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Prophenoloxidase activation and antimicrobial peptide expression induced by the recombinant microbe binding protein of *Manduca sexta*

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Abstract

Manduca sexta microbe binding protein (MBP) is a member of the β -1,3-glucanase-related protein superfamily that includes Gram-negative bacteria-binding proteins (GNBPs), β -1,3-glucan recognition proteins (β GRPs), and β -1,3-glucanases. Our previous and current studies showed that the purified MBP from baculovirus-infected insect cells had stimulated prophenoloxidase (proPO) activation in the hemolymph of naïve and immune challenged larvae and that supplementation of the exogenous MBP and peptidoglycans (PGs) had caused synergistic increases in PO activity. To explore the underlying mechanism, we separated by SDS-PAGE naïve and induced larval plasma treated with buffer or MBP and detected on immunoblots changes in intensity and/or mobility of hemolymph (serine) proteases [HP14, HP21, HP6, HP8, proPO-activating proteases (PAPs) 1–3] and their homologs (SPH1, SPH2). In a nickel pull-down assay, we observed association of MBP with proHP14 (slightly), β GRP2, PG recognition protein-1 (PGRP1, indirectly), SPH1, SPH2, and proPO2. Further experiments indicated that diaminopimelic acid (DAP) or Lys PG, MBP, PGRP1, and proHP14 together trigger the proPO activation system in a Ca^{2+} -dependent manner. Injection of the recombinant MBP into the 5th instar naïve larvae significantly induced the expression of several antimicrobial peptide genes, revealing a possible link between HP14 and immune signal transduction. Together, these results suggest that the recognition of Gram-negative or -positive bacteria via their PGs induces the melanization and Toll pathways in *M. sexta*.

Graphical abstract

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Keywords

insect immunity; Lys- or DAP-type peptidoglycan; pattern recognition; hemolymph protease system; melanization; Toll pathway

1. Introduction

Serine protease (SP) pathways coordinate innate immune responses in insect hemolymph (Kanost and Jiang, 2015), which include melanization catalyzed by phenoloxidases (POs), Spätzle-induced synthesis of antimicrobial peptides (AMPs), and cytokine-mediated changes in hemocyte behaviors. In *Drosophila melanogaster*, genetic studies indicate that ModSP, Grass, and Spätzle-processing enzyme (SPE) are responsible for activation of the Spätzle-Toll-Dorsal/Dif pathway that induces AMP expression, whereas SP7, MP1 and Haya are involved in generating active PO for melanization (Veillard et al., 2016). In *Tenebrio molitor*, MSP, SAE (SPE activating enzyme), and SPE form a cascade pathway to activate Spätzle that induces AMP production (Park et al., 2010). The SPE also proteolytically activates proPO in the presence of *T. molitor* SPH1 oligomers. Remarkably, this serine protease homolog is also activated by SPE. In *Manduca sexta*, hemolymph proteases HP14, HP21, and PAP2/3 form a cascade to activate proPOs in the presence of a high M_r complex of SPH1 and SPH2 (Jiang et al., 2010). HP6 activates proHP8 and proPAP1 (An et al., 2009), HP8-processed Spätzle stimulates AMP synthesis (An et al., 2010), and PAP1 generates active POs (Wang and Jiang, 2008). However, it is unclear how proHP6 is activated and whether or not proHP14 activation is related to the generation of active HP6.

In these processes, *Drosophila* ModSP, *Tenebrio* MSP, or *Manduca* HP14 acts as an initiation protease of the respective pathway via autoactivation after polysaccharides on microbial surfaces are recognized by specific binding proteins. For instance, a complex of Lys/DAP-peptidoglycan, peptidoglycan recognition protein (PGRP), and Gram-negative bacteria-binding protein (GNBP) induces MSP autoactivation in *T. molitor* (Yu et al., 2010; Kim et al., 2008). *Drosophila* ModSP is likely activated in the same manner by Lys-PG, PGRP-SA, and GNBP1 (Buchon et al., 2009). Like Lys-PGs of most Gram-positive bacteria, β -1,3-glucan of fungi recognized by GNBP3 leads to the Toll pathway activation (Gobert et al., 2003; Gottar et al., 2006), although the involvement of ModSP is not fully established. *M. sexta* proHP14 is activated by a complex of β -1,3-glucan and its recognition protein (β GRP2) (Wang and Jiang, 2006; Takahashi et al., 2015) and by Lys-PGs alone (Ji et al., 2004). So far, it is unclear how Gram-negative bacteria or their DAP-type PGs activate melanization in *M. sexta*. Interestingly, incubation of naïve larval hemolymph with exogenous PGRP1 or microbe binding protein (MBP) in the absence of microbial elicitor causes a concentration-dependent increase of PO activity (Sumathipala and Jiang, 2010; Wang et al., 2011). PGRP1 recognizes *Escherichia coli* and *Bacillus megaterium* DAP-PGs and *Micrococcus luteus* Lys-PG; MBP strongly binds to DAP-PG, lipoteichoic acid (LTA), and lipopolysaccharide (LPS). Each specific binding stimulates a PO activity increase significantly higher than the sum of those caused by the elicitor and binding protein alone. To explore mechanisms underlying such synergism, we examined the hemolymph proteins induced by immune challenge and their changes after the SP system was activated by

