glycosylation sites are marked with an *asterisk*. The Arg-Gly-Asp sequence is indicated with ▲▲▲▲▲▲▲.

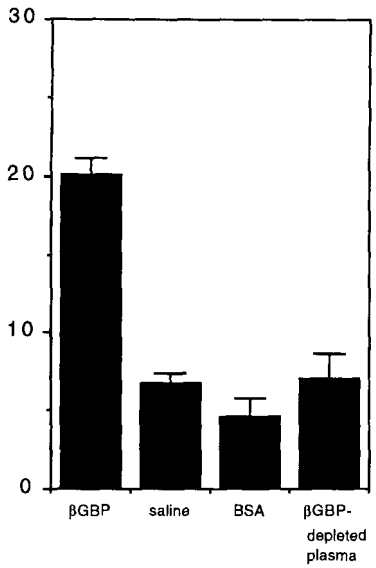

FIG. 4. **Opsonic effect of βGBP on crayfish hyaline hemocytes.** The *numbers* denote the percentage hemocytes containing ingested yeast particles. The number of particles per hemocyte varied between 1 and 4. The average number of particles per hemocyte was the same in all treatments.

of the yeast with 200 μg/ml bovine serum albumin or with crayfish plasma depleted of βGBP did not result in an increased uptake of the fungal cells. Thus, the βGBP functioned as an opsonin in these experiments and increased the uptake of yeast cells by the isolated blood cells.

DISCUSSION

This study reports the molecular cloning of a protein (βGBP) which after binding to 1,3-β-D-glucans mediates different defense reactions in crayfish. Some noticeable features of the βGBP sequence are: the lack of a signal peptide, the near absence of cysteine residues, and several short repeated sequences such as Glu-Asn-Phe-Glu-Thr, Asp-Ile-Asn-Ile-Ser, Glu-Gly-Lys-Leu-Val, and Asp-Lys-Val-*X*-Thr-Leu, all four of which occur twice in the βGBP open reading frame.

The crayfish βGBP is not similar to any other known protein as judged from data bank comparisons. In particular, it is noticeable that βGBP is not similar to other lectins, which perhaps is not surprising since no lectins with specific ligand requirement for 1,3-β-D-glucopyranosyl units have been sequenced. However, two short sequence motifs exhibit some sequence similarity with the active site of microbial 1,3–1,4-β-D-glucan 4-glucanohydrolases (27) in the βGBP sequence (Fig. 5). Although speculative, this could mean that the βGBP has evolved from one or two β-glucanase genes and then, possibly, as the result of our glucanase assay indicates, lost its capacity to hydrolyze 1,3-β-D-glucans.

Factor G, which mediates the glucan-triggered pathway of the horseshoe crab coagulation cascade, has recently been cloned. That protein, in contrast to the crayfish βGBP, contains a serine proteinase domain and is dimeric (5). The βGBP clearly has a primary structure different from factor G. There are no evident similarities between the primary structure of the βGBP and mammalian proteins able to bind microbial polysaccharides such as the mannan-binding protein (28) or the LPS-binding protein (29). The mannan-binding protein is a C-type lectin (30), which in addition to its carbohydrate binding domain contains collagen-like repeats (28). Thus, the similarity between βGBP and these binding proteins from mammals is probably only functional in that all these factors have in common is that they specifically bind to glycans or lipids commonly

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I T D I A H I D I E G G F K - V G P F Q P   479  (1)
E R I Y I E I D G E S S L L T L K K F H L   934  (2)
G T P W D E I D I E F L G K D T T K V Q F   149  (3)
G R P W V E V D I E V L G K N P G S F Q S    67  (4)
G T Q W D E I D I E F L G K D T T K V Q F   118  (5)
N N P W D E I D I E F L G K D T T K V Q F   151  (6)
          *   *   *
```

FIG. 5. **Alignment of two glucanase-like stretches of crayfish βGBP with the active site of bacterial 1,3–1,4-β-D-glucan 4-glucanohydrolases.** Sequences (*1*) and (*2*) are from the βGBP, and (*3*) to (*6*) are glucanohydrolases from *Bacillus licheniformis* (34), *Fibrobacter succinogenes* (35), *Bacillus macerans* (36), and *Clostridium thermocellum* (37), respectively. The *numbering* is based on the sequence of each protein. *Boxed* residues occur in the same position in βGBP and in one or several glucanohydrolases. One gap has been introduced in the βGBP sequence. The positions of Glu^{134}, Glu^{138}, and Asp^{136} in the *B. licheniformis* enzyme (27) are indicated with *asterisks*. These residues were suggested to act as catalytic nucleophile, general acid catalyst and to affect the pK_a of the catalytic reaction, respectively (27).

present in fungal or bacterial cell walls. This binding will result in increased phagocytic uptake of the microbial cells (Fig. 4) (31, 32). The murine plasma LPS-binding protein, for example, will mediate phagocytosis after binding to the lipid A core of the LPS (32). In addition, several other immune responses are mediated by the LPS-binding protein such as the synthesis of tumor necrosis factor α (33).

Thus, both in mammals and arthropods pattern recognition of characteristic macromolecules present on the surface of pathogens paves the way to the activation of immunocompetent cells. Studies on how 1,3-β-D-glucans bring about different immune reactions via the βGBP and the hemocyte membrane receptor in invertebrates may therefore shed light on the evolution of innate immunity.

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