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Recognition of microbial molecular patterns and stimulation of prophenoloxidase activation by a β -1,3-glucanase-related protein in *Manduca sexta* larval plasma

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Abstract

Detection of pathogenic invaders is the essential first step of a successful defense response in multicellular organisms. In this study, we have identified a new member of the β -1,3-glucanase-related protein superfamily from the tobacco hornworm *Manduca sexta*. This protein, designated microbe binding protein (MBP), is 61% identical in sequence to *Bombyx mori* Gram-negative bacteria binding protein, but only 34–36% identical to *M. sexta* β -1,3-glucan recognition protein-1 and 2. Its mRNA levels were strongly up-regulated in hemocytes and fat body of immune challenged larvae, along with an increase in concentration of the plasma protein. We expressed *M. sexta* MBP in a baculovirus-insect cell system. The purified protein associated with intact bacteria and fungi. It specifically bound to lipoteichoic acid, lipopolysaccharide, diaminopimelic acid-type peptidoglycans (DAP-PGs) from *Escherichia coli* and *Bacillus subtilis*, but less so to laminarin or Lys-type PG from *Staphylococcus aureus*. The complex binding pattern was influenced by other plasma factors and additional microbial surface molecules. After different amounts of MBP had been incubated with larval plasma on ice, a concentration-dependent increase in phenoloxidase (PO) activity occurred in the absence of any microbial elicitor. The activity increase was also observed in the mixture of plasma and a bacterial or fungal cell wall component. The prophenoloxidase (proPO) activation became more prominent when DAP-PGs, *Micrococcus luteus* Lys-PG, or lipoteichoic acid was included in the mixture of MBP and plasma. Statistic analysis suggested that a synergistic enhancement of proPO activation was caused by an interaction between MBP and these elicitors, but not *S. aureus* Lys-PG, lipopolysaccharide, curdlan, or laminarin. These data indicate that *M. sexta* MBP is a component of the surveillance mechanism and, by working together with other pattern recognition molecules and serine proteinases, triggers the proPO activation system.

Keywords

pattern recognition; hemolymph protein; melanization; insect immunity; β GRP; GGBP; serine proteinase cascade

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Introduction

Innate immunity is the first line of defense against pathogen infection in all animals including insects (Gillespie et al., 1997; Strand, 2008). It is triggered by host proteins that recognize conserved surface determinants of microbes, such as lipopolysaccharide (LPS) or peptidoglycans (PGs) from bacteria and β -1,3-glucan from fungi. These pattern recognition receptors (PRRs) bind invading cells and initiate rapid cellular and humoral responses. Complex signaling pathways become active and induce the production of antimicrobial peptides and other defense molecules (Regan et al., 2009; Lemaitre and Hoffman, 2007). Extracellular serine proteinase cascades coordinate multiple immune responses: one leading to spätzle processing and Toll pathway activation; another causing proteolytic activation of prophenoloxidase (proPO). Active phenoloxidase (PO) then generates quinones that polymerize to form melanin (Nappi and Christensen, 2005). Reactive compounds such as 5,6-dihydroxyindole directly kill pathogenic invaders (Zhao et al., 2007; Kohler et al., 2007).

Biochemical and genetic studies have revealed more than 10 groups of PRRs in invertebrates, including hemolin, peptidoglycan recognition proteins (PGRPs), β -1,3-glucan recognition proteins (β GRPs), C-type lectins, galectins, fibrinogen-related proteins, thioester-containing proteins, Nimrods, scavenger receptors, and Down syndrome cell adhesion molecules (Yu et al., 2002; Lemaitre and Hoffman, 2007). These proteins bind to surface moieties of pathogens or parasites and initiate a coordinated defense reaction. One of these groups is composed of proteins with a region similar in sequence to β -glucanases from bacteria, fungi, sea urchins, and insects. Some of its members, due to differences in binding properties, are designated β GRPs, LPS- and β -1,3-glucan-binding protein, or Gram-negative bacteria-binding proteins (GNBPs) (Lee et al., 1996; Ochiai and Ashida, 2000; Ma and Kanost, 2000; Lee et al., 2000; Kim et al., 2000; Jiang et al., 2004). Other members of the family have similar names but their binding specificities have not yet been experimentally established.

To better understand the molecular basis of innate immunity in *Manduca sexta*, we did a pyrosequence analysis of expressed sequence tags for hemolymph proteins (Zou et al., 2008). Of the 218 newly discovered cDNA contigs, six encoded polypeptides similar to regions of *Bombyx mori* GGBP (Lee et al., 1996). In this work, we isolated cDNA clones of the GGBP homolog, characterized the recombinant protein from a baculovirus expression system, and explored its immunological functions. We studied its transcriptional changes in larval hemocytes and fat body after an immune challenge. This protein, named *M. sexta* microbe binding protein (MBP), specifically bound to microbial cell wall components and triggered proPO activation. Together with other PRRs discovered in the same insect, it contributes to a surveillance mechanism by which the host can detect microbial incursions and stimulate defense reactions.

2. Materials and Methods

2.1. Insect rearing, immune challenge, and cDNA synthesis

M. sexta eggs were purchased from Carolina Biological Supply and the larvae were reared on an artificial diet (Dunn and Drake, 1983). Each of day 2, 5th instar larvae was injected with 30 μ l H₂O, formaldehyde-killed *Escherichia coli* (2×10^7 cells) or *Micrococcus luteus* (20 μ g) (Sigma-Aldrich, M3770). Hemolymph samples were collected from cut prolegs of the larvae 24 h later and centrifuged at 5,000g for 5 min to remove hemocytes. Total RNA samples were isolated from hemocytes and dissected fat body using TRIZOL Reagent (Life Technologies, Inc). The samples (1 μ g each) were incubated with 1 $\times$ gDNA Wipeout buffer (Qiagen) in a 14 μ l reaction at 42°C for 2 min and chilled on ice immediately. For cDNA

synthesis, Quantiscript reverse transcriptase (1 μ l), 5 $\times$ Quantiscript RT buffer (4 μ l) and RT primer mix (1 μ l) (Qiagen) were added to each treated RNA sample, incubated at 42°C for 30 min, and heated at 95°C for 3 min to inactivate the enzyme.