recombinant MBP. Isolation of the proteins associating with MBP by nickel affinity chromatography led to their identification by a panel of antibodies against defense proteins of *M. sexta*. Based on the results, we designed and conducted experiments to find out how DAP-PG and MBP trigger the protease pathway initiation. To test how MBP may be related to other immune responses, we injected the recombinant protein into hemocoel, observed drastic changes in AMP mRNA levels, and discussed connections between melanization and Toll pathway activation in *M. sexta*.

2. Methods and materials

2.1. Insect rearing, bacterial injection, and hemolymph collection

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet (Dunn and Drake, 1983). Each day 2, 5th instar larva was injected with a mixture of *E. coli* (2×10^7 cells), *M. luteus* (20 μ g) (Sigma-Aldrich), and curdlan (20 μ g, insoluble β -1,3-glucan from *Alcaligenes faecalis*) (Sigma-Aldrich) in 30 μ l H₂O. Hemolymph was collected from cut prolegs of three larvae 24 h later, centrifuged at 5000g for 5 min to remove hemocytes, and supernatants were pooled as induced plasma (IP). Similarly, control plasma (NP) was prepared from three day 3, 5th instar naïve larvae. After incubation with exogenous MBP, the two plasma samples were used to detect changes in hemolymph proteins and MBP-associated immune factors after incubation with exogenous MBP.

2.2. Production of *M. sexta* MBP, PGRP1, and proHP14

M. sexta MBP and PGRP1 were expressed and purified from the baculovirus-infected insect cells as described before (Sumathipala and Jiang, 2010; Wang et al., 2011). *M. sexta* proHP21 and proPAP2 were prepared previously (Wang and Jiang, 2007). For producing full-length proHP14, the longest cDNA was amplified using primers J578 (5' - CCCGAATTCAGTGTGAAAGAAG) and J503 (5' - ACTCTCGAGATACTCGTCGGTCCA) (Wang and Jiang, 2010). After agarose gel electrophoresis, the DNA fragment was recovered and cloned into pGEM-T (Promega). Following sequence verification, the cDNA was retrieved from the vector by *EcoRI*-*XhoI* double digestion and subcloned into the same sites in the pMFH6 (Lu and Jiang, 2008). Proteins expressed using this vector contain a hexahistidine tag at the carboxyl-terminus. A baculovirus stock was generated from the recombinant plasmid (proHP14/pMFH6) by site-specific transposition in order to infect *Sf21* cells under optimal conditions. The secreted proenzyme was isolated from the conditioned medium to test its autoactivation in the presence of PGs and other proteins. The procedure for isolating the proHP14 deletion mutants (Wang and Jiang, 2010) was followed to enrich and purify the proenzyme for functional analysis.

2.3. Stimulation of proPO activation in induced hemolymph by the recombinant MBP alone or with elicitors

Five-microliter aliquots of 1:10 diluted cell-free hemolymph from immune challenged larvae (day 2, 5th instar) were incubated with 0, 10, 20, 40, 60, 80, and 100 ng MBP or BSA (all in 1.0 μ l) and buffer (18 μ l, 20 mM Tris-HCl, pH 7.5, 0.001% Tween-20, 1 mM CaCl₂) at room

temperature for 10 min prior to PO activity measurement using dopamine as a substrate (Jiang et al., 2003). The experiment was repeated with 0, 50, 100, 200, 300, 400, 500, 600, 800, and 1000 ng MBP or BSA in 1.0 μ l. To test a possible synergistic effect of MBP-elicitor interaction on proPO activation, 5 μ l of 1:10 diluted plasma from induced larvae was incubated with the buffer, elicitor, MBP, or both on ice for 30 min prior to PO activity measurement. The elicitors include DAP-PGs of *E. coli* (InvivoGen) and *B. mageterium*, Lys-PG of *M. luteus*, and LTA of *Staphylococcus aureus* (InvivoGen). PO activities were compared to reveal synergistic enhancements.

