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Characterization of a Novel *Manduca sexta* beta-1, 3-glucan recognition protein (β GRP3) with Multiple Functions

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Abstract

Recognition of pathogens by insect pattern recognition receptors is critical to mount effective immune responses. In this study, we reported a new member (β GRP3) of the β -1, 3-glucan recognition protein (β GRP) family from the tobacco hornworm *Manduca sexta*. Unlike other members of the *M. sexta* β GRP family proteins, which contain an N-terminal small glucan binding domain and a C-terminal large glucanase-like domain, β GRP3 is 40–45 residues shorter at the N-terminus and lacks the small glucan binding domain. The glucanase-like domain of β GRP3 is most similar to that of *M. sexta* microbe binding protein (MBP) with 78% identity. β GRP3 transcript was mainly expressed in the fat body, and both its mRNA and protein levels were not induced by microorganisms in larvae. Recombinant β GRP3 purified from *Drosophila* S2 cells could bind to several Gram-negative and Gram-positive bacteria and yeast, as well as to laminarin (β -1, 3-glucan), mannan, lipopolysaccharide (LPS), lipoteichoic acid (LTA), and meso-diaminopimelic acid (DAP)-type peptidoglycan (PG), but did not bind to Lysine-type PG. Binding of β GRP3 to laminarin could be competed well by free laminarin, mannan, LPS and LTA, but almost not competed by free PGs. Recombinant β GRP3 could agglutinate *Bacillus cereus* and *Escherichia coli* in a calcium-dependent manner and showed antibacterial (bacteriostatic) activity against *B. cereus*, novel functions that have not been reported for the β GRP family proteins before. *M. sexta* β GRP3 may serve as an immune surveillance receptor with multiple functions.

Keywords

β GRP; microbial cell walls; antibacterial; agglutination; innate immunity

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1. Introduction

The innate immune system is the first line of defense against pathogenic invaders in animals (Mogensen, 2009; Sukhithasri et al., 2013) and relies on surveillance molecules, named pattern recognition receptors (PRRs), to recognize pathogen-associated molecular patterns (PAMPs) present in pathogens but not in the hosts (Charroux et al., 2009; Charroux and Royet, 2010; Kanost et al., 2004; Lemaitre and Hoffmann, 2007). PRRs include C-type lectins, β -1, 3-glucan recognition/binding proteins (β GRPs/BGRPs) and Gram-negative bacteria binding proteins (GNBPs), peptidoglycan recognition proteins (PGRPs), Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors, nucleotide-binding oligomerization domain (NOD) receptors, and Dectin receptors (Pal and Wu, 2009; Takeuchi and Akira, 2010). Examples of PAMPs include lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycans (PG) from bacteria and β -1, 3-glucan from fungi (Rao and Yu, 2010). Upon binding to PAMPs, PRRs can stimulate humoral and cellular immune responses, such as phagocytosis, nodule formation, encapsulation and melanization, synthesis of antimicrobial peptides and activation of the prophenoloxidase (proPO) system (Jiang et al., 2010; Kanost et al., 2004; Thompson et al., 2011). In insect hemolymph, activation of serine proteinase cascade leads to the proteolytic activation of proPO to active phenoloxidase (PO) (Gupta et al., 2005; Jiang et al., 2010). PRRs such as C-type lectins, β GRPs/BGRPs and PGRPs can stimulate proPO activation in hemolymph when binding to various PAMPs (Jiang et al., 2004; Lee et al., 2004; Ma and Kanost, 2000; Rao and Yu, 2010; Wang et al., 2011).

Gram-negative bacteria binding protein (GNBP) was first characterized as a 50-kDa hemolymph protein from the silkworm *Bombyx mori* that can bind to *Escherichia coli* (Lee et al., 1996). GNBPs actually belong to the β -1, 3-glucan recognition protein (β GRP/BGRP) family, which is one of the major pattern recognition receptors that can bind to β -1, 3-glucans on bacteria and fungi (Hughes, 2012; Jiang et al., 2004; Lee et al., 1996; Ma and Kanost, 2000; Wang et al., 2011). Members of the β GRP family proteins contain a small (~100 residues) N-terminal glucan binding domain and a large (~350 residues) C-terminal glucanase-like domain that lacks key residues in the active sites for glucanase activity (Ma and Kanost, 2000; Ochiai and Ashida, 2000). The small glucan binding domains of β GRPs bind to β -1, 3-glucan with a mechanism different from that of glucanase-like domains (Dai et al., 2013; Kanagawa et al., 2011; Mishima et al., 2009; Takahasi et al., 2009). β GRPs have been identified in invertebrates, including insects and crustaceans, and they can bind to microbial cell wall components, leading to activation of proPO (Cerenius et al., 1994; Duvic and Soderhall, 1990; Vargas-Albores et al., 1996; Vargas-Albores et al., 1997; Zheng and Xia, 2012). In the tobacco hornworm *Manduca sexta*, β GRP1 and β GRP2 have been identified and they can greatly stimulate proPO activation after binding to laminarin (β -1, 3-glucan) (Jiang et al., 2004; Ma and Kanost, 2000). *M. sexta* microbe binding protein (MBP), a β -1, 3-glucanase related protein, binds to bacteria and fungi, and MBP alone weakly stimulates proPO activation, but can significantly activate proPO when combined with different microbial elicitors (Wang et al., 2011). An inducible GNBP was purified from the silkworm, *B. mori* (Hughes, 2012; Lee et al., 1996), and silkworm β GRP can bind to β -1, 3-glucan to initiate activation of the proPO cascade (Ochiai and Ashida, 2000). *Drosophila*

DGNBP-1 can bind to LPS and β -1, 3-glucan and enhance immune gene expression induced by LPS and β -1, 3-glucan (Kim et al., 2000). *Anopheles gambiae* GNBP are involved in anti-*Plasmodium* responses (Warr et al., 2008).

