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Innate Immunity in a Pyralid Moth

FUNCTIONAL EVALUATION OF DOMAINS FROM A β-1,3-GLUCAN RECOGNITION PROTEIN*

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Invertebrates, like vertebrates, utilize pattern recognition proteins for detection of microbes and subsequent activation of innate immune responses. We report structural and functional properties of two domains from a β-1,3-glucan recognition protein present in the hemolymph of a pyralid moth, *Plodia interpunctella*. A recombinant protein corresponding to the first 181 amino-terminal residues bound to β-1,3-glucan, lipopolysaccharide, and lipoteichoic acid, polysaccharides found on cell surfaces of microorganisms, and also activated the prophenoloxidase-activating system, an immune response pathway in insects. The amino-terminal domain consists primarily of an α-helical secondary structure with a minor β-structure. This domain was thermally stable and resisted proteolytic degradation. The 290 residue carboxyl-terminal domain, which is similar in sequence to glucanases, had less affinity for the polysaccharides, did not activate the prophenoloxidase cascade, had a more complicated CD spectrum, and was heat-labile and susceptible to proteinase digestion. The carboxyl-terminal domain bound to laminarin, a β-1,3-glucan with β-1,6 branches, but not to curdlan, a β-1,3-glucan that lacks branching. These results indicate that the two domains of *Plodia* β-1,3-glucan recognition protein, separated by a putative linker region, bind microbial polysaccharides with differing specificities and that the amino-terminal domain, which is unique to this class of pattern recognition receptors from invertebrates, is responsible for stimulating prophenoloxidase activation.

Pattern recognition receptors (PRRs)[1] are biosensor proteins that complex with pathogen-associated molecular patterns (PAMPs) and subsequently transduce signals necessary for activation of an appropriate immune response. Protein families of invertebrate PRRs (1) include β-1,3-glucan recognition proteins (βGRPs) and Gram-negative bacterial-binding proteins (GNBPs) (2–12), peptidoglycan recognition proteins (13–15), lipopolysaccharide (LPS)-binding proteins and C-type lectins (16–22), complement-like proteins (23, 24), hemolin (4, 25–27), and scavenger receptor proteins (28, 29). Within these protein families, PRR interactions with microbes activate both cellular and humoral aspects of innate immunity. Cellular responses include hemocyte participation in phagocytosis, nodule formation, or encapsulation (30). Humoral immune responses may involve the activation of distinct proteinase-regulated pathways that result in the production of antimicrobial peptides, activation of the prophenoloxidase (PPO), and formation of clots via a coagulation pathway. The PPO-activating system is involved in the production of melanin that forms at the site of wounds and on the surface of pathogens and parasites (31, 30). PPO is present in the hemolymph as a zymogen that is activated by a prophenoloxidase-activating proteinase. Prophenoloxidase-activating proteinases themselves appear to be activated via proteolytic cleavage (32–38).

The *Plodia interpunctella* βGRP (PiβGRP) was originally identified as a soluble 53-kDa plasma protein that binds β-1,3-glucan, lipopolysaccharide, and lipoteichoic acid and subsequently activates the PPO cascade (12). A cDNA clone encoding PiβGRP was isolated and found to belong to a family of PRRs identified from *Manduca sexta* (βGRPs 1 and 2), *Bombyx mori* (βGRP and GNBP), *Drosophila melanogaster* (GNBPs 1–3), *Hyphantria cunea* (GNBP), and *Eisenia foetida* (coelomic cytolytic factor). PiβGRP is expressed in fat body and is constitutively present in the hemolymph. In this study, we describe structural and functional properties of recombinant proteins corresponding to putative amino- and carboxyl-terminal domains from PiβGRP.

EXPERIMENTAL PROCEDURES

Expression of Recombinant βGRP and Deletion Constructs—The full-length 471-residue βGRP (without secretion signal peptide) was expressed using an Invitrogen pTrcHis2-TOPO® expression vector in *Escherichia coli* and purified as outlined previously (12). Two amino-terminal deletions mutants composed of 118 and 181 residues that corresponded to amino-terminal domains reported in *B. mori* and *M. sexta*, respectively (8, 9), were constructed (Fig. 1*A*). The 118 and 181 residue recombinant proteins are referred to as 118N and 181N, respectively. A carboxyl-terminal domain (290C) consisting of 290 residues was produced similarly. All of the recombinant proteins were expressed as fusion proteins with a c-*myc* and a six-histidine tag at their carboxyl terminus comprising 32 additional residues.

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectroscopy was conducted by using a Jasco J-720 spectropolarimeter. All of the proteins were diluted to 3–4 μM in 10 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and scanned from 260 to 180 nm at 20-nm min^{-1}. The mean molar residue ellipticity (θ) values were calculated by inputting the raw CD data θ into the equation $(\theta) = \theta/(10 \times l \times C_r)$, where l is the path length in centimeters and C_r is the molar concentration of the protein multiplied by number of residues.

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The complete amino acid sequence of PiβGRP can be accessed through NCBI Protein Database under NCBI accession number AAM95970.

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[1] The abbreviations used are: PRRs, pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; βGRP, β-1,3-glucan recognition protein; GNBPs, Gram-negative bacterial-binding proteins; LPS, lipopolysaccharide; rβGRP, recombinant *P. interpunctella* βGRP; PPO, prophenoloxidase; CD, circular dichroism; LTA, lipoteichoic acid; BSA, bovine serum albumin; SPR, surface plasmon resonance; PiβGRP, *P. interpunctella* βGRP; ANOVA, analysis of variance.

