A β1,3-Glucan Recognition Protein from an Insect, Manduca sexta, Agglutinates Microorganisms and Activates the Phenoloxidase Cascade

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Pattern recognition proteins function in innate immune responses by binding to molecules on the surface of invading pathogens and initiating host defense reactions. We report the purification and molecular cloning of a cDNA for a 53-kDa β1,3-glucan-recognition protein from the tobacco hornworm, Manduca sexta. This protein is constitutively expressed in fat body and secreted into hemolymph. The protein contains a region with sequence similarity to several glucanases, but it lacks glucanase activity. It binds to the surface of and agglutinates yeast, as well as Gram-negative and Gram-positive bacteria. β1,3-Glucan-recognition protein in the presence of laminarin, a soluble glucan, stimulated activation of prophenoloxidase in plasma, whereas laminarin alone did not. These results suggest that β1,3-glucan-recognition protein serves as a pattern recognition molecule for β1,3-glucan on the surface of fungal cell walls. After binding to β1,3-glucan, the protein may interact with a serine protease, leading to the activation of the prophenoloxidase cascade, a pathway in insects for defense against microbial infection.

Pattern recognition molecules serve as biosensors for detection of invading pathogens in the innate immune systems of vertebrate and invertebrate animals (1). They also play a crucial role in regulating the adaptive immune reactions carried out by vertebrate lymphocytes (2). Pattern recognition molecules bind to certain pathogen-associated molecular patterns that are not found in the host, such as lipopolysaccharide or peptidoglycan from bacterial cell walls and molecules that are not found in the host, such as lipopolysaccharide or peptidoglycan from bacterial cell walls. Upon binding to the foreign invaders, pattern recognition proteins trigger defense pathways such as the complement system in vertebrates (2) and the prophenoloxidase (PPO) activation pathway in insects and other arthropods (3).

The PPO activation pathway, like the complement system, involves a protease cascade. Determining the molecular mechanisms by which pattern recognition proteins differentiate non-self from self and transduce signals that stimulate defensive responses is a key to understanding the regulation of innate immune systems.

Some pattern recognition proteins from mammals, such as the cellular receptor CD14, the mannose-binding protein, the first component of complement (C1q), and the macrophage mannose receptor and scavenger receptor have been well characterized (4). Recent investigations of pattern recognition molecules from insects have identified a group of C-type lectins, which bind bacterial lipopolysaccharides and stimulate antibacterial responses of hemocytes or activate the phenoloxidase pathway (5–8). A peptidoglycan recognition protein has also been discovered in the silkworm (9, 10). A 19-kDa hemolymph protein is constitutively expressed in the fat body, epithelial cells, and hemocytes of naive silkworms and can be induced by bacterial challenge. Binding of peptidoglycan to peptidoglycan recognition protein can trigger the PPO defense pathway (9). A cDNA which encodes a similar bacteria-inducible peptidoglycan recognition protein has been cloned from the moth, Trichoplusia ni (11). Furthermore, proteins homologous to the insect peptidoglycan recognition proteins have been identified in human and mouse and are expressed in bone marrow and spleen (10, 11), indicating that this peptidoglycan recognition protein is conserved from insects to humans.

Fungal infections can be recognized by proteins which bind to cell wall β1,3-glucans, a molecular pattern specific for fungi. A group of similar β1,3-glucan-binding proteins from crustaceans and fungi have been isolated, and a cDNA for one of them was cloned from the crayfish, Pacifastacus leniusculus (12–15). The crayfish protein can induce spreading and degranulation of crayfish granular hemocytes when bound to β1,3-glucan (16). An insect β1,3-glucan-recognition protein has been purified from the silkworm, Bombyx mori (17). Binding of this 62-kDa protein with β1,3-glucan triggers the PPO pathway in plasma. Several β1,3-glucan-binding proteins have been purified from cockroach species (18–20). These proteins also enhance PPO activation triggered by soluble β1,3-glucan. However, so far no cDNA or amino acid sequence for an insect β1,3-glucan recognition protein has been reported.

In this paper, we describe the purification and cDNA cloning of a β1,3-glucan-recognition protein (GRP) from the tobacco hornworm, M. sexta. It is synthesized in the fat body and secreted into hemolymph. The deduced amino acid sequence of GRP shows similarity with bacterial and sea urchin glucanases (21), clotting factor G subunit α from a horseshoe crab (22), earthworm CCF-1 (23), and also with a Gram-negative bacteria-binding protein from the silkworm (24). M. sexta GRP at physiological concentration caused aggregation of yeast and
bacteria. Finally, we report that GRP can trigger the activation of the PPO pathway upon binding to soluble β1,3-glucan.

