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cDNA cloning, purification, properties, and function of a β-1,3-glucan recognition protein from a pyralid moth, *Plodia interpunctella*

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Abstract

Microorganisms possess distinctive biochemical or molecular patterns on their cell surfaces, such as those formed by the lipopolysaccharides, lipoteichoic acids, and/or peptidoglycans of bacteria and the β-1,3-glucans of fungi. Pattern recognition proteins that bind to these surface moieties have been implicated in the activation of the innate immune response in insects and other invertebrates. We report the purification and cloning of a cDNA for a 53-kDa β-1,3-glucan recognition protein (βGRP) from the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae). βGRP cDNA contains an open reading frame that encodes 488 amino acids, of which the first 17 residues comprise the secretion signal peptide. The calculated molecular mass of the 471-residue mature protein is 53,311 Da. The protein consists of a carboxyl-terminal domain that is similar to other recognition proteins from invertebrates, β-1,3-glucanases from bacteria, and a β-1,3-glucanase from the sea urchin, *Strongylocentrotus purpuratus*. The amino-terminus of βGRP shares sequence similarity with other invertebrate recognition molecules and the β-1,3-glucanase from *S. purpuratus*. Affinity purification of a 53-kDa protein and subsequent sequencing of a peptide produced by tryptic cleavage confirmed the presence of the βGRP in *P. interpunctella* larval hemolymph. RT-PCR analysis indicates that βGRP is constitutively expressed in all life-stages, with no detectable induction following exposure of wandering larvae to microbial elicitors. Northern blot analysis indicates that the 1.8-kb βGRP transcript is transcribed within the fat body. Recombinant βGRP retains β-1,3-glucan-binding activity, binds to lipopolysaccharide and lipoteichoic acid in vitro, causes aggregation of microorganisms, and activates the prophenoloxidase cascade in the presence of soluble β-1,3-glucan. These data support the hypothesis that the 53-kDa βGRP functions to recognize pathogen surface molecules as nonself and subsequently activates insect innate immune responses.

Keywords: Insect immunity; Innate immunity; Pattern recognition proteins; Indianmeal moth; β-1,3-glucan; Prophenoloxidase; Hemolymph; Immune response; Insect

1. Introduction

An immune response requires an initial recognition event that signals the host organism of an impending threat. Pattern recognition proteins function to initiate the host immune response upon binding to non-self pathogen-associated molecular patterns (PAMPs). PAMPs are molecules present on the surface of microorganisms, typically as structural components, and are not found within the host organism (Medzhitov and Janeway, 1997). PAMPs for which pattern recognition proteins have been identified include β-1,3-glucan and mannans from fungi, lipopolysaccharide (LPS) and flagellin from Gram-negative bacteria, lipopeptides from mycobacteria, lipoteichoic acid (LTA) and peptidoglycan from Gram-positive bacteria, and unmethylated CpG DNA from bacteria.

Pattern recognition molecules serve as biosensors in the activation of innate immune responses in both vertebrates and invertebrates. Families of pattern recognition receptors characterized in insects and other arthro-

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Nomenclature

AMV-RT avian myeloblastosis virus reverse transcriptase
βGRP β-1,3-glucan recognition protein
BSA bovine serum albumin
CIP calf intestinal phosphatase
CCF coelomic cytolytic factor
DMSO dimethyl sulfoxide
DTT dithiothreitol
EDTA ethylenediamine tetraacetate
GNBP Gram-negative bacterial binding protein
HPLC high performance liquid chromatography
IPTG isopropylthio-β-galactoside
LPS lipopolysaccharide
LTA lipoteichoic acid
MALDI-MS matrix assisted laser desorption ionization mass spectrometry
PAGE polyacrylamide gel electrophoresis
PAMP pathogen-associated molecular pattern
PBS phosphate-buffered saline
PGRP peptidoglycan recognition protein
PIPES 1,4-piperazinediethanesulfonic acid
PPO prophenoloxidase
PRP pattern recognition protein
PTU phenylthiourea
RLM-RACE RNA ligase-mediated rapid amplification of cDNA ends
RT-PCR reverse transcription polymerase chain reaction
SDS sodium dodecyl sulfate
SPH serine protease-like homolog
TAP tobacco acid pyrophosphatase
TBS Tris-buffered saline

pods include β-1,3-glucan recognition proteins (βGRPs) and Gram-negative bacterial binding proteins (GNBPs) (Ma and Kanost, 2000; Ochiai and Ashida, 2000; Kim et al., 2000; Shin et al., 1998; Beschin et al., 1998; Bilej et al., 2001; Sritunyalucksana et al., 2002; Lee et al., 1996; Dimopoulos et al., 1997), peptidoglycan recognition proteins (PGRPs) (Kang et al., 1998; Ochiai and Ashida, 1999; Werner et al., 2000), LPS-binding proteins and C-type lectins (Jomori and Natori, 1991; Kawasaki et al., 1996; Shin et al., 1998; Koizumi et al., 1999; Theopold et al., 1999; Yu et al., 1999; Yu and Kanost, 2000), complement-like proteins (Lagueux et al., 2000; Levashina et al., 2001), a scavenger receptor protein (Pearson et al., 1995; Rämet et al., 2001), and hemolin (Sun et al., 1990; Ladendorff and Kanost, 1991; Shin et al., 1998; Yu and Kanost, 2002). βGRPs from several arthropods have been implicated in the activation of a protease cascade that leads to prophenoloxidase activation (Söderhäll et al., 1988; Beschin et al., 1998; Ochiai and Ashida, 2000; Ma and Kanost, 2000; Lee et al., 2000; Bilej et al., 2001) and to activate induction of antimicrobial peptide genes (Kim et al., 2000).

In this paper, we describe the purification, cDNA cloning, expression, and properties of a β-1,3-glucan recognition protein from the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), a major agricultural insect pest of stored and/or processed commodities. Evidence for agglutination of bacteria and yeast, binding to several PAMP molecules, and activation of the PPO activating system by the recombinant protein near physiological concentration is also presented.

2. Materials and methods

2.1. Insects

A field strain of *Plodia interpunctella* was collected from shelled corn in El Paso, Illinois, in November of 1998 and was reared in the laboratory on a diet of cracked wheat, wheat shorts, wheat germ, Brewer's yeast, honey, glycerin, and water. Cultures were maintained at 27 °C and 50–55% relative humidity in 12 h light:dark. Wandering 5th instar larvae that have finished

feeding and that are preparing to pupate were used for all experiments.

2.2. Plasma collection

Larvae were surface sterilized in ice-cold 95% ethanol, placed on ice, and hemolymph was collected by cutting the third pro-leg with sterile micro-scissors and drawing hemolymph into a gel-loading micropipet tip. Two to 3 μL of hemolymph could be obtained from a single larva, each weighing ~15 mg. Hemolymph was dispensed into a few crystals of phenylthiourea (PTU) to prevent melanization. Hemolymph samples were centrifuged at 10,000 *g* for 5 min at 4 °C to pellet hemocytes. Cell-free plasma was diluted 1:50 with distilled water, and protein concentration determined with Coomassie Plus Protein Assay Reagent (Pierce) with bovine serum albumin as a standard.

2.3. Immunoblot analysis

For immunoblot analysis, proteins were separated on Novex 10% Bis-Tris NuPAGE 1.0-mm thick SDS polyacrylamide gels, transferred to nitrocellulose membranes, which were blocked with 5% (w/v) nonfat dry milk in 1 × TBS - T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20), and incubated with rabbit antiserum against *Manduca sexta* βGRP-1 or -2 (1:2000 dilution) (Ma and Kanost, 2000; Yu et al., 2002). Antibody binding was observed by a color reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit IgG (BioRad). MultiMark protein molecular weight standards (Novex) were run simultaneously.

2.4. Affinity purification of βGRP

Curdlan (an insoluble β-1,3-glucan preparation from Sigma) was used as an affinity matrix to purify βGRP from *P. interpunctella* plasma by a procedure similar to that of Ochiai and Ashida (1988) and Ma and Kanost (2000). Hemolymph (200 μL) was collected from 100 larvae into an equal volume of 1× 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) containing 2 mM *p*-aminobenzamidine and 0.5 mg/mL PTU. After centrifugation at 12,000 *g* for 15 min at 4 °C plasma was incubated with 10 mg of curdlan in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) for 0.5 h on ice with occasional mixing. The plasma/curdlan mixture was centrifuged at 12,000 *g* for 5 min at 4 °C to obtain the unbound fraction. The curdlan pellet was washed five times with 1 mL of PBS (from which the final wash supernatant gave an $OD_{280} < 0.005$), once with 1 mL of PBS containing 0.5 M NaCl, followed by three additional 1-mL washes of PBS. Bound protein was eluted from the curdlan precipitate by addition of 50 μL 1× SDS - NuPAGE sample buffer (Novex) containing 50 mM DTT and heated for 10 min at 95 °C. After centrifugation at 12,000 *g* for 10 min at 4 °C, the supernatant was analyzed by SDS-PAGE and immunoblotting.

2.5. Internal amino acid sequence determination

Attempts to obtain sequence from the amino terminus of the affinity-purified βGRP were unsuccessful, indicating a blocked amino-terminus. The affinity-purified βGRP (10–20 pmol) was subjected to in-gel trypsin digestion, peptide purification and identification, and sequencing at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University, New Haven, CT. The purity of HPLC-peptide peaks was determined using MALDI-MS. Peptide sequencing was performed on an Applied Biosystems Procise 494 cLc instrument equipped with an on-line HPLC for the identification of the resulting phenylthiohydantoin (Pth) amino acid derivatives from Edman degradation.

2.6. cDNA isolation and sequencing

A *P. interpunctella* larval whole-body cDNA library in λ Uni-Zap XR vector (Stratagene) (Zhu et al., 2000) was screened using rabbit antiserum against *M. sexta* βGRP2 (Yu et al., 2002) as a probe. Approximately 7 × 10^5 λ plaques in *E. coli* strain XL1-Blue MRF′ were screened as described by Sambrook et al. (1989). Homogeneous positive phage plaques were obtained following secondary and tertiary screens. Subcloning was performed by in vivo excision of the pBluescript phagemid from the Uni-ZAP XR vector according to the manufacturer's instructions. The positive clone containing the longest insert (1.9-kb) was sequenced on both strands by the DNA Sequencing and Genotyping Facility at Kansas State University, Manhattan, KS. Complete sequencing of the cloned insert was obtained using T3 and T7 vector primers as well as the specific sense (3bgrp5, 5′ AAAGTCTGTCTGCCAGGGAA 3′) and antisense (4bgrp3, 5′ GGAGGCCGAGTGATAGGAA 3′) primers.