2.4. Changes in hemolymph proteins caused by the recombinant MBP

The control plasma (10 μ l NP) was incubated for 30 min at room temperature with 10 μ l MBP (0.1 mg/ml) and 100 μ l buffer (20 mM Tris-HCl, pH 7.5, 0.001% Tween-20, 1 mM CaCl₂, 0.01% 1-phenol-2-thiourea (PTU)) or with 110 μ l of the buffer only. The induced plasma (10 μ l IP) was incubated with the buffer with or without MBP under the same conditions for 15 min. The reaction mixtures were treated with SDS sample buffer and 1/10 of the samples were resolved by SDS-PAGE in ten groups. Following electrotransfer and blocking, the ten blots were separately incubated with 1:2000 diluted polyclonal antisera against HP14, HP21, PAP1, PAP2, PAP3, HP6, HP8, SPH1, SPH2, and proPO2 as primary antibodies. The antibody-antigen complexes were detected using 1:3000 diluted goat-anti-rabbit antibodies conjugated to alkaline phosphatase (Bio-Rad).

2.5. Detection of hemolymph proteins associated with the MBP

In order to identify proteins that bind MBP, the induced plasma (150 μ l) was incubated with 10 μ l BSA (10 μ g), MBP (10 μ g), or MBP (10 μ g) and *E. coli* PG (10 μ g) in 140 μ l buffer (25 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.01% PTU) on ice for 1 h. The mixtures were separately passed through three Ni-NTA agarose spin columns (Qiagen) by centrifugation at 1000g for 2 min. After repeating the binding step for five times using flow-through from the previous cycle, each column was washed for three times with 0.5 ml 50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.001% Tween-20. Bound proteins were eluted by heating at 95°C for 5 min in 100 μ l 1 $\times$ SDS sample buffer. Aliquots (5 μ l) were separated by SDS-PAGE under reducing condition and subjected to immunoblot analysis using 1:2000 diluted polyclonal antisera against β GRP1, β GRP2, PGRP1, immulectin-2, HP14, HP21, HP1, HP6, HP8, PAP1, PAP2, PAP3, SPH1, SPH2, and proPO2.

2.6. Autoactivation of proHP14 induced by PG recognition

To study possible self-cleavage of proHP14, the proenzyme (0.15 μ g in 1 μ l) was incubated with MBP (0.3 μ g in 1 μ l), PGRP1 (0.3 μ g in 1 μ l), *E. coli* PG (1.0 μ g in 1 μ l), and buffer (20 μ l, 20 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 0.001% Tween-20) at 37°C for 90 min. In the controls, one or more of the components were replaced by the buffer. The reaction mixtures were treated by SDS sample buffer with or without dithiothreitol, separated by 10% SDS-PAGE, and subjected to immunoblot analysis using 1:2000 diluted HP14 antiserum as primary antibody and 1:3000 diluted goat-anti-rabbit IgG conjugated to alkaline phosphatase as secondary antibody. Ca²⁺-dependence of the activation was examined by including 10 mM EDTA in the reaction. To inspect possible differences in the extent of proHP14 auto-

proteolysis, Lys-PG of *S. aureus* (InvivoGen) was incubated with MBP, PGRP1 and proHP14 as described above for *E. coli* PG. To test if the cleaved HP14 can activate proPAP2 via HP21, *E. coli* PG (1 µg), MBP (0.3 µg), PGRP1 (0.3 µg), proHP14 (0.1 µg), proHP21 (0.1 µg), proPAP2 (0.2 µg), and the buffer were incubated at 37°C for 1 h. In the control reactions, *E. coli* PG, proHP21, or other components were replaced by same volume of the buffer. Amidase activity generated in the reaction mixtures were measured using 150 µl of 100 µM Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR_pNA) in a microplate assay (Wang and Jiang, 2007).

2.7. Induction of AMP expression by the recombinant MBP

Sterile buffer (50 µl, 20 mM Tris-HCl, 50 mM NaCl, pH 7.5) and MBP (10 µg in the buffer) were injected into hemocoel of three day 1, 5th instar naïve larvae in the control and treatment groups, respectively. Fat body was dissected 24 h later for total RNA isolation using TRIZOL Reagent (Life Technologies, Inc). The pooled RNA samples (1 µg each) were used for cDNA synthesis and quantitative real-time PCR according to Wang et al (2011). The cDNA samples were normalized in preliminary PCRs using specific primers for *M. sexta* ribosomal protein S3: j055 (5'-CTCAGGCCGAGTCTTTGAGATACA) and j057 (5'-ACATAGTCATTGCATGGGTCACCG). Each 15 µl PCR mixture contained about 40 ng cDNA template, 4.5 pmoles of each primer, and 7.5 µl FastStart Universal SYBR Green Master (Roche Life Science). Thermal cycling conditions were: 95°C for 30 s; 53°C for 30 s; 72°C for 20 s for a total of 50 cycles. The primers were: j1070 (5'-GCAGGCGACGACAAGAAC) and j1071 (5'-ATGCGTGTGGTAAGAGTAGC) for attacin-1, j1072 (5'-CCGTGTTTTATTCTTCGTCTTC) and j1073 (5'-AATCCTTTGACCTGCACCC) for cecropin-6; j1074 (5'-GCAAGTCGGCAACAATGG) and j1075 (5'-ACCCTGTCCTGTCAAGTTTG) for gloverin; j1076 (5'-GTGTGCCTCGTGGAGAATG) and j1077 (5'-ATGCCTTGGTGATGTCGTC) for lysozyme; j1078 (5'-TGCTTTCTTTAACCCTTTGTCCTC) and j1079 (5'-TATTCTAACACAGCCTATAATGCG) for moricin-1. Relative abundances of the AMP transcripts were calculated based on 2^{-C_t} , where $C_t = (C_{t, AMP} - C_{t, rpS3})_{IF} - (C_{t, AMP} - C_{t, rpS3})_{CF}$.