**EXPERIMENTAL PROCEDURES**

**Insects—**M. Sexta eggs were originally obtained from Carolina Biological Supply. Larvae were reared on an artificial diet as described by Dunn and Drake (25).

**Affinity Purification of GRP for Antiserum Production—**Hemolymph (100 ml) was collected from day 3 fifth instar M. sexta larvae. Cell-free hemolymph (plasma) was prepared by diluting the hemolymph 1:2 in an anticoagulant buffer (4 mM NaCl, 40 mM KCl, 0.1% polyvinylpyrrolidone, 1.9 mM PIPES, 4.8 mM citric acid monohydrate, 13.6 mM sodium citrate, 4 mM EDTA, 5% sucrose, pH 6.8) and centrifuging at 12,000 × g for 15 min to remove hemocytes. The plasma (100 ml) was incubated with 0.5 g of curdlan (an insoluble β1,3-glucan preparation, Sigma) pre-equilibrated with PBS (0.01 M phosphate buffer, 0.02% NaN3, 0.15 M sodium chloride, and 0.137 M potassium chloride, pH 7.4, at 25 °C) at room temperature for 20 min with mixing and then centrifuged (12,000 × g, 5 min). The pellet wash was washed with the same buffer until the supernatant was colorless. The 20–35% saturated ammonium sulfate fraction was pooled and applied to a hydroxyapatite column (3 × 3 cm) pre-equilibrated with PBS (0.01 M phosphate buffer, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 200 μM dNTP, and 2 units of *Thermus aquaticus* DNA polymerase (Invitrogen) and then incubated with 5 ml of PBS and 1 ml of 6% sodium dodecyl sulfate at 100 °C for 30 min, followed by centrifugation at 18,000 × g for 30 min. The supernatant was dialyzed against SDS-PAGE gel and used as an antigen for the production of a rabbit antiserum (Cocalico Biologicals, Reamstown, PA).

**Western Blot Analysis—**For immunoblot analysis of proteins after separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% dry skim milk, and then incubated with rabbit antiserum to GRP (1:1000 dilution). Antibody binding was visualized by a color reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit IgG (Bio-Rad). To estimate the concentration of GRP in hemolymph, 1-μl samples of plasma were separated by SDS-PAGE followed by immunoblotting as described above. The immunoreactive bands were digitized using a Kodak DC120 digital camera, and band intensities were measured using one-dimension Image Analysis Software (Kodak Digital Science). Using known concentrations of purified, native GRP were used to produce a standard curve, for determining GRP concentration in plasma samples. Plasma from larvae of several insect species including the Indian mealmoth *Plodia interpunctella*, the fruit fly *Drosophila melanogaster*, and the tobacco budworm *H. virescens* (obtained from the Department of Entomology, Kansas State University) was collected and analyzed by SDS-PAGE and Western blotting, using antiserum to *M. sexta* GRP.

**Purification of Native GRP—**One hundred and fifty ml of hemolymph were collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation. The 20–35% saturated ammonium sulfate fraction was collected as described above, and then fractionated by ammonium sulfate precipitation.
was dissected, washed with PBS, and then incubated at 28 °C in 2 ml of EX-CELL 405 (JRH Biosciences) insect cell culture medium containing penicillin (100 units/ml) and streptomycin (100 µg/ml) in wells of a 24-well plate with shaking at 100 rpm. At different times of incubation (0, 7, 11, 29, 40, and 64 h), 20 µl of the medium was removed and stored at −80 °C for later analysis. These samples were then analyzed for the presence of GRP by Western blotting using antibody to GRP as described above.

**Computer Analysis of Sequence Data**—Preliminary sequence editing and analysis was performed using IBI pestull programs. The amino acid sequence deduced from the cDNA was used to search the nonredundant peptide sequence data base and the translated sequence tag data base (National Center for Biotechnology Information) with the blast program (30). The most similar protein sequences were retrieved and aligned with the GRP sequence using the pileup program from the GCG Sequence Analysis Software Package 7.3.1 (31).

Recombinant GRP—PCR was used to generate a GRP cDNA fragment encoding amino acid residues 1–468, which was cloned into the NcoI and HindIII sites of *E. coli* expression vector H6-pQE-60 (32) and used to transform *E. coli* strain XL1Blue. Expression of GRP in this vector yields a protein with an amino-terminal sequence of Met-(His)-Ala-Met-Gly-Leu-Glu-Val. Maximum expression of the recombinant protein (0.1 mg/ml *E. coli* culture) was obtained from cultures in Luria-Bertani medium containing 100 µg/ml ampicillin, with shaking for 20 h at 37 °C. The bacteria from 100 ml of culture were centrifuged at 10,000 × *g* for 5 min and then resuspended in 10 ml of 8 M urea, 100 mM sodium phosphate, pH 6.8, 200 mM sodium chloride, 10 mM β-mercaptoethanol (buffer B) to lyse the bacteria. After centrifugation at 12,000 × *g* for 15 min, the supernatant was used for purification of recombinant GRP (H6GRP) by Ni2+-affinity chromatography.