2.7. 5′-RACE

The 5′-end of the *P. interpunctella* βGRP cDNA was obtained from RNA ligase-mediated rapid amplification of 5′-cDNA ends (RLM-RACE) using the GeneRacer™ Kit (Invitrogen). Total RNA from ten larvae was obtained following homogenization of whole larvae in guanidine thiocyanate, extraction of RNA by phenol:-chloroform, and precipitation in isopropanol (Titus, 1991). The RNA pellet was resuspended in guanidine thiocyanate solution and precipitated consecutively in

isopropanol and ethanol. Total RNA (5 μg) was initially treated for 1 h at 50 °C with calf intestinal phosphatase (CIP) which removes the 5′-phosphate on truncated mRNAs as well as on all non-mRNAs. The RNA was extracted in phenol:chloroform and precipitated in ethanol as described in the GeneRacer™ protocol. Dephosphorylated RNA was treated with tobacco acid pyrophosphatase (TAP) for 1 h at 37 °C to remove the 5′-cap from intact, full-length mRNA. The RNA was again extracted with phenol:chloroform and precipitated in ethanol. The GeneRacer™ RNA Oligo (5′ CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA 3′) was ligated to the dephosphorylated, decapped RNA using T4 RNA ligase at 37 °C for 1 h, and the RNA was extracted with phenol:chloroform and precipitated in ethanol. mRNA was reverse transcribed using a *P. interpunctella* βGRP gene specific primer (4bgrp3) and Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) at 42 °C for 1 h. The 5′-end of the βGRP cDNA was amplified by PCR using the sense GeneRacer™ 5′ Primer (5′CGACTGGAGCACGAGGACACTGA3′) and nested antisense *P. interpunctella* βGRP gene specific primer (5bgrp3, 5′ TTCCAGCGTGGAAGCGAGACTCCAACAG 3′). The gel-purified PCR product was cloned into the pCR4-TOPO vector and used to transform OneShot Chemically Competent *E. coli* (Invitrogen). Plasmid DNA was purified by using the QIAprep Plasmid Prep Kit (Qiagen). Both strands of cloned DNA were sequenced by using T3 and T7 vector primers.

2.8. Computer analysis of sequence data

Comparison of the *P. interpunctella* βGRP cDNA sequence against the non-redundant public sequence database was made using BLASTX (Altschul et al., 1990). Sequence analysis tools of the ExPASy Molecular Biology Server of Swiss Institute of Bioinformatics, including Translate, SignalP, and Compute pI/MW, were used to analyze the deduced βGRP protein sequence. Similar protein sequences were aligned with βGRP using CLUSTALW provided by the European Bioinformatics Institute (EBI). *P. interpunctella* βGRP was aligned individually with other protein sequences using GAP from SeqWeb (Version 2) (Accelrys) to calculate percent identity and similarity. TreeView (Version 1.6.6) was used to generate a phylogenetic tree from CLUSTALW guide tree data (Page, 1996). N- and O-glycosylation predictions were made using the NetNGlyc 1.0 and NetOGlyc 2.0 prediction servers found at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/).

2.9. RT-PCR expression analysis

Total RNA was extracted from different developmental stages, including embryos (1d, $n \approx 1500$), late 4th instar larvae (13d, $n = 30$), early 5th instar (14d, $n = 12$), late 5th instar (15d, $n = 10$), wandering 5th instar larvae (16d, $n = 10$), prepupae (17d, $n = 10$), pupae (18d, $n = 10$) and adults (28d, ♂ $n = 15$ and ♀ $n = 10$) as described above for 5′ RACE. Similarly, total RNA was obtained 20 h after injection of wandering 5th instar larvae with saline, *E. coli* (~10^6 cells), *Micrococcus lysodeikticus* (~10^6 cells), or *Saccharomyces cerevisiae* (~10^6 cells). The first strand cDNA synthesis was catalyzed from 5 μg of total RNA using an oligo-dT primer and SuperScript II RNase H^- reverse transcriptase (Gibco BRL Life-Technologies). RT-PCR was conducted using the specific sense and antisense *P. interpunctella* βGRP primers, 3bgrp5 and 4bgrp3, respectively. Control RT-PCR experiments were conducted using 1RPS75 (5′ ACAACAAGAAGTCAATCATCATCTA 3′) and 2RPS73 (5′ TTGTGCTCAATGGTGGTCTGTTG 3′) sense and antisense gene specific primers from *P. interpunctella* ribosomal protein S7. The PCR reaction mixture contained 1 μl target cDNA, 5 μl 10× PCR buffer (100 mM Tris-HCl pH 9.0 containing 0.5 M KCl and 1% Triton X-100), 3.0 μl of 25 mM $MgCl_2$, 2.0 μl of 2.5 mM of each dNTP in H_2O, 0.5 μl of each 50 μM primer (βGRP and control reactions conducted in separate sample tubes), 2.5 μl dimethyl sulfoxide (DMSO), 1.25 units of Taq DNA polymerase (Promega), and H_2O to obtain a final volume of 50 μl. The DNA was initially denatured for 3 min at 94 °C, and the PCR amplification included 20-, 30-, or 40-cycles of 30 s denaturing at 94 °C, 30 s annealing at 49 °C, and 1 min extension at 72 °C in an Eppendorf Mastercycler Gradient thermal controller (Brinkmann Instruments). A sample without RNA was run simultaneously as a negative control for RT-PCR. A positive control for PCR was performed using both RPS7 and βGRP plasmid DNA. RT-PCR products were separated on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and photographed over UV light.

2.10. Northern blot analysis

Total RNA was isolated from *P. interpunctella* larvae using guanidine thiocyanate extraction and was precipitated with isopropanol (Titus, 1991). mRNA was obtained from the whole larval total RNA sample and from fat bodies dissected from 29 larvae using a Micro-FastTrack 2.0 mRNA isolation kit from Invitrogen. Northern blot analysis was performed by using the NorthernMax kit from Ambion. BrightStar Biotinylated RNA Millenium Markers (Ambion), fat body mRNA (2 μg), and whole-larval mRNA (3 μg) were diluted in 3× formaldehyde loading dye containing 0.1 μg ethidium bromide and heated at 65 °C for 10 min. RNA samples were separated on 1% denaturing agarose gel, blotted onto Ambion's BrightStar-Plus nylon membrane, and cross-linked to the membrane using a UV-crosslinker at

1200 μJ/cm^2. A biotin-14-CTP βGRP RNA probe was made from a linearized βGRP/pGEM-T EZ plasmid using Ambion's MAXIscript SP6 in vitro transcription kit. The blot was prehybridized in ULTRAhyb at 68 °C for 2 h and hybridized in 0.1 nM probe in ULTRAhyb for 2 h at 68 °C. The blot was washed twice for 5 min at room temperature in Ambion's Low Stringency Wash buffer (equivalent to 2× SSC) followed by two 15 min washes at 68 °C in Ambion's High Stringency Wash buffer (equivalent to 0.1× SSC). Detection of the biotin-labeled βGRP RNA probe was conducted using Ambion's BrightStar BioDetect kit and exposing the blot to Kodak BIOMAX-ML film for less than 1 min.

2.11. Production of recombinant βGRP

PCR was used to generate βGRP cDNAs encoding amino acid residues 1-471, which were cloned into the *E. coli* expression vectors H_6-pQE-60 (Lee et al., 1994) and pTrcHis2-TOPO (Invitrogen). Insertion of the correct sequence into each expression vector was confirmed by sequencing of each plasmid.

For expression using the H_6-pQE-60 vector, the PCR primers 9bgrp5 (5′-CCATGGGACAGCCGCGTG-CGCAGCAGTAC 3′) and 8bgrp3 (5′-AAGCTTCAGCGCATAGACTCTGACATA 3′) were used to add *Nco*I and *Hind*III restriction sites to the 5′ and 3′ ends of the βGRP cDNA, respectively. The gel-purified PCR product was cloned into a pGEM-T EZ plasmid (Promega), cleaved with *Nco*I and *Hind*III, and the gel-purified fragment was ligated into the H_6-pQE-60 vector such that the six-histidine tag was at the amino-terminus and in frame with βGRP. Transformed *E. coli* (strain XL1 blue) were grown in 0.5 L of LB containing 0.05 mg/mL ampicillin at 37 °C until A_{600} of 0.5 was reached. Expression of recombinant βGRP (rβGRP) was induced by the addition of IPTG to a final concentration of 1.0 mM. Bacteria were incubated at 37 °C for 6 h with vigorous shaking and collected by centrifugation. Cell pellets were resuspended in guanidinium lysis buffer (6 M guanidine hydrochloride, 0.02 M sodium phosphate, 0.5 M sodium chloride, pH 7.8), incubated at room temperature for 0.5 h with gentle rocking, and sonicated at 30% maximum output (142.5 W) using a VirSonic 475 Ultrasonic Cell Disrupter (VirTis). The clear-lysate supernatant was collected following centrifugation. Purification of the His-tagged rβGRP from the clear lysate was conducted using Invitrogen's ProBond Purification System under denaturing conditions. Two mL of ProBond Ni^{2+} resin (Invitrogen) was equilibrated in denaturing binding buffer (8 M urea, 0.02 M sodium phosphate, 0.5 M NaCl, pH 7.8) following the manufacturer's instructions. Chromatography was conducted following a batch elution method in which the pH was decreased incrementally. Two 5-mL aliquots of the cell clear lysate were mixed with the equilibrated ProBond column for 15 min each at room temperature with constant end-over-end mixing. The unbound fractions were collected from the top of the column following centrifugation at 500 rpm for 2 min at room temperature. The affinity column was washed twice with 4 mL of pH 7.8 denaturing binding buffer, followed by three washes of 4 mL of pH 6.0 denaturing wash buffer, and three washes of 4 mL of pH 5.3 denaturing wash buffer. The bound protein was eluted with 10 mL of pH 4.0 denaturing elution buffer. Removal of urea was conducted using a Slide-A-Lyzer 10K-dialysis cassette (Pierce) with two dialysis steps. Each dialysis step consisted of at least 12 h and was conducted at 4 °C against 1 L of buffer. The first dialysis buffer contained 0.01 M Tris-HCl, pH 8 with 0.1% Triton X100 and 2.5 M urea, whereas the second dialysis buffer lacked urea.