In this paper, we reported the characterization and functional analysis of β GRP3, a new member of the *M. sexta* β GRP family. We investigated tissue distribution of β GRP3 transcript and induced expression of β GRP3 mRNA in fat body, hemocytes and midgut as well as β GRP3 protein in hemolymph by immune challenge. We also expressed and purified recombinant β GRP3 from *Drosophila* S2 cells and studied binding of β GRP3 to microorganisms and to various microbial cell wall components, including laminarin (β -1, 3-glucan), mannan, LPS, LTA, meso-diaminopimelic acid (DAP)-type and Lysine-type PGs. Interestingly, we found that β GRP3, which is 40–45 residues shorter at the N-terminus and lacks the small glucan binding domain, possessed novel properties with calcium-dependent agglutinating activity against *Bacillus cereus* and *E. coli* and strong antibacterial (bacteriostatic) activity against *B. cereus*.

2. Materials and methods

2.1 Insect rearing and *Drosophila* S2 cell line

M. sexta eggs were purchased from Carolina Biological Supply (Burlington, NC). Larvae were reared on artificial diet at 25°C (Dunn and Drake, 1983), and the fifth instar larvae were used for the experiments. *D. melanogaster* Schneider S2 cells were purchased from American Type Culture Collection (ATCC).

2.2 cDNA cloning of β GRP3 and sequence analysis

An *M. sexta* EST sequence was predicted to encode a partial BGRP-like protein (Accession number: GR922389.1). We then designed primers based on the EST sequence to clone the full-length cDNA. Briefly, total RNA was prepared from the fat body of day 3 naïve larvae using TRIzol® Reagent (T9424, Sigma–Aldrich). For reverse transcription, total RNA was treated with RQ1 RNase-free DNase I (Promega) at 37°C for 30 min to remove contaminated genomic DNA, and DNase was inactivated by heating to 65°C for 20 min. Reverse transcription was performed using oligo(dT) primer (Promega) and ImProm-II reverse transcriptase (Promega) following the manufacturer's instructions. *M. sexta* β GRP3 full-length cDNA was cloned using the forward primer β GRP3-F1: 5'-ACG ACT CGA TCA CAA GCA AC-3' and the reverse primer β GRP3-R1: 5'-CAG AAC TTG AGC ATG GCT TT-3'. 5' and 3' RACE reactions were performed using smarter race kit (Clontech). The opening reading frame (ORF) of β GRP3 was predicted from the nucleotide sequence using DNAMAN (Lynnon Corporation, Quebec, Canada). BLASTP (<http://www.ncbi.nlm.nih.gov/>) was used to search homologous β GRP, BGRP or GNBP protein sequences. Multiple sequence alignment was performed by ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) with protein sequences retrieved from the NCBI database using default settings, and the phylogenetic tree was generated from the conserved regions of 18 proteins (not including the N-terminal regions) by Neighbor-Joining method with bootstrap of 1,000 replications using MEGA5 (Tamura et al., 2011). Figures were made from one representative set of data with the GraphPad Prism software (GraphPad, CA).

Significance of difference was determined by one way ANOVA followed by a Tukey's multiple comparison test using the same software (GraphPad, CA).

2.3 Expression and purification of recombinant β GRP3 and GFP

Recombinant β GRP3 was expressed in *E. coli* and purified for production of polyclonal antibody in rabbit. The cDNA sequence encoding mature β GRP3 (residues 24–441) was obtained by PCR using primers β GRP3-F2 (5'-CAT GCC ATG GTT TAT CGG TCC CGT TCC ACG TCT TTG-3') and β GRP3-R2 (5'-CCC AAG CTT TTA CAG CGC GAC TAC TTT GAC ATA GTC-3'). PCR fragment was purified by agarose gel electrophoresis, digested with *Nco* I and *Hind* III enzymes, ligated into the *Nco* I/*Hind* III-digested expression vector H6pQE-60 (Lee et al., 1994) and then transformed into competent *E. coli* XL1-Blue cells. Recombinant plasmids were prepared from positive clones and confirmed by restriction enzyme digestion and DNA sequencing. Single bacterial colony on petri dish plates containing recombinant plasmid DNA was inoculated into LB medium containing ampicillin (100 μ g/ml) and incubated at 37°C overnight. The overnight culture was diluted 1:100 in LB medium and incubated at 37°C to OD₆₀₀=0.8 and then isopropyl-D-thiogalactoside (IPTG) (0.5 mM final concentration) was added to induce protein expression for another 6 h at 37°C. Bacterial cells were harvested by centrifugation and lysed with the lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% Triton X-100, 2 mg/ml lysozyme), and recombinant protein was purified using Ni-NTA agarose beads (Qiagen) following the manufacturer's instructions. The purified protein was further separated on 12% SDS-PAGE and the gel slice containing recombinant β GRP3 was used as an antigen to produce rabbit polyclonal antiserum at Cocalico Biologicals, Inc (Pennsylvania, USA).

Recombinant β GRP3 was also expressed in *Drosophila* S2 cells and purified. The cDNA sequence encoding mature β GRP3 (residues 24–441) was amplified by PCR using primers β GRP3-F3 (5'-GGA AGA TCT TAT CGG TCC CGT TCC ACG TCT TTG A-3') and β GRP3-R3 (5'-CCG CTC GAG CAG CGC GAC TAC TTT GAC ATA GTC-3'). The PCR product was recovered by agarose gel electrophoresis-Wizard® SV Gel and PCR Clean-Up System (A9285, Promega), subcloned into T-Easy vector (A1360, Promega), and recombinant plasmid was isolated. After digested with *Bgl* II/*Xho* I, cDNA fragment was recovered and inserted into *Bgl* II/*Xho* I digested expression vector pMT/BiP/V5-His A (V413020, Invitrogen) using T4 DNA ligase (M0202L, NEB). Green fluorescent protein (GFP) in the expression vector pMT/BiP/V5-His/GFP (V413020, Invitrogen) was used as a control protein. Recombinant expression vectors were purified using PureYield™ Plasmid Miniprep System (A1222, Promega) according to the manufacturer's instruction and the insert sequences were confirmed by DNA sequencing. These plasmids were used to transfect S2 cells and generate stable S2 cell lines following the manufacturer's instructions.