Proteolysis and Thermal Stability of Recombinant Proteins—Recombinant *P. interpunctella* βGRP (rβGRP), 118N, 181N, and 290C were digested with equal amounts (2 μg) of bovine trypsin or chymotrypsin in 20 mM Tris-HCl, pH 8.5, for 1 h at 25 °C. Following incubation, samples were heat-treated in SDS sample buffer to inactivate the proteinase and separated by SDS-PAGE. In a second test, the proteolysis of rβGRP through time was observed by incubating 7.2 μg of rβGRP with 2.9 μg of trypsin or chymotrypsin at 25 °C in 20 mM Tris-HCl, pH 8.5, in a total volume of 120 μl. At appropriate time intervals (0, 0.01, 0.5, 1, 3, 6, and 24 h), a 20-μl aliquot was removed, added to SDS sample buffer, and heated in a 70 °C water bath for 10 min to inactivate the proteinases. In both experiments, proteins were transferred to nitrocellulose membranes after SDS-PAGE and immunoblot analysis was conducted using rabbit antiserum against *M. sexta* βGRP2.

Heat stability was determined by incubating an aliquot of each recombinant protein diluted to 0.12 mg ml^{-1} in 10 mM Tris-HCl, pH 8, containing 0.1% Triton X-100 in a boiling water bath for 10 min. Heat-treated samples were centrifuged at 10,000 × *g* for 5 min at 4 °C, and supernatants containing heat-stable protein were collected. Precipitates were resuspended and centrifuged. After an additional wash, pellets containing heat-labile proteins were resuspended in 125 μl of the Tris buffer and analyzed as above. Curdlan binding activity of heat-treated recombinant Piβ GRP and domains was determined as described below.

Aggregation of Microorganisms—Recombinant proteins were tested for agglutination of fluorescein-labeled *E. coli* (K12 strain), *Staphylococcus aureus* (Wood strain without protein A), and *Saccharomyces cerevisiae* (12). Samples containing BSA or buffer alone were also analyzed. Multiple images obtained with a SPOT digital camera from Diagnostic Instruments (Sterling Heights, MI) were used to determine the degree of aggregation.

Binding of Recombinant βGRP and Domains to Curdlan—Curdlan (an insoluble β-1,3-glucan, Sigma) was used as an affinity matrix to assess β-1,3-glucan binding of recombinant proteins. Proteins (10 μg of rβGRP, 15 μg of 118N, 12 μg of 181N, and 20 μg of 290C) were incubated with 10 mg of curdlan equilibrated in phosphate-buffered saline (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 curdlan-protein mixture was centrifuged at 10,000 × *g* for 5 min at 4 °C, and the pellet was washed. Bound proteins were eluted from the curdlan precipitate by the addition of 50 μl of 1 × SDS-NuPAGE sample buffer (Novex, San Diego, CA) containing 50 mM dithiothreitol followed by heating for 10 min at 95 °C. The slurry was centrifuged at 10,000 × *g* for 5 min at 4 °C, and equal volumes of supernatants were analyzed by SDS-PAGE with Coomassie Blue protein staining and by immunoblotting.

Competition for binding curdlan by rβGRP in the presence of laminarin was tested by incubating 30 μg of recombinant protein with or without laminarin (0.5 mg) prior to the addition to curdlan (10 mg in 0.5 ml of phosphate-buffered saline). Following incubation for 30 min on ice with occasional mixing, curdlan was precipitated by centrifugation and the precipitates were washed. Protein was eluted from curdlan by heat treatment in SDS sample buffer, and equivalent amounts of supernatants were analyzed.

Binding of Recombinant βGRP and Domains to Laminarin—Laminarin (a soluble β-1,3-glucan, Sigma) was biotinylated by following the protocols modified from Takaki *et al.* (39) and Shinohara *et al.* (40). 10 tubes, each containing 26 nmol of laminarin and 52 nmol of biotin-biotinamidocaproyl hydrazide-hydrazide in 30% CH_3CN in a total of 10 μl, were incubated at 90 °C for 2 h. Samples were pooled and incubated with 0.5 ml of UltraLink immobilized streptavidin in binding buffer (0.1 M NaH_2PO_4/Na_2HPO_4 and 150 mM NaCl, pH 7) at 4 °C for 1 h. The resin was centrifuged at 2,500 × *g* for 1 min, washed, and then suspended in 0.5 ml of buffer. Each recombinant protein sample (40 μg) was added to the laminarin-affinity resin (50 μl) and incubated at 4 °C for 0.5 h. The resin was washed three times, resuspended in 50 μl of 1× SDS-PAGE sample buffer, and placed into a boiling water bath for 10 min. The eluted proteins were analyzed by SDS-PAGE with Coomassie Blue protein staining and immunoblot analysis. The specificity of the binding to laminarin (and not the affinity resin itself) was tested by preparing immobilized-streptavidin resin with biotin-biotinamidocaproyl hydrazide-hydrazide but without laminarin as the ligand and assaying as above.