The pTrcHis2-TOPO expression vector was used to produce a rβGRP containing a carboxyl-terminal six-histidine tag. The primers 7bgrp5 (5′-GGATCCCAGCCGCGTGCGCAGCAGTAC 3′) and 8bgrp3 were used to generate a βGRP PCR product that was gel-purified and cloned into the pTrcHis2-TOPO vector. Transformation of TOP10 One-Shot *E. coli* (Invitrogen) was performed, and the βGRP/pTrcHis2-TOPO plasmid was sequenced using pTrcHis-Forward and -Reverse primers to confirm correct insertion. The βGRP PCR product was inserted into the pTrcHis2-TOPO plasmid such that it was in frame with the *c-myc* epitope and six-histidine tag present at the carboxyl-terminus of βGRP. A single βGRP-containing transformant colony was selected and grown in a 5 mL LB starter culture containing 0.05 mg/mL ampicillin and 0.5% glucose at 37 °C. Expression of the rβGRP was performed at 37 °C in a 1.5 L LB culture containing 0.05 mg/mL ampicillin. Overexpression, extraction, and purification of 6HisC-rβGRP was conducted as outlined above.

2.12. Binding of recombinant βGRP to PAMPs

Purified recombinant proteins 6HisC- and 6HisN-rβGRP were tested for their ability to bind to β-1,3-glucan (curdlan), lipopolysaccharide (LPS), and lipoteichoic acid (LTA). Binding of rβGRP to β-1,3-glucan was assessed by curdlan co-precipitation similar to the method used to purify the soluble protein from plasma as above. Binding of 6HisC-rβGRP to LPS and LTA was assayed using microplate binding assays modified from Tobias et al. (1989) and Yu and Kanost (2000, 2002). Microplate wells were coated with 2 μg of LPS or LTA and assayed for binding to rβGRP using rabbit primary antiserum against *M. sexta* βGRP2 and goat anti-rabbit IgG conjugated with alkaline phosphatase. Alkaline phosphatase activity, which is proportional to the binding of rβGRP to the PAMP, was quantified in a microplate reader (BioTek Instruments) using an alka-

line phosphatase substrate kit from BioRad. rβGRP concentrations of 0, 1.25, 2.5, 5, 10, 15, 25, and 50 μg/mL were assayed. To validate the specificity of binding to LPS or LTA, 5 μg/mL rβGRP was pre-incubated with 0, 0.1, 1, 10, 50, 100, 250, or 500 μg/mL free competitor (LPS or LTA). Fifty μL of the pre-incubated solution was added to wells pre-coated with LPS or LTA and assayed for in vitro binding as described above.

2.13. Aggregation of microorganisms by rβGRP

To investigate potential functions of βGRP, experiments were conducted to test whether recombinant protein can bind to and cause aggregation of the yeast *S. cerevisiae*, the Gram-positive bacterium *Staphylococcus aureus*, and the Gram-negative bacterium *E. coli*. rβGRP (2.5 μg) in 10 mM Tris-HCl, pH 8, with 0.1% Triton X100 was incubated with 10 μL of fluorescein-conjugated *S. cerevisiae* (1×10^8 cells/mL), *S. aureus* (Wood strain without protein A, 1×10^9 cells/mL), or *E. coli* (K12 strain, 5×10^8 cells/mL) (all from Molecular Probes) in PBS at room temperature for 0.5 h. A 1-μL aliquot was applied to a microscope slide and aggregation was observed using an Olympus BX60 System microscope with a reflected light fluorescence attachment. Images were obtained using a SPOT digital camera from Diagnostic Instruments.

2.14. Activation of the PPO pathway by rβGRP

6HisC-rβGRP was tested for its ability to activate *M. sexta* prophenoloxidase in the presence of a soluble β-1,3-glucan (laminarin). Phenoloxidase activation was assayed using a method modified from Ma and Kanost (2000). Thirty μl of 6HisC-rβGRP (0.13 mg/mL in 10 mM Tris-HCl, pH 8 and 0.01% Triton X-100) was mixed with 10 μL of *M. sexta* plasma with or without laminarin (1 mg/mL final concentration) in wells of a microplate. The volume of each sample well was brought up to 130 μL with 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8. After incubation for 20 min at room temperature, 20 μL of 15 mM dopamine was added to each well. Phenoloxidase activity was determined by measuring the absorbance at 490 nm at one-min intervals for 30 min using a BioTek EL340 microplate reader equipped with Kc^3 software. PO activity corresponded to the slope of initial linear regions from each curve. The final working concentration of 6HisC-rβGRP was approximately 30 μg/mL or 0.5 μM. Control samples were run simultaneously and either lacked 6HisC-rβGRP or contained 0.5 μM BSA. Values represent the mean ± standard error of data from three sets of triplicates on a single pooled plasma sample collected from five *M. sexta* day 3 larvae (5th instar). Statistical analysis for PPO activation with or without laminarin was performed using the SAS PROC *t*-test at $\alpha = 0.05$.

3. Results

3.1. Identification of a β-1,3-glucan-binding protein from P. interpunctella larval hemolymph

A 53-kDa polypeptide present in both *M. sexta* and *P. interpunctella* plasma was cross-reactive with antisera made against *M. sexta* βGRP2 (Fig. 1). Rabbit antisera made against *M. sexta* βGRP1 also cross-reacted with a polypeptide with a similar molecular mass (data not shown). Because βGRP is of relatively low abundance in plasma (estimated concentration is 20–30 μg/mL) and because the molecular size of the βGRP is similar to that of hemolymph storage proteins found in *P. interpunctella* larvae, a protein band corresponding to βGRP was not visible after Coomassie staining of SDS-PAGE gels (Fig. 1, Lane 2).

3.2. Purification and analysis of P. interpunctella βGRP

After incubation of larval plasma with curdlan, the insoluble pellet was washed extensively with buffer. The

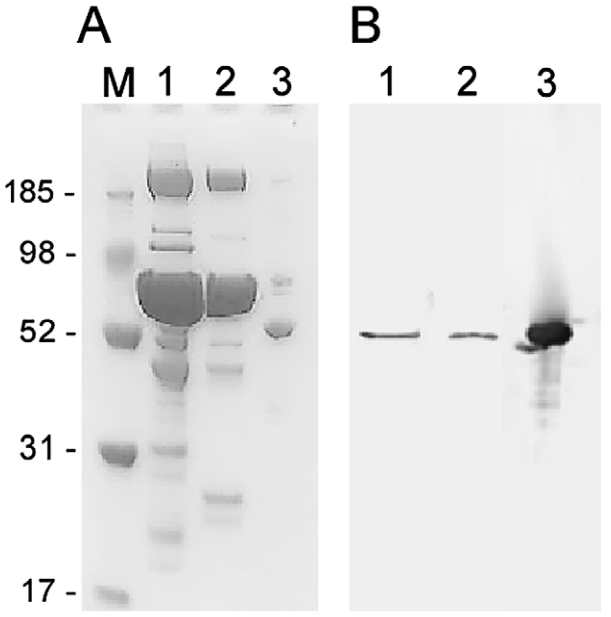

Fig. 1. SDS-PAGE and immunoblot analysis of larval plasma and purified *P. interpunctella* βGRP. SDS-PAGE gel stained with Coomassie Blue (A) and immunoblot (B) of *M. sexta* plasma (Lane 1), *P. interpunctella* plasma (Lane 2), and βGRP affinity-precipitated from *P. interpunctella* plasma by using curdlan (Lane 3). For gel stained with Coomassie (A), ~15 μg total protein from *M. sexta* plasma and *P. interpunctella* plasma were loaded in Lanes 1 and 2, respectively. For immunoblot (B), 10 μg total protein from *M. sexta* plasma and 5 μg total protein from *P. interpunctella* plasma was loaded in Lanes 1 and 2, respectively. Fifteen- and 5-μL of βGRP obtained from curdlan precipitated plasma were loaded in Lane 3 of the stained gel and immunoblot, respectively. Protein molecular weight standards are shown in Lane M.

major plasma proteins did not bind to curdlan and were found in the initial wash fractions (data not shown). Washing of the curdlan pellet with buffer containing 0.5 M NaCl did not result in the elution of additional plasma proteins. However, proteins with an approximate molecular mass of 200-, 94-, 53-, and 40-kDa were eluted from the curdlan precipitate by heat treatment in SDS solution under reducing conditions (Fig. 1). The 53-kDa polypeptide was the most abundant protein and it also cross-reacted with an anti-serum to *M. sexta* βGRP2. Repeated attempts to directly sequence the 53-kDa polypeptide via Edman degradation failed, indicating that the amino-terminus of the mature protein was blocked. The 200-, 94-, and 40-kDa proteins were not recognized by *M. sexta* βGRP2 antibody.

Approximately 2 μg of βGRP was purified from 0.2 mL of *P. interpunctella* plasma. The βGRP band from SDS-PAGE containing approximately 2 μg of protein (19 pmol) was excised and subjected to in-gel proteolytic hydrolysis for peptide sequencing as described in *Materials and methods*. MALDI-MS analysis of peptides generated from trypsin digestion and purified by HPLC revealed a fraction that contained a single, homogeneous peptide with a molecular mass of 1,091.3 Da. The peptide was sequenced and found to consist of the ten residues AQQYVVPSAK. The predicted molecular mass of this peptide was 1091.242 Da, which was in excellent agreement with the size determined by mass spectrometry.

3.3. cDNA cloning and analysis of deduced βGRP protein sequence

A cDNA clone isolated from an initial screen of the *P. interpunctella* expression library by using antiserum made to *M. sexta* βGRP2 consisted of 1917 nucleotides, but it was incomplete at its 5′-end. 5′-RACE was utilized to obtain the missing 91 nucleotides. The complete *P. interpunctella* βGRP cDNA clone contains a 34-nucleotide 5′ noncoding region, an open reading frame of 1464 nucleotides, and a 3′ untranslated sequence of 510 nucleotides (Fig. 2). The open reading frame encodes 488 amino acids, of which the first 17 residues comprise a predicted secretion signal peptide. The ten-residue peptide with sequence AQQYVVPSAK that was obtained by digestion and sequencing of the curdlan-precipitated 53-kDa protein corresponds exactly to residues 4–13 encoded by the cDNA (Fig. 2). βGRP contains two putative N-linked glycosylation sites at asparagine residues 356 and 436. However, NetNGlyc 1.0 predicted that only Asn 356 is glycosylated. NetOGlyc 2.0 predicted putative O-glycosylation sites at threonines 110, 114, 119, 120, 123, and 136 and at serine 115. The calculated molecular mass of the 471-residue mature protein is 53,311 Da, with a predicted pI of 5.94.