To purify recombinant β GRP3 and GFP, copper sulfate (final concentration of 250 μ M) was added to stable S2 cells to induce expression of β GRP3 or GFP. Cell culture medium was collected starting at 24 h after protein expression for several days by collecting culture medium every day and re-suspending the cells with fresh medium, and recombinant β GRP3 and GFP were purified by affinity chromatography using anti-V5 agarose beads (A7345, Sigma-Aldrich) as described previously (Xu et al., 2012; Zhong et al., 2012). Fractions were

analyzed by 10% SDS-PAGE, and those containing recombinant β GRP3 or GFP were combined and desalted using D-salt™ Excellulose™ GF-5 desalting column (#1851850, Pierce) pre-equilibrated with H₂O. Recombinant β GRP3 or GFP was eluted with water, and fractions containing β GRP3 or GFP were pooled and concentrated for the following assays.

2.4 Tissue distribution and expression of β GRP3 mRNA in response to immune challenge

To determine tissue distribution of β GRP3 mRNA, day 2 fifth instar *M. sexta* naïve larvae were used in the following experiments. Larvae were injected with 50 μ l of heated-killed *E. coli* DH5a, *Serratia marcescens*, *Staphylococcus aureus*, *Bacillus subtilis* (5×10^7 cells/larva) or yeast (*Saccharomyces cerevisiae*) (10^7 cells/larva), or H₂O (as a control), hemocytes, fat body, midgut, epidermis and testis were collected at 24 h post-injection and washed 3 times in anti-coagulant (AC) saline (4 mM NaCl, 40 mM KCl, 8 mM EDTA, 9.5 mM citric acid monohydrate, 27 mM sodium citrate, 5% sucrose, 0.1% polyvinyl pyrrolidone, 1.7 mM PIPES). Total RNAs were isolated from these tissues using TRIzol® Reagent, and cDNAs were prepared from total RNAs (1 μ g from each tissue) in a 25- μ l reaction as described above. Real-time PCR was performed with SYBR Premix (Takara) on a 7500 system (Applied Biosystems) using primers β GRP3-F4 (5'-AAG AAG CGA CGG CTC TGC TTG G-3') and β GRP3-R4 (5'-TGG ACA GCC AGG AGT TGG TGC-3') for β GRP3 gene, and RPS3-F (5'-GTT GCG AGG TGG TGG TTT C-3') and RPS3-R (5'-CCG TTC TTG CCC TGT TGG TC-3') for the control ribosomal protein S3 (*rpS3*) gene, and the reaction conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Then dissociation curve analysis was performed. Data from three replicates of each sample were analyzed with SDS software (ABI) using a comparative method (2^{-Ct}) using *rpS3* gene as an internal control gene. Expression in naïve or water-injected larvae was set to 1.

2.5 Time-course induction of β GRP3 protein in hemolymph

To determine time-course induction of β GRP3 protein in hemolymph by Western blot analysis, day 2 fifth instar naïve larvae were injected with *E. coli*, *Micrococcus luteus* or *S. cerevisiae* as described above. Hemolymph (~50 μ l) was collected every 2 h after injection (at least 4 larvae in each group). Hemolymph samples were centrifuged at 3000 g for 10 min and cell-free plasma samples were collected. Equal volumes of plasma samples (from at least 4 larvae) were mixed and 0.5 μ l mixed plasma samples were analyzed by 10% SDS-PAGE. Western blot was performed using rabbit anti- β GRP3 antiserum (1:2000 dilution) and goat anti-rabbit IgG conjugated to alkaline phosphatase (1:10,000 dilution) (Sigma-Aldrich).

To determine the concentration of β GRP3 protein in hemolymph, cell-free plasma samples from fifth instar naïve or *E. coli*-injected larvae were collected and pooled (from at least 30 larvae in each group). The amounts of β GRP3 in plasma samples were estimated by comparing the intensity of endogenous β GRP3 in the plasma samples with that of recombinant β GRP3 purified from *Drosophila* S2 cells by Western blot analysis using myImage Analysis V1.1 (Thermo Scientific).

2.6 Binding of β GRP3 to microbial cell wall components

To test binding of β GRP3 to microbial cell wall components, plate ELISA assays were performed. Ultrapure TLR grade lipopolysaccharide (LPS) and peptidoglycan (PG) from *E. coli* K12 (LPS-K12 and PG-K12), lipoteichoic acid (LTA) and PG from *B. subtilis* (LTA-BS and PG-BS) and *S. aureus* (LTA-SA and PG-SA) were from Invivogen. Mannan and laminarin (β -1, 3-glucan) were from Sigma-Aldrich. Briefly, wells of a flat bottom 96-well plate (Costar, Fisher) were coated with different microbial components (2 μ g/well) as described previously (Yu and Kanost, 2000; Yu et al., 2005). The plates were placed overnight at room temperature until the water evaporated completely, heated to 60°C for 30 min, and then blocked with 200 μ l/well of 1 mg/ml BSA in Tris buffer (TB) (50 mM Tris-HCl, 50 mM NaCl, pH 8.0) for 2 h at 37°C. The plates were rinsed and increasing concentrations of purified recombinant β GRP3 or GFP (a control protein) in TB containing 0.1 mg/ml BSA were added to the coated plates (50 μ l/well). For competitive binding assay, recombinant β GRP3 (diluted to 2 μ g/ml) was pre-incubated with increasing concentrations of free microbial components for 1 h at room temperature as described previously (Yu et al., 2005) and then added to the laminarin-coated plates. Binding of recombinant proteins to microbial components was allowed to occur for 3 h at room temperature. Then, the plates were incubated with monoclonal anti-polyhistidine antibody (Sigma-Aldrich) (1:2,000 in TB containing 0.1 mg/ml BSA) (100 μ l/well) overnight at 4°C, followed by incubation with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich) (1:3,000 in TB containing 0.1 mg/ml BSA) (100 μ l/well) for 2 h at 37°C. The plates were rinsed, *p*-nitrophenyl phosphate (1 mg/ml in 10 mM diethanolamine, 0.5 mM $MgCl_2$) was added (50 μ l/well), and absorbance at 405 nm of each well was determined every minute for 30 min using a microtiter plate reader (Bio-Tek Instrument, Inc.). The data were analyzed by the GraphPad Prism software.