Affinity Constant Determination via Surface Plasmon Resonance (SPR)—A BIAcore® 3000 SPR biosensor (BIAcore AB, Uppsala, Sweden) was used to measure kinetic interactions between laminarin and the recombinant proteins. Laminarin (195 nmol) was biotinylated and injected directly onto a streptavidin-coated sensor chip SA (BIAcore AB). As a control, biotin-biotinamidocaproyl hydrazide-hydrazide was similarly injected onto a different flow cell of the SA sensor chip to account for nonspecific interactions. A series of concentrations of rβGRP, 118N, 181N, and 290C ranging from 0.5 nM to 2 μM prepared in 10 mM HBS-P (10 mM HEPES, pH 7.4 with 150 mM NaCl and 0.005% Tween 20) was injected over the surface of the sensor chip at a flow rate of 15 μl min^{-1}. Proteins were injected at the same rate onto both the control (immobilized biotin) and test (immobilized laminarin) flow cells at 25 °C over 6 min, and the interaction was monitored as the change in SPR response. After sample injections, flow cells were washed with HBS-P buffer and dissociation of analyte (protein) from the immobilized ligand was monitored continuously over 15 min at a flow rate of 15 μl min^{-1}. At the end of each cycle, the regeneration of the chip was accomplished with two successive 30-s washes of 50 mM H_3PO_4 at a flow rate of 30 μl min^{-1}. The chip was equilibrated with HBS-P prior to the next analysis. Both association rate constants (k_a) and dissociation rate constants (k_d) for the laminarin:protein interactions were obtained by fitting background-corrected SPR binding data to the 1:1 Langmuir binding model within the BIAevaluation software version 3.1. The association constant (K_A) was subsequently determined as k_a/k_d. The statistical analysis of association constants for each protein was performed by using the SAS PROC ANOVA at $\alpha = 0.05$ (41).

Binding of Recombinant βGRP and Domains to Lipopolysaccharide and Lipoteichoic Acid—Binding to LPS and LTA was determined by using microplate binding assays modified from Tobias *et al.* (42) and Yu

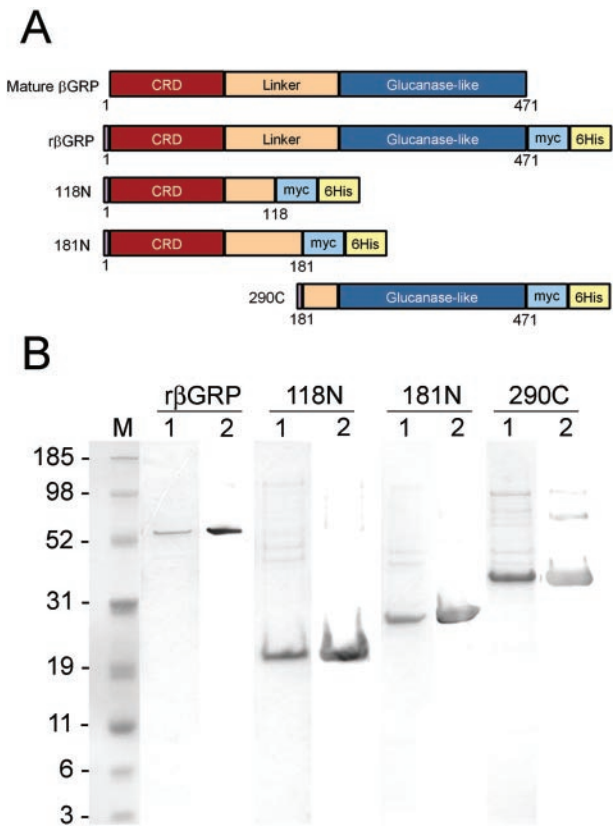

FIG. 1. **Expression of *P. interpunctella* βGRP and domains in *E. coli*.** *A*, βGRP truncation constructs expressed in *E. coli* as fusion proteins in-frame with a c-*myc* and a six-histidine tag at the carboxyl terminus of each recombinant protein. Each recombinant protein contains an additional 32 residues: three residues at the amino terminus (Met-Ala-Leu) and 29 residues at the carboxyl terminus (comprising the c-*myc* tag, the six-histidine tag, and linker regions). The approximate locations of amino-terminal carbohydrate recognition domain, putative linker region, and carboxyl-terminal glucanase-like domain are shown. rβGRP comprised the entire mature polypeptide (471 amino acid residues) excluding the putative secretion signal peptide. 118N and 181N consisted of the first 118 and 181 amino-terminal residues of the mature βGRP, respectively. The carboxyl-terminal construct, 290C, consisted of the carboxyl-terminal 290 amino acid residues of βGRP beginning with Val-182. *B*, recombinant proteins (rβGRP, 118N, 181N, and 290C) expressed in *E. coli*. Proteins were separated by SDS-PAGE and analyzed by Coomassie Blue staining (*lanes 1*) and immunoblot analysis (*lanes 2*). Protein molecular weight standards are shown in *lane M*.

and Kanost (22, 27). LPS (from *E. coli* 0111:B4, smooth strain LPS, Sigma) or LTA (from *S. aureus*) suspensions (40 μg ml^{-1}) were used to coat wells of a microplate (Continental Lab Products, San Diego, CA). After blocking, rβBRP, 118N, 181N, and 290C at concentrations of 0, 0.024, 0.047, 0.094, 0.19, 0.28, 0.47, and 0.94 μM in TB (50 mM Tris-HCl, pH 8, with 50 mM NaCl) containing 0.1 mg ml^{-1} BSA were added to LPS- or LTA-coated wells and incubated for 3 h at 25 °C. After washing and incubation with rabbit antiserum made against *M. sexta* βGRP2, wells were treated with secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase, Bio-Rad). Alkaline phosphatase activity against *p*-nitrophenyl phosphate, which is proportional to LPS or LTA binding activity, was detected at 405 nm. To validate the specificity of binding to LPS or LTA, 5 μg ml^{-1} rβGRP or 181N was preincubated with 0, 0.1, 1, 10, 50, 100, 250, or 500 μg ml^{-1} free competitor (LPS or LTA). 50 μl of the solution was added to wells precoated with LPS or LTA and assayed.