A BLAST search, multiple sequence analysis, and phylogenetic tree analysis of the βGRP cDNA sequence revealed that it was most similar to a family of pattern recognition proteins, which includes other βGRPs and GNBPs (Fig. 3). Alignments of the translated *P. interpunctella* βGRP protein sequence with those of *M. sexta* βGRP2 (AY135522), *B. mori* βGRP (BAA92243), *M. sexta* βGRP1 (AAF44011), *Drosophila melanogaster* GNBP3 (AAF50349), *B. mori* GNBP (AAB40946), *Hyphantria cunea* GNBP (AAD09290), and *Eisenia foetida* coelomic cytolytic factor 1 (CCF-1) (AAC35887) gave identities of 63, 55, 55, 44, 39, 39 and 34%, respectively. These results indicated that this family of pattern recognition proteins is evolutionarily conserved in many insects and other invertebrates.

Members of the βGRP family possess a carboxyl-terminal region that shares significant sequence similarity with the catalytic domain of β-1,3-glucanases from the sea urchin, *Strongylocentrotus purpuratus* (JC6141) and from various bacteria (AAC60453, AAA22474, AAA60459, AAC69707, CAA61884, CAA88008, AAC25554, and AAC44371). The *P. interpunctella* βGRP shares 35% identity and 43% similarity with the *S. purpuratus* glucanase and 18–34% identity and 34–44% similarity with the catalytic domains from various bacterial β-1,3-glucohydrolyases. The CCFs from two annelids, *E. foetida* and *Lumbricus terrestris*, have been shown to function as pattern recognition proteins but only share significant sequence similarity with the carboxyl-terminal glucanase-like region of βGRPs. Like the CCFs, *Anopheles gambiae* GNBP (CAA04496) and several putative GNBPs (EAA07723, EAA07705, and EAA07707) possess sequence similarity with glucanases and the carboxyl-terminus of the βGRPs. The *P. interpunctella* βGRP N-terminal domain, which consists approximately of the first 120 residues, possesses sequence similarity only to the amino-terminal region found in other βGRPs. The insect βGRP/GNBP family contains only four conserved cysteines, which are located near the amino terminal end of the carboxyl-terminal glucanase-like domain.

3.4. Expression of βGRP in P. interpunctella

Northern blot analysis of mRNA obtained from larval fat body and whole body extracts revealed that the βGRP probe hybridized to a ~1.8-kb RNA band (Fig. 4A). The size of the transcript is near the expected size obtained from cDNA cloning and is consistent with previous findings that βGRP is synthesized in insect fat body (Ma and Kanost, 2000). RT-PCR was utilized to determine the relative abundance of the βGRP and RPS7 transcripts following immune-challenge. PCR conducted with cDNA obtained from RNA from wandering-stage larvae 20 h following inoculation with *E. coli*, *M. lysodeikticus*, or *S. cerevisiae* did not exhibit a detectable increase in βGRP relative to controls (Fig. 4B). PCR conducted by

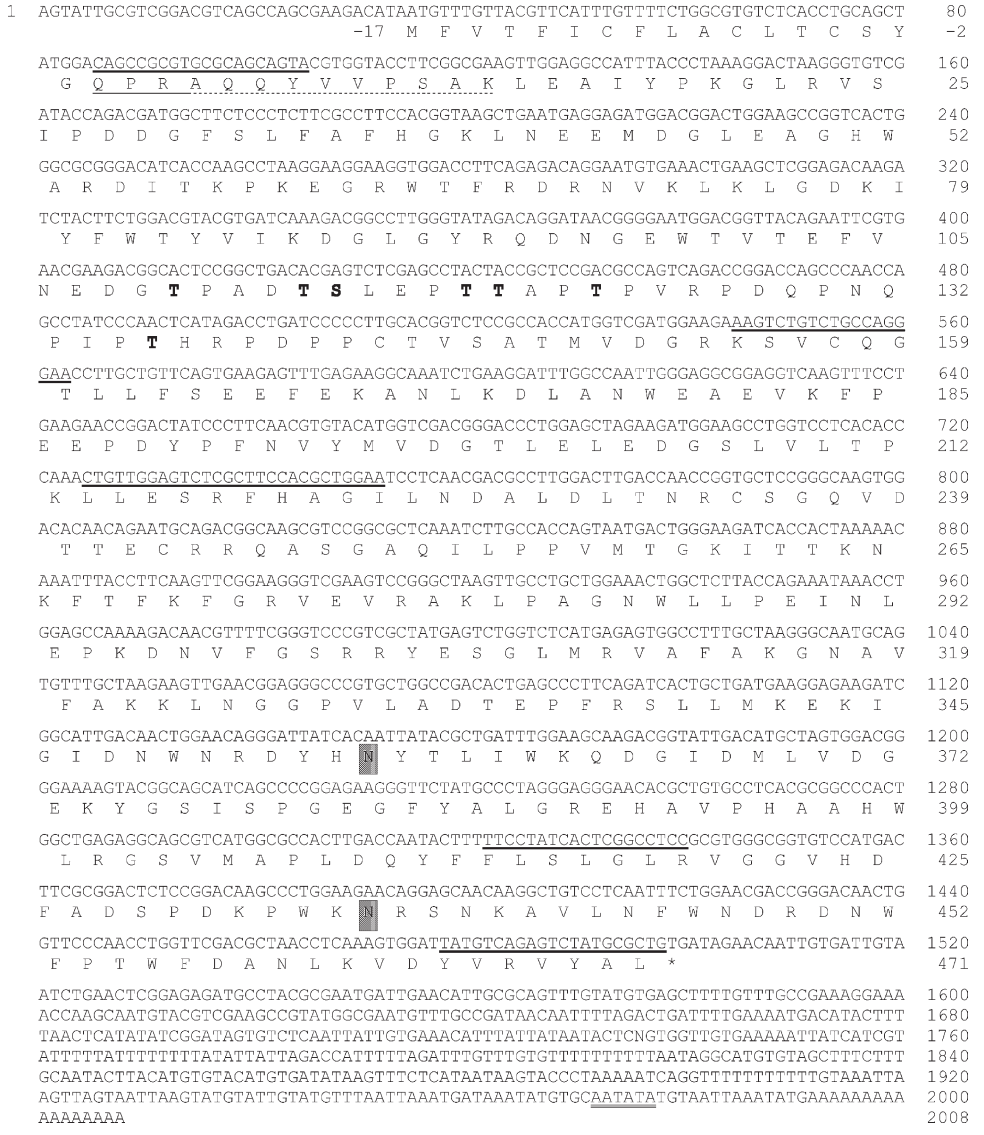

Fig. 2. Nucleotide and deduced amino acid sequence of *P. interpunctella* βGRP. The cDNA nucleotide sequence (1-2008) is shown above the deduced amino acid sequence (-17-471). The amino terminus of the mature protein is underlined with a single line and is assigned positive numbers. The putative signal peptide is assigned negative numbers. The amino acid sequence underlined with a dotted line was confirmed through peptide sequencing of a tryptic digest of the purified protein. Putative N-linked glycosylation sites are indicated as shaded residues and putative O-linked glycosylation sites are indicated in bold. Nucleotide sequences underlined with single, bold lines indicate positions of sense PCR primers including 3bgrp5 (545–563), 7bgrp5 (86–105), and 9bgrp5 (86–105) and antisense PCR primers including 4bgrp3 (1322–1340), 5bgrp3 (725–752), and 8bgrp3 (1478–1498). A putative polyadenylation sequence, AATATA, is double underlined, and the termination codon TGA is marked with an asterisk.

using 20-, 30-, or 40-cycles showed no significant difference in the level of βGRP transcript following the immune challenges. Immunoblot analysis of larval plasma samples inoculated with *S. cerevisiae* and collected 24 h post inoculation also failed to show any increased protein expression of βGRP compared to naïve or mock-injected larvae (data not shown). We cannot rule out the possibility that *P. interpunctella* βGRP may be upregulated at earlier or later times post inoculation.

RT-PCR was conducted to determine the presence and relative abundance of the βGRP transcript throughout the lifecycle of *P. interpunctella*. RT-PCR analysis revealed that the transcript is present in embryos, larvae, prepupae, pupae, and adults (Fig. 4C). The level of βGRP transcript did not appear to change throughout development when compared to controls.

3.5. Production of recombinant βGRP

An *E. coli*-heterologous expression system was used to produce sufficient amounts of rβGRP for functional studies. Recombinant βGRP was produced using H_6-pQE-60 or pTrcHis2-TOPO expression plasmids that result in fusion proteins with a six-histidine tag at either the amino-terminus (6HisN-rβGRP) or at the carboxyl-terminus (6HisC-rβGRP) of the expressed proteins,