2.7 Binding of β GRP3 to microorganisms

Binding of recombinant β GRP3 to microorganisms was performed using a published method (Du et al., 2009) with slight modifications. Briefly, Gram-negative *E. coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, Gram-positive *S. aureus*, *B. subtilis*, *M. luteus* and *Bacillus cereus*, as well as *S. cerevisiae* were cultured overnight. The microorganisms were pelleted, washed twice with Tris-buffered saline (TBS) (25 mM Tris-HCl, 2.7 mM KCl, 137 mM NaCl, pH 7.5), then thoroughly resuspended in TBS containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$. Purified recombinant β GRP3 or GFP (as a control) (each at 2 μ g in 50 μ l) were added to 50 μ l bacteria (4×10^8 cells/ml) or yeast (4×10^7 cells/ml) with rotation for 1 h at room temperature. The microorganisms were pelleted and the supernatants were collected as unbound proteins. These microorganisms were washed four times with TBS, subjected to elution with 7% SDS (100 μ l) for 10 min, washed in 1 ml TBS four times, and finally lysed in lysis buffer. Samples of the unbound proteins, TBS wash, 7% SDS elution and microbial lysates (to detect tightly bound proteins) were subjected to immunoblotting analysis using monoclonal anti-V5 antibody (V8012, Sigma-Aldrich).

2.8 Agglutination of microorganisms by β GRP3

E. coli, *S. marcescens*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *B. cereus*, *M. luteus*, and *S. cerevisiae* were used for the agglutination assays. Aliquots (3.5 μ l) of bacteria (3×10^9 cells/ml) and yeast (2.5×10^8 cells/ml) suspensions were mixed with purified recombinant β GRP3 or GFP protein (final concentration of 8 μ g/ml) in a total of 25 μ l TBS containing 2 mM CaCl_2 and 1 mM MgCl_2 or 5mM EDTA. The mixtures were incubated at room temperature for 1 h and then observed by microscopy as described previously (Yu et al., 1999; Yu et al., 2006).

2.9 Antimicrobial activity assay

The antimicrobial inhibition zone assay was performed according to a published protocol (Hultmark, 1998). A single colony of *E. coli*, *S. marcescens*, *B. cereus*, *B. subtilis*, or *S. cerevisiae* was grown overnight in LB broth (for bacteria) or YPD medium (for *S. cerevisiae*) at 37°C. Overnight bacterial cultures were then sub-cultured in LB medium and *S. cerevisiae* culture was sub-cultured in YPD medium until mid-log phase ($\text{OD}_{600} \sim 0.6$). Fifteen microliters (15 μ l) of log-phase cultures were diluted into 20 ml LB or YPD medium containing 1.5% agarose (kept in a 45°C heating block), mixed well and spread immediately on a petri dish. After the medium was solidified, 5-mm wells were punched in the agarose. Buffer, ampicillin, or purified β GRP3 was added into each well. The plates were incubated at 37°C overnight, and the diameters of inhibition zones were measured and recorded.

B. cereus growth curves were also determined in the presence of recombinant β GRP3 and various microbial components as described previously (Rao et al., 2012). Briefly, overnight *B. cereus* culture was sub-cultured in LB medium until mid-log phase, centrifuged at 1000 g for 10 min at 4°C, washed once in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and diluted to $\text{OD}_{600} = 10^{-3}$ with LB medium. The diluted *B. cereus* cultures (75 μ l/well) was mixed with purified β GRP3 alone (final concentration of 2 μ g/ml), β GRP3 mixed with different microbial components (final concentration of 20 μ g/ml), or water (Control) in 96-well plates, and the total volume was adjusted to 100 μ l with LB medium. *B. cereus* cultures were incubated at 37°C with 220 rpm shaking. OD_{600} was measured every hour by PowerWave XS plate reader (Bio-Tek, USA). Bacterial growth curves were generated using the Graphpad Prism software (GraphPad, CA).

3. Results

3.1 cDNA cloning and sequence analysis of *M. sexta* β GRP3

An *M. sexta* EST sequence was found to encode a partial BGRP-like protein (Accession number: GR922389.1), which is different from *M. sexta* β GRP1 and β GRP2 proteins reported previously (Jiang et al., 2004; Ma and Kanost, 2000). To clone the new β GRP homologue, we designed gene-specific primers based on the EST sequence. The full-length cDNA sequence of *M. sexta* β GRP3 was obtained by 5' and 3' RACE. The complete ORF of β GRP3 is 1323 nucleotides long encoding a protein of 441 amino acids with a putative signal peptide of 23 residues. The calculated molecular mass of mature β GRP3 is 48.9 kDa with an isoelectric point (pI) of 8.33. There are four potential N-linked glycosylation sites in β GRP3 (Asn⁴⁶, Asn⁷⁹, Asn¹²⁴ and Asn¹⁷⁷), and the glycosyl hydrolase like domain is

located close to the C-terminus of β GRP3 (Fig. S1). β GRP3 is ~40–45 residues shorter at the N-terminus compared to *M. sexta* β GRP1 (487 residues), β GRP2 (482 residues) and microbe binding protein (MBP) (482 residues), and it lacks the small glucan binding domain (Fig. S2). The N-terminal region (~57 residues, not including the 23-residues putative signal peptide) of β GRP3 shows no similarity to any sequence in the NCBI database (data not shown).