Approximately 5 mg of recombinant H6GRP was mixed with 5 ml of Ni-agarose resin (Qiagen) for 12 h at room temperature. This mixture was then packed in a column and washed with 15 ml of buffer B. Then the column was washed with a 60-ml gradient from 8 M urea in Buffer B to 0 M urea in 100 mM sodium phosphate, pH 6.8, 200 mM NaCl, 2 mM glutathione, 0.02 mM oxidized glutathione, 10% glycerol (buffer C) at 1 ml/min. Finally, the column was eluted with 100 mM imidazole in buffer C. Three-ml fractions were collected and analyzed by SDS-PAGE. Fractions containing purified H6GRP were stored at 4 °C. Immediately before use, samples were passed through a Sephadex G-25 column (PD-10, Amersham Pharmacia Biotech) to change the buffer to 50 mM Tris, pH 7.6. One milliliter of 10,000 cells/ml of larval plasma; lanes 2 and 4, 0.7 µg of GRP purified under nondenaturing conditions. Lanes 1 and 2 are stained with Coomassie Blue; lanes 3 and 4 are detected by Western blotting using antibody to GRP.

**RESULTS**

Identification of a β1,3-Glucan-binding Protein from *M. sexta* Larval Hemolymph—To identify hemolymph proteins that can bind to β1,3-glucan, curdlan (an insoluble β1,3-glucan preparation) was used as an affinity matrix to purify proteins from *M. sexta* larval plasma. After incubating curdlan with plasma and washing the curdlan with buffer, the bound proteins were eluted by treatment with SDS and β-mercaptoethanol at 95 °C for 10 min. When this sample was analyzed by SDS-PAGE, a major protein with a molecular mass of approximately 53 kDa was observed (Fig. 1A). This protein was later named β1,3-glucan-recognition protein (GRP). GRP accounted for more than 50% of the total protein eluted from curdlan. Several other weaker bands were also observed, including a band at ~17 kDa. GRP bound so tightly to curdlan that it could not be eluted without denaturing in SDS. Treatments with acid (pH 1.5), high salt concentration (2 M NaCl), or 8 M urea did not elute GRP from curdlan. Approximately 3 mg of denatured GRP was obtained from 100 ml of hemolymph by this affinity method.

GRP eluted from curdlan was further purified by SDS-
and subjected to next purification step. A larval plasma. Buffer with a scale on the right axis calculate an apparent native mass of 73 kDa for GRP. Arrows pooled fraction from the ConA column. Washing with the same buffer, the column was eluted with 0.5 M methylgraphic step are described under "Experimental Procedures." To the 200 mM at pH 7.6, GRP eluted at the end of the gradient (Fig. 2). No additional GRP was eluted when the column was out as described under "Experimental Procedures" using sin-1,3-glucan recognition protein from plasma under nondenaturing conditions. Conditions for each chromatographic step are described under "Experimental Procedures." Solid lines show the absorbance at 250 nm; broken lines show the gradient of elution buffers with a scale on the right axis. The bars represent the fractions that contained GRP (detected by Western blot analysis) which were pooled and subjected to next purification step. A, Q-Sepharose Fast Flow column chromatography of a 20–35% saturated ammonium sulfate fraction of larval plasma. B, concanavalin A-agarose column chromatography. The column was pre-equilibrated with 50 mM Tris, 20 mM NaCl, pH 7.6. After washing with the same buffer, the column was eluted with 0.5 M methyl-α-D-mannopyranoside. C, hydroxyapatite column chromatography of the pooled fraction from the ConA column. D, gel filtration HPLC of purified GRP. The indicated elution times of standard proteins were used to calculate an apparent native mass of 73 kDa for GRP. Arrows indicate peak positions of standard proteins and GRP.