2.2. cDNA cloning and sequence analysis

BLASTX (<http://www.ncbi.nlm.nih.gov/>) was employed to search the non-redundant protein sequence database with the putative GNBPs (Zou et al., 2008). Search results were used to determine the order, orientation, and open reading frame of the EST contigs. Based on manual analysis of conserved regions, primers j973 (5'-TACCTAACGTATCCATACATG, forward) and j974 (5'-CCGACGAGTAATGTGGAGAGC, reverse) were synthesized for PCR amplification of a cDNA fragment of *M. sexta* MBP. Same amounts of cDNA from fat body and hemocytes of naïve and bacteria-injected larvae were used in a preliminary PCR experiment to identify the tissue and immune status with the highest mRNA level. The reactions (25 μ l) contained 100 ng cDNA template, 10 pmol of each primer, 2.5 U *Taq* DNA polymerase, and commercial buffer with dNTPs. The thermal cycling conditions were 35 cycles of 94°C, 30s; 50°C, 30s; 72°C, 50s. Following gel electrophoresis, PCR product at the expected size was extracted and cloned into pGEM-T Easy (Promega). Plasmid DNA was isolated from the transformants and examined for correct insert size and sequence. The cDNA fragment was retrieved by *Eco*RI digestion, separated by electrophoresis, purified from the gel slice, and labeled with [α -³²P]-dCTP (3000 Ci/mmol) using Multiprime DNA Labeling System (GE Healthcare Life Science). The resulting probe was used to screen the *M. sexta* induced fat body cDNA library (Jiang et al., 2005) according to Sambrook and Russell (2001). Positive plaques were purified to homogeneity and subcloned by *in vivo* excision of the pBluescript phagemids. Plasmid DNA was isolated and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

Nucleotide sequences were assembled and extended at the 5' end by incorporating singleton sequences from our EST projects (Zou et al., 2008; Zhang et al., unpublished data). Following a BLASTX search of GenBank, some related protein sequences were retrieved from GenBank for multiple sequence alignment and phylogenetic analysis using ClustalX2 (<http://www.ebi.ac.uk/tools/clustalw2>). An open gap penalty of 10, an extension gap penalty of 1, a Blossum matrix, and neighbor-joining algorithm were used for the multiple sequence alignment and tree construction. Phylogram was displayed using Treeview X (<http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/>).

2.3. Quantitative polymerase chain reaction (qPCR)

The four cDNA samples were normalized in preliminary PCRs using specific primers for *M. sexta* ribosomal protein S3 (rpS3): j055 (5'-CTCAGGCCGAGTCTTTGAGATACA) and j056 (5'-ACTTCATGGACTTGGCTCTCTGAC). Each 15 μ l reaction mixture contained about 50 ng cDNA template, 4.5 pmol of each primer, and 7.5 μ l FastStart Universal SYBR Green Master (Roche Applied Science). Thermal cycling conditions were 94°C, 30 s; 53°C, 30 s; 72°C, 30 s for a total of 50 cycles. The templates, after being adjusted to contain equal amount of the internal standard, were used for qPCR analyses of rpS3 and MBP levels under the identical conditions. The MBP-specific primers (j291: 5'-CCCTTCGTGTCATACCAGCATCTA and j292: 5'-ATGTCCGCACCATCTACCTGTATC) were similar in melting temperature to those of j055 and j056. Relative abundances of MBP transcripts were calculated based on $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t,MBP} - C_{t,rpS3})_X - (C_{t,MBP} - C_{t,rpS3})_{CH}$ (X: CH, IH, CF, or IF).

2.4. Construction of expression plasmid MBP/pMFH6

Primers j279 (5'-CCATGGGAATTCCTAACGTATCCATACATGC) and j280 (5'-AAGCTTACTCGAGCGGACTACTTTGACGTAG) were used to amplify a cDNA fragment encoding the mature MBP in a 25 µl PCR reaction containing 5 ng Q4 cDNA (Fig. 1) and 0.5 µl 50× Advantage cDNA polymerase mix (Clontech). The thermal cycling conditions were 35 cycles of 94°C, 30s; 50°C, 30s; 68°C, 120s, followed by 3 min of incubation at 68°C. The gel-purified PCR product was cloned into pGEM-T and completely sequenced to ensure an error-free insert. The cDNA segment, retrieved by digestion with *EcoRI* and *XhoI*, was inserted into the same sites of plasmid pMFH6 (Lu and Jiang, 2008), which allows recombinant protein synthesized in insect cells under the control of polyhedrin promoter, secreted into media using the honeybee mellitin signal peptide, and purified on a Ni-NTA column via the carboxyl-terminal hexahistidine tag.

2.5. Baculovirus generation, insect cell infection, and MBP purification

In vivo transposition of the expression cassette, selection of bacterial colonies and isolation of recombinant bacmid DNA were conducted as per manufacturer's protocols (Life Technologies, Inc). The initial viral stock was obtained by transfecting *Spodoptera frugiperda* Sf9 cells with a bacmid DNA-CellFECTIN mixture and the titer was improved through serial infections. The V₅ viral stock, containing highest level of baculoviruses ($1\sim2\times10^8$ pfu/ml), was stored at -70°C for further experiments. Sf9 cells (at 2.4×10^6 cells/ml) in 500 ml of Sf-900™ III serum-free medium (Life Technologies, Inc) were infected with the baculovirus stock at a multiplicity of infection of 10 and grown at 27°C for 84 h with gentle agitation (100 rpm). After the cells were removed by centrifugation at 5,000g for 10 min, the supernatant was diluted with equal volume of H₂O and loaded onto a 20 ml dextran sulfate-Sepharose CL-6B beads equilibrated in buffer A (10 mM potassium phosphate, pH 6.4). After washing with 100 ml buffer A, bound proteins were eluted with a linear gradient of 0-1.0 M NaCl in buffer A at 1.0 ml/min for 100 min. Column fractions (4.0 ml/tube) were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining or immunoblot analysis using 1:200 diluted, affinity-purified rabbit anti-(His)₆ IgG as the first antibody. The MBP fractions were combined and loaded onto a 1-ml Ni²⁺-NTA agarose column equilibrated with 10 mM imidazole in buffer B (0.005% Tween-20, 0.3 M NaCl, 50 mM potassium phosphate, pH 8.0). Bound proteins were eluted with a linear gradient of 10-250 mM imidazole in buffer B at 0.5 ml/min for 60 min. Fractions (1.3 ml/tube) were analyzed, pooled, and precipitated with solid ammonium sulfate at 80% saturation. After centrifugation at 15,000g for 30 min, the protein pellet was dissolved in 1.0 ml, 50 mM NaCl, 20 mM Tris-HCl, pH 7.5 and dialyzed against the same buffer overnight. A rabbit polyclonal antiserum was raised against 0.5 mg of the purified MBP and the remaining protein (1.5 mg) was aliquoted and stored at -80°C.