BLASTP search showed that the glucanase-like domain of *M. sexta* β GRP3 is most similar to that of *M. sexta* MBP (78% identity), a β -1, 3-glucanase related protein (Wang et al., 2011), *Hyphantria cunea* β -1, 3-glucan binding protein (63% identity) (Shin et al., 1998) and *B. mori* BGRP-2 (63% identity) (Lee et al., 1996), followed by *Helicoverpa armigera* BGRP-3 (61% identity) (Pauchet et al., 2009), *Ostrinia furnacalis* BGRP (60% identity), *Papilio xuthus* Gram-negative bacteria binding protein-3 (GNBP-3) (58% identity). *M. sexta* β GRP3 also shows similarity to *M. sexta* β GRP1 (36% identity) (Ma and Kanost, 2000), β GRP2 (35% identity) (Jiang et al., 2004) and β -1, 3-glucanase (36% identity), but lacks the four key residues (W, E, D, E) required for glucanase activity (Fig. 1A) (Ma and Kanost, 2000). Therefore, β GRP3 may serve as a pattern recognition receptor in the immune system of *M. sexta*.

The β GRP family proteins have been identified in different invertebrate species. The phylogenetic tree constructed based on the glucanase-like domains of 17 β GRP related proteins and *M. sexta* β -1, 3-glucanase showed that *M. sexta* β GRP3 and microbe binding protein (Ms-MBP) are clustered in the same clade, β GRP1 and β GRP2 are in a different clade, while *M. sexta* β -1, 3-glucanase is in a distant clade (Fig. 1B). These results suggest that the common ancestor of *M. sexta* β GRP family proteins diverged into three clades with possible functional variances.

3.2 Expression profiles of β GRP3 mRNA and protein

We expressed *M. sexta* β GRP3 in *E. coli* and purified the recombinant protein for production of rabbit polyclonal antibody. We also expressed and purified V5-tagged β GRP3 and GFP from *Drosophila* S2 cells for functional study. Recombinant β GRP3 purified from S2 cells migrated close to 58 kDa, which is larger than the calculated mass of 48.9 kDa, probably due to the addition of V5-His tag and post-translational modifications (Fig. 2). Recombinant β GRP3 could be recognized by both monoclonal anti-V5 and polyclonal anti- β GRP3 antibodies (Fig. 2B and C).

The mRNA level of β GRP3 in different tissues of *M. sexta* naïve larvae was determined by real-time PCR, and the results showed that β GRP3 transcript was mainly expressed in fat body at a significantly higher level than in hemocytes, midgut and other tissues (Fig. 3A). Expression of β GRP3 mRNA in fat body, hemocytes and midgut was not significantly up-regulated in response to microbial challenges (Fig. 3B–D), which is similar to that of *M. sexta* β GRP1 (Ma and Kanost, 2000). We also determined β GRP3 protein concentration in hemolymph by Western blot. The results showed that β GRP3 protein concentration in the hemolymph of *E. coli*, *M. luteus* and *S. cerevisiae* injected larvae did not change significantly from 0 to 12 h post-injection (Fig. 4A–C). To estimate the concentration of endogenous β GRP3 protein in hemolymph, Western blot was performed with increasing

volumes of hemolymph from naïve and *E. coli*-injected larvae along with increasing amounts of purified recombinant β GRP3, and endogenous β GRP3 protein was estimated at ~260 μ g/ml in the hemolymph of naïve larvae and ~255 μ g/ml in the hemolymph of *E. coli*-injected larvae (Fig. 4D and E). These results suggest that both β GRP3 transcript and protein levels were not induced by immune challenges.

3.3 Binding of β GRP3 to microbial cell wall components and microorganisms

To determine whether recombinant β GRP3 can bind to different microbial cell wall components, plate ELISA assay was performed. Compared to the control GFP protein, β GRP3 could bind to LPS-K12 and PG-K12 from *E. coli* K12, LTA-BS and PG-BS from *B. subtilis*, mannan and laminarin from fungi, and LTA-SA from *S. aureus* (Fig. 5), but did not bind to PG-SA from *S. aureus* (Fig. 5D). To confirm binding of β GRP3 to microbial components is specific, a competitive binding assay was performed. Binding of β GRP3 to laminarin was well competed by free laminarin, mannan, LPS-K12, LTA-SA and LTA-BS, but only slightly competed by PG-K12 and PG-BS (Fig. 5I), indicating that β GRP3 has broad binding spectra to microbial cell wall components with different affinities.

To further confirm broad binding spectra of β GRP3, a direct binding of β GRP3 to different microorganisms was performed. We first tested binding of GFP and β GRP3 to *E. coli* and found that GFP did not bind to *E. coli* and remained in the unbound fraction (Fig. 6A), but β GRP3 bound tightly to *E. coli* since only a small fraction of β GRP3 was eluted by 7% SDS and more β GRP3 remained in the *E. coli* lysates (Fig. 6D). We then tested binding of GFP and β GRP3 to several Gram-negative and Gram-positive bacteria as well as yeast. GFP did not bind to any of the microorganisms tested (Fig. 6B and C), but β GRP3 bound to all eight microorganisms tested (Fig. 6E and F). More β GRP3 protein bound to *E. coli* (*Ec*), *P. aeruginosa* (*Pa*), *S. aureus* (*Sa*) and *B. subtilis* (*Bs*) than to the other four microorganisms (Fig. 6E and F). Binding of β GRP3 to *S. marcescens* (*Sm*), *S. aureus* (*Sa*), *B. subtilis* (*Bs*), *M. luteus* (*Ml*), and *S. cerevisiae* (*Sc*) seems to be stronger since little or no β GRP3 was eluted by 7% SDS and more β GRP3 remained in microbial lysates (comparing Fig. 6E and F). These results suggest that β GRP3 is able to bind to multiple types of PAMPs on various microorganisms.