3. Results

3.1. Elicitation of proPO activation in induced plasma by the recombinant MBP

M. sexta MBP purified from the baculovirus-infected insect cells stimulated proPO activation in plasma from the naïve 5th instar larvae (Wang et al., 2011). To test whether or not the microbe-independent process occurs in induced plasma, we collected hemolymph from larvae injected with killed bacteria, incubated the IP with recombinant MBP (0 to 100 ng), and detected a large increase in PO activity. In the negative control of IP mixed with different amounts of BSA, PO activity was much lower (Fig. 1A). In another control, the MBP's β-1,3-glucanase-like domain from the same expression system did not cause any proPO activation (data not shown). To test if further increases in MBP level would cause a reduction in proPO activation, as exhibited in naïve larval hemolymph (Wang et al., 2011), we repeated the experiment with a broader range of MBP amounts (50 to 1000 ng) and observed a PO activity peak (17.5 U) at 200 ng of exogenous MBP, similar to the result

obtained using NP. When excess amounts of MBP were added, PO activity gradually reduced to a basal level of 1.3 U at 800 ng. Since MBP is not a PO inhibitor, we interpret this concentration-related change in PO activity as result of nonproductive distribution of a limiting factor in the system (see discussion below). Based on the published results (Wang et al., 2011), we measured PO activities in induced plasma samples incubated with buffer, with exogenous MBP, with *E. coli*, *B. megaterium* DAP-PG, *M. luteus* Lys-PG or *S. aureus* LTA, and with both MBP and an elicitor (Fig. 1B). Statistical analysis indicated the co-presence of MBP and these elicitors synergistically enhanced proPO activation under the experimental conditions, suggesting that MBP had interacted with these elicitors. In contrast, there was no significant enhancement in proPO activation in the reaction containing both MBP and *B. subtilis* DAP-PG (data not shown).

3.2. Detection of hemolymph protein changes in the control and induced plasma

To explore the activation mechanism, we examined the cell-free hemolymph samples (NP and IP for naive and induced plasma) incubated with buffer or the recombinant MBP. SDS-PAGE and immunoblot analysis revealed the following changes (Fig. 2): 1) Level of proHP14 (75 kDa band, slightly lower than the one in lanes 1–4) increased at 24 h after the bacterial injection. The proenzyme disappeared in NP and reduced in IP after MBP treatment. The regulatory domain of HP14 appeared at 45 kDa and its catalytic domain (30 kDa) was hardly recognized by the polyclonal antibodies (Wang and Jiang, 2010); 2) ProHP21 level increased after the immune challenge. Like proHP14, proHP21 disappeared in NP and reduced in IP after MBP incubation. While this is consistent with the proHP21 activation by HP14 (Wang and Jiang, 2007), we only detected a faint band at the correct size of HP21 catalytic domain in NP; 3) Induced proPAP2 and proPAP3 also reduced or disappeared after the treatment. Increases in the weak bands of the dual clip domains are indicative of their proteolytic processing by HP21 (Wang and Jiang, 2007; Gorman et al., 2007); 4) In comparison, the intense bands of PAP1 clip domain clearly exhibited the immune induction of proPAP1 and its activation by HP6 (An et al., 2009). The catalytic domain of PAP1 was poorly recognized by its polyclonal antibodies, as shown previously (Wang and Jiang, 2008); 5) After the immune challenge, there was a small increase in proHP6 that decreased and disappeared in IP and NP after MBP had been added, respectively. The immuno-reactive band (*c.a.* 75 kDa) likely represented the covalent complex of a serpin and the HP6 catalytic domain (Tong et al., 2005); 6) The increase in proHP8 level and its activation by HP6 (An et al., 2009) were also supported by the immunoblot data; 7) The proSPH1 and proSPH2 levels did not increase much at 24 h after the immune challenge but their proteolytic activation by PAP3 (Wang et al., 2014) and PAP1 (Wang and Jiang, 2008) significantly increased after MBP incubation. 8) A part of proPO2 (and perhaps proPO1 in the heterodimer) was activated by PAP1, PAP2 and PAP3 in the presence of SPH1 and SPH2 (Yu et al., 2003; Jiang et al., 2010). Similar amounts of the PO covalent complexes formed in NP and IP after incubation with MBP. Taken together, these results indicated that nearly all known members of the immune SP-SPH system (Kanost and Jiang, 2015) were activated in response to the exogenous MBP.