2.6. Binding of MBP to microbial cells, insoluble PGs and curdlan

Single colonies of *M. luteus*, *Staphylococcus aureus*, *Bacillus megaterium*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *E. coli* were individually grown in 3.0 ml LB medium at 37°C at 220 rpm until OD₆₀₀ was close to 1.0. *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Candida albicans* were cultured in 3.0 ml YEPD medium at 28°C for 16 h at 250 rpm. After centrifugation at 2,500g and washing twice with buffer D (20 mM Tris-HCl, pH 7.5, 20 mM NaCl), cells were resuspended in Tris buffered saline (TBS, 137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.6) to $\sim1\times10^9$ bacteria and $\sim1\times10^8$ fungi per ml. *Beauveria bassiana* was cultured on a YPG plate (0.2% yeast extract, 1% peptone, 2% glucose, 1.7% agar) at 25°C for 3 days. After hyphae and conidia were harvested from the plate using sterile water, the cells were washed as described above. Purified MBP (0.5 µg) in 50 µl buffer D containing 1 mM benzamidine and 0.01% 1-phenyl-2-thiourea (PTU) was mixed with the pellet from 200 µl cell suspension at 4°C for 1

h. After centrifugation at 4,000g for 15 min, the supernatant was treated with 5× SDS sample buffer and 1/5 of it analyzed as the unbound fraction. The cell pellet was washed three times with buffer D (200 µl each), suspended with 20 µl of 2× SDS buffer, heated at 95 °C for 5 min, and 1/4 of it analyzed as the bound fraction. Following 10% SDS-PAGE and electro-transfer, immunoblot analysis was performed using 1:3000 diluted MBP antiserum as the first antibody. Intensities (I) of MBP bands in the bound and unbound fractions were quantified using Kodak Digital Analysis Software to estimate the binding extent:

$$4 \times I_{\text{bound}} \div (4 \times I_{\text{bound}} + 5 \times I_{\text{unbound}})$$

Similarly, insoluble DAP-PGs from *E. coli* K12 (0.5 mg, InvivoGen), *B. megaterium* (2.0 mg) and *B. subtilis* (2.0 mg), insoluble Lys-PGs from *M. luteus* and *S. aureus* (2.0 mg each) (Sumathipala and Jiang, 2010), and insoluble β -1,3-glucan from *Alcaligenes faecalis* (i.e. curdlan, 2.0 mg, Sigma-Aldrich) were mixed at 4 °C for 1 h with 0.5 µg MBP in 50 µl of buffer D containing benzamidine and PTU. After centrifugation at 16,000g for 15 min, the supernatants and pellets were obtained for SDS sample treatment, 10% SDS-PAGE, and immunoblot analysis under the same conditions described above for cells.

To test the binding of endogenous MBP, 10 µl of plasma from induced larvae and 70 µl buffer D containing benzamidine and PTU were mixed with the pellet from 200 µl cell suspension, 2.0 mg insoluble PG (0.5 mg *E. coli* PG), or 2.0 mg curdlan at 4 °C for 1 h. After centrifugation and washing, supernatants and pellets were obtained for sample treatment, electrophoretic separation, and immunoblot analysis under similar conditions described above. Since one eighth of the unbound and one half of the bound fractions were examined, binding extent was calculated as: $I_{\text{bound}} / (I_{\text{bound}} + 4 \times I_{\text{unbound}})$.

2.7. Plate assay of MBP binding to soluble ligands

Ultrapure LPS of *Porphyromonas gingivalis*, *B. subtilis* lipoteichoic acid (LTA), *E. coli* DAP-PG, *S. aureus* Lys-PG (all from InvivoGen) and *Laminaria digitata* laminarin (Sigma-Aldrich) (50 µl, 40 ng/µl) were applied to wells of a 96-well microplate and air dried overnight at room temperature. The plate was incubated at 60°C for 30 min to fix the ligands, and the wells were blocked with 200 µl of 1.0 mg/ml bovine serum albumin (BSA) in TBS at 37°C for 2 h. After washing, MBP (100 ng in 50 µl TBS containing 0.1 mg/ml BSA) was added to the wells and the plate incubated at room temperature for 2 h. A competition assay was performed to test whether the binding was a result of specific interaction between the protein and ligand: aliquots of MBP (100 ng) were individually incubated with the ligands (20 µg) in 50 µl TBS for 1 h at room temperature in the presence of 0.1 mg/ml BSA, and the mixtures were added to wells coated with the same ligands and incubated for 2 h at room temperature. After washing, 100 µl of 1:1000 diluted MBP antiserum in TBS containing 0.1 mg/ml BSA was incubated with the bound antigen for 2 h at 37°C. Following the washing step, 100 µl of 1:1500 diluted goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) (Bio-Rad) in TBS containing 0.1 mg/ml BSA and 0.1% NaN₃ was added to react with the first antibody at room temperature overnight. Then wells were washed four times with TBS and once with 0.5 M MgCl₂, 10 mM diethanolamine. Aliquots (50 µl) of *p*-nitrophenyl phosphate (1.0 mg/ml in the diethanolamine buffer) were added to the wells and absorbance at 405 nm was monitored in the kinetic mode on a VersaMax microplate reader (Molecular Devices). One unit of AP activity is defined as the amount of enzyme causing an increase of 0.001 absorbance unit per minute at room temperature.

To determine the effect of MBP concentration on specific binding of LPS, LTA and *E. coli* DAP-PG, the ELISA binding assay was repeated three times under the identical conditions, except a series of MBP solutions at 0, 0.4, 0.8, 1.2, 1.6, 2.0, 4.0, 8.0, 12, 16, and 20 ng/µl were separately incubated with or without a ligand (20 µg) before reacting with the same

ligand immobilized on the well surface. The AP activity differences between total binding (*i.e.* without competition) and nonspecific binding (*i.e.* with competition) were plotted against MBP concentrations. Nonlinear regression analysis was performed using Prism 3.0 (GraphPad Software Inc.) and single binding site model to calculate K_d and r^2 .

2.8. Elicitor-independent and -dependent proPO activation by MBP at different concentrations

Aliquots of cell-free hemolymph (5 μ l, 1:10 diluted) from day 2, fifth instar naïve larvae were incubated with 20 μ l BSA (0, 200, 400, 600, 800 ng) or MBP (0, 50, 100, 200, 300, 400, 600, 800 ng) in 0.001% Tween-20, 1 mM CaCl_2 , 20 mM Tris-HCl, pH 7.5, for 1 h on ice. PO activity was determined using dopamine as a substrate on a 96-well microplate reader (Jiang et al., 2003). Absorbance at 470 nm was monitored in the kinetic mode, and one unit of PO activity is equal to the amount of enzyme yielding an increase of 0.001 absorbance units per min.