Activation of the PPO Pathway in the Presence of Laminarin—A method described previously by Ma and Kanost (8) and Fabrick *et al.* (12) was used to test ability of the recombinant proteins to activate *M. sexta* PPO in the presence of soluble β-1,3-glucan (laminarin). Protein samples (0.5 μM) were incubated with 150 μg of laminarin and mixed with 10 μl of *M. sexta* plasma. Phenoloxidase activity was determined by measuring the absorbance at 490 nm. Values represent the means ± S.E. of data from three sets of triplicate measurements on a single pooled plasma sample collected from five *M. sexta* 5th Instar (day 3) larvae. PPO activation was analyzed for significance by using the SAS PROC Student's *t* test at $\alpha = 0.05$ (41).

RESULTS

Production of Recombinant Full-length and Truncated βGRP—PiβGRP is 471 amino acids long with an amino-terminal region of ~120 residues, a putative linker region, and a carboxyl-terminal region (~220 residues) that is similar in sequence to β-1,3-glucanases (12). The amino-terminal portion had no significant sequence similarity to any proteins other than the amino terminus of proteins that belong to the βGRP/GNBP family. The structural and functional properties of the *P. interpunctella* βGRP were analyzed by producing a full-length rβGRP, two amino-terminal truncation mutants (118N and 181N) that lack the glucanase-like domain, and a carboxyl-terminal truncation mutant (290C) that includes only the glucanase-like domain (Fig. 1*A*). Purified recombinant proteins were obtained in milligram quantities from 0.5–1.0 liters of bacterial cultures. SDS-PAGE analysis with Coomassie Blue staining indicated that each recombinant protein solution contained a single major protein band (Fig. 1*B*). The molecular masses of the recombinant proteins (as estimated by SDS-PAGE) were 54-, 20-, 24-, and 36-kDa for rβGRP, 118N, 181N, and 290C, respectively.

Structural Properties—rβGRP and its domains appear to be folded as indicated by CD spectroscopy (Fig. 2) and possess binding and biological activities. CD spectroscopy of 118N and 181N indicates that these proteins contain primarily an α-helical secondary structure with a minor β-structure. Both proteins exhibit CD spectra reminiscent of a protein containing primarily α-helix with a positive absorbance peak near 192 nm and a negative peak near 208 nm. Proteins containing only α-helix also possess a second negative absorption peak near 222 nm, which is not clearly evident in the spectra obtained from 118N and 181N, indicating that the recombinant proteins may possess other forms of secondary structure (*i.e.* β-sheet or

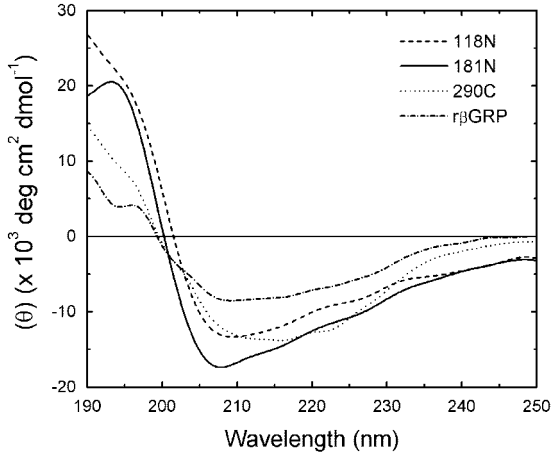

FIG. 2. **Circular dichroism spectroscopy of recombinant proteins.** CD spectra were obtained for recombinant βGRP and domains in 10 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 at 25 °C using a Jasco J-720 spectropolarimeter.