FIG. 2. Purification of β1,3-glucan recognition protein from plasma under nondenaturing conditions. Conditions for each chromatographic step are described under "Experimental Procedures." Solid lines show the absorbance at 250 nm; broken lines show the gradient of elution buffers with a scale on the right axis. The bars represent the fractions that contained GRP (detected by Western blot analysis) which were pooled and subjected to next purification step. A, Q-Sepharose Fast Flow column chromatography of a 20–35% saturated ammonium sulfate fraction of larval plasma. B, concanavalin A-agarose column chromatography. The column was pre-equilibrated with 50 mM Tris, 20 mM NaCl, pH 7.6. After washing with the same buffer, the column was eluted with 0.5 M methyl-α-D-mannopyranoside. C, hydroxyapatite column chromatography of the pooled fraction from the ConA column. D, gel filtration HPLC of purified GRP. The indicated elution times of standard proteins were used to calculate an apparent native mass of 73 kDa for GRP. Arrows indicate peak positions of standard proteins and GRP.

Purification and Analysis of GRP—to obtain nondenatured GRP for functional studies, GRP was purified from fifth instar larval plasma by a series of chromatographic steps that did not involve binding to curdlan. The purification procedure consisted of ammonium sulfate fractionation, followed by column chromatographic separations using Q-Sepharose, ConA, and hydroxyapatite (Fig. 2). The 20–35% ammonium sulfate fraction contained about 50% of the total GRP in plasma. This step removed most of the storage proteins, which are present at high concentration in the plasma. GRP bound to Q-Sepharose at pH 7.6 in a buffer containing 50 mM Tris and 20 mM NaCl. When the column was eluted with a sodium chloride gradient from 20 to 200 mM at pH 7.6, GRP eluted at the end of the gradient (Fig. 2A). No additional GRP was eluted when the column was washed with 1 mM sodium chloride. Fractions from the Q-Sepharose column containing GRP were combined and applied to a ConA column. GRP did not bind to the ConA column and was present in the flow-through fractions. However, other glycoproteins that did bind to ConA were separated from GRP in this step. Pooled fractions from the ConA column were applied to a hydroxyapatite column pre-equilibrated with 10 mM sodium phosphate buffer, pH 6.8. GRP eluted near the end of a sodium phosphate gradient from 10 to 100 mM.

When the fractions containing GRP were analyzed by SDS-PAGE, GRP appeared as a single band with an apparent molecular mass of 53 kDa (Fig. 1A, lane 2). Analysis by MALDI mass spectrometry gave a mass of 53,583 Da for GRP. Approximately 0.3 mg of pure GRP was obtained from 100 ml of plasma. When this purified GRP was analyzed by gel filtration HPLC, it eluted as a single peak, with an elution volume consistent with a native molecular mass of about 70 kDa. This result suggests that GRP exists as a monomer in solution.

The amino-terminal sequence of GRP isolated as described above was identical to that obtained from the protein eluted from curdlan. To obtain sequence information from an internal peptide fragment, GRP was treated with formic acid to hydrolyze between Asp and Pro residues. The resulting peptide fragments were separated by two-dimensional gel electrophoresis and transferred to a polyvinylidene difluoride membrane. From one of the peptide spots on the membrane with a mass of approximately 25 kDa, an amino acid sequence of 17 residues was determined by Edman degradation (Fig. 3).

cDNA Cloning and Deduced Protein Sequence—The amino-terminal sequence of GRP and the internal amino acid sequence of the fragment derived from formic acid hydrolysis were used to design degenerate oligonucleotide primers for PCR, to amplify a fragment of a GRP cDNA. PCR was carried out as described under “Experimental Procedures” using single-stranded cDNA synthesized from fat body mRNA as a template. A 700-base pair PCR product was obtained and cloned.
into a plasmid vector, and this cloned fragment was used as a hybridization probe to screen a larval fat body cDNA library. Forty-eight positive clones were isolated from a screen of approximately 3 x 10⁷ plaques. The GRP cDNA clone (pGRP9) containing the longest insert (approximately 1.6 kilobases) was sequenced.

The sequence of pGRP9 contains a 47-nucleotide 5' noncoding region, an open reading frame of 1461 nucleotides, and a 3'-untranslated sequence of 56 nucleotides (Fig. 3). The open reading frame encodes 487 amino acid residues. The first 19 amino acid residues make up a secretion signal peptide, and the amino-terminal sequence of the mature protein begins at residue 20. The internal amino acid sequence of 17 residues determined from a peptide obtained after hydrolysis of GRP with formic acid was consistent with the deduced sequence between residues 240 and 256 (Fig. 3). The calculated molecular mass of the 468-residue mature protein is 52,335 Da, which is 1,247 Da less than the mass determined by mass spectrometry. There are two putative N-linked glycosylation sites in the carboxyl-terminal region of the protein. After GRP was treated with N-glycosidase F, it migrated slightly faster than untreated GRP in SDS-PAGE analysis (Fig. 4), indicating that this glycosidase removed approximately 1 kDa of N-linked carbohydrate from the protein. There are five Cys residues in the mature protein. Therefore, GRP must contain at least one free Cys that is not involved in a disulfide bond. The calculated pl of the mature GRP is 5.0. This is in agreement with the results of two-dimensional gel electrophoresis, in which the pl of GRP was estimated to be approximately 5.5 (data not shown).