To test a possible synergistic effect of MBP-elicitor interaction on proPO activation, 5 μ l of 1:10 diluted plasma from naïve larvae was incubated with 20 μ l of buffer (#1), elicitor (1.0 μ g in buffer: #2), MBP (100 ng in buffer: #3), or both (1.0 μ g elicitor + 100 ng MBP in buffer: #4) on ice for 40 min. The elicitors include insoluble DAP-PGs of *E. coli*, *B. megaterium* and *B. subtilis*, insoluble Lys-PGs of *M. luteus* and *S. aureus*, LPS, LTA, insoluble curdlan, and laminarin. PO activities were measured and plotted as mean $\pm$ SEM ($n=3$) in bar graphs, along with those of the controls (#1, plasma only; #2, plasma and elicitor; #3, plasma and MBP). Since interaction of plasma factors with elicitor (#2 - #1) and co-presence of plasma and exogenous MBP (#3 - #1) both led to proPO activation, an interaction of elicitor and exogenous MBP in plasma (#4 - #1) was expected to increase proPO activation to a level significantly higher than the sum of the two components [(#2 - #1) + (#3 - #1)]. Therefore, PO activities of #4 and (#2 + #3 - #1) were directly compared using unpaired t-test to reveal possible synergistic enhancement caused by elicitor-MBP interaction.

3. Results

3.1. cDNA cloning and structural features of *M. sexta* MBP

Our analysis of immunity-related expressed sequence tags (Zou et al., 2008) revealed six *M. sexta* cDNA contigs encoding polypeptide sequences similar to regions of *B. mori* GGBP. To investigate a possible role of the putative GGBP in defense responses, we designed specific primers and amplified the cDNA fragment, which was labeled with [α - ^{32}P]-dCTP to screen 1.2×10^5 plaques of an induced fat body cDNA library. Each of the positive clones (Q2, Q3, and Q4) contained regions 75~99% identical to the contigs, but none included an intact open reading frame (ORF) (Fig. 1). We searched our EST databases using the 5' end of the longest clone (Q4) and identified eight unassembled pyrosequence reads. These reads allowed a 5'-end extension of 34 nucleotides, but the ORF was still incomplete. Only the first nucleotide of the start codon (ATG) was missing, as suggested by a sequence comparison with GGBPs from other moths (data not shown).

The mature protein, now designated *M. sexta* MBP, is 462 residues long, with calculated molecular mass and isoelectric point of 51,482 Da and 5.78, respectively. Potential *N*- and *O*-linked glycosylation sites are present in the sequence at N^4VS , N^{90}LS , N^{198}ET , T^{123} , and S^{389} . Six Cys residues reside at positions 118, 131, 212, 219, 317 and 376. The first four, well conserved in this family of proteins, may form two disulfide bonds to stabilize the structure.

3.2. Sequence similarity and phylogenetic relationships

A BLASTP search of GenBank indicated that MBP is similar in sequence to *M. sexta* β GRP1 (identity: 36%; similarity: 51%) (Ma and Kanost, 2000), and β GRP2 (identity: 34%; similarity: 50%) (Jiang et al., 2004), *B. mori* GNBPs (identity: 61%; similarity: 74%) (Lee et al., 1996), *Hyphantria cunea* GNBPs (identity: 65%; similarity: 76%) (Shin et al., 1998), *Helicoverpa armigera* and *M. sexta* β -1,3-glucanases (identity: 34%; similarity: 47% for both) (Pauchet et al., 2009; Zhang et al., unpublished data). The MBP, GNBPs and β GRP sequences lacked the catalytic residues (Glu, Asp, Glu) present in the two β -1,3-glucanases and five *Anopheles gambiae* GNBPs (Jiang et al., 2004). The phylogenetic tree (Fig. 2) consists of four clades. The most basal clade, closer to bacterial and fungal glucanases (data not shown), includes putative β -1,3-glucanases from the moths, mosquito and beetle, which lack the first ~110 residues of β GRPs or GNBPs. Acquisition of the amino-terminal extension and loss of the catalytic residues likely occurred before the separation of the other three clades. Two clades are represented by *B. mori* GNBPs and *D. melanogaster* GNBPs, and the third clade includes moth β GRPs, *D. melanogaster* and *T. castaneum* GNBPs, and *A. gambiae* GNBPs.

3.3. Induced expression of *M. sexta* MBP gene

We performed a qPCR experiment to test whether or not the MBP mRNA level increased in the larval fat body or hemocytes upon bacterial injection (Fig. 3A). The lowest MBP transcript level was observed in hemocytes from naïve larvae, and there was a ~40-fold increase in hemocytes after the immune challenge. The basal level of MBP mRNA in fat body, 4.2-fold higher than induced hemocytes, further increased 6.3-fold after the bacterial injection. This indicated that fat body is the major site for MBP synthesis at 24 h post injection.

A polyclonal antiserum raised against the recombinant MBP recognized multiple bands on the immunoblot of plasma proteins separated by SDS-PAGE (Fig. 3B). The top band (~60 kDa), strongly recognized by β GRP2 antibodies (data not shown), seemed to represent β GRP2 and/or β GRP1 cross-reacted by MBP antibodies, whereas apparent molecular masses of the lower bands (53-55 kDa) were larger than the calculated one of mature *M. sexta* MBP (51.5 kDa). These size increases are consistent with the prediction that the MBP is heterogeneously glycosylated at multiple sites (Fig. 1). Low levels of the binding proteins (*i.e.* MBP and β GRP1/2) were detected in plasma samples from larvae injected with water and the protein levels became higher in plasma from larvae injected with *E. coli* or *M. luteus*. The estimated MBP concentrations in uninduced and induced plasma are 15-20 and 60-80 μ g/ml, respectively. These results are in agreement with the qPCR analysis of MBP transcripts (Fig. 3A), as well as published data on β GRP2 mRNA and protein levels (Jiang et al., 2004). On the other hand, *M. sexta* β GRP1 gene expression is not affected by immune status of larvae (Ma and Kanost, 2000) and its protein product migrated slightly faster than β GRP2, suggesting the doublet is not β GRP1 or β GRP2.

3.4. Production and characterization of the recombinant MBP

We produced *M. sexta* MBP as a (His)₆-tagged protein in the baculovirus-insect cell system. The secreted protein in the medium was efficiently captured by cation exchange chromatography on a dextran sulfate-Sepharose column and eluted as a single sharp peak. Protein contaminants were removed from the enriched MBP pool as flow-through fractions of the Ni²⁺-NTA column. The affinity-purified protein migrated as a single band at 55 kDa on an SDS-polyacrylamide gel, recognized by MBP antibodies (Fig. 4). There was no large difference in gel mobility under the reducing or non-reducing condition. Therefore, quaternary association of MBP, if any, is not maintained by an interchain disulfide bond.