3.4 Agglutination of *E. coli* and *B. cereus* by β GRP3

To test whether β GRP3 also has agglutinating activity against microorganisms, purified recombinant β GRP3 or GFP was incubated with eight microorganisms, and agglutination of microbial cells was observed under microscope. GFP did not agglutinate any of the eight microorganisms tested (Fig. 7A–C and data not shown), but β GRP3 could agglutinate *E. coli* and *B. cereus* (Fig. 7D and E), but not *S. cerevisiae* (Fig. 7F) and the other five bacteria (data not shown), and agglutination of *E. coli* and *B. cereus* was calcium-dependent (Fig. 7G and H). In addition, *E. coli* cells formed aggregates in the presence of β GRP3 (Fig. 7D), while *B. cereus* cells were cross-linked by β GRP3 to form large networks (Fig. 7E).

3.5 Antibacterial activity of β GRP3 against *B. cereus*

Since β GRP3 could bind to microorganisms (Fig. 6) and agglutinate *E. coli* and *B. cereus* (Fig. 7), it may have antimicrobial activity and an inhibition zone assay was performed.

Ampicillin and buffer were used as positive and negative controls. Ampicillin showed high activity against *E. coli* and relatively high activity against *B. subtilis*, but recombinant β GRP3 did not show activity against *E. coli* or *B. subtilis* (Fig. 8A and B). Interestingly, ampicillin was not active against *B. cereus*, but β GRP3 showed strong activity against *B. cereus* in a dose-dependent manner (Fig. 8C). Both ampicillin and β GRP3 did not have activity against *S. cerevisiae* (Fig. 8D). To test whether binding of microbial components to β GRP3 may inhibit its activity against *B. cereus*, bacterial growth curves were determined in presence of β GRP3 alone or β GRP3 mixed with microbial components. The results showed that β GRP3 alone almost completely inhibited *B. cereus* growth ($OD_{600} = 0.104$ compared to $OD_{600} = 1.013$ in the control group (water) at 20 h after incubation). LPS-K12, LTA-BS, LTA-SA, laminarin, mannan and PG-BS could all completely block the antibacterial activity of β GRP3 against *B. cereus* ($OD_{600} = 0.998 - 1.087$ compared to $OD_{600} = 1.013$ in the control group) (Fig. 8E). PG-K12 also significantly blocked the activity of β GRP3 against *B. cereus* growth ($OD_{600} = 0.805$ vs. $OD_{600} = 0.104$) (p-value < 0.001), but *B. cereus* growth was still significantly inhibited compared to the control group ($OD_{600} = 0.805$ vs. $OD_{600} = 1.013$) (p-value < 0.001) (Fig. 8E).

4. Discussion

Invertebrates lack the adaptive immune system and rely on pattern recognition receptors (PRRs) as surveillance molecules to detect microorganisms and mount immune responses. These surveillance molecules circulate in the animal body to recognize pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009; Pal and Wu, 2009; Sukhithasri et al., 2013). β -1, 3-glucan recognition proteins (β GRPs/BGRPs) are a family of proteins containing a small N-terminal glucan binding domain and a large C-terminal glucanase-like domain, which lacks key residues for glucanase activity (Fig. 1A) but still can bind to β -1, 3-glucan. In this study, we have cloned a new member (β GRP3) of the *M. sexta* β GRP family, and investigated functions of β GRP3 in innate immunity.

M. sexta β GRP1 and β GRP2 transcripts are mainly expressed in fat body. β GRP1 mRNA is expressed in both feeding and wandering stages of larvae, while β GRP2 transcript is only expressed in the wandering stage larvae (Jiang et al., 2004; Ma and Kanost, 2000). We found that β GRP3 mRNA was predominantly expressed in fat body of naïve larvae (Fig. 3A). Expression of β GRP3 transcript in fat body, hemocytes and midgut as well as β GRP3 protein in hemolymph was not induced by different immune challenges (Figs. 3B–D and 4), which is similar to that of *M. sexta* β GRP1 but different from that of β GRP2 (Jiang et al., 2004; Ma and Kanost, 2000). *M. sexta* MBP mRNA is mainly expressed in fat body and is significantly induced by immune challenge, and MBP protein concentration in hemolymph also increases after immune stimulations (Wang et al., 2011). These results indicate that the β GRP family proteins are differentially regulated by development and immune challenge.

Recombinant β GRP3 had a broad binding spectrum to microbial components (Fig. 5) and microorganisms (Fig. 6). Broad binding spectra of insect PRRs, including members of the β GRP family, PGRPs and C-type lectins, have been reported. For example, two beetle 1, 3- β -D-glucan-binding proteins can recognize β -1, 3-D-glucan and peptidoglycan (Lee et al., 2004). *M. sexta* C-type lectins can bind to several different microbial components, including

LPS, LTA and PG (Yu et al., 2005; Yu et al., 1999; Yu and Kanost, 2000; Yu et al., 2006). *Drosophila* DGNBP-1 can bind to LPS and β -1, 3-glucan (Kim et al., 2000). *M. sexta* β GRP1 can bind to *E. coli*, *M. luteus* and *S. cerevisiae* (Ma and Kanost, 2000), β GRP2 can bind to laminarin and LTA but not LPS (Jiang et al., 2004), and MBP can bind to LPS, LTA and DAP-type PG (Wang et al., 2011). Thus, the β GRP family proteins may have broad binding spectra for various pathogens but differ in binding affinities. Less *M. sexta* β GRP3 bound to DAP-type PGs than to LPS, LTA and laminarin (Fig. 5), which is consistent with the results of competitive binding assay that binding of β GRP3 to laminarin was only slightly competed by PGs but completely competed by LPS, LTA and laminarin (Fig. 5I).

