Insect Biochemistry and Molecular Biology 70 (2016) 10–23

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Mechanisms of nodule-specific melanization in the hemocoel of the silkworm, *Bombyx mori*

Min Shu, Dingze Mang, Gege Sun Fu, Shiho Tanaka, Haruka Endo, Shingo Kikuta, Ryoichi Sato*

Graduate School of Bio-Application and Systems Engineering, Tokyo University of Agriculture and Technology, Naka-cho, Koganei, Tokyo 184-8588, Japan

ARTICLE INFO

Article history:
Received 21 September 2015
Received in revised form
26 November 2015
Accepted 15 December 2015
Available online 18 December 2015

Keywords:
Nodule
Melanization
Pattern recognition receptors
Serine proteinase
Serine proteinase homolog
Bombyx mori

ABSTRACT

In the insect immune system, nodules are known to be a product of the cellular response against microorganisms and may be a preferential target for melanization. However, the mechanism of nodule-preferential melanization remains to be explored. In this study, we identified several mechanisms of nodule-preferential melanization by analyzing congregation and the activation of several factors involved in the prophenoloxidase (proPO)-activating system in the silkworm, *Bombyx mori*. Microorganism-binding assays revealed that *B. mori* larval plasma have an effective invading microorganism-surveillance network consisting of at least six pattern-recognition receptors (PRRs). We also found that a hemolymph serine proteinase, BmHP14, can bind to *Saccharomyces cerevisiae*. Pull-down assays showed that PRR C-type lectins form protein complexes with serine proteinase homologs, BmSPH1 and BmSPH2, which leads to the activated forms of BmSPH1 and BmSPH2 being gathered on microorganisms and trapped in nodules. Immunostaining analysis revealed that most factors in the proPO-activating system and some factors in the triggering system for antimicrobial peptide production exist in the granules of hemocytes which can gather in nodules. Western blot analysis showed that factors in the proPO-activating system are congregated in formed nodules by their concentration in plasma and aggregating hemocytes.

1. Introduction

Insects lack adaptive immunity, but they do have a highly efficient innate immune system for fighting off invading pathogens (Vilmos and Kurucz, 1998). This system consists of cellular responses such as phagocytosis, encapsulation, and nodule formation, which are mediated by circulating hemocytes (Eleftherianos et al., 2007; Marmaras and Lampropoulou, 2009; Schmidt et al., 2001). Their immune system also consists of humoral responses, such as the synthesis of antimicrobial peptides (AMPs) and the activation of the prophenoloxidase (proPO) system (Cerenius and Söderhäll, 2004; Fauvarque and Williams, 2011; Hultmark, 2003).

Many previous studies on the humoral response focused on the molecular mechanisms of proPO activation, discovering that the recognition of invading pathogens was a critical step in immune responses (An and Kanost, 2010; Wang et al., 2011). The recognition process is mediated by several groups of proteins known as pattern recognition receptors (PRRs) or pattern recognition proteins (PRPs) (hereafter, referred to as PRRs), which can specifically recognize and bind to highly conserved pathogen-associated molecular patterns (PAMPs) such as peptidoglycan (PGN), lipoteichoic acid (LTA), lipopolysaccharide (LPS), β-1, 3-glucan, and mannan, which is found on the cell walls of bacteria and fungi (Medzhitov and Janeway, 2002; Sumathipala and Jiang, 2010; Yu and Kanost, 2002). Genetic and biochemical studies have revealed more than 10 groups of PRRs in invertebrates, including β-1, 3-glucan recognition proteins (βGRPs), peptidoglycan recognition proteins (PGRPs), C-type lectins, hemocytins, Hemolin, fibrinogen-related proteins, galectins, Nimrods, thioester-containing proteins (TEPs), scavenger receptors, Down syndrome cell adhesion molecules

Abbreviations: AMPs, antimicrobial peptides; BmLBP, *Bombyx mori* lipopolysaccharide binding protein; BmMBP, *B. mori* multibinding protein; HPs, serine proteinases; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PAMPs, pathogen-associated molecular patterns; PGRP, peptidoglycan recognition protein; proPO, prophenoloxidase; PRRs, pattern recognition receptors; ROS, reactive oxygen species; SPHs, serine proteinase homologs; βGRPs, β-1, 3-glucan recognition proteins.