3.5. Binding properties of *M. sexta* MBP

After the purified MBP (0.5 μ g) was incubated with $\sim 2 \times 10^8$ live bacteria for 1 h, about 1/4-1/20 of it bound to the cells (Fig. 5B): *M. luteus* (G+), *S. aureus* (G+), *B. subtilis* (G+, DAP-PG) and *E. coli* (G-) bound more MBP than *B. megaterium* (G+, DAP-PG), *K. pneumoniae* (G-), *P. aeruginosa* (G-) and *S. typhimurium* (G-) did. The same pattern was observed after induced plasma had been incubated with the bacterial cells (Fig. 5A). Under the experimental conditions, the binding of purified MBP to fungal cells was more complete: about 1/4-2/3 of MBP associated with the four strains tested (*S. cerevisiae* > *B. bassiana* > *P. pastoris*, *C. albicans*) (Fig. 5D). In contrast, MBP in plasma from immune challenged larvae did not bind to the fungal cells (Fig. 5C). Competition for a similar binding site by β GRPs, which did not occur to bacteria (Fig. 5A), may be responsible for the lack of binding to fungi by endogenous MBP. An extra band at ~ 50 kDa was detected by the MBP antibodies in some bound fractions of the plasma (Fig. 5A). This band could be a processing product of β GRPs or MBP. BSA did not bind bacteria, fungi, PGs, or curdlan (data not shown).

To find out which molecules on the microbial surface are responsible for the binding, we separately incubated the recombinant MBP with insoluble PGs from *M. luteus* (Lys-type), *S. aureus* (Lys-type), *B. megaterium* (DAP-type), *B. subtilis* (DAP-type), *E. coli* (DAP-type), and insoluble β -1,3-glucan (*i.e.* curdlan) from *A. faecalis*. We detected little binding to PGs from *M. luteus* or *B. megaterium* (Fig. 5F). Incomplete binding happened to the other polysaccharides in the order of curdlan > *B. subtilis* DAP-PG > *E. coli* DAP-PG, *S. aureus* Lys-PG. Likewise, MBP in the induced plasma bound to curdlan, *B. subtilis* and *E. coli* DAP-PGs, but less so to *M. luteus* and *S. aureus* Lys-PGs (Fig. 5E). Since *M. luteus* PG itself did not bind to purified MBP, we interpret its binding to endogenous MBP as indirect (*i.e.* via other plasma factors). In contrast, binding of MBP to *B. megaterium* DAP-PG, if any, was below the detection limit (Fig. 5, E and F).

In order to account for the binding of purified MBP to the bacteria, whose PGs are either not directly responsible (*e.g.* *M. luteus*) or inaccessible (a thin layer of PG in Gram-negative bacteria lies under the outer membrane that contains LPS and O-antigens), we tested whether or not other cell surface components such as LTA and LPS may contribute to the total binding. Using an ELISA-based, semiquantitative binding assay, we observed specific bindings of *M. sexta* MBP to soluble LPS, LTA, *E. coli* DAP-PG, *S. aureus* Lys-PG, and laminarin coated onto the plate (Fig. 6A). The most dramatic reduction of total binding occurred in the competition assays involving LTA, LPS, and *E. coli* DAP-PG, whereas half of the binding to soluble *S. aureus* Lys-PG and laminarin was nonspecific. To estimate binding constants, we repeated the competition test by incubating various amounts of MBP with LTA, LPS, or DAP-PG coated to wells. We found 50% of the specific binding occurred at a MBP concentration of 21.1 ± 5.6 nM for LTA, 37.7 ± 9.0 nM for LPS, and 43.4 ± 6.8 nM for *E. coli* DAP-PG (Fig. 6, B-D) according to the one-site binding model. Curve-fitting using two-site binding model yielded poor results (data not shown).

3.6. Role of *M. sexta* MBP in proPO activation

To test its possible role in the proPO activation system, we first incubated the purified MBP with diluted cell-free hemolymph from naïve larvae and observed a concentration-dependent increase in proPO activation in the absence of bacterial elicitor (Fig. 7). The PO activity reached its peak (15.2 U) at an added MBP concentration of 12 μ g/ml and rapidly decreased to a low level (4.9 U) when excess amounts of the binding protein were present. A similar increase in PO activity occurred in induced plasma after MBP was supplemented (data not shown). However, there was no such response after BSA was added to the plasma.

We further tested whether or not exogenous MBP could enhance proPO activation triggered by various elicitors. Since MBP alone caused proPO activation, 1:10 diluted plasma from naïve larvae was incubated on ice for 40 min with buffer, elicitor, MBP, or both elicitor and MBP at low levels. Under these conditions, low PO activity was detected in plasma (#1) and the mixture of plasma and elicitor (#2) (Fig. 8, panels A-G). While proPO activation occurred in plasma after a small amount of MBP was added (#3), higher PO activity was detected after plasma, MBP, and the elicitor had been incubated for 40 min (#4). The increases were statistically significant ($p < 0.05$) for insoluble DAP-PGs from *E. coli*, *B. subtilis*, and *B. megaterium*, as well as Lys-PG from *M. luteus* (Fig. 8, A-D), suggesting that exogenous MBP had interacted with these four PGs and other plasma factors and such interactions synergistically enhanced proPO activation. In contrast, PO activity increase was statistically insignificant ($p > 0.05$) when *S. aureus* Lys-PG or LPS was used (Fig. 8, panels E and F). Curdlan and laminarin bind MBP (Fig. 5 and Fig. 6) but did not cause substantial enhancement in proPO activation (#4 - #1), when compared with the sum of elicitor- and MBP-caused proPO activation [(#2 - #1) + (#3 - #1)] (Fig. 8, panels H and I). In contrast, LTA specifically interacted with MBP (Fig. 6) and enhanced proPO activation to a level significant higher than MBP and LTA alone did (Fig. 8, panel G).

4. Discussion

4.1. Evolution of the superfamily of β -glucanase-related proteins

It is now clear that β GRPs, GNBPs and β -1,3-glucanases in insects are a superfamily of proteins that recognize and even hydrolyze polysaccharides from fungi and bacteria. While the naming of β GRPs and β -1,3-glucanases is supported by experimental data (Ochiai and Ashida, 2000; Ma and Kanost, 2000; Jiang et al., 2004; Pauchet et al., 2009), other members (e.g. *D. melanogaster* GNBPs, *A. gambiae* GNBPs) may have been incorrectly designated (Buchon et al., 2009). In our opinion, the entire superfamily should be named β GRPs, standing for β -1,3-glucanase-related proteins, to better reflect their conserved structural unit and evolution history. This group of proteins arose from duplication of an ancient β -glucanase gene and function diversification. One gene copy maintained its original function by encoding active enzyme and it expanded in some insect lineages such as cockroaches (Genta et al., 2003), termites (Lin et al., 2008) and mosquitoes (Warr et al., 2008) but was lost in the fruit flies (Sackton et al., 2007). The other gene copy acquired an amino-terminal extension of ~110 residues and lost the residues essential for the hydrolytic activity. Neofunctionalization of the latter, facilitated by additional gene duplications, gave rise to all the receptor-type molecules for sensing the presence of microbes (Fig. 2): the first descendant probably yielded GNBPs in clades 2 and 3, whereas its sister gene evolved to β -1,3-glucan recognition protein (“ β GRP”) in clade 4. *B. mori* GGBP, *H. cunea* GGBP and *M. sexta* MBP belong to clade 2; *Drosophila* GGBP1 and 2; *Anopheles* GGBP-A1, *Tribolium* GGBP2 and others comprise clade 3. These two clades are more closely related to each other than to clade 1 (“ β -glucanase”) or 4 (“ β GRP”) and could be categorized together as “GGBP”, named after its first member *B. mori* GGBP. Note that the names in quotation represent phylogenetic grouping, which may or may not reflect actual binding function. The major sequence differences in *D. melanogaster* GGBP1 and 2, both existing in eleven other *Drosophila* species (Sackton et al., 2007) suggests another duplication resulted in this gene pair at a time before the genus *Drosophila* emerged but after flies diverged from mosquitoes. Another gene duplication seemed to occur before the radiation of moth species, giving rise to β GRP1 and β GRP2 in many lepidopteran insects (Fig. 2). The evolutionary history of β -glucanase-related proteins in arthropods proposed here is further supported by other phylogenetic analyses (Lin et al., 2008; Pauchet et al., 2009).