**Sequence Comparisons**—Searching of the GenBank sequence data bases indicated that the GRP open reading frame has significant sequence similarity with bacterial β-glucanases and with several invertebrate proteins that contain glucanase...
like regions. The carboxyl-terminal region (residues 174–442) was similar to a group of \( \beta_1,3 \)-glucanases, with the highest degree of similarity among these to enzymes from a sea urchin and from \textit{Bacillus circulans} (Fig. 5). The amino-terminal region was similar to several proteins from invertebrates, which also contain a carboxyl-terminal glucanase-like sequence. \textit{GRP} was similar throughout its sequence to a Gram-negative bacteria-binding protein (GNBP) from the silkworm, \textit{B. mori} (24) (42% identity), and to homologous putative GNBP's from the fall webworm, \textit{Hyphantria cunea} (33) (39% identity), and a mosquito \textit{Anopheles gambiae} (34) (38% identity). Partial amino acid sequences available from cDNAs corresponding to expressed sequence tags (EST) from \textit{B. mori} and from \textit{Drosophila melanogaster} also were quite similar to \textit{GRP}. \textit{M. sexta} GRP is 59% identical to the protein encoded by the \textit{B. mori} EST (accession number AU004243) but only 37% identical to the corresponding amino-terminal region of \textit{B. mori} GNBP, which suggests that this EST, rather than GNBP, is the silkworm ortholog of \textit{M. sexta} GRP. In addition to these insect proteins, \textit{GRP} is similar to sequences of coelomic cytolytic factor 1 (CCF-1), which is a glucan-binding protein from an earthworm, \textit{Eisenia fetida} (23) (34% identity) and to a \( \beta_1,3 \)-glucanase from a sea urchin, \textit{Strongylocentrotus purpuratus} (21) (34% identity) (Fig. 5). These proteins are similar to \textit{GRP} in both the glucanase domain and the amino-terminal region, although the amino-terminal extension is much shorter in CCF-1 than in the sea urchin glucanase or the insect proteins.

The most conserved region of the glucanase-like domain of \textit{GRP} and the similar invertebrate proteins is in a sequence that aligns with a region near the active site of bacterial \( \beta_1,3 \)-glucanases and \( \beta_1,3–1,4 \)-glucanases (Fig. 5). However, \textit{GRP} lacks four conserved residues that line the active site of such enzymes, and in particular has nonconservative replacements of two Glu residues that are believed to act as the catalytic residues responsible for cleaving \( \beta_1,3 \)- or \( \beta_1,4 \)-glycosidic bonds in the bacterial glucanases (35). This observation is consistent with the fact that we have not detected any \( \beta_1,3 \)-glucanase activity associated with \textit{GRP} (data not shown).

In addition to the sequences discussed above, BLAST searches using the \textit{GRP} sequence identified a number of sequences related to \textit{GRP} and to bacterial glucanases. The PILEUP program (31) was used to align the sequence of \textit{GRP} with the most similar proteins identified by searching the GenBank database with the BLAST program. These include a group of proteins from invertebrates, all of which contain a carboxyl-terminal region with similarity to bacterial glucanases. \textit{Ms-GRP}, \textit{M. sexta} \( \beta_1,3 \)-glucan-recognition protein (AF177982); \textit{Bm-EST}, an expressed sequence tag from \textit{B. mori} (AU004243); \textit{Bm-GNBP}, \textit{B. mori} Gram-negative bacteria-binding protein (L38591); \textit{Hc-GNBP}, \textit{H. cunea} putative Gram-negative bacteria-binding protein (AF023916); \textit{Dm-EST1}, two expressed sequence tags (5' and 3' ends) from \textit{D. melanogaster} (AI257586, AI109637); \textit{Ag-GNBP}, \textit{A. gambiae} putative Gram-negative bacteria-binding protein (AJ001042); \textit{Sp-GLUC}, \textit{S. purpuratus} \( \beta_1,3 \)-glucanase (U49711); \textit{Ef-CCF-1}, \textit{E. foetida} coelomic cytolytic factor 1 (AF030028). Two bacterial glucanases are also shown in this alignment: \textit{Bc-GLUC}, \textit{B. circulans} \( \beta_1,3 \)-glucanase A1 (P23903), the bacterial glucanase most similar to \textit{M. sexta} GRP; \textit{Bm-GLUC}, \textit{B. macerans} endo-1,3-1,4-\( \beta \)-glucanase (P23904), a related glucanase whose structure has been determined by x-ray crystallography (35). Residues conserved in all of the invertebrate sequences are marked with *, and residues identical in at least 4 of the invertebrate sequences are marked with +. Residues corresponding to the active site of the \textit{B. macerans} glucanase are marked with #. Positions in \textit{M. sexta} GRP which are identical to at least one of the bacterial glucanases are underlined. Numbering corresponds to the \textit{M. sexta} GRP sequence as shown in Fig. 3. In the sequences derived from expressed sequence tags, regions that have not yet been sequenced are shown with ; symbols. A marks the approximate site of proteolytic cleavage of \textit{GRP} to produce the 17-kDa fragment shown in Fig. 1.
amounts of GRP for functional studies, GRP was expressed as elicitors (24, 33, 34). which are synthesized in response to exposure to microbial GNBP and homologous proteins from (Fig. 7 B molymph. The level of GRP mRNA in fat body did not increase with synthesis of GRP by fat body and secretion into he-