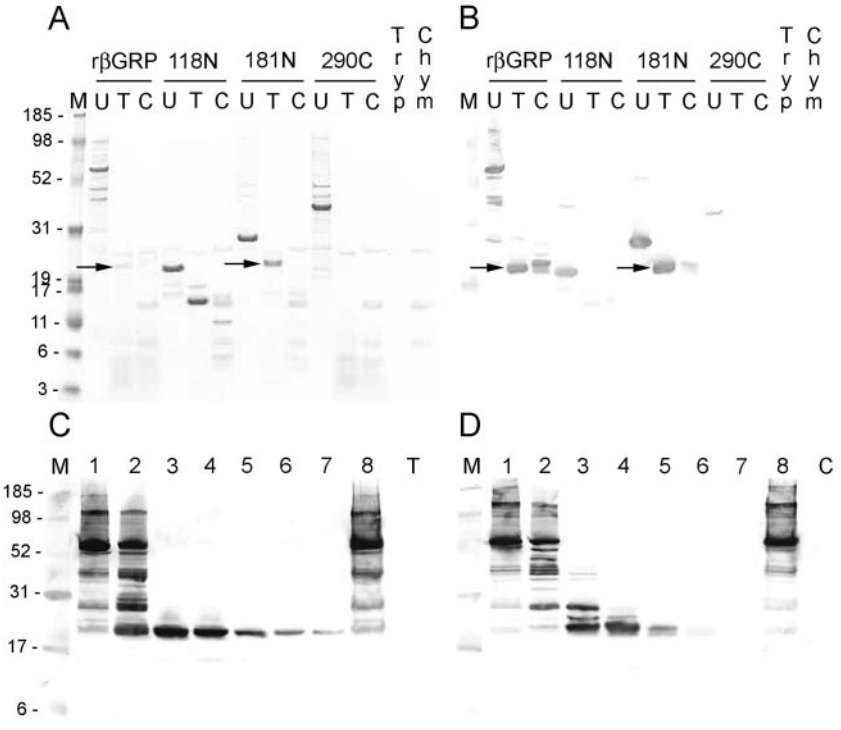

FIG. 3. **Proteinase digestion of rβGRP and its domains.** Recombinant proteins treated with trypsin or chymotrypsin for 1 h were separated on SDS-PAGE gels and analyzed by Coomassie Blue protein staining (*A*) and immunoblot analysis (*B*). Untreated, trypsin-treated, and chymotrypsin-treated proteins are shown in *lanes U*, *T*, and *C*, respectively of the Coomassie Blue-stained gel (*A*) and immunoblot (*B*). rβGRP was also treated with proteinase for different time periods, separated on SDS-PAGE gels, and transferred to nitrocellulose membranes, and the blots were probed with anti-βGRP sera. Immunoblots *C* and *D* correspond to rβGRP treated with trypsin and chymotrypsin, respectively. *Lanes 1–7* for immunoblots *C* and *D* correspond to proteinase-treated rβGRP at 0, 0.01, 0.5, 1, 3, 6, and 24 h, respectively. rβGRP 24-h controls without proteinase are shown in *lane 8*. Trypsin and chymotrypsin controls (without rβGRP) are shown in *lanes T* and *C*, respectively. *Lane M* contains protein molecular weight standards.

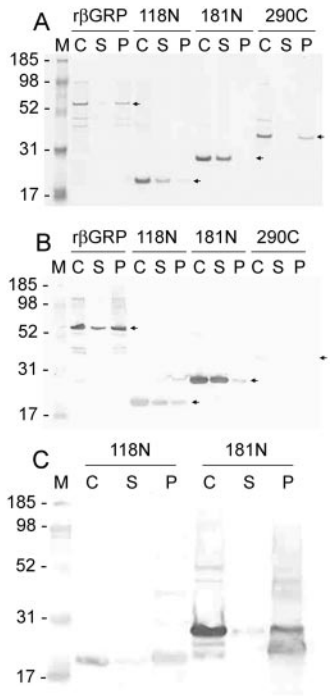

FIG. 4. **Thermal stability of amino-terminal domain from *P. interpunctella* βGRP.** SDS-PAGE with Coomassie Blue protein staining (*A*) and immunoblot analysis (*B*) of rβGRP, 118N, 181N, and 290C following heat treatment. Equal volumes (14.2 of 125 μl total) of the heat-soluble and heat-precipitated samples were loaded in *lanes S* and *P*, respectively. Untreated proteins (0.85 μg for Coomassie Blue staining and 0.5 μg for immunoblotting) were loaded as controls in *lanes C*. *Lane M* contains protein molecular weight standards. *Arrows* indicate position of protein bands on gel or immunoblot. *C*, immunoblot of curdlan binding assay following heat treatment. Both heat-treated 118N and 181N bound curdlan. Proteins that co-precipitated with curdlan are shown in *lanes P*, whereas supernatants are indicated as *S*. Heat-soluble supernatants are shown *lane C*.

turns). The CD spectra of rβGRP and 290C were complicated and could not be easily interpreted, indicating that these proteins contained a mixture of secondary structure. We believe that the proteins were folded correctly because we could detect various functional activities in both proteins.

The amino-terminal domain of βGRP was resistant to both proteolysis and thermal denaturation. The treatment of rβGRP or 181N with trypsin or chymotrypsin for 1 h produced a 20-kDa protein fragment that cross-reacted with antisera made against *M. sexta* βGRP2 (Fig. 3, *A* and *B*). Amino acid sequencing of this fragment revealed a single polypeptide with sequence (AQQYVV) corresponding to very near the amino-terminal end (residues 4–9) of *P. interpunctella* βGRP (12). The 20-kDa fragment resisted degradation by trypsin for at least 24 h and by chymotrypsin for up to 6 h (Fig. 3, *C* and *D*). 118N did not resist degradation and was hydrolyzed to smaller peptides by both proteinases within 1 h. Both trypsin and chymotrypsin completely hydrolyzed 290C within 1 h of treatment.

Heat treatment and recovery of recombinant proteins indicated that the amino-terminal domain of βGRP is highly resistant to thermal denaturation (Fig. 4, *A* and *B*). Recombinant proteins 118N and 181N both remained in solution after treatment at 100 °C for 10 min, suggesting a highly stable conformation. In addition, 118N and 181N retained their ability to bind to the β-1,3-glucan, curdlan, following heat-treatment (Fig. 4*C*). rβGRP and 290C were found primarily in the heat-denatured pellet, indicating that the carboxyl-terminal domain of βGRP is heat-labile.

Aggregation of Microbes—Aggregation of *S. cerevisiae*, *S. aureus*, and *E. coli* was observed in the presence of physiological concentrations of rβGRP (Table I). Incubation with rβGRP resulted in large multicellular aggregates of each microorganism with very few individual cells visible. In contrast, only a few large aggregates of yeast or *E. coli* were observed in the presence of 118N and 181N. 290C did not cause significant aggregation of any of the microbes. We previously showed that the agglutination of yeast by rβGRP was concentration-dependent and reversible in the presence of soluble β-1,3-glucan (12). Thus, the full-length rβGRP is required for substantial microbial aggregation.

TABLE I
In vitro agglutination of fluorescein-labeled microorganisms by recombinant P. interpunctella βGRP and the amino- and carboxyl-terminal domains

Degree of aggregation was scored as −, +, ++, or +++ for no agglutination detected, few visible cell aggregates, substantial cell aggregates present, and high numbers of cell aggregates visible, respectively.