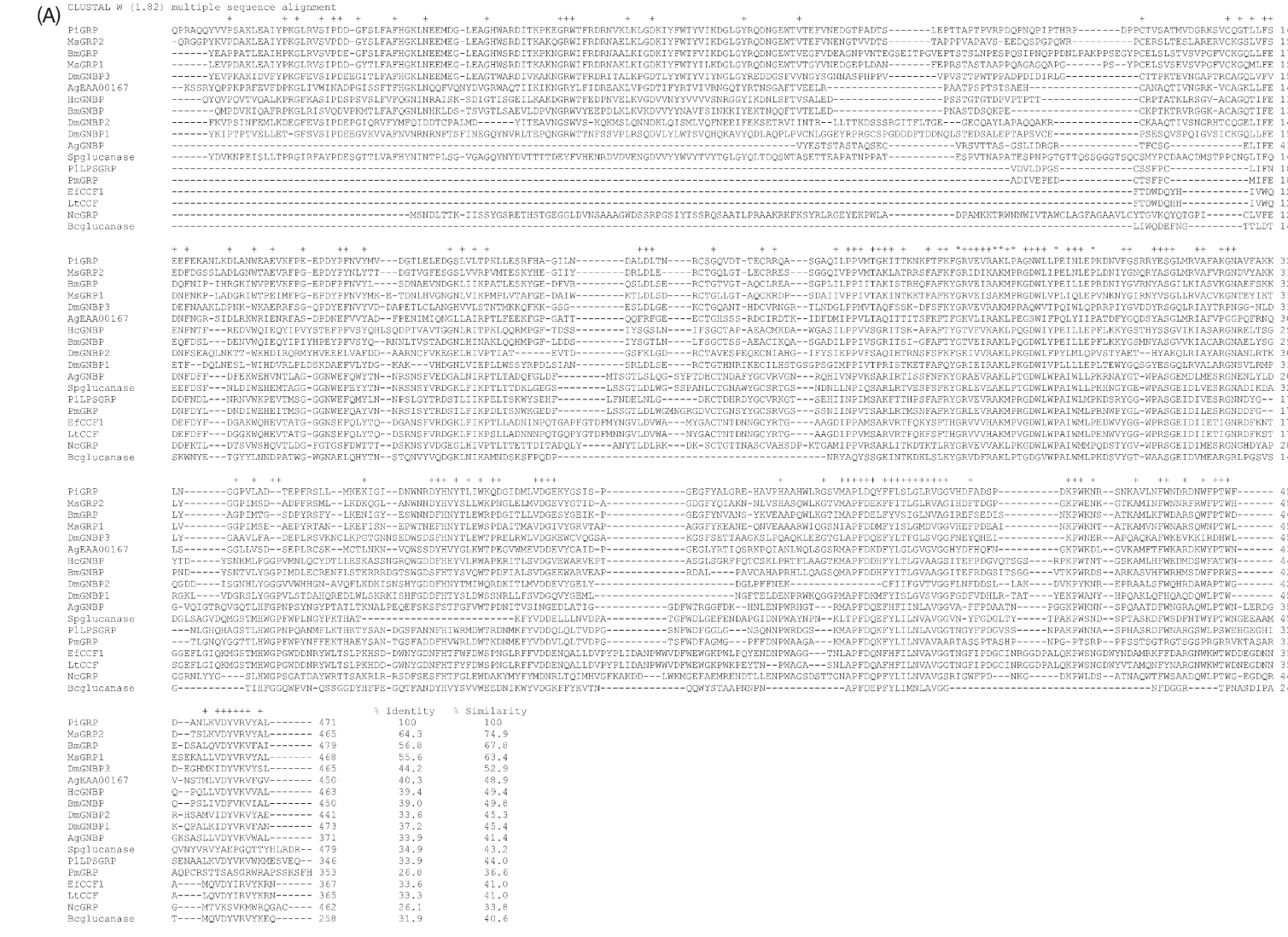

Fig. 3. Sequence alignment and phylogenetic tree analysis of *P. interpunctella* βGRP with other βGRP/GNBP family members and glucanases. (A) The CLUSTALW multiple sequence alignment program was used to align the *P. interpunctella* βGRP amino acid sequence with 17 other protein sequences found within the GenBank database possessing the lowest BLAST E-value scores. Residues conserved in all of the sequences are marked with ∗, and residues identical in at least nine of sequences are marked with +. Sequence identities and similarities were determined using the GAP alignment tool from SeqWeb. (B) Phylogenetic distances were obtained from a CLUSTALW alignment of 22 sequences and a tree was constructed using TreeView. The aligned sequences are as follows: PiGRP, *P. interpunctella* βGRP (AF532603); MsGRP2, *M. sexta* βGRP2 (AY135522); BmGRP, *B. mori* βGRP (BAA92243); MsGRP1, *M. sexta* βGRP1 (AAF44011); DmGNBP3, *D. melanogaster* GNBP3 (AAF50349); AgEAA00167, putative *A. gambiae* βGRP (EAA00167); HcGNBP, *H. cunea* GNBP (AAD09290); BmGNBP, *B. mori* GNBP (AAB40946); DmGNBP2, *D. melanogaster* GNBP2 (AAF33850); DmGNBP1, *D. melanogaster* GNBP1 (AAF33849); AgGNBP, *A. gambiae* GNBP (CAA04496); Spgluc, *S. purpuratus* β-1,3-glucanase (JC6141); PlLPSGRP, *Pacifastacus leniusculus* LPS/βGRP (CAB65353); PmGRP, *Penaeus monodon* βGRP (AAM21213); EfCCF1, *E. foetida* coelomic cytolytic factor 1 (AAC35887); LtCCF, *Lumbricus terrestris* CCF (AAL09587), NcGRP, *Neurospora crassa* βGRP-like protein (CAC28724), and Bcgluc, *Bacillus circulans* β-1,3-glucanase domain (AAA22474). Four additional *A. gambiae* sequences (including the putative GNBPs EAA04713, EAA07723, EAA07705, and EAA07707) are shown in the phylogenetic tree. SignalP was used to predict signal peptide sequences, which were removed from sequences (if applicable) prior to alignment.

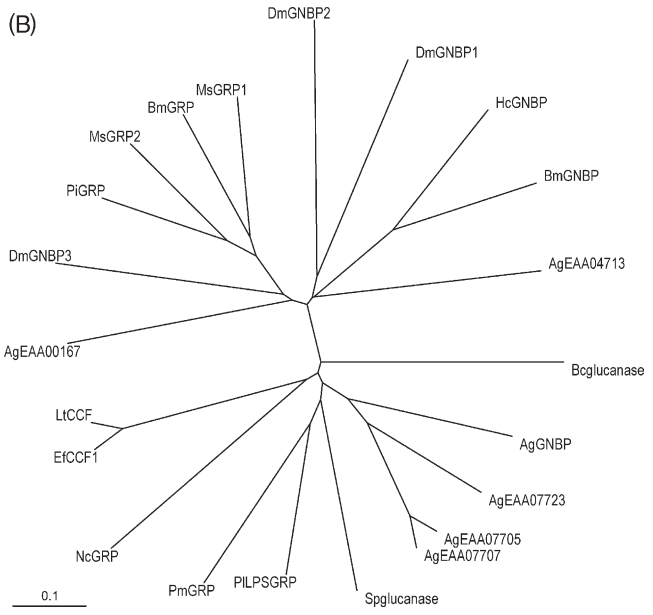

Fig. 3. *Continued*

respectively. rβGRP produced by using either expression vector was insoluble in the buffer extract obtained from *E. coli* following sonication. Sonication of *E. coli* pellets in buffer containing guanidinium hydrochloride resulted in solubilization of the rβGRP. Purification of rβGRP was facilitated by Ni^{2+}-affinity chromatography in buffer containing 8 M urea. The recombinant proteins remained soluble following the removal of urea by dialysis.

SDS-PAGE and immunoblot analysis of the expressed proteins revealed that the eluted fractions consisted of a single major protein with a molecular mass of approximately 54-kDa that strongly cross-reacted with antiserum in immunoblots (Fig. 5). However, the amino-terminal tagged rβGRP (6HisN-rβGRP) obtained from Ni^{2+}-affinity chromatography was not pure and contained smaller polypeptides that cross-reacted with anti-

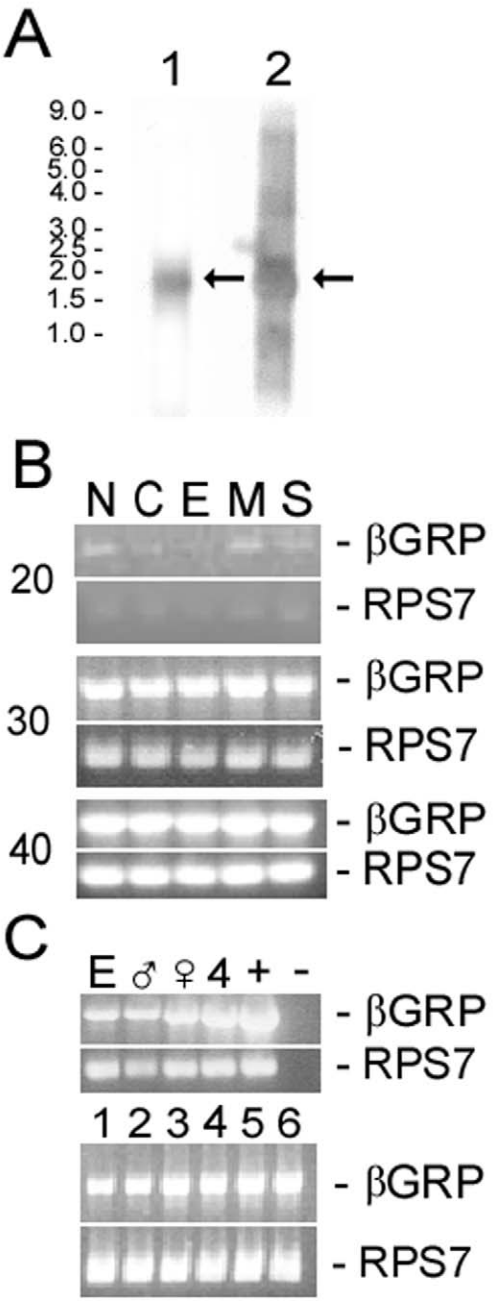

Fig. 4. Expression of βGRP in *P. interpunctella*. (A) Two μg *P. interpunctella* fat body mRNA (Lane 1), and 3 μg *P. interpunctella* whole-larval mRNA (Lane 2) were separated by electrophoresis on a 1% denaturing agarose gel and blotted and cross-linked onto a nylon membrane. The blot was hybridized with biotin-labeled βGRP RNA probe and detected by using Ambion's BrightStar BioDetect kit. (B) Relative expression levels of βGRP in wandering-stage *P. interpunctella* larvae 20-h post inoculation with *E. coli* (E), *M. lysodeikticus* (M), and *S. cerevisiae* (S) were determined by using RT-PCR. Naïve (N) and saline-injected (C) controls were run simultaneously. Twenty, 30, or 40 PCR cycles were conducted to observe differences in βGRP expression compared to that of a RPS7 control. (C) RNA obtained from *P. interpunctella* at different developmental stages was analyzed for βGRP transcripts by using RT-PCR. *P. interpunctella* life-stages that were tested include embryos (E), adult males (♂), adult females (♀), 4th instar larvae (1), early 5th instar larvae (2), 5th instar larvae (3), wandering-5th instar larvae (4), pre-pupae (5), and pupae (6). A negative (-) RT-PCR control reaction, which lacked template, and positive (+) RT-PCR control reactions, which contained plasmid DNA harboring RPS7 and βGRP insertions, were run simultaneously.

βGRP2 in immunoblots. These truncated forms of rβGRP were most likely produced by incomplete translation within *E. coli*, as proteinase inhibitors did not prevent their production. Truncated polypeptides were also present in the 6HisC-βGRP *E. coli* extracts, but they were not retained during the Ni^{2+}-affinity chromatography step (Fig. 5). A total of 1 mg of 6HisN-rβGRP (containing truncation products) was obtained from 0.5 L of expression culture, whereas 2 mg of purified 6HisC-rβGRP was obtained from 1.5 L of expression culture.