4.2. *M. sexta* MBP binding pattern affected by microbial surface molecules and plasma factors

M. sexta MBP exhibited a broad binding specificity by associating with LPS, LTA, *E. coli* DAP-PG, *S. aureus* Lys-PG, and laminarin (Fig. 6). While 50% of the specific binding of LPS, LTA, and DAP-PG occurred in the range of 20–50 nM, the binding to Lys-PG and laminarin was less specific. The low binding of MBP to laminarin seems to support our proposal that “GNBP” represents a transition state from “ β -glucanase” to “ β GRP” (Fig. 2). Single-domain β -1,3-glucanases bind β -1,3-glucan to exert its catalytic activity. The amino-terminal extension of *B. mori*, *Plodia interpunctella*, and *Tenebrio molitor* β GRPs is responsible for binding to the glucan (Takahasi et al., 2009; Fabrick et al., 2004; Lee et al., 2009). Based on our findings, neither of the two domains in MBP specifically binds laminarin at a high level. Instead, “GNBP” members may have evolved unique binding properties not known to exist in “ β -glucanase” or “ β GRP” families and, therefore, it would be interesting to assign the specific binding activities (LPS, LTA, and DAP-PG) to either of the two domains of MBP.

So far, binding properties of β -glucanase-related proteins have been mainly characterized in members of the “ β GRP” clade, including *B. mori* β GRP1 (Ochiai and Ashida, 2000; Takahasi et al., 2009), *M. sexta* β GRP1 (Ma and Kanost, 2000) and β GRP2 (Jiang et al., 2004), *P. interpunctella* β GRP (Fabrick et al., 2004), and *T. molitor* GNBP3 (Lee et al., 2009). Specific binding to β -1,3-glucan is established for all these proteins and, in some cases, assigned to their amino-terminal domain (~110 residues). Full-length *P. interpunctella* β GRP and its fragment (residues 1–181) specifically bind LPS and LTA. Interestingly, the crayfish LPS- and β -1,3-glucan-binding protein, a member of the “ β -glucanase” clade that lacks the amino-terminal β -sandwich structure (data not shown), exhibits the LPS- and β -1,3-glucan-binding activities. In the “GNBP” clade, *B. mori* GNBP binds to Gram-negative bacteria *Enterobacter cloacae* but its target(s) are unclear. *D. melanogaster* GNBP1 recognizes LPS and β -1,3-glucan to induce antimicrobial peptide synthesis (Kim et al., 2000). Surprisingly, this GNBP, by working together with *Drosophila* PGRP-SA, plays a key role in the defense against Gram-positive bacterial infection through the Toll pathway (Gobert et al., 2003; Pili-Floury et al., 2004). While molecular mechanism of the dual detection remains controversial in the fly (Buchon et al., 2009), this and the previous studies (Sumathipala and Jiang, 2010) revealed some interesting features of *M. sexta* MBP and PGRP1. Our future investigations will be focused on understanding, at the domain level, how both proteins recognize bacteria and trigger the serine proteinase system for proPO and Toll pathway activation.

The results of our ELISA assays (Fig. 6) agreed well with those of the pull-down tests using purified MBP and insoluble polysaccharides. 1) MBP bound *E. coli* and *B. subtilis* DAP-PGs, *S. aureus* Lys-PG, but not *M. luteus* Lys-PG (Fig. 5F). Structural changes in *B. megaterium* DAP-PG (e.g. cross-linking extent, glucosamine deacetylation), in comparison with *B. subtilis* DAP-PG, may have prevented MBP binding. 2) Purified MBP strongly bound to curdlan (Fig. 5F), a β -1,3-glucan lacking branches, but not much to laminarin, β -1,3-glucans with β -1,6-branching. In comparison, *P. interpunctella* β GPP bound laminarin and its amino-terminal fragments (118N and 181N) co-precipitated with curdlan (Fabrick et al., 2004). 3) The specific recognition of LTA or LPS by MBP was at least partly responsible for the association of purified MBP to bacteria (Fig. 5B), since *M. luteus* PG was not directly bound by MBP and DAP-PGs of the Gram-negative bacteria were not exposed to MBP. On the other hand, the association of MBP with fungal cells (Fig. 5D) was consistent with its recognition of β -1,3-glucans, especially unbranched ones.

Binding of MBP to microbes is affected not only by their cell wall components but also by plasma factors. For instance, *M. luteus* Lys-PG somehow was bound by MBP in the induced

plasma (Fig. 5E). Because the purified Lys-PGs did not interact with purified MBP, we suspect the observed binding was indirect. Endogenous MBP associated with fungi less than the purified protein did, probably due to the strong competition of β GRPs for the same site (Fig. 5C). Plasma MBP bound to bacteria more than fungi under the current conditions due to the lack of such competition, since we detected trace amount of β GRPs in the pellets of *M. luteus*, *S. aureus* and *B. subtilis* (Fig. 5A). The ~50 kDa immunoreactive band present in the bound fractions of *M. luteus*, *S. aureus*, *B. subtilis*, and *E. coli* may be caused by proteolysis of MBP or β GPPs.

4.3. *M. sexta* MBP and melanization

A connection between MBP and the proPO activation system was supported by the finding that adding the recombinant protein to larval plasma concentration-dependently induced proPO activation in the absence of a microbial elicitor (Fig. 7). Elicitor-independent enhancement of melanization also occurred after *M. sexta* PGRP1 had been incubated with plasma from naïve larvae (Sumathipala and Jiang, 2010). Spontaneous melanization was observed when PGRP-LE was over-expressed in *Drosophila* (Takehana et al., 2002). Constitutively present at a low level in hemolymph (Fig. 3), MBP molecules may form clusters only when pathogen surface is detected, as shown in *T. molitor* PGRP-SA (Park et al., 2007). Added MBP increased its plasma concentration and may have triggered self-association, a process favoring interactions with other components of the proPO activation system. We hypothesize that further pathogen-independent clustering above a critical concentration might hinder binding of the system components and, thus, down-regulate melanization (Fig. 7).