Protein	Degree of aggregation		
	S. cerevisiae	*S. aureus*	*E. coli*
BSA	−	−	−
rβGRP	+++	+++	+++
118N	+	−	+
181N	+	−	+
290C	−	−	−

Binding to Microbial Polysaccharides—rβGRP, 118N, and 181N co-precipitated with curdlan (Fig. 5*A*), suggesting that the first ~100 amino-terminal residues of PiβGRP comprise a β-1,3-glucan binding domain (or carbohydrate recognition domain). Neither 290C (Fig. 5*A*) nor BSA (data not shown) co-precipitated with the insoluble β-1,3-glucan. However, all of the recombinant 1,3-glucan recognition protein constructs (including 290C) bound to biotinylated laminarin immobilized on streptavidin-agarose (Fig. 5*B*). Thus, the carboxyl-terminal glucanase-like domain of PiβGRP has the capacity to bind to laminarin (a β-1,6-branched β-1,3-glucan) but not to the linear β-1,3-glucan, curdlan. Excess laminarin reduced the binding of rβGRP to curdlan, indicating that laminarin and curdlan bind to similar regions within the protein (Fig. 5*C*).

We further characterized the binding of the 1,3-glucan recognition protein domains to laminarin by surface plasmon resonance analysis. A single flow cell on a streptavidin-coated sensor chip SA was coated with either biotin (control) or biotinylated-laminarin and subsequently utilized for all of the analyses. Immobilization of biotin onto the control flow cell resulted in a net change of ~40 response units. Injection of biotinylated laminarin resulted in a net change of ~600 response units that appeared stable, indicating the immobilization of a fixed amount of laminarin. A typical sensorgram for the injection of rβGRP over the control and bound-laminarin flow cell is shown in Fig. 5*D*. The binding constants of the recombinant proteins are summarized in Table II. All four recombinant proteins bound to the chip containing immobilized laminarin but did not interact with a CM5 dextran chip surface (data not shown), which consists of only the dextran linker. Corrections for nonspecific binding were made by the subtraction of data obtained from the biotin-control flow cell for each protein. rβGRP had a higher affinity for laminarin (K_A of 3.8×10^8 M^{-1}) than the truncated mutants 118N, 181N, or 290C, although the differences were not statistically significant by ANOVA at $\alpha = 0.05$.

The binding of rβGRP[2] and 181N to LPS or LTA immobilized in wells of microplates was concentration-dependent and saturable (Fig. 6). rβGRP reached saturation at ~0.2 μM for both LPS and LTA, whereas 181N reached a maximum at ~0.5 μM. The binding of rβGRP and 181N to LPS and LTA was specific

[2] Data published in Ref. 12.

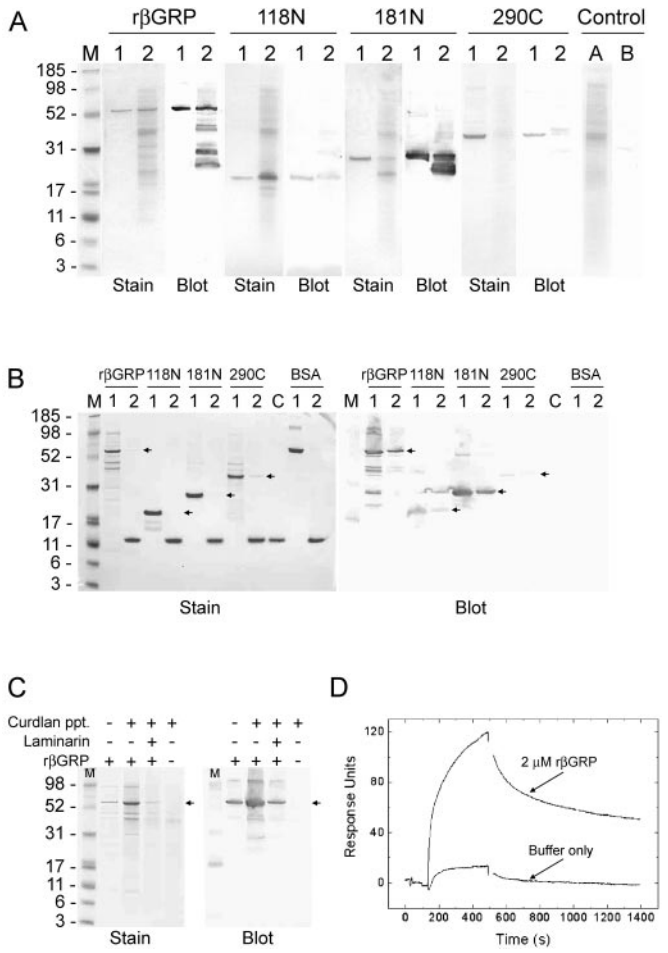

FIG. 5. **Binding of rβGRP and domains to β-1,3-glucan.** *A*, binding of rβGRP, 118N, 181N, and 290C to β-1,3-glucan as determined by co-precipitation with insoluble curdlan and analysis on SDS-PAGE gels stained with Coomassie Blue (*Stain*) or detected by immunoblotting (*Blot*). *Lanes 1* contain 0.35 μg of protein prior to the addition to curdlan, and *lanes 2* contain indicated protein sample eluted from curdlan precipitated by heat treatment in SDS solution. Curdlan controls, without addition of protein, are shown with *lanes A* and *B*, corresponding to a Coomassie Blue-stained gel and an immunoblot, respectively. Protein molecular weight standards are shown in *lane M*. *B*, biotinylated-laminarin was immobilized onto streptavidin-agarose resin and used to monitor the binding of rβGRP, 118N, 181N, and 290C to the β-1,6-branched β-1,3-glucan. Proteins eluted from the column were analyzed on SDS-PAGE gels stained with Coomassie Blue (*Stain*) or detected by immunoblotting (*Blot*). *Lanes 1* contain protein prior to the addition to the affinity resin (2.5 μg in the stained gels and 1 μg for immunoblots), and *lanes 2* contain indicated bound protein sample eluted from the resin. BSA was run as a negative binding control. A control without the addition of protein is shown in *lane C*. Protein standards were loaded in *lanes M*. *C*, curdlan binding activity of rβGRP was monitored by SDS-PAGE analysis with Coomassie Blue staining (*Stain*) or immunoblotting (*Blot*) in the presence of laminarin. rβGRP (30 μg) with or without 0.5 mg of laminarin was incubated with 10 mg of curdlan, washed, and eluted by treatment in boiling water with SDS gel loading buffer. Samples were loaded onto gels as indicated. Controls included rβGRP (0.7 μg) and supernatant obtained from curdlan without addition of recombinant protein. Protein standards were run in *lane M*. *D*, surface plasmon resonance sensorgram illustrating interaction of rβGRP (2 μM) with immobilized-laminarin SA chip.