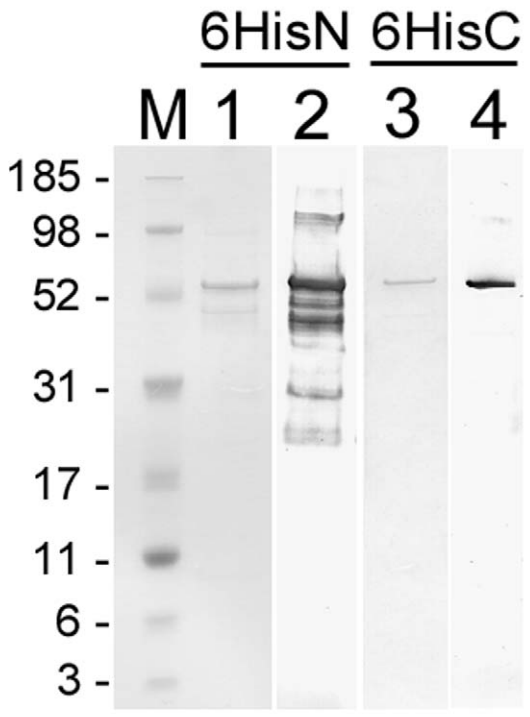

Fig. 5. Production of recombinant *P. interpunctella* β-1,3-glucan recognition protein. SDS-PAGE and immunoblot analysis of recombinant βGRP purified by Ni^{2+}-affinity chromatography under denaturing conditions. Lanes 1 and 3 are stained with Coomassie Blue; lanes 2 and 4 are detected by immunoblotting using antibody to *M. sexta* βGRP2. Lanes 1 and 2 contain 0.2 μg of 6HisN-rβGRP; lanes 3 and 4 contain 0.1 μg of 6HisC-rβGRP. Protein molecular weight standards are shown in Lane M.

3.6. Binding of recombinant βGRP to PAMPs

Both 6HisN- and 6HisC-rβGRP were co-precipitated by curdlan, demonstrating that the recombinant proteins were folded and possessed β-1,3-glucan binding activity (Fig. 6A). The truncated polypeptides present in the 6HisN-rβGRP sample also bound β-1,3-glucan. It appears that the curdlan used to co-precipitate the proteins and to ascertain carbohydrate-binding activity may contain contaminating proteinases, because protein fragments were observed in the curdlan-precipitated 6HisC-rβGRP sample, which were not present otherwise. We could detect little or no binding of 6HisC-rβGRP to chitin or cellulose (data not shown).

Specific binding to LPS and LTA by 6HisC-rβGRP was monitored using immunosorbent microplate assays. 6HisC-rβGRP bound to LPS and LTA in a dose dependent manner that reached saturation at approximately 10 μg/mL (Fig. 6B). Binding to LPS and LTA is specific, as free competitor (LPS or LTA) reduced binding of rβGRP to bound LPS- or LTA-ligand, respectively (Fig. 6B inset). These results indicated that βGRP bound to surface moieties commonly associated with microbial pathogens.

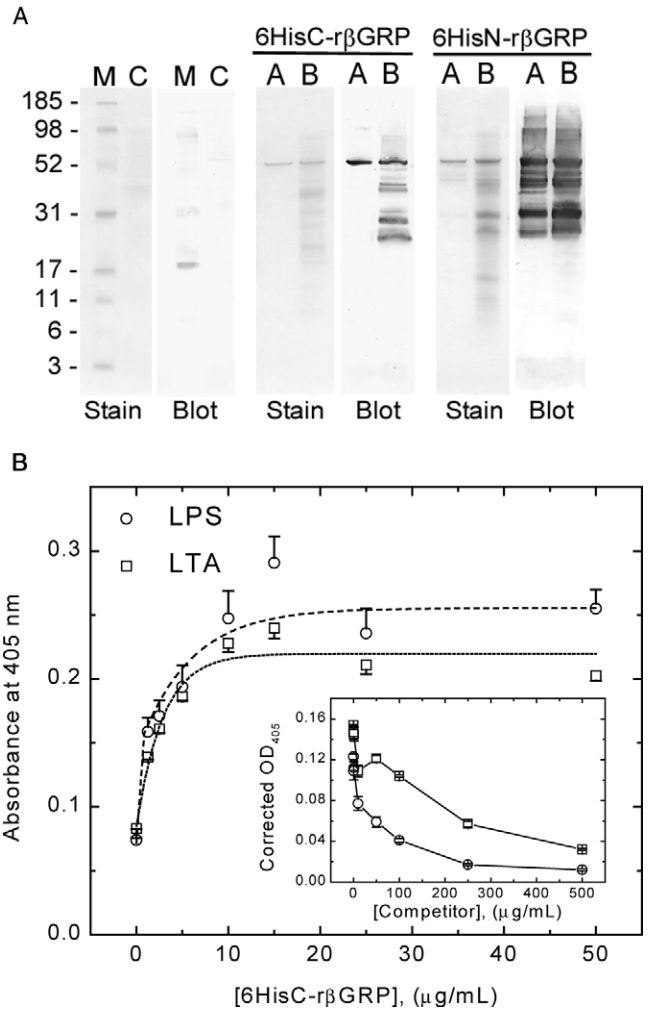

Fig. 6. In vitro binding of recombinant *P. interpunctella* βGRP to PAMPs. (A) Binding of 6HisN- or 6HisC-rβGRP to β-1,3-glucan as assessed by co-precipitation with curdlan and analysis on SDS-PAGE gels stained with Coomassie Blue (Stain) or detected by immunoblotting (Blot). Lanes labeled A contain 0.1 μg of 6HisC-rβGRP or 0.2 μg of 6HisN-rβGRP (as indicated above gels and blots) prior to addition to curdlan. Lanes labeled B contain indicated protein sample eluted from curdlan precipitate by heat treatment in SDS solution. Curdlan controls, without addition of protein, are shown in lanes labeled C. Protein molecular weight standards are shown in Lane M. (B) Binding of 6HisC-rβGRP to immobilized LPS or LTA. rβGRP prepared at different concentrations was added to LPS- or LTA-coated microtiter plates and incubated for 3 h at room temperature. The binding assay was performed as described in *Materials and methods*. rβGRP binding to LPS is shown with open circles, whereas LTA-binding is indicated with open squares. Broken lines represent non-linear regression curves. The inset shows inhibition of binding by rβGRP (5 μg/mL) to immobilized LPS or LTA in the presence of different concentrations of free competitor (LPS or LTA). Each point represents the mean ± S.E. for $n = 9$.

3.7. Aggregation of microorganisms by rβGRP

The presence of 1 μM of either 6HisC- or 6HisN-rβGRP with fluorescein-labeled *S. cerevisiae*, *S. aureus*, or *E. coli* caused substantial aggregation compared to controls containing buffer or BSA (Fig. 7A and Table 1).

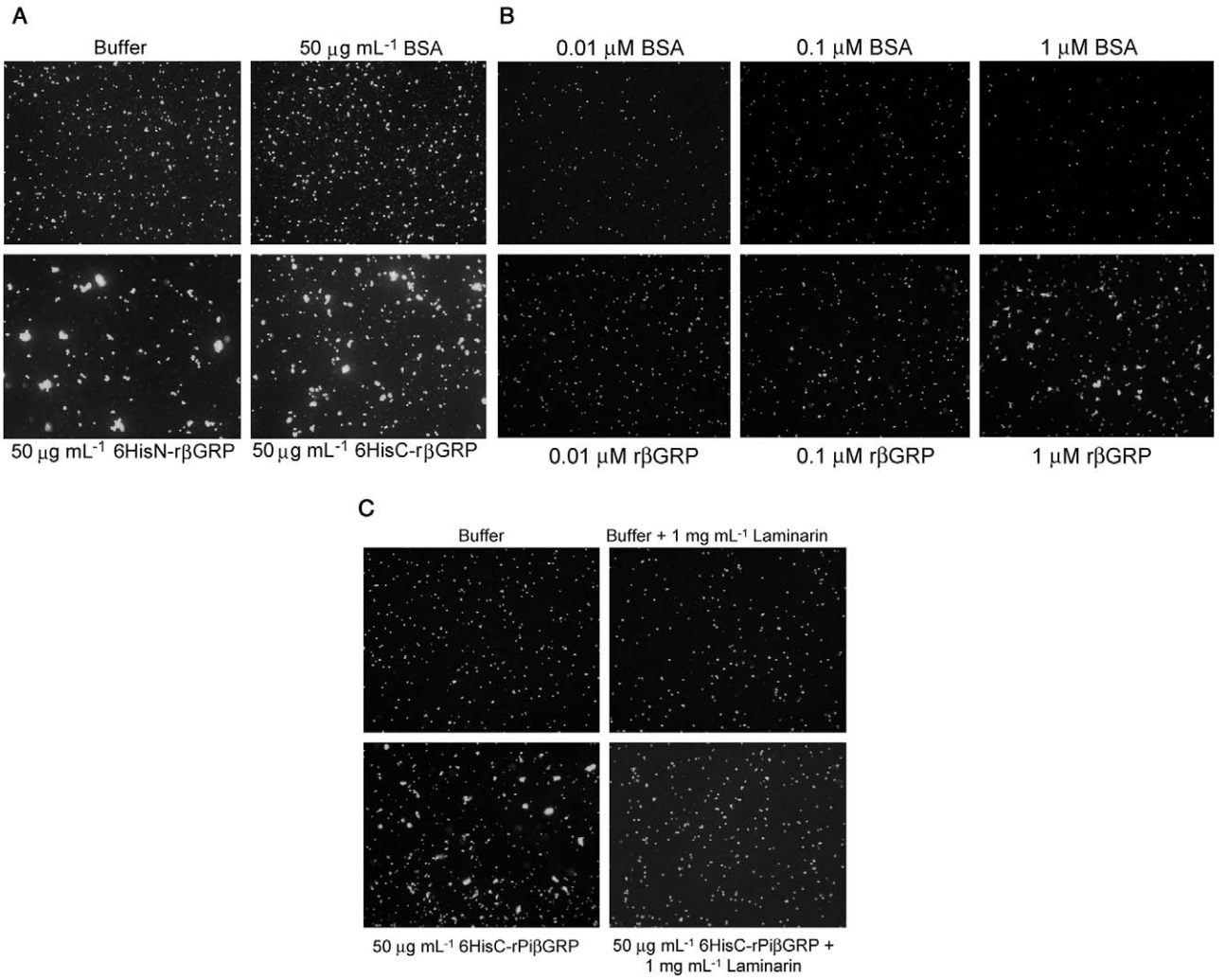

Fig. 7. Agglutination of yeast by recombinant βGRP. 6HisC- and 6HisN-rβGRP cause in vitro agglutination of fluorescein-labeled *S. cerevisiae* (A) that is dose dependent (B) and is inhibited by the presence of excess β-1,3-glucan (C). (A) Buffer, BSA (50 μg/mL), or recombinant βGRP (6HisN- or 6HisC-rβGRP) were incubated with fluorescein-conjugated *S. cerevisiae* (1×10^8 cells/mL) in PBS. After incubation for 30 min at room temperature, cells were observed by fluorescence microscopy. (B) Different concentrations (0.01, 0.1, and 1 μM) of BSA or 6HisC-rβGRP were incubated with labeled yeast cells prior to observation of aggregation. (C) Fluorescein-labeled yeast cells incubated in buffer, buffer with laminarin (1 mg/mL), 6HisC-rβGRP (50 μg/mL), or 6HisC-rβGRP with laminarin were analyzed for aggregation by using fluorescence microscopy.