* Corresponding author. Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Koganei 2-24-16, Tokyo 184-8588, Japan.
E-mail address: ryoichi@cc.tuat.ac.jp (R. Sato).

http://dx.doi.org/10.1016/j.ibmb.2015.12.005
0965-1748/

(Dscams), Eaters, and Drapers (Tanaka et al., 2008; Wang et al., 2011; Yu et al., 2002). Upon binding to the invading microorganisms, a part of PRRs trigger the activation of the proPO cascade, which involves serine proteinases (SPs or HPs; hereafter, referred to as HPs) and serine proteinase homologs (SPHs) (An et al., 2009; Gupta et al., 2005; Lu and Jiang, 2008; Wang and Jiang, 2006). Some activated HPs involved in the proPO cascade, including *Manduca sexta* (tobacco hornworm) HP6, cooperate with other HPs, such as MsHP8 to regulate the Toll pathway for AMP production (An et al., 2009). More than 20 HPs have been identified in the hemolymph of *M. sexta*, but only the functions of HP14, HP21, HP6, and HP8 are known (An et al., 2009; Ji et al., 2004; Wang and Jiang, 2006, 2007, 2010).

In terms of cellular responses, hemocytes recognize invading microorganisms by directly interacting with PAMPs located on the surface of invading pathogens or indirectly through interaction with PRRs–PAMPs complexes in the plasma (Au et al., 2004; Eleftherianos et al., 2007; Lavine and Strand, 2002; Ohta et al., 2006; Ribeiro and Brehélin, 2006). Inter- and intra-cellular signaling events then coordinate effector cell responses, such as phagocytosis and nodule formation (Eleftherianos et al., 2007; Lavine and Strand, 2002; Suzuki et al., 2011). Although insect innate immune system is further subdivided into cellular and humoral responses, recent evidences have implied that the proPO-activating system (the melanization responses) is intimately associated with the generation of factors which stimulate cellular responses by aiding phagocytosis, encapsulation and nodulation (Cerenius et al., 2008; Nappi and Vass, 1993; Richman and Kafatos, 1996; Suzuki et al., 2011). In the *Tnebrio molitor* proPO-activating system, melanization reaction was indicated to generate antimicrobial activity which is supposed to depend on the production of microbicidal reactive oxygen species (ROS) (Kan et al., 2008). Although melanization is a highly effective system for killing invading microorganisms, it is also harmful to the insects themselves, because ROS can injure not only invading pathogens but also insect tissues. However, melanization responses are faster and stronger in nodules than in hemolymph (Sakamoto et al., 2011). Therefore, the hypothesis that the melanization of hemolymph is not the main objective but rather a byproduct of nodule melanization cannot be ruled out (Tokura et al., 2014). On the other hand, if the melanization system evolved to enhance the role of nodules, this suggests that nodules must have many mechanisms to implement the preferential melanization of nodules.

In the previous report, we showed that addition of melanization substrate l-3, 4-dihydroxyphenylalanine (DOPA) to the freshly-formed nodules promoted nodule melanization drastically, suggesting that melanization-related proteins are involved in the freshly-formed nodules (Sakamoto et al., 2011). Moreover, we confirmed that BmproPO1 is located in nodules and is produced by aggregated hemocytes, and further suggested that BmLBP, BmSPH1 and BmSPH2 regulate melanization of *Escherichia coli*-induced nodule through protein complex formation (Tokura et al., 2014). In this study, we explored the mechanism of congregation of melanization-related proteins in *Bombyx mori* larvae nodules. Specifically, we investigated the expression patterns of 11 putative melanization-related proteins, including six *B. mori* PRRs (BmPRRs), two *B. mori* SPHs (BmSPHs), and three *B. mori* HPs (BmHPs). We also investigated network of major BmPRRs involved in the surveillance of invading pathogens, C-type lectin-dependent congregation routes to nodules of proPO-controlling factors, *B. mori* serine proteinase homologs BmSPH1 and BmSPH2, stickiness-dependent congregation routes to nodules of BmHP14, and the production of melanization-related factors in hemocytes. These investigations revealed two origins of melanization-related factors in nodules and ways by which those factors are transported into nodules.