While this non-physiological increase of melanization provided clues for the mode of action of MBP, it led to complications in calculating the synergistic effect of MBP-elicitor binding on PO activity increase (Fig. 8). Small amount of elicitor did not enhance proPO activation in 1:10 diluted plasma (#2), as compared with the plasma control (#1). A low level of added MBP increased PO activity (#3), and additional DAP-PGs, LTA or *M. luteus* Lys-PG caused significant enhancement in proPO activation (Fig. 8, A-D and G, #4 vs. #3). The synergistic enhancement should be attributed to the association of DAP-PGs or LTA with exogenous MBP and other pathway components. In contrast, β -1,3-glucans, *S. aureus* Lys-PG, or LPS did not cause any statistically significant increase under the experimental conditions (Fig. 8, E, F, H, and I). These results are consistent with the binding data (Fig. 5), except in three cases: *B. megaterium* DAP-PG was hardly bound by MBP and, perhaps, a small yet specific binding generated a significant enhancement in proPO activation; LPS specifically and strongly associated with MBP but the binding may not induce a particular conformation required for the system activation; curdlan-MBP binding caused a small decrease in proPO activation, possibly due to a competition of the elicitor with endogenous β GRPs. As we know, interaction of curdlan with β GRP1/ β GRP2 causes autoactivation of proHP14 and triggered proPO activation pathway (Wang and Jiang, 2006 and 2010).

4.4. Concluding remarks

We have isolated cDNA clones of *M. sexta* MBP, a member of the “GNBP” clade of β -1,3-glucanase-related protein superfamily. This plasma protein is mainly produced by fat body and is highly induced upon immune challenge. The recombinant MBP exhibits different levels of specific binding to LPS, LTA, DAP-PG, Lys-PG and β -1,3-glucan, accounting for its association with bacteria and fungi. Other plasma factors also affect microbe recognition by endogenous MBP. Addition of purified MBP leads to proPO activation in the absence of microbial elicitors, whereas only certain specific interactions of MBP and elicitor lead to synergistic enhancement of melanization. *M. sexta* MBP is a component of the immune surveillance, mainly detecting LTA and DAP-PG to trigger proPO activation. At present,

domain-specific binding properties of MBP are not known. Neither is it understood why specific binding by MBP does not always further enhance proPO activation.

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The abbreviations used are

βGRP	β-1,3-glucan recognition protein
MBP	microbe-binding protein
GNBP	Gram-negative bacteria-binding protein
PGRP	peptidoglycan recognition protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
DAP	diaminopimelic acid
PRR	pattern recognition receptor
ORF	open reading frame
PO and proPO	phenoloxidase and its precursor
qPCR	quantitative polymerase chain reaction
BSA	bovine serum albumin
AP	alkaline phosphatase
PAGE	polyacrylamide gel electrophoresis
DTT	dithiothreitol

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1 TGGCTGCGTGGTGGCGGACGTGTGATCACGTTGTTTTGTGTGTTCACTTGACGTGCGGTCAAATACCTAACGTATCCATACATGCAT 9

91 ACAAAACCAAGGTTTATTAGTGTTCGATACCCGATGGACAAACATAGGCCTGTCGCTTCCATGGCAACATTAAACGGGGTATTATA 39

181 AAAATGACGTCGGTACAATAGCGGAGAGGCTCTCAAGCAAGAAGCGGATGTGGATCTACGAAGACCCGAATTGGAGTTGAAAGTGG 39

271 GTGACGTCATGCACTATTATGTTTCGTGTGCGATTAAATAGTAAAGGCTACTGAAGGATAATTGAGCTTCACTGTCAAGAACTGGAAG 69

361 ACAGGTCTAGCGTGACTGGTCCGATCCGGCCCGGAACGGTGTGTCGGGAGGATTGTAAGCCCACTCGTACTAAAGTTCGCGCCGGGTTCG 129

451 CTGCGCCGGACAGACTCTCTTGAAGACAACCTCGACATGTTGGACGAGAGCGTGTGGCAGATTGAGCAGTACATACCTAACACCCATC 159

541 CCGAGTATCCCTTCGTGTATACAGCATCTAGTACTGATCAGACCGTCACGACCGCGGTGGCAGCGTCGGGATCACCCCAAACATGC 189

631 AACAGGACTTACAGGATTACAAATGAAACCATATTGTCTGGCAGCTTAATTGTTTGTGGTGCAGCTCATCTGCAGATTCTGTGTC 219

721 TGATACAGGTAGATGGTGGGACATCTTGGCCGAGTGGCGAGCGCGCATCACTCCAAGACCTTCGCGTTCACGTAGGCAAGGTGT 249

811 ACGTGCGAGCAAACTGCCAAGGAGACTGGTGTATCCAGAAATCTTCTGGAGCCATTTTACATAAGTACGGTTCGTACATTACT 279

901 CGTCGGGGTTATAAGATAGCAACTGCTCGCGCAATAGACAACCTTACTACGACCAATGATTACAGTAATAAGGTGCTGTACGGAG 309

991 GACCTATAATGGACTTGGCGTGTGAGAGACACTGCTGTCCACCAAGCTGAGGAGAGACGGATCCGCTTGGGGGACGACTTCCACCTGT 339

1081 ATTCATTAGATGGACTCCAGAGCACATAGTCTAATGGTGGATGATGAGGAATGGGCGGAGTGGAGCCGACGGCGAGCGGTCTGGAG 369

1171 GCCAGTTTCCCGCTCTCGAGGCACTGCCACAGATGTTGCTACGCGCGGACGTCGATGGCTCCCTTCGATGATTACTTCTACCTAA 399

1261 CTCTGGGCGTGGCGGCTGGCAGCATCACAGAGTTCGGGATGGCTCGGTACCGTCGGGACAGGATAAGCCATGGCGCAACAGCGCTC 429

1351 GGAAGCCATGCTCACTTCTGGCAGGACAACTCTTGGCTGCCTACCTGGCTGCTGTCCCGGCTTGAAGTCGACTACGTCAAAGTAG 459

1441 TCGCGCTCTAATATACGAGATTCTAGTAAAAAATTT 459

V A L *

Fig. 1. Nucleotide and deduced amino acid sequences of *M. sexta* MBP

Amino acid residues, shown in one-letter abbreviations below the cDNA sequence, are aligned with the second nucleotide of each codon. Residues in the predicted signal peptide (*double underlined*) are assigned negative numbers whereas residues in the mature protein are assigned positive numbers. Putative *N*- and *O*-linked glycosylation sites are marked “×” and “+”, respectively. Cys residues (*double underlined*) are in *bold*, and the stop codon is indicated by “*”. Starting sites of the positive clones (Q2 through Q4) and pyrosequence reads (1 through 8) are *double underlined* and marked with their names. Nucleotide differences between Q4 and other sequences are shaded on top, with the nonsynonymous substitution shaded below. The j279 and j280 primer binding sites for recombinant expression in the baculovirus system are *underlined*. Nucleotides 5–251, 215–710, 452–626, 711–990, 1107–1296, 1265–1462 correspond to contigs 6004 (99% identity), 6422 (96%), 6134 (82%), 4565 (91%), 1635 (75%) and 3092 (78%), respectively.