Table 1
In vitro agglutination of fluorescein-labeled microorganisms[a]

	Degree of aggregation			
	Buffer	BSA	6HisN-rβGRP	6HisC-rβGRP
S. cerevisiae	–	–	+++	+++
E. coli	–	+	+++	+++
S. aureus	–	–	+++	++

[a] Degree of aggregation indicated as "−, +, ++, or +++" for no aggregation detected, few visible cell aggregates, substantial cell aggregates present, and high numbers of cell aggregates visible, respectively.

Aggregation of yeast was concentration-dependent, with appreciable aggregation occurring between 6HisC-rβGRP concentrations of 0.1-1.0 μM (Fig. 7B). From immunoblot analysis, we predicted that the physiological concentration of βGRP in 5th instar larvae was approximately 20–30 μg/mL or 0.4-0.6 μM. Therefore, aggregation of yeast and bacteria did occur at physiological concentrations of βGRP. Aggregation of yeast by 6HisC-

rβGRP could be inhibited in the presence of excess soluble laminarin (a β-1,3-glucan), indicating that βGRP is bound to β-1,3-glucans on the surface of yeast (Fig. 7C). These data showed that at physiological concentrations, *P. interpunctella* βGRP binds to and causes aggregation of microorganisms, which could enhance the immune response signal and provide more efficient clearing of the infection by hemocytes.

3.8. Activation of PPO pathway by rβGRP

rβGRP was tested for its ability to interact with soluble β-1,3-glucan and enhance activation of PPO. Because *P. interpunctella* plasma is difficult to obtain in large quantities without activating PPO, *M. sexta* plasma was used for PPO activation assays. As controls, laminarin alone or BSA with laminarin did not activate PPO (Fig. 8) (although at much longer incubation times, laminarin alone does stimulate PPO activation, perhaps through interaction with endogenous *M. sexta* GRP). Incubation of 6HisC-rβGRP with plasma did result in activation of PPO when compared to controls. However, when 6HisC-rβGRP was incubated with plasma containing laminarin, PO activity increased approximately 3-fold above that of 6HisC-rβGRP alone. This activation by 6HisC-rβGRP with laminarin was significantly higher than that without laminarin ($t = 10.4$, $P < 0.0001$). It is possible that the PO activity present in samples with rβGRP and no laminarin is due to contaminating LPS present from the purification of the protein from *E. coli*. These data suggested that βGRP functions to survey the insect hemocoel for foreign microbes (fungi) and that the binding of βGRP to a PAMP (β-1,3-glucan) results in activation of a proteinase in the PPO activation cascade.

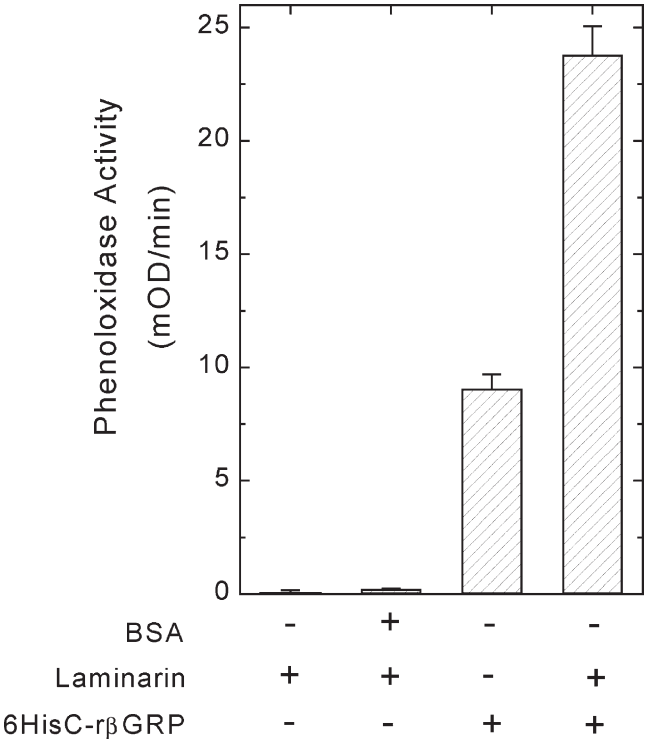

Fig. 8. Activation of the prophenoloxidase pathway by *P. interpunctella* rβGRP. Ten microliters of pooled plasma from five *M. sexta* larvae was incubated with 6HisC-rβGRP with- or without 1 mg/mL laminarin for 20 min in the wells of a microtiter plate. Phenoloxidase activity was determined by using the substrate dopamine. The final concentration of 6HisC-rβGRP was 30 μg/mL or 0.5 μM. Control samples run simultaneously either lacked 6HisC-rβGRP or contained 0.5 μM BSA. Values represent the mean ± S.E. of data from three tests of triplicates conducted using a single pooled plasma sample.

4. Discussion

Several families of pattern recognition proteins have been identified in insects, including βGRPs, PGRPs, C-type lectins, hemolin, a scavenger receptor, and complement-like proteins (Levashina et al., 2001; Rämet et al., 2001; Yu et al., 2002). βGRPs from several insects have been shown to bind to PAMPs and subsequently activate the prophenoloxidase pathway or to activate induction of antimicrobial peptide genes. In the present study, a 53-kDa polypeptide with affinity for β-1,3-glucan was purified from Indianmeal moth plasma and its cDNA was cloned. The 53-kDa soluble protein strongly cross-reacted with antisera generated against βGRPs from *M. sexta*. The calculated molecular mass of the 471-residue mature protein is 53,311 Da, consistent with the size of βGRP purified from plasma. The amino-terminus of the predicted mature βGRP begins with glutamine, which is consistent with our observation that the N-terminus is blocked, as glutamine is readily cyclized to pyroglutamate, which cannot be processed by Edman degradation. The sequence of a trypsin fragment from βGRP matched exactly with the deduced sequence for residues 4-13 of the βGRP cDNA clone, indicating that we have indeed isolated a cDNA corresponding to the purified βGRP. βGRP from *P. interpunctella* possesses two putative N-glycosylation sites and several O-glycosylation sites, but glycosylation of this βGRP has not yet been studied.

The full-length *P. interpunctella* cDNA possesses sequence similarity to other βGRPs and GNBPs, including two βGRPs from *M. sexta*, a βGRP from *B. mori*, three βGRPs from *D. melanogaster*, a putative βGRP from *A. gambiae*, and GNBPs from *B. mori*, *H. cunea*, and *A. gambiae*. In addition, the carboxyl-terminus of *P. interpunctella* βGRP (approximately the final 300 residues) shares significant sequence similarity to β-1,3-glucanases from various bacteria and a glucanase from the sea urchin, *S. purpuratus*. The carboxyl-terminus also shares similarity with pattern recognition proteins called CCFs from the annelids *E. foetida* and *L. terrestris*. βGRPs from other insects lack β-1,3-glucanase

activity (Ma and Kanost, 2000; Ochiai and Ashida, 2000). The *P. interpunctella* βGRP is also predicted to lack glucanase activity because like other βGRPs and GNBPs, it lacks the active site catalytic residues. However, even though the glucanase catalytic residues are conserved in CCF-1 from *E. foetida*, it too lacks apparent glucohydrolase activity (Beschin et al., 1998).

βGRP in other lepidopteran insects is expressed primarily in fat body (Ma and Kanost, 2000; Ochiai and Ashida, 2000). *P. interpunctella* βGRP also appears to be expressed in fat body. RT-PCR experiments indicate that *P. interpunctella* βGRP is expressed in embryos, larvae, prepupae, pupae, and adults. It is unknown if βGRP is provided to embryos through maternal transfer or if βGRP functions in other facets of embryogenesis. βGRP synthesis was not induced following immune challenge, as detected by RT-PCR and immunoblotting. It is possible that minor differences in βGRP transcript level did occur, but were not detected by our analysis. Other insect βGRPs including *M. sexta* βGRP1, *M. sexta* βGRP2, and *B. mori* βGRP are similarly expressed in insect larvae prior to immune challenge. However, expression of *M. sexta* βGRP2 and *B. mori* βGRP as well as other members of this family is upregulated following challenge with bacteria or yeast (Ma and Kanost, unpublished data; Ochiai and Ashida, 2000; Lee et al., 1996; Shin et al., 1998; Dimopoulos et al., 1997). The constitutive occurrence of some PRPs may keep the insect in a constant state of readiness by providing immediate detection of an impending infectious threat, whereas the induction of some PRPs provides additional recognition capability until the infection has been cleared.

Recombinant *P. interpunctella* βGRP was biologically active. It bound to PAMPs associated with fungi and bacteria, including β-1,3-glucan, lipopolysaccharide, and lipoteichoic acid but not to β-1,4-linked carbohydrates including chitin or cellulose. The binding of rβGRP to LPS and LTA in vitro was concentration-dependent, saturable, and could be decreased by competition with free ligand. rβGRP also bound very tightly to insoluble β-1,3-glucan. The broad binding specificity of βGRP provides the insect with a single pattern recognition molecule that has overlapping recognition capabilities. *P. interpunctella* rβGRP not only binds to the surface molecules of microorganisms, but it also can cause agglutination of yeast and bacteria. Lectins cause agglutination of cells by binding to surface molecules through multiple binding sites (Chen et al., 1995; Wilson et al., 1999). It is unknown if βGRPs possess multiple binding sites for microbial surface compounds or whether the βGRPs interact to form oligomers, which could also result in agglutination of cells. We suggest that one function of βGRP is to cause aggregation of microorganisms in vivo, which could improve the efficiency of clearance by hemocytes through phagocytosis or nodule formation. Large aggregates of microorganisms may also more effectively stimulate or enhance other immune response signals as opposed to individual cells dispersed throughout the hemocoel.

Prophenoloxidase (PPO) is commonly activated in hemolymph of insects and other arthropods following infection by microorganisms (Ashida and Brey, 1998). PPO is activated through proteolytic cleavage by a proteinase, which itself is also activated via proteolytic cleavage. It is currently unknown how many proteinases participate in the cascade that results in PPO activation. Regulation of the proteinases involved in activating PPO is critical for homeostasis of the insect, as hyperactivated PO could result in production of cytotoxic compounds, such as electrophilic quinones. In contrast, an arthropod in which the PPO pathway is compromised and cannot be easily activated could provide an environment in which microbes would thrive and cause sepsis and eventual death.

