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Characterization of a novel *Manduca sexta* beta-1, 3-glucan recognition protein (βGRP3) 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 (βGRP3) of the β-1, 3-glucan recognition protein (βGRP) family from the tobacco hornworm *Manduca sexta*. Unlike other members of the *M. sexta* βGRP family proteins, which contain an N-terminal small glucan binding domain and a C-terminal large glucanase-like domain, βGRP3 is 40–45 residues shorter at the N-terminus and lacks the small glucan binding domain. The glucanase-like domain of βGRP3 is most similar to that of *M. sexta* microbe binding protein (MBP) with 78% identity. βGRP3 transcript was mainly expressed in the fat body, and both its mRNA and protein levels were not induced by microorganisms in larvae. Recombinant βGRP3 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 βGRP3 to laminarin could be competed well by free laminarin, mannan, LPS and LTA, but almost not competed by free PGs. Recombinant βGRP3 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 βGRP family proteins before. *M. sexta* βGRP3 may serve as an immune surveillance receptor with multiple functions.

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 (βGRPs/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, βGRPs/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

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(βGRP/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 βGRP 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 βGRPs 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). βGRPs 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, 1997; Zheng and Xia, 2012). In the tobacco hornworm *Manduca sexta*, βGRP1 and βGRP2 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 βGRP 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* GNBPs are involved in anti-*Plasmodium* responses (Warr et al., 2008).

In this paper, we reported the characterization and functional analysis of βGRP3, a new member of the *M. sexta* βGRP family. We investigated tissue distribution of *βGRP3* transcript and induced expression of *βGRP3* mRNA in fat body, hemocytes and midgut as well as βGRP3 protein in hemolymph by immune challenge. We also expressed and purified recombinant βGRP3 from *Drosophila* S2 cells and studied binding of βGRP3 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 βGRP3, 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. *Drosophila melanogaster* Schneider S2 cells were purchased from American Type Culture Collection (ATCC).

2.2. cDNA cloning of βGRP3 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* βGRP3 full-length cDNA was cloned using the forward primer βGRP3-F1: 5′-ACG ACT CGA TCA CAA GCA AC-3′ and the reverse primer βGRP3-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 βGRP3 was predicted from the nucleotide sequence using DNAMAN (Lynnon Corporation, Quebec, Canada). BLASTP (http://www.ncbi.nlm.nih.gov/) was used to search homologous βGRP, 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 1000 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 βGRP3 and GFP

Recombinant βGRP3 was expressed in *E. coli* and purified for production of polyclonal antibody in rabbit. The cDNA sequence encoding mature βGRP3 (residues 24–441) was obtained by PCR using primers βGRP3-F2 (5′-CAT GCC ATG GTT TAT CGG TCC CGT TCC ACG TCT TTG-3′) and βGRP3-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 *Hin*d III enzymes, ligated into the *Nco* I/*Hin*d 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 μg/ml) and incubated at 37 °C overnight. The overnight culture was diluted 1:100 in LB medium and incubated at 37 °C to $OD_{600} = 0.8$ 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 lyzed 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 βGRP3 was used as an antigen to produce rabbit polyclonal antiserum at Cocalico Biologicals, Inc (Pennsylvania, USA).

Recombinant βGRP3 was also expressed in *Drosophila* S2 cells and purified. The cDNA sequence encoding mature βGRP3 (residues 24–441) was amplified by PCR using primers βGRP3-F3 (5′-GGA AGA TCT TAT CGG TCC CGT TCC ACG TCT TTG A-3′) and βGRP3-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