3.3. Proteins associated with the recombinant MBP during proPO activation in hemolymph

Unlike the recombinant MBP, endogenous MBP did not cause spontaneous melanization under normal physiological condition. Upon pathogen recognition, the latter likely binds to bacteria and undergoes a conformational change that recruits hemolymph proteins and triggers the SP-SPH system. To understand this process, we took advantage of the hexahistidine tag in the recombinant MBP and used it as a bait to capture its associating proteins by a nickel pull-down assay. In comparison to the BSA control, there were more β GRP2, proHP14, SPH1, SPH2 and proPO2 eluted from the MBP column (Fig. 3). The increase in proHP14 was very small and it is unclear whether or not its association with MBP was direct. Since HP14 is an initiation protease of the SP-SPH system (Ji et al., 2004; Wang and Jiang, 2006; Takahashi et al., 2015), this association prompted us to test if MBP binding to microbes and proHP14 could cause the zymogen activation that triggers the protease cascade. The proteolytic activation of proSPH1 and proSPH2 have caused their disappearance at the precursor positions (Fig. 3). In contrast, there was no discernable difference in levels of β GRP1, PGRP1, immulectin-2, HP1, HP6, HP8, HP1, HP21, PAP1–3 (data not shown) in the eluted samples between BSA control and MBP. Interestingly, when soluble DAP-PG from *E. coli* was included in the reaction mixture, we detected PGRP1 in the bound fraction (Fig. 3, last panel, lane 3). Apparently, the DAP-PG had brought PGRP1 and MBP together.

3.4. Cleavage activation of proHP14 induced by dual recognition of PGs by MBP and PGRP1

The changes in IP elicited by MBP and the MBP-proHP14 association led us to postulate that recognition of bacterial surface components by MBP and other surveillance proteins causes HP14 autoactivation that triggers the entire SP-SPH system. To test this hypothesis, we cloned the full-length cDNA of proHP14 into pMFH6, constructed the recombinant baculovirus to infect *Sf21* cells, and isolated the secreted zymogen in the conditioned medium. The purified protein and L₁ truncation mutant of proHP14 (Wang and Jiang, 2010) migrated to the same position on the SDS polyacrylamide gel (Fig. S1), suggesting that the first low density lipoprotein receptor class A repeat (LDLa) was cleaved after R⁸SRR⁶¹ by an intracellular processing enzyme (Wang and Jiang, 2006). The 75 kDa truncated protein contained a small amount of the 45 kDa fragment (Fig. 4A, lane 1) corresponding to the four LDLa repeats, one Sushi and one Wonton domain (Wang and Jiang, 2010). The regulatory heavy chain is linked via a disulfide bond to the 30 kDa catalytic domain hardly recognized by the HP14 antibodies.

In light of the findings in *Drosophila* (Gobert et al., 2003) and *Tenebrio* (Yu et al., 2010) and the DAP-PG-mediated MBP-PGRP1 association (Fig. 3, the last panel), we tested whether or not DAP-PG, PGRP1 and MBP alone or their mixtures may induce proHP14 autoactivation. *M. sexta* PGRP1 and MBP both specifically recognize DAP-PGs of Gram-negative bacteria and the Gram-positive genus *Bacillus* (Sumathipala and Jiang, 2010; Wang et al., 2011). Incubation of *E. coli* DAP-PG, PGRP1, or MBP with proHP14 caused a small increase in intensity of the 45 kDa band (Fig. 4A, lanes 2–4). Further increases in proHP14 auto-processing (lanes 5–7) occurred after any two of the three components had been mixed with the zymogen. Near complete cleavage (lane 8) was detected after all four were present

in the same reaction. These data demonstrated the cooperation of MBP and PGRP1 in recognizing the DAP-PG and inducing proHP14 autoactivation. In the absence of DTT, the two chains of HP14 remained attached and migrated to an apparent M_r of 65 kDa (Fig. 4B, lane 4), slower than proHP14 did (lane 3). The uncleaved and unreduced zymogen had a more compact structure with an apparent M_r of 60 kDa. The autoactivation of proHP14 is Ca^{2+} -dependent, since chelation of the ion by EDTA abolished the process almost completely (Fig. 4C). We further examined the extent of auto-proteolysis induced by *S. aureus* Lys-PG and found about 60% of the total proHP14 had been cleaved, much lower than that caused by *E. coli* DAP-PG (Fig. 4D). To reconstitute the beginning steps of proPAP2 activation, we generated active HP14 by incubating *E. coli* DAP-PG, MBP, PGRP1 and proHP14 but HP14, a chymotrypsin-like enzyme that cuts next to Leu, did not activate proPAP2 unless proHP21 was also included (Fig. 4E). Similar to proHP14, proHP21 has a Leu¹⁵² before its proteolytic activation site and HP21 activated by HP14 has a trypsin-like specificity that cleaves after Lys¹⁵³ in proPAP2 (Wang and Jiang, 2007). PAP2 has an amidase activity that hydrolyzes IEARpNA but HP21 doesn't (data not shown). In another control, the mixture of MBP, PGRP1 proHP14, proHP21 and proPAP2 did not generate PAP2 to hydrolyze the synthetic substrate.