The glucanase domain, widespread in evolution, appears to have evolved in the insects (Fig. 6). In all of these sequences, the putative glucanase active site residues are conserved, in contrast to GRP and the other sequences with lower but still significant similarity to the conserved region near the glucanase active site. These include β,1,3-glucanases from bacteria and plants, an expressed sequence tag from the cloned cDNA. No band was detected in RNA derived from RNA standards. The approximate size of the mRNA was confirmed by probing a duplicate blot with 32P-labeled cDNA for ribosomal protein S3 (29). The approximate size of the mRNA was derived from RNA standards. B. RNA samples from fat body of fifth instar larvae injected with a mixture of E. coli and yeast 24 h earlier were analyzed by Northern blotting as described above. Control larvae were injected with PBS. The bands intensities were quantified using one-dimensional Image Analysis Software (Kodak Digital Science), and the ratio of GRP mRNA to ribosomal protein S3 mRNA was calculated. Bars represent the mean ± S.E. (n = 5).

Expression of GRP in vivo—In Northern blot analysis, the GRP cDNA hybridized to a 1.5-kilobase nucleotide band in RNA samples from larval fat body, consistent with the size expected from the cloned cDNA. No band was detected in RNA from hemocytes or integument (Fig. 7A). These results indicate that fat body is the primary site of GRP synthesis, as is true for most insect hemolymph proteins. Furthermore, when fat body was cultured in vivo, GRP was first detected in the culture medium after 7 h and its concentration gradually increased up to 64 h in culture (data not shown). This result is also consistent with synthesis of GRP by fat body and secretion into hemolymph. The level of GRP mRNA in fat body did not increase significantly after larvae were injected with bacteria and yeast (Fig. 7B). This lack of inductibility distinguishes GRP expression from that of three similar proteins from insects, B. mori GNBP and homologous proteins from H. cunea and A. gambiae, which are synthesized in response to exposure to microbial elicitors (24, 33, 34).

Production of Recombinant GRP—To obtain sufficient amounts of GRP for functional studies, GRP was expressed as a recombinant protein in E. coli. Recombinant GRP (H6GRP), which contains an amino-terminal 6-histidine tag, was insoluble in the buffer extract obtained after lysis of E. coli by sonication. It could be dissolved in 8 M urea and was purified by affinity chromatography. After H6GRP bound to the Ni2+ affinity column, it was renatured using a linear gradient of urea from 8 M to 0 M, and then eluted by washing with 100 ml imidazo-

The H6GRP preparation was soluble and contained a single band at 53 kDa when analyzed by SDS-PAGE and Coo-

The concentration of GRP in plasma is approxi-

mately 30 μg/ml (determined by densitometry analysis of Western blot samples as described under “Experimental Procedures”). These results indicate that such binding would occur at physiological GRP concentrations.

Because H6GRP was able to bind to bacteria and yeast, we tested whether H6GRP can aggregate these microorganisms. The presence of H6GRP at 50 μg/ml caused significant aggre-

agation of S. cerevisiae, E. coli, and S. aureus, whereas bovine serum albumin used as a control at the same concentration did not lead to aggregation of these organisms (Fig. 9). These results suggest that one function of GRP may be to aggregate invading microorganisms, leading to more efficient clearance by hemocytes.