as free LPS and LTA effectively reduced the binding of the protein to the bound ligand. No significant binding was observed for 118N or 290C with LPS or LTA (data not shown). However, it is difficult to compare binding between the proteins because the antibody generated against full-length *M. sexta* βGRP2 does not recognize the proteins equally. Note that in Fig. 5*A*, unequal amounts of protein were loaded to achieve similar levels of detection on immunoblots.

TABLE II

BIAcore surface plasmon resonance affinity determination for rβGRP and its domains to immobilized laminarin

Each value was determined as described under "Experimental Procedures" and is expressed as the mean ± S.E. for three independent binding studies. No statistical significance was observed for values within each column as determined by ANOVA at $\alpha = 0.05$.

Protein	k_a	k_d	K_A
	$M^{-1}\ s^{-1}$	s^{-1}	M^{-1}
rβGRP	$6.3 \pm 4.0 \times 10^3$	$3.8 \pm 3.1 \times 10^{-5}$	$3.8 \pm 2.6 \times 10^8$
118N	$2.5 \pm 1.1 \times 10^3$	$3.1 \pm 2.1 \times 10^{-5}$	$1.2 \pm 0.6 \times 10^8$
181N	$8.9 \pm 8.1 \times 10^2$	$9.2 \pm 1.0 \times 10^{-7}$	$1.1 \pm 1.0 \times 10^8$
290C	$1.1 \pm 1.0 \times 10^3$	$2.1 \pm 1.0 \times 10^{-5}$	$2.9 \pm 2.2 \times 10^7$

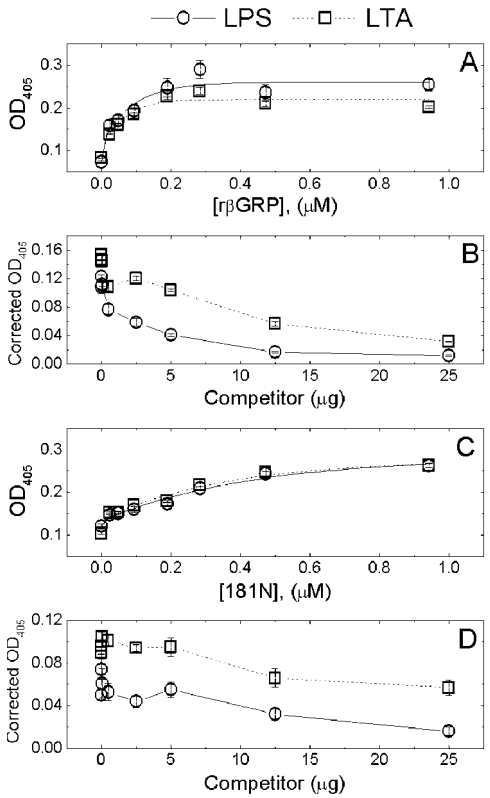

FIG. 6. **Binding of rβGRP and 181N to LPS and LTA.** Binding of rβGRP (*A*) and 181N (*C*) to immobilized LPS or LTA. Different protein concentrations of rβGRP or 181N were prepared in TB buffer containing 0.1 mg ml^{-1} BSA, added to wells containing LPS or LTA, and incubated for 3 h at 25 °C. rβGRP (*B*) and 181N (*D*) bound LPS or LTA specifically as increasing amounts of free LPS or LTA prevented binding of the proteins to coated wells. Binding to LPS is shown with *open circles*. Binding to LTA is indicated with *open squares*. Each point represents the mean ± S.E. from three individual measurements.

Activation of Prophenoloxidase—rβGRP, 118N, and 181N stimulated PPO activation when added to *M. sexta* plasma, but a significantly greater (~2.5-fold) stimulation of PPO activation occurred when 1 mg ml^{-1} laminarin was included in the reaction mixture with these proteins (Fig. 7). 290C did not activate PPO with or without laminarin. Laminarin alone or with bovine serum albumin did not result in significant PPO activation (although a longer incubation time with laminarin alone did stimulate PPO activation). No additive or synergistic effects were observed in samples that contained both 181N and 290C.