In this study, we showed that recombinant *P. interpunctella* βGRP participated in the activation of PPO. rβGRP preincubated with soluble β-1,3-glucan and added to insect plasma showed a significant activation of PPO above controls or rβGRP alone. This result indicated that *P. interpunctella* βGRP may interact with β-1,3-glucan to form an "activated" PRP, which can propagate the appropriate stimulus (proteinase signaling pathway), culminating in the activation of prophenoloxidase. At present, we do not understand how PRPs that are bound to PAMPs cause activation of a proteinase or proteinases that participate in PPO activation. The binding of βGRP to glucan or other PAMPs may result in a conformational change, thereby "activating" βGRP and conferring an ability to interact with other plasma proteins that may participate in activating immune responses, including the PPO cascade and/or the synthesis of antimicrobial peptides. These plasma factors that facilitate such immune pathways may include proteinases or other proteins that can recruit proteinases. Serine protease-like homologs (SPHs) have recently been identified as important factors for activation of insect phenoloxidase (Kwon et al., 2000; Yu et al., 2003). A complex of proteins formed by interactions between PRPs and other plasma factors may be involved in PPO activation. A similar mechanism has been suggested for vertebrate complement activation through the lectin-mediated pathway in which proteinases (MBL-associated serine proteases) are recruited to the PRP (mannan binding lectin) to form a complex necessary for propagation of the cascade (Wallis and Dodd, 2000; Thielens et al., 2001). Further studies on binding interactions between βGRP (as well as its individual domain subunits) with PAMPs and other plasma proteins should provide a greater understanding of the function of pattern recognition molecules in invertebrate innate immunity.

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Mention of a commercial or proprietary product does not constitute a recommendation by the USDA.

The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number AF532603.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Ashida, M., Brey, P.T., 1998. In: Brey, P.T., Hultmark, D. (Eds.), Molecular Mechanisms of Immune Responses in Insects. Chapman & Hall, New York, pp. 135–172.

Beschin, A., Bilej, M., Hanssens, F., Raymakers, J., Van Dyck, E., Revets, H., Brys, L., Gomez, J., De Baetselier, P., Timmermans, M., 1998. Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade. J. Biol. Chem. 273, 24948–24954.

Bilej, M., De Baetselier, P., Van Dijck, E., Stijlemans, B., Colige, A., Beschin, A., 2001. Distinct carbohydrate recognition domains of an invertebrate defense molecule recognize Gram-negative and Gram-positive bacteria. J. Biol. Chem. 276, 45840–45847.

Chen, C., Durrant, J., Newton, R.P., Ratcliff, N.A., 1995. A study of novel lectins and their involvement in the activation of the prophenoloxidase system in *Blaberus dicoidalis*. Biochem. J 310, 23–31.

Dimopoulos, G., Richman, A., Müller, H.M., Kafatos, F.C., 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. Proc. Natl. Acad. Sci. USA 94, 11508–11513.

Jomori, T., Natori, S., 1991. Molecular cloning of cDNA for lipopolysaccharide-binding protein from the hemolymph of the American cockroach, *Periplaneta americana*. J. Biol. Chem. 266, 13318–13323.

Kang, D., Liu, G., Lundstrom, A., Gelius, E., Steiner, H., 1998. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. Proc. Natl. Acad. Sci. USA 95, 10078–10082.

Kawasaki, K., Kubo, T., Natori, S., 1996. Presence of the Periplaneta lectin-related protein family in the American cockroach *Periplaneta americana*. Insect Biochem. Molec. Biol 26, 355–364.

Kim, Y.S., Ryu, J.H., Han, S.J., Choi, K.H., Nam, K.B., Jang, I.H., Lemaitre, B., Lee, W.J., 2000. Gram-negative bacterial-binding protein, a pattern recognition receptor for lipopolysaccharide and β-1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. J. Biol. Chem. 275, 32721–32727.

Koizumi, N., Imamura, M., Kadotani, T., Yaoi, K., Iwahana, H., Sato, R., 1999. The lipopolysaccharide-binding protein participating in hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains. FEBS Letters 443, 139–143.

Kwon, T.H., Kim, M.S., Choi, H.W., Joo, C.H., Cho, M.Y., Lee, B.L., 2000. A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity in the coleopteran insect, *Holotrichia diomphalia* larvae. Eur. J. Biochem 267, 6188–6196.

Ladendorff, N.E., Kanost, M.R., 1991. Bacteria-induced protein P4 (hemolin) from *Manduca sexta*: A member of the immunoglobin superfamily which can inhibit hemocyte aggregation. Arch. Insect Biochem. Physiol 18, 285–300.

Lagueux, M., Perrodou, E., Levashina, E.A., Capovilla, M., Hoffmann, J.A., 2000. Constitutive expression of a complement-like protein in Toll and JAK gain-of-function mutants of *Drosophila*. Proc. Natl. Acad. Sci. USA 97, 11427–11432.

Lee, W.J., Lee, J.D., Kravchenko, V.V., Ulevitch, R.J., Brey, P.T., 1996. Purification and molecular cloning of an inducible Gram-negative bacteria-binding protein from the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. USA 93, 7888–7893.

Lee, E., Linder, M.E., Gilman, A.G., 1994. Expression of G-Protein α Subunits in *Escherichia coli*. Methods Enzymol 237, 146–164.

Lee, S.Y., Wang, R., Söderhäll, K., 2000. A lipopolysaccharide- and β-1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 275, 1337–1343.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., Kafatos, F.C., 2001. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. Cell 104, 709–718.

Ma, C., Kanost, M.R., 2000. A β1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. J. Biol. Chem. 275, 7505–7515.

Medzhitov, R., Janeway, C.A., 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell 91, 295–298.

Ochiai, M., Ashida, M., 1988. Purification of a β-1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, *Bombyx mori*. J. Biol. Chem. 263, 12056–12062.

Ochiai, M., Ashida, M., 1999. A pattern recognition protein for peptidoglycan: Cloning the cDNA and the gene of the silkworm, *Bombyx mori*. J. Biol. Chem. 274, 11854–11858.

Ochiai, M., Ashida, M., 2000. A pattern recognition protein for β-1,3-glucan: The binding domain and the cDNA cloning of β-1,3-glucan recognition protein from the silkworm, *Bombyx mori*. J. Biol. Chem. 275, 4995–5002.

Page, R.D.M., 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12, 357–358.

Pearson, A., Lux, A., Krieger, M., 1995. Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 92, 4056–4060.

Rämet, M., Pearson, A., Manfruelli, P., Li, X., Koziel, H., Göbel, V., Chung, E., Krieger, M., Ezekowitz, R.A.B., 2001. Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity 15, 1027–1038.

Sambrook, R., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. , 2nd Ed. Cold Springs Harbor Lab. Press, Plainview, NY.

Shin, S.W., Park, S.S., Park, D.S., Kim, M.G., Kim, S.C., Brey, P.T., Park, H.Y., 1998. Isolation and characterization of immune-related genes from the fall webworm, *Hyphantria cunea*, using PCR-based differential display and subtractive cloning. Insect Biochem. Molec. Biol 28, 827–837.

Söderhäll, K., Rögener, W., Söderhäll, I., Newton, R.P., Ratcliffe, N.A., 1988. The properties and purification of a *Blaberus craniifer* plasma protein which enhances the activation of haemocyte prophenoloxidase by a β 1,3-glucan. Insect Biochem 18, 323–330.

Sritunyalucksana, K., Lee, S.O., Söderhäll, K., 2002. A β-1,3-glucan binding protein from the black tiger shrimp, *Penaeus monodon*. Devel. Comp. Immunol 26, 237–245.

Sun, S.C., Lindström, I., Boman, H.G., Faye, I., Schmidt, O., 1990.

Hemolin: An insect-immune protein belonging to the immunoglobin superfamily. Science 250, 1729–1732.

Theopold, U., Rissler, M., Fabbri, M., Schmidt, O., Natori, S., 1999. Insect glycobiology: A lectin multigene family in Drosophila melanogaster. Biochem. Biophys. Res. Comm 261, 923–927.

Thielens, N.M., Cseh, S., Thiel, S., Vorup-Jensen, T., Rossi, V., Jensenius, J.C., Arlaud, G.J., 2001. Interaction properties of human mannan-binding lectin (MBL)-associated serine proteases-1 and -2, MBL-associated protein 19 and MBL. J. Immunol. 166, 5068–5077.

Titus, D., 1991. Promega Protocols and Application Guide. , 2nd Ed. Promega Corporation, Madison, WI.

Tobias, P.S., Soldau, K., Ulevitch, R.J., 1989. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. J. Biol. Chem. 264, 10867–10871.

Wallis, R., Dodd, R.B., 2000. Interaction of mannose-binding protein with associated serine proteases: effects of naturally occurring mutations. J. Biol. Chem. 275, 30962–30969.

Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., Hultmark, D., 2000. A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 97, 13772–13777.

Wilson, R., Chen, C., Ratcliffe, N.A., 1999. Innate immunity in insects: the role of multiple, endogenous serum lectins in the recognition of foreign invaders in the cockroach, *Blaberus dicoidalis*. J. Immunol. 162, 1590–1596.

Yu, X.Q., Gan, H., Kanost, M.R., 1999. Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase. Insect Biochem. Molec. Biol 29, 585–597.

Yu, X.Q., Kanost, M.R., 2000. Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to Gram-negative bacteria. J. Biol. Chem. 275, 37373–37381.

Yu, X.Q., Kanost, M.R., 2002. Binding of hemolin to bacterial lipopolysaccharide and lipoteichoic acid: An immunoglobulin superfamily member from insects as a pattern-recognition receptor. Eur. J. Biochem. 269, 1827–1834.

Yu, X.Q., Zhu, Y.F., Ma, C., Fabrick, J.A., Kanost, M.R., 2002. Pattern recognition proteins in *Manduca sexta* plasma. Insect Biochem. Molec. Biol 32, 1287–1293.

Yu, X.Q., Jiang, H., Wang, Y., Kanost, M.R., 2003. Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. Insect Biochem. Molec. Biol 33, 197–208.

Zhu, Y.C., Kramer, K.J., Dowdy, A.K., Baker, J.E., 2000. Trypsinogen-like cDNAs and quantitative analysis of mRNA levels from the Indianmeal moth, *Plodia interpunctella*. Insect Biochem. Molec. Biol 30, 1027–1035.