Activation of the PPO Pathway by GRP in the Presence of Laminarin—Activation of PPO in insect plasma can be trig-

gered by β,1,3-glucan (17, 36, 37). We performed experiments to determine whether interaction of GRP with β,1,3-glucan might participate in activation of this pathway. M. sexta larval plasma was diluted 1:2 in anticoagulant buffer, which decreased the endogenous GRP concentration from 30 to 10 μg/ml. This diluted plasma was incubated with different amounts of the β,1,3-glucan laminarin and 100 μg/ml H6GRP at room
temperature for 20 min, and then the phenoloxidase activity in the plasma was determined (Fig. 10). Laminarin alone (1 mg/ml) did not trigger the PPO pathway within 20 min. However, when laminarin (1 or 0.1 mg/ml) combined with 100 mg/ml H6GRP was added to plasma, PPO activity significantly increased. H6GRP alone stimulated a much smaller degree of PPO activation. These results suggest that GRP serves as a biosensor for β1,3-glucan in insect defense and that binding of GRP to fungi may lead to activation of a proteinase in the PPO activation cascade.

**DISCUSSION**

We have purified from hemolymph of the tobacco hornworm, *M. sexta*, a 53-kDa protein that binds very tightly to β1,3-glucans. We have named this protein GRP, which is the name used for a very similar protein from hemolymph of silkworms (17). We first isolated GRP by taking advantage of its affinity to curdlan, an insoluble β1,3-glucan, but this protein bound so tightly to curdlan that it could only be eluted by treatment with SDS. We then developed a method to purify GRP that did not require denaturing conditions. GRP is present in hemolymph...
at all developmental stages of *M. sexta* at approximately 30 μg/ml (0.6 μM). In immunoblot analysis using antiserum to *M. sexta* GRP, we have detected an immunoreactive band at ~50 kDa in plasma from other insect species, including two lepidopterans: *P. interpunctella* and *H. virescens*; and a dipteran:* D. melanogaster*, suggesting that the presence of a similar GRP in plasma may be expected in other insects. *M. sexta* GRP has a sequence very similar to that of a β1,3-glucan-recognition protein isolated from hemolymph of the silkworm,* B. mori* (17), which is a 62-kDa protein that binds very strongly to curdlan and is involved in phenoloxidase activation.3

We isolated a cDNA clone that encodes GRP from a larval fat body library. The amino acid sequence of GRP, deduced from the nucleotide sequence of the cloned cDNA, indicated that the protein is composed of two regions, which appear to represent amino-terminal and carboxyl-terminal domains. The carboxyl-terminal domain is similar in sequence to β1,3- and β1,3,1,4-glucanases from bacteria (38) and to a β1,3-glucanase from a sea urchin (21), but GRP had no detectable β1,3-glucanase activity. Two catalytic Glu residues, conserved in the active site of the bacterial glucanases (35) are replaced with Leu and Cys in *Manduca* GRP, which explains the absence of glucanase activity (Fig. 4). Thus, GRP may be an evolutionary descendent of a glucanase that has lost its catalytic activity but has maintained the ability to bind to β1,3-glucans. Clotting factor G from a horseshoe crab,* Tachypleus tridentatus*, also contains a region with similarity to these glucanases (Fig. 6), and it is possible that binding of β1,3-glucans to this domain triggers the activation of the enzyme’s proteolytic subunit (22).

Although no sequences similar to the glucanase-like domain of GRP appear to have been found so far in vertebrates, the discovery of a homologous sequence in an ascidian (a primitive chordate) (39) suggests that such glucan-binding proteins may exist in vertebrates as well.

A group of proteins from insects and from an earthworm are also similar in sequence to GRP. They contain the carboxyl-terminal glucanase-like domain and also a unique amino-terminal domain. These include a GNBP from hemolymph of the silkworm,* B. mori*. (24). GNBP-like sequences have also been identified in the fall webworm,* H. cunea* (33), and a mosquito,* A. gambiae* (34), although these two proteins are known so far only from cDNA sequences and have not been tested for function. A partial *B. mori* sequence encoded by an expressed sequence tag cDNA is more similar to *M. sexta* GRP than is *B. mori*, GNBP (Fig. 5), suggesting that this cDNA may encode the silkworm GRP.

A protein called CCF-1 from an earthworm,* E. foetida*, also has a sequence similar to that of GRP (23). CCF-1 binds to β1,3-glucan and bacterial lipopolysaccharide and thus may function in a manner similar to GRP. Although the glucanase active site residues are conserved in CCF-1 (Fig. 5), it also lacks apparent glucohydrolase activity (23). The catalytic Glu residues are also conserved in *A. gambiae* GNBP (Fig. 5), but whether it can function as a glucanase is unknown.