DISCUSSION

PRRs play an essential role in the activation of invertebrate innate immune pathways including those regulated by proteinase cascades such as the horseshoe crab-clotting reaction, the

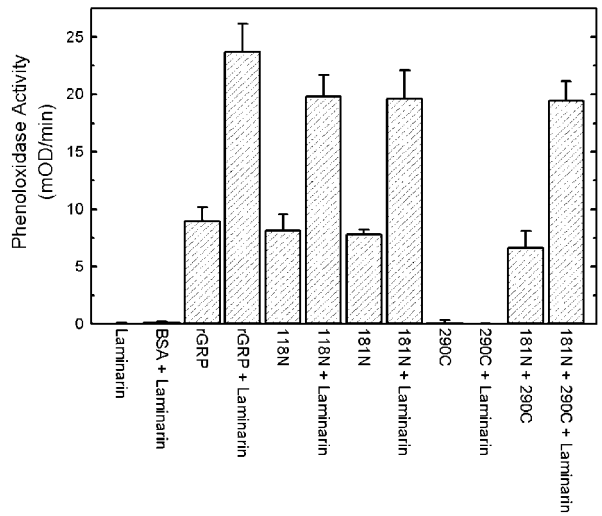

FIG. 7. **Activation of the prophenoloxidase pathway by *P. interpunctella* rβGRP and domains.** Ten microliters of pooled plasma from five *M. sexta* larvae was incubated with rβGRP, 118N, 181N, 290C, or 181N and 290C with or without 1 mg ml^{-1} laminarin for 20 min in wells of a microtiter plate. Phenoloxidase activity was determined by using the substrate dopamine. The final protein concentration for each sample was 0.5 μM. Phenoloxidase activity was significantly higher with rβGRP, 118N, and 181N in the presence of laminarin than without (rβGRP, $t = 5.70, p < 0.0047$; 118N, $t = 5.14, p < 0.0068$; 181N, $t = 4.80, p < 0.0087$). Controls either lacked protein or contained 0.5 μM BSA. Values represent the mean slope ± S.E. of triplicates from three tests conducted with a single pooled plasma sample.

Toll pathway in *Drosophila*, the prophenoloxidase-activating system in insects and other arthropods, and the processing of a cytokine that causes the spreading of insect hemocytes (43–47). These signaling pathways are ancient in origin and conserved in many organisms. The horseshoe crab blood-clotting pathway is controlled by a proteinase cascade in which the PRRs possess both carbohydrate recognition domains, which bind to PAMPs, and proteinase domains that are activated autocatalytically (50). The PPO pathway involves the proteolytic activation of prophenoloxidase to phenoloxidase, which results in the production of melanin or other compounds that function in protective and/or signaling capacities. Various PAMPs, including β-1,3-glucan can trigger the activation of insect PPO (48–55). PRRs including βGRPs, peptidoglycan recognition proteins, and C-type lectins have been shown to stimulate PPO activation (1). In *Drosophila*, the antifungal peptide, drosomycin, is up-regulated by the action of the *Toll* pathway and requires the extracellular proteinase processing of spaetzle, a ligand for Toll. A peptidoglycan recognition proteins has been shown recently to activate this pathway in the presence of Gram-positive bacteria (but not fungi) (56). The mechanism by which PRRs, bound to PAMPs, activate proteinases remains unknown.

Ochiai and Ashida (9) and Ma and Kanost (8) found that polypeptides corresponding to the amino-terminal domain of two lepidopteran βGRPs bound to curdlan (an insoluble β-1,3-glucan), whereas the carboxyl-terminal domain did not. Similarly, we demonstrated that a recombinant protein corresponding to the amino-terminal domain of *P. interpunctella* βGRP (the first 118 residues) could bind to curdlan, whereas no binding was detected with the carboxyl-terminal glucanase-like domain. However, the glucanase-like domain of *P. interpunctella* βGRP has an affinity to laminarin (a soluble β-1,3-glucan) as determined by affinity precipitation and SPR. Although laminarin and curdlan both contain β-(1→3)-linked D-glucopyranose backbones, the two polysaccharides differ. Laminarin contains β-(1→6) side chain branching (one branch approximately every 20 glucose units) and has an average molecular mass of 7.7×10^3 Da (57). In contrast, curdlan is a linear polymer of β-1,3-glucan with no side chain substitutions and is generally a heterogeneous mixture of polymer sizes (average degree of polymerization of 450) (58). It is likely that these structural differences allow for binding by the carboxyl-terminal domain of βGRP to laminarin but not curdlan. Laminarin and curdlan apparently compete for a common binding site or sites on rβGRP, because excess laminarin decreased the amount of protein co-precipitated with curdlan.

Some insect PRRs function to opsonize microorganisms within the hemocoel, thereby activating or amplifying cellular immune responses (1, 59). Aggregation of microorganisms *in vivo* may enhance immune signaling pathways or provide superior targets for cellular immunity. In this study, we showed that a full-length rβGRP was necessary for agglutination of microbes, whereas deletion mutants of the protein were not effective. Aggregation of cells requires that agglutinins have at least two binding sites or can form self-oligomers. Because the truncated protein domains did not agglutinate microbes, they most probably have only one binding site. Although rβGRP and/or its domains bound to surface compounds of various types of microorganisms and caused aggregation of these microbes, we could not demonstrate any direct anti-microbial activity associated with these proteins (data not shown).

The amino-terminal domain for *P. interpunctella* βGRP has several unique physical characteristics. The first 118 residues of Piβ GRP were sufficient for binding β-1,3-glucan and for the activation of PPO, whereas the glucanase-like domain has less affinity toward some PAMPs and could not activate PPO. Furthermore, the amino-terminal domain is highly resistant to degradation by proteolysis and is resistant to heat denaturation. The folded structure of this domain must be extremely stable. Although 181N does possess a pair of cysteines (Cys-143 and Cys-157) that might participate in a disulfide bond, 118N lacks cysteine residues; thus, a disulfide linkage is not responsible for the thermal stability. Whereas no tertiary structure is available for proteins similar to βGRPs, we found that the amino-terminal recombinant truncations of Piβ GRP have a predominantly α-helical secondary structure. Further studies of these domains should reveal structural features required for their stability as well as what motifs are important for binding carbohydrates and mediating immunological functions.

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