3.5. Expression of AMP genes caused by injection of the recombinant MBP

It is known that PG injection induces AMP expressions. To explore a possible link between proHP14 activation and AMP induction, we injected the purified MBP into hemocoel, isolated RNA from fat body of the larvae 24 h later, and measured AMP transcript levels by quantitative RT-PCR. As shown in Fig. 5, the mRNA levels of attacin-1, cecropin-6, gloverin, lysozyme, and moricin-1 were 53, 58, 6.7, 3.5, 830-fold higher than the buffer-injected larvae, respectively. However, it remains unclear whether this activation is through the HP14 pathway or other MBP interaction proteins.

4. Discussion

In this study, we first demonstrated that adding the recombinant MBP to the induced hemolymph of *M. sexta* elicited melanization, a universal antimicrobial response of insects and crustaceans. Since MBP already existed in the challenged larvae but did not have the same effect, this seems to be an experimental artifact but provides important insights into the enzyme system. As a matter of fact, this phenomenon is not limited to MBP or induced plasma (Wang et al., 2011). Exogenous *M. sexta* PGRP1 led to proPO activation in the plasma of naïve larvae in the absence of a microbial elicitor (Sumathipala and Jiang, 2010). So did the purified recombinant proHP1a or proHP1b with a C-terminal hexahistidine tag (Yang et al., 2016). Overexpression of PGRP-LE caused melanization in the *Drosophila* larvae (Takehana et al., 2002). These results suggest that some abundant components of the proPO activation system tend to trigger spontaneous melanization, when they oligomerize. Also, the recombinant proteins enriched by Ni-NTA beads may be easier than the same proteins lacking the affinity tag to align with each other for association. This is consistent with the notion that molecular clustering is essential for triggering a local defense reaction (Yu et al., 2003; Park et al., 2007; Takahashi et al., 2014). On the contrary, unrelated proteins (e.g. BSA, Fig. 1), natural proHP14 and proHP1 (Wang and Jiang, 2006; Yang et al., 2016),

recombinant proPAP1 lacking an affinity tag (Wang et al., 2001), and proSPH1&2 monomers (Lu and Jiang, 2008; Piao et al., 2005) did not cause proPO activation. Although the criteria (being an abundant system component and an affinity-purified recombinant protein) accounted for the positive and negative results, further tests are needed to validate or falsify the hypothesis.

Takahashi et al. (2015) reported that the LDLa repeats of *M. sexta* proHP14 interact with the C-terminal glucanase-like domain of β GRP2 that binds to β -1,3-glucans (e.g. laminarin, zymosan) through its N-terminal recognition domain. Indirectly associating with the fungal polysaccharides, neighboring proHP14 molecules may cleave one another to generate active HP14. HP14 leads to sequential activation of the downstream proenzymes (proHP21, proPAP2&3, proSPH1&2, and proPOs) to stimulate melanization. This model can also be used to explain why proPO activation decreased after exogenous MBP exceeds a certain concentration (Fig. 1). MBP is a homolog of β GRP2 with the same domain structure (Wang et al., 2011). As the amount of proHP14 in plasma is limited, an increase in oligomeric MBP leads to higher levels of the activated HP14 and other active proteins in the cascade at first (Fig. 2). Above certain threshold, however, the association of proHP14 with excessive recombinant MBP oligomers is expected to be too sparse to cross-activate each other. In other words, the unproductive distribution leads to lower proPO activation.

In the pull-down experiment, there was a discernable increase in β GRP2 (but not β GRP1) (Fig. 3), indicating that MBP may associate with β GRP2 directly or indirectly in the absence of DAP-PG and this binding may have other consequences. In comparison, the PGRP1 association occurred only when DAP-PG was present (Fig. 3), which may increase the recognition specificity. Due to the reasons discussed above, recombinant proHP14 may also have a tendency to associate and cleave one another during affinity purification or concentration, but the LDLa repeats seemed to block the latter process to some extent (Fig. 4A, lane 1, 1–3% of the total). In general, PG bindings by the LDLa repeats, Sushi domain, and Wonton domain (Wang and Jiang, 2010) increased the chance of proHP14 in an open conformation for cross-cleavage (lane 4) and led us to inaccurately name HP14 “pattern recognition serine protease” (Ji et al., 2004). Adding MBP and PGRP1 further enhanced the proHP14 auto-proteolysis in the absence (lanes 2 and 3) and presence (lanes 5 and 6, 1/10 to 1/5) of DAP-PG. While affinity-purified recombinant MBP and PGRP1 led to more proHP14 auto-processing (lane 7, about 1/5 of the total), this process was nearly complete when DAP-PG, MBP, PGRP1, and proHP14 were present simultaneously (lane 8). Together with the previous studies (Wang and Jiang, 2006; Takahashi et al., 2015), these new results (Fig. 4) clearly indicated that HP14 is an entry point of the *M. sexta* proPO activation system for both fungal and bacterial infection.