2. Materials and methods

2.1. Collection of plasma, hemocytes fractions and nodules

Silkworms, *B. mori* (hybrid strain, Kinshu × Showa), were reared on an artificial diet (Silkmate 2 M; Nihon-Nosanko, Yokohama, Japan) containing chloramphenicol (Wako, Osaka, Japan) at 25 °C. Fifth-instar 3–4 days larvae after molting were swabbed with 70% ethanol and bled by proleg puncture using a sterile needle. Hemolymph was collected and diluted 3 times with insect physiological saline (IPS; 150 mM NaCl, 5 mM KCl, 0.1 M Tris–HCl, 1 mM $CaCl_2 \cdot 2H_2O$, pH 6.8) mixed with 1 × concentration protease inhibitor (EDTA-free; Roche, Mannheim, Germany) and centrifuged at 800 g for 15 min at 4 °C. Cell free supernatant was collected as a plasma fraction. Low concentration of protease inhibitor could prevent autoactivation of several hemolymph proteins during collection and centrifugation of the hemolymph, but did not inhibit completely the protein activations in the microorganism-binding assay. Hemocytes pellet was washed twice with IPS by repeating the centrifugation. As previously reported (Sakamoto et al., 2011), nodules induced to form in the hemocoel of *B. mori* fifth-instar larvae were dissected 30 min after injection of CBB stained *E. coli*, *Micrococcus luteus* and *Saccharomyces cerevisiae* cells (10^6 cells in 10 μl) into the hemocoel using a Microliter Syringe (Hamilton, Reno, NV, USA). In order to remove the surrounding plasma and loosely bound hemocytes, collected nodules were washed twice with IPS containing high concentration of protease inhibitor. Shapes and purities of nodules collected by this method were previously shown in Sakamoto et al. (2011).

2.2. Preparation and cultivation of bacteria and yeast cells

Gram-positive bacteria *M. luteus* (IAM1056) and Gram-negative bacteria *E. coli* (K-12W3110) were cultured in Luria Bertani (LB) medium (peptone 10 g, yeast extract 5 g, NaCl 5 g, distilled water 1 l). *S. cerevisiae* (IAM4125) was cultured in yeast and malt (YM) medium (glucose 10 g, peptone 5 g, yeast extract 3 g, malt extract 5 g, distilled water 1 l). Microorganisms grown in the logarithmic phase were collected by centrifugation at 8000 rpm for 20 min at 4 °C, washed twice with IPS and fixed in 4% paraformaldehyde (Wako, Osaka, Japan) with gentle shaking for 90 min. The fixed cells were collected by centrifugation at 3300 rpm for 20 min at 4 °C, then washed 5 times with IPS and resuspended in IPS to store at 4 °C.

2.3. Determination of B. mori orthologue factors

B. mori orthologues of *M. sexta* HP1 (hemocyte protease 1, AF017663), HP3 (hemocyte protease 3, AF017665), HP5 (hemolymph proteinase 5, AAV91003), HP6 (hemolymph proteinase 6, AAV91004), HP8 (hemolymph proteinase 8, AAV91006), HP9 (hemolymph proteinase 9, AAV91007), HP10 (hemolymph proteinase 10, AAV91008), HP14 (pattern recognition serine proteinase, AY380790), HP17 (hemolymph proteinase 17, AAV91014), HP18 (hemolymph proteinase 18, AAV91016), HP19 (hemolymph proteinase 19, AAV91017), HP21 (hemolymph proteinase 21, AAV91019) which have been proved or predicted to participate in proPO-activating system or AMP-synthesizing system (Jiang et al., 2005) were identified using TBLASTN search systems of the KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/) and DDBJ (DNA Data Bank of Japan, http://www.ddbj.nig.ac.jp/intro-e.html). Sequences with the highest identity to corresponding *M. sexta* HP (MsHP) were designated BmHP1 (AB436162), BmHP3 (BGIBMGA013013), BmHP5 (AK380629), BmHP6 (AK384444), BmHP8 (ABB58762), BmHP9 (DQ443346), BmHP10 (AK378029),