The β GRP family proteins are mainly involved in activation of the proPO system (Fabrick et al., 2003; Jiang et al., 2004; Ma and Kanost, 2000; Ochiai and Ashida, 2000), and some β GRPs can also aggregate microorganisms (Beschlin et al., 1998; Fabrick et al., 2003; Jiang et al., 2004). Like most β GRP proteins, *M. sexta* β GRP3 could activate proPO in plasma (Zhong X and Yu X, unpublished results), more importantly, it possess some unique properties that have not been reported for the β GRP family proteins: 1) calcium-dependent agglutination of *E. coli* and *B. cereus* (Fig. 7), which is similar to that of C-type lectins (Yu et al., 2005; Yu et al., 1999; Yu and Kanost, 2000; Yu et al., 2006), and 2) direct antibacterial (bacteriostatic) activity against *B. cereus* that is resistant to ampicillin (Fig. 8C). Compared to *M. sexta* β GRP1, β GRP2 and MBP, β GRP3 is ~40–45 residues shorter at the N-terminus and lacks the small glucan binding domain (Fig. S2). BlastP search showed that the shorter N-terminal domain (~57 residues) of *M. sexta* β GRP3 does not have a hit in the NCBI database (data not shown), indicating that the N-terminus of β GRP3 is not a truncated form of the small glucan binding domain but a new sequence that may have unknown functions. We speculate that the shorter N-terminal domain of β GRP3 may be involved in protein-protein interactions for formation of dimers or oligomers, which are required for agglutination of bacterial cells, since β GRP3 only contains a glucanase domain. Such a protein-protein interaction may also be calcium-dependent. The coelomic cytolytic factor 1 (CCF-1) of earthworm *Eisenia fetida*, which is a member of the β GRP family, also lacks the small N-terminal glucan binding domain, but it can still agglutinate Gram-negative bacteria, indicating that the N-terminal glucan binding domain is not absolutely required for agglutination (Beschlin et al., 1998). Antibacterial activity of β GRP3 is due to bacteriostatic effect but not bactericidal effect as the activity of β GRP3 was completely blocked by free laminarin, mannan, LPS, LTA and PG-BS, and significantly blocked by PG-K12 (Fig. 8E). Future work is to understand how β GRP3 agglutinates bacterial cells and exerts antibacterial activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- *M. sexta* β -1, 3-glucan recognition protein 3 (β GRP3) was mainly expressed in fat body of naïve larvae.
- Expression of both β GRP3 mRNA and protein was not induced by microbial challenge.
- Recombinant β GRP3 bound to laminarin, LPS, LTA and DAP-type PG, as well as to several bacteria and yeast.
- β GRP3 could agglutinate *E. coli* and *Bacillus cereus* in a calcium-dependent manner.
- β GRP3 showed strong bacteriostatic activity against *B. cereus* which is resistant to ampicillin.

Fig. 1. Multiple sequence alignment of the active sites of glucanases and some βGRP family proteins and phylogenetic tree of the βGRP family proteins

Glucanase-related proteins and the βGRP family proteins were retrieved from the NCBI database, multiple sequence alignment was performed by ClustalW2, and only residues around the active site of glucanase were shown (A). The Glycine residue conserved in all sequences and the two highly conserved hydrophobic residues are shaded. Residues corresponding to the active site (**W**, **E**, **D** and **E**) of the *Bacillus macerans* glucanase are shown in bold. A phylogenetic tree of 17 members of the βGRP family proteins and *M. sexta* beta-1, 3-glucanase (based on the large glucanase-like domains, see Fig. S3) was constructed by MEGA5 using the Neighbor-Joining method with bootstraps of 1,000. Ms-βGRP1, *M. sexta* beta-1, 3-glucan recognition protein 1 (AF177982); Ms-βGRP2, *M. sexta* beta-1, 3-glucan recognition protein 2 (AAN10151); Ms-βGRP3, *M. sexta* beta-1, 3-glucan recognition protein 3 (ADK39022); Ms-MBP, *M. sexta* microbe binding protein (GNBP-like) (ADT82662); Ms-Glucanase, *M. sexta* beta-1, 3-glucanase (AEV66276); Af-BGRP, *Apis florea* beta-1, 3-glucan-binding protein (XP_003694585); Hc-BGRP, *Hyphantria cunea* beta-1, 3-glucan-binding protein (O96363.1); Bm-GNBP, *Bombyx mori* beta-1, 3-glucan recognition protein 2 precursor (NP_001037450); Ha-BGRP3, *Helicoverpa armigera* beta-1, 3-glucan recognition protein 3 (ACI32828.1); Px-GNBP3, *Papilio xuthus* Gram-negative bacteria binding protein 3 (BAM19646.1); Dp-BGRP, *Danaus plexippus* beta-1, 3-glucan-binding protein (EHJ65186.1); Pr-BGRP3, *Pieris rapae* beta-1, 3-glucan recognition protein 3 (ACI32823.1); SI-BGRP2, *Spodoptera littoralis* beta-1, 3-glucan recognition protein 2 (ACI32819.1); Cq-GRP, *Culex quinquefasciatus* beta-1, 3-glucan recognition protein (XP_001850624.1); Dw-GK12242, *Drosophila willistoni* GK12242 (XP_002068068.1); Gm-GNBP, *Glossina morsitans* Gram-negative binding protein 1-like protein (ABC25063.1); Aa-GRP, *Aedes aegypti* AAEL007626-PA (EAT40654.2); Ad-GRP, *Anopheles darlingi* hypothetical protein (EFR19153.1).

Fig. 2. Analysis of recombinant GFP and β GRP3 purified from *Drosophila* S2 cells
V5-tagged recombinant GFP and β GRP3 proteins were purified from *Drosophila* S2 cells and analyzed by SDS-PAGE (1.5 μ g each protein) (A) and Western blot (0.2 μ g each protein) using monoclonal anti-V5 (B) or polyclonal anti- β GRP3 (C) antibody. Lane 1, GFP; lane 2, β GRP3.