The role of HP14 and its orthologs (MSP and ModSP) as initiation SPs is highly conserved in the holometabolous insects (Kanost and Jiang, 2015; Park et al., 2010; Veillard et al., 2016). *M. sexta* proHP14 is activated in response to fungal infection via β GRP1 or β GRP2 (Wang and Jiang, 2006 and 2010). Likewise, *D. melanogaster* and *T. molitor* GNBPs detect fungal β -1,3-glucans to generate active ModSP and MSP (Gottar et al., 2006; Buchon et al., 2009; Roh et al., 2009). In response to Gram-positive bacterial infection, *Drosophila* GGBP1 and PGRP-SA may recognize their Lys-PGs to cause proModSP autoactivation (Gobert et

al., 2003; Buchon et al., 2009). Similar to *M. sexta* MBP and PGRP1, *T. molitor* GNBP1 and PGRP-SA together bind DAP-PGs or *S. aureus* Lys-PG to induce proMSP autoactivation (Yu et al., 2010).

The proHP14 auto-proteolysis may lead to AMP up-regulation (Fig. 5) or just concur with the latter. In other words, we have not yet established a direct link between them so far. Based on the results of our *in vitro* assays (Figs. 1–4), we propose that the entire SP-SPH system is triggered in response to the exogenous MBP or PGRP1 in the absence or presence of PGs. In addition to the HP14-HP21-PAP2/3 branch, this system also includes HP6 that activates proHP8, HP8 that cleaves pro-Spätzle, and Spätzle that triggers the Toll pathway (An et al., 2009 and 2010). In addition, HP6 generates PAP1 to reinforce proPO activation caused by PAP2 or PAP3 in the presence of SPH1&2 (Fig. 6). Recently, we provided evidence that proHP1 may adopt an active conformation (proHP1*) to activate proHP6 in the presence of other plasma factors (Yang et al., 2016). To test whether or not proHP1 may associate with MBP somehow, we examined the plasma proteins eluted from the BSA- and MBP-treated Ni-NTA beads. Unfortunately, we did not detect proHP1 in the MBP-bound sample (data not shown). Further research is needed to find out how MBP injection induced the AMP induction, possibly via HP6 and HP8.

5. Conclusions

Supplementation of the purified MBP elicited proPO activation in a concentration-dependent manner in cell-free hemolymph from the immune challenged larvae of *M. sexta* in the absence of microbial elicitor. Adding DAP/Lys PGs and LTA from certain bacteria enhanced melanization synergistically, suggesting that MBP specifically recognized these microbe-associated molecular patterns to initiate local immune responses. Several plasma factors (HP14, HP21, HP6, HP8, PAP1-3, SPH1, SPH2, POs) were activated by limited proteolysis during this process. β GRP2, PGRP1, proHP14, SPH1, SPH2, and proPOs directly or indirectly associated with the recombinant MBP to different extents. Using the purified MBP, PGRP1, proHP14 and PGs, we demonstrated that the PG-induced proHP14 cross-activation via both recognition proteins and partial cleavage activation occurred when one or two components were absent. The AMP synthesis induced by MBP may not rely directly on the proHP14 autoactivation. The roles of HP14 and its orthologs are strikingly similar in holometabolous insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AP	alkaline phosphatase
AMP	antimicrobial peptide
BSA	bovine serum albumin
βGRP	β-1,3-glucan recognition protein
NP and IP	control and induced plasma from naïve and immune challenged larvae
DAP	diaminopimelic acid
DTT	dithiothreitol
GNBP	Gram-negative bacteria-binding protein
HP	hemolymph protease
LDLa	low density lipoprotein receptor class A repeat
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MBP	microbe binding protein
MSP/ModSP	modular serine protease
PG and PGRP	peptidoglycan and its recognition protein
PO and proPO	phenoloxidase and its precursor
PAP	proPO activating protease
PTU	1-phenol-2-thiourea
PRR	pattern recognition receptor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP and SPH	serine protease and their noncatalytic homolog
SPE and SAE	Spätzle processing enzyme and SPE activating enzyme

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Highlights

- Synergistic stimulation of proPO activation by MBP and DAP-PG
- Identification of hemolymph proteins changed by and associated with exogenous MBP
- Autoactivation of proHP14 induced by DAP-PG, MBP and PGRP1
- Possible relationship between proHP14 activation and AMP synthesis