The insect proteins related to GRP as well as the earthworm CCF-1 and the sea urchin β1,3-glucanase contain an amino-terminal domain that lacks similarity to glucanases or to any other sequences currently in the Genbank data base. This amino-terminal domain (residues 1–173 in *M. sexta* GRP) has a molecular mass of approximately 17 kDa in *M. sexta* GRP and in the related invertebrate proteins, except for CCF-1 and the *A. gambiae* protein, in which this region is somewhat smaller (Fig. 5). We identified a 17-kDa polypeptide from *M. sexta* plasma that bound to curdlan and had an amino-terminal sequence identical to that of GRP (Figs. 1 and 5). This polypeptide appears to represent the amino-terminal domain of GRP and perhaps is a product of endogenous proteolytic activity in plasma. We observed that this species accumulated upon storage of plasma, which is consistent with the hypothesis that it is a degradation product of GRP and that the two domains are linked by a region that is sensitive to proteolysis. Although the function of this domain is not known, it appears that it can bind to curdlan independently from the carboxyl-terminal glucanase-like domain and thus may have its own glucan-binding site.

Northern blot analysis indicated that GRP mRNA is present in fat body (a tissue analogous to mammalian liver) and not in other tissues tested, including hemocytes. Cultured fat body released GRP into the medium, which is consistent with the hypothesis that fat body is the primary source of GRP in plasma. A significant difference between *M. sexta* GRP and the insect GNBPd is that synthesis of the GNBPd is strongly induced by microbial infection (24, 33, 34), whereas GRP is constitutively expressed and not induced as an acute phase response protein.

GRP bound to the surface of yeast cells, which contain β1,3-glucans in their cell walls, and to Gram-negative and Gram-positive bacteria.* E. foetida* CCF-1 also was shown to bind to β1,3-glucan and bacterial lipopolysaccharide (23). The ability of GRP to bind to microbial surfaces suggests that it may function as a pattern recognition molecule for detection of bacterial or fungal pathogens. Incubation of GRP with yeast or bacteria caused them to aggregate, which could improve the efficiency of clearance of these microorganisms by hemocytes through phagocytosis or nodule formation (40).

Activation of prophenoloxidase in hemolymph is a commonly observed response to microbial infection in insects and many other invertebrates (3). When GRP preincubated with the soluble β1,3-glucan, laminarin, was added to diluted plasma, the prophenoloxidase activation pathway, which involves a serine proteinase cascade (3) was triggered, and active phenoloxidase was released from hemocytes. We observed that this species accumulated upon storage of plasma, which is consistent with the hypothesis that it is a degradation product of GRP and that the two domains are linked by a region that is sensitive to proteolysis. Although the function of this domain is not known, it appears that it can bind to curdlan independently from the carboxyl-terminal glucanase-like domain and thus may have its own glucan-binding site.

Proteins that bind to β1,3-glucans and stimulate phenoloxidase activation have been identified in other invertebrate species and appear to fall into several classes. A 100-kDa β1,3-glucan-binding protein, which enhances activation of phenoloxidase has been isolated from a crayfish, *P. leniusculus* (12). Proteins very similar to the crayfish glucan-binding protein have also been identified in shrimp (13, 14). These 100-kDa proteins from crustaceans appear to represent one family of invertebrate β1,3-glucan-binding proteins that function as pattern recognition molecules. Two different types of β1,3-glucan-binding proteins have been isolated from hemolymph of cockroaches from the genus *Blaberus*. An approximately 90-kDa protein isolated from *B. craniifer*, with subunits of 63 and 52 kDa, binds to laminarin and enhances activation of proPO from a hemocyte lysate (18). A different type of β1,3-glucan-specific lectin isolated from *B. discoidalis* is a hexamer of 80- and 82-kDa subunits, and appears to be a member of the hexamerin family of arthropod hemolymph proteins (20). Similar proteins have been isolated from plasma of a

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3 C. Ma, unpublished results.

M. Ochiai and M. Ashida, personal communication.
group of different cockroach species (19). These cockroach β1,3-glucan-specific lectins, in combination with laminarin, are also able to activate the phenoloxidase system (20).

\textit{M. sexta} GRP, \textit{B. mori} GRP, and the earthworm protein CCF1 are members of another family of circulating proteins able to activate the phenoloxidase system (20). Glucan-specific lectins, in combination with laminarin, are also thought to occur in the lectin-mediated pathway for complement activation (41). Further study of these β1,3-glucan-recognition proteins in arthropods should lead to a better understanding of the function and evolution of pattern recognition molecules that bind to polysaccharides on the surface of microbial pathogens and stimulate innate immune responses.

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