Fig. 2. Phylogenetic relationships among glucanase-related proteins in insects

Multiple sequence alignment and tree construction were performed as described in *Section 2.2*, using sequences of *M. sexta* MBP (HQ007028), β GRP1 (AF177982), β GRP2 (AY135522), *B. mori* GNBP (L38591 or β GRP2), β GRP1, β GRP3, and β GRP4 (a putative β -glucanase) (Tanaka et al., 2008), *Plodia interpunctella* β GRP (AAM95970), *Hyphantria cunea* GNBP (AF023916), *D. melanogaster* GNBP1 (AAF33849), GNBP2 (NP_524141) and GNBP3 (AAF33851), *Anopheles gambiae* GNBP1 (XP_308984), A2 (XP_320146), B1 (ABU80041, one of the five putative β -glucanases), *Tribolium castaneum* GNBP1, GNBP2, GNBP3, and β -glucanases of *Spodoptera frugiperda* (ABR28478) and *Helicoverpa armigera* (ABU98621). The bootstrap values (>600 in 1000 trials) are indicated at nodes.

Fig. 3. Expression profile of *M. sexta* MBP

A) As described in *Section 2.3*, total RNA samples of hemocytes (H) and fat body (F) from naïve larvae (C) and larvae injected with formaldehyde-killed *E. coli* (I) 24 h ago were reverse transcribed to cDNA, normalized with ribosomal protein S3, and analyzed by qPCR using gene-specific primers. Relative abundances of MBP transcripts were calculated based on average C_t values from three repeats and plotted as a bar graph (mean $\pm$ SEM). **B)** Plasma samples (1.0 μ l each) from day 3, fifth instar larvae injected with H₂O, *E. coli*, or *M. luteus* (3 larvae per treatment) 24 h ago were subjected to 10% SDS-PAGE and immunoblot analysis using 1:2000 diluted MBP antiserum as the first antibody. Sizes and positions of the molecular weight makers are indicated on the left. Arrowhead and arrow indicate β GRP2/1 and MBP, respectively.

Fig. 4. 10% SDS-PAGE analysis of purified *M. sexta* MBP from baculovirus-infected insect cells
A) Coomassie blue staining: 1.0µg MBP treated with SDS sample buffer in the presence or absence of dithiothreitol (DTT); **B)** immunoblot analysis: 0.2µg MBP treated with SDS and DTT was separated by gel electrophoresis, electro-transferred onto nitrocellulose membrane, and detected using diluted MBP antiserum. Positions and sizes of the molecular weight makers are indicated on the right.

Fig. 5. Immunoblot analysis of MBP in the free and bound fractions of the mixtures of microbial cells (or their insoluble cell wall components) and induced plasma (or purified MBP)

As described in *Section 2.6*, endogenous and exogenous MBP was incubated with microbial cells or their surface molecules. Unbound (free) and bound fractions were separated and analyzed using MBP antibodies. MBP is indicated by an arrow, β GRP2/1 is marked by a triangle ($\blacktriangle$), and an apparent proteolytically processed product is labeled with *.

A) Induced plasma + bacteria: *upper panel*, free MBP in the supernatant after incubating with the bacteria (first letters of genus and species names listed), *lower panel*, bound MBP; **B)** purified MBP + bacteria: *upper and lower panels* were obtained using the same bacteria as those in **A)**; **C)** induced plasma + fungi; **D)** purified MBP + fungi; **E)** induced plasma + curdlan/PG; **F)** purified MBP + curdlan/PG. Note the disappearance of β GRP2/1 (**C** and **E**) in the supernatant of fungi/curdlan and its appearance at same position in the bound fraction.

Fig. 6. Association of soluble microbial cell wall components with purified MBP measured by ELISA

As described in *Section 2.7*, recombinant MBP was incubated with soluble *E. coli* DAP-PG, *S. aureus* Lys-PG, LPS, LTA, and laminarin immobilized on a 96-well microplate. The binding was detected via an ELISA and shown as mean $\pm$ SEM ($n = 3$). **A)** Total (*open bar*) and non-specific (*closed bar*) bindings, represented by alkaline phosphatase (AP) activities, correspond to MBP samples pre-incubated with buffer or excess amount of elicitor, respectively. **B-D)** AP activity differences ($\circ$), representing specific (*i.e.* total – nonspecific) bindings, are plotted against concentrations of MBP used in the competitive binding assay. The data are analyzed by curve fitting based to one-site binding model. **B)** *E. coli* DAP-PG, $r^2=0.91$; **C)** LPS, $r^2=0.82$; **D)** LTA, $r^2=0.77$.

Fig. 7. Concentration-dependent increase in proPO activation caused by purified *M. sexta* MBP in the absence of microbial elicitor

As described in *Section 2.8*, aliquots of cell-free hemolymph from day 2, fifth instar naïve larvae were incubated with MBP (●) or BSA (□) on ice for 1 h. PO activities were determined and shown as mean $\pm$ SEM ($n=3$). The concentration of endogenous MBP in the mixture was estimated to be 60~80 nM.

Fig. 8. Roles of *M. sexta* MBP in proPO activation

As described in Section 2.8, 5 μ l of 1:10 diluted plasma from naïve larvae were incubated with buffer (#1), 1 μ g elicitor (insoluble DAP-PG and Lys-PG, LPS, LTA, insoluble curdlan, or laminarin, #2), 0.1 μ g purified MBP (#3), or elicitor and MBP (#4) on ice for 40 min. PO activities were measured and plotted as bars (mean $\pm$ SEM, n = 3) for each treatment. Since interaction of plasma factors with elicitor (#2 - #1) and co-presence of plasma and exogenous MBP (#3 - #1) both lead to proPO activation, an interaction of elicitor and exogenous MBP in plasma (#4 - #1) is anticipated to increase proPO activation to a level significantly higher than the sum of the two components [*i.e.* (#2 - #1) + (#3 - #1)]. To detect a possible synergistic effect of elicitor-MBP interaction, the PO activity changes represented by (#4 - #1) and (#2 + #3 - #1), or simply #4 and (#2 + #3 - #1), are compared using unpaired t-test. An asterisk (*) indicates that #4 is significantly higher than #2 + #3 - #1 ($p < 0.05$).