Fig. 1. Increase of proPO activation in induced hemolymph caused by recombinant *M. sexta* MBP in the absence (A) or presence (B) of microbial elicitor

(A) As described in Section 2.3, aliquots of diluted cell-free hemolymph from immune challenged larvae were incubated with 0 to 100 ng (*left* panel) and 50 to 1000 ng (*right* panel) MBP or BSA. PO activities are shown as mean $\pm$ SEM ($n = 3$). The concentration of endogenous MBP in the mixture was estimated to be 24 to 32 nM, using known amounts of the purified recombinant MBP as standards running side-by-side with the plasma samples on SDS-PAGE followed by immunoblot with MBP antibodies. (B) 5 μ l of 1:10 diluted plasma from induced larvae were incubated with buffer (#1), 20 ng purified MBP (#2), 1 μ g elicitor (*E. coli* soluble DAP-PG, *B. megaterium* insoluble DAP-PG, *M. luteus* insoluble Lys-PG, or *S. aureus* soluble LTA, #3), or elicitor and MBP both (#4) prior to PO activity measurement. 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)]. Therefore, the PO activity changes represented by (#4 – #1) and (#2 + #3 – 2 $\times$ #1), or simply #4 and (#2 + #3 – #1), are compared using Student's t-test. An asterisk (*) indicates that #4 is significantly higher than #2 + #3 – #1 ($p < 0.05$), supporting a synergistic effect of the elicitor-MBP interaction.

Fig. 2. Detection of changes in intensity and mobility of hemolymph proteins in control (NP) and induced (IP) plasma from *M. sexta* larvae

As described in *Section 2.4*, NP or IP was incubated with buffer (lane 1 for NP, lane 3 for IP) or MBP (lane 2 for NP, lane 4 for IP). Following SDS-PAGE and electrotransfer, protein blots were separately incubated with diluted polyclonal antisera, as indicated in the bottom of each panel. The eight M_r marker proteins are shown as short bars with their sizes indicated on the left. Positions of the precursor proteins (*arrowheads*), cleavage products (*arrow* for catalytic/protease-like domain and *circle* for regulatory chain), putative serine protease-serpin complexes (*asterisk*), and PO oligomers (*diamond*) are indicated. Some of the cross-reactive bands are unclear.

Fig. 3. Hemolymph proteins associated with MBP

As described in *Section 2.5*, induced plasma was incubated with BSA (lane 1) or MBP (lane 2) for 1 h at 4°C before affinity chromatography. Bound proteins were eluted, aliquoted, and separated by 10, 12 or 15% SDS-PAGE, and subjected to immunoblot analysis using specific polyclonal antibodies indicated in the bottom of each panel. Each lane is equivalent to the bound proteins from 7.5 μ l of the IP. Positions of the M_r markers are shown as short bars with their sizes marked. Intact protein, *arrowhead*; protease-like domain, *arrow*; regulatory chain, *circle*. The 55 kDa band (marked ●) crossreacted by the diluted β GRP2 and PGRP1 antiserum is MBP. In lane 3 of the last panel, the binding to MBP was performed in the presence of *E. coli* DAP-PG.

Fig. 4. Ca^{2+} -dependent activation of *M. sexta* proHP14 by PGs recognized by MBP and PGRP1 and HP14-induced proPAP2 activation via HP21
As described in *Section 2.6*, the purified MBP, PGRP1, proHP14, *E. coli* PG, and buffer were incubated at 37°C for 90 min, along with the control reactions. The reaction mixtures were treated with 1×SDS sample buffer in the presence (**A** and first two lanes of **B**) or absence (last two lanes of **B**) of DTT, separated by 10% SDS-PAGE, and subjected to immunoblot analysis using HP14 antiserum as the primary antibody. EDTA at a final concentration of 10 mM was included in the reaction to test whether or not Ca^{2+} chelation blocks proHP14 cleavage activation (**C**). Lys-PG from *S. aureus* (**D**: right lane) or DAP-PG from *E. coli* (**D**: middle lane) was incubated with proHP14, MBP, and PGRP1 to test possible difference in the extent of proHP14 auto-proteolysis. Like the laminarin-βGRP2 binary complex, the ternary complex of DAP-PG, MBP and PGRP1 caused proHP14 auto-proteolysis, HP14 activated proHP21, HP21 activated proPAP2, and PAP2 hydrolyzed IEARpNA (**E**).

Fig. 5. Up regulation of antimicrobial protein genes by injection of MBP

As described in *Section 2.7*, buffer or MBP was injected into larvae and fat body was dissected 24 h later for total RNA isolation, cDNA synthesis, and qPCR analysis. Relative abundances of AMP transcripts were calculated based on average Ct values from two technical repeats and plotted as a bar graph (mean $\pm$ SEM).

Fig. 6.
A model for proHP14 auto-proteolysis and HP14-triggered activation of the SP-SPH system in *M. sexta*.