Fig. 3. Tissue distribution and expression of β GRP3 mRNA in response to immune challenge
 (A) Tissue distribution of β GRP3 mRNA. Expression of β GRP3 mRNA in hemocytes, fat body, midgut, epidermis and testis of the 5th instar *M. sexta* naïve larvae was determined by real-time PCR and normalized to *rpS3* gene. (B–D): Expression of β GRP3 mRNA in fat body (B), hemocytes (C), and midgut (D) after immune challenge. Expression of β GRP3 mRNA in hemocytes, fat body and midgut of the 5th instar *M. sexta* naïve larvae or larvae injected with H₂O, *E. coli*, *S. marcescens*, *S. aureus*, *B. subtilis*, or *S. cerevisiae* at 24 h post-injection was determined by real-time PCR. The bars represent the mean of three individual measurements $\pm$ SEM. Relative expression of β GRP3 mRNA in naïve larval hemocytes (A), or in tissues of naïve larvae (B–D) was set as 1. Comparing expression of β GRP3 mRNA in different tissues (A) or after microbial injections, identical letters among tissues or treatments indicate not significant difference ($p > 0.05$), while different letters indicate significant difference ($p < 0.05$) determined by one way ANOVA followed by a Tukey's multiple comparison test.

Fig. 4. Expression of β GRP3 protein in hemolymph in response to immune challenge

The 5th instar *M. sexta* naïve larvae were injected with *E. coli* (A), *M. luteus* (B), or *S. cerevisiae* (C), and hemolymph samples were collected at 0, 2, 4, 6, 8, 10 and 12 h post-injection as described in the Materials and Methods. Cell-free mixed plasma samples (0.5 μ l each) were subjected to Western blot analysis using rabbit anti- β GRP3 antibody. (D) and (E): Concentration of endogenous β GRP3 in hemolymph. Increasing amounts of purified recombinant β GRP3 (lanes 1–4) or increasing volumes of mixed plasma samples (lanes 5–8) from naïve larvae (D) or *E. coli*-injected larvae (E) were analyzed by Western blot using rabbit anti- β GRP3 antibody. Lanes 1–4: 10, 25, 50 and 75 ng purified recombinant β GRP3, respectively; lanes 5–8: 0.1, 0.2, 0.3 and 0.4 μ l cell-free mixed plasma samples, respectively.

Fig. 5. Binding of β GRP3 to microbial cell wall components

Increasing concentrations of recombinant β GRP3 or GFP purified from *Drosophila* S2 cells were added to 96-well plates coated with LPS-K12 (A), PG-K12 (B), LTA-SA (C), PG-SA (D), LTA-BS (E), PG-BS (F), mannan (G) or laminarin (H), and binding of recombinant proteins to microbial components was determined by plate ELISA using anti-V5 antibody. Recombinant β GRP3 (2 μ g/ml) was pre-incubated with increasing amounts of free microbial components and then added to the laminarin-coated 96-well plates (I), and binding of β GRP3 to laminarin was determined by plate ELISA assay. The figures showed total binding of recombinant proteins to microbial components. Each point represents the mean of three individual measurements $\pm$ SEM, and the lines in (A–H) represent nonlinear regression calculation of one-site binding curves for β GRP3 (solid line) and GFP (dotted line).

Fig. 6. Binding of β GRP3 to microorganisms

Microorganisms were incubated with recombinant GFP (A–C) or β GRP3 (D–F) at room temperature for 1 h, washed with TBS, and bound proteins were eluted with 7% SDS. The remaining microorganisms were lysed in lysis buffer. Recombinant proteins in each fraction were detected by Western blot using anti-V5 antibody. (A) and (D): Binding of recombinant GFP (A) and β GRP3 (D) to *E. coli*. Lane 1: purified recombinant proteins (0.2 μ g each); lane 2: unbound proteins; lane 3: the fourth TBS wash; lane 4: 7% SDS elution; lane 5: *E. coli* lysates. In lanes 2–5, equivalent volumes to lane 1 were loaded in each lane. (B, C, E and F): Binding of GFP and β GRP3 to various microorganisms. Only SDS elution fractions (B and E) and microbial lysates after SDS eluting (C and F) were analyzed by Western blot using anti-V5 antibody. *Ec*: *E. coli*, *Pa*: *P. aeruginosa*, *Sm*: *S. marcescens*, *Sa*: *S. aureus*, *Bs*: *B. subtilis*, *Ml*: *M. luteus*, *Bc*: *B. cereus*, *Sc*: *S. cerevisiae*.

Fig. 7. Agglutination of microorganisms by β GRP3

Purified recombinant GFP or β GRP3 was incubated with *E. coli*, *B. cereus* or *S. cerevisiae* in TBS containing 2 mM CaCl_2 and 1 mM MgCl_2 (A–F) or 5 mM EDTA (G and H) at room temperature for 60 min, samples of microbial cells were examined by microscopy. Bar = 20 μm .

Fig. 8. Antibacterial activity of β GRP3 against *B. cereus*

Antibacterial activity of recombinant β GRP3 was determined by inhibition zone assay (A–D) and broth micro-dilution assay (E) as described in the Materials and Methods. Buffer and different amounts of ampicillin (Amp) were used as controls in the inhibition zone assay, the plates were incubated at 37°C overnight, and the diameters of inhibition zones were measured and recorded (A–D). Diluted *B. cereus* culture was incubated with recombinant β GRP3 alone (2 μ g/ml) or β GRP3 (2 μ g/ml) mixed with different free microbial components (20 μ g/ml each) in 96-well plates at 37°C, and OD₆₀₀ was recorded every hour up to 22 h. The points represent the mean of four individual measurements $\pm$ SEM. Asterisks indicate significant difference in OD₆₀₀ values at 20 h among the control, β GRP3, and β GRP3+PG-K12 groups ($p < 0.001$) determined by one way ANOVA followed by a Tukey's multiple comparison test.