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Pattern recognition proteins in *Manduca sexta* plasma

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Abstract

Recognition of nonself is the first step in mounting immune responses. In the innate immune systems of both vertebrates and arthropods, such recognition, termed pattern recognition, is mediated by a group of proteins, known as pattern recognition proteins or receptors. Different pattern recognition proteins recognize and bind to molecules (molecular patterns) present on the surface of microorganisms but absent from animals. These molecular patterns include microbial cell wall components such as bacterial lipopolysaccharide, lipoteichoic acid and peptidoglycan, and fungal β-1,3-glucans. Binding of pattern recognition proteins to these molecular patterns triggers responses such as phagocytosis, nodule formation, encapsulation, activation of proteinase cascades, and synthesis of antimicrobial peptides. In this article, we describe four classes of pattern recognition proteins, hemolin, peptidoglycan recognition protein, β-1,3-glucan recognition proteins, and immulectins (C-type lectins) involved in immune responses of the tobacco hornworm, *Manduca sexta*.

Keywords: C-type lectin; Immulectin; Hemolymph; Peptidoglycan-binding protein; β-1,3-Glucan-binding protein; Hemolin; Phenoloxidase

1. Introduction

A critical first step in any immune response is the recognition of an invading organism as foreign. Once such recognition has taken place, it may trigger a protective response involving blood cells or soluble plasma proteins. Innate immune systems in both mammals and arthropods utilize proteins known as pattern recognition proteins or receptors, which perform a surveillance function by binding to molecules (molecular patterns) common to the groups of microorganisms, but absent from animals (Janeway, 1989; Hoffmann et al., 1999). Pattern recognition proteins lack the binding specificity of antibodies, and instead function by binding to classes of polysaccharides, such as lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan, and β-1,3-glucans, present on the surface of bacteria or fungi. This type of recognition leads to rapid and broad responses to infection, typical of innate immune systems. Immune responses, which have been characterized in insects include phagocytosis, nodule formation, encapsulation, proteolytic activation of phenoloxidase, and induced synthesis of antimicrobial proteins and peptides (Gillespie et al., 1997; Ashida and Brey, 1998; Bulet et al., 1999). We have been investigating immune responses of a lepidopteran insect, the tobacco hornworm, *Manduca sexta*, with a particular interest in immune pathways involving serine proteinases (Kanost et al., 2001). We have identified and characterized a number of pattern recognition proteins by protein purification and cDNA cloning methods and have begun to understand some of their functions (Table 1). These proteins from *M. sexta* plasma appear to participate in several immune responses, including phagocytosis, nodule formation, and activation of phenoloxidase. We describe in this article some of our results related to four groups of pattern recognition proteins from *M. sexta* plasma.

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Abbreviations:
βGRP, β-1,3-glucan recognition protein; GNBP, Gram-negative bacteria-binding protein; Ig, immunoglobulin; IML, immulectin; LGBP, LPS- and glucan-binding protein; MBL, mannose-binding lectin; PGRP, peptidoglycan recognition protein

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Table 1
Pattern recognition proteins in plasma of *M. sexta*

Protein	Molecular mass (kDa)	Domain structure	Concentration (naive) (μg/ml)	Concentration (bacteria-induced) (μg/ml)	Microbial polysaccharide recognized
PGRP	19	Phage lysozyme-like domain	2	60	Peptidoglycan
IML-1	36	Two C-type lectin domains	Not detectable	1	LPS
IML-2	35	Two C-type lectin domains	18	75	LPS
IML-3	35	Two C-type lectin domains	12	16	?
IML-4	35	Two C-type lectin domains	10	10	?
Hemolin	48	Four Ig domains	35	1500	LPS, lipoteichoic acid
Glucan-recognition protein-1	53	*N*-terminal glucan-binding domain, C-terminal glucanase-like domain	30	30	β-1,3-Glucan, lipoteichoic acid
Glucan-recognition protein-2	54	*N*-terminal glucan-binding domain, C-terminal glucanase-like domain	0	30	β-1,3-Glucan, lipoteichoic acid

2. Hemolin

Hemolin is a 47 kDa protein composed of four I-set type immunoglobulin (Ig) domains that are most similar to those in neural cell adhesion molecules (Sun et al., 1990; Ladendorff and Kanost, 1991; Faye and Kanost, 1998; Su et al., 1998). So far, hemolin has been detected only in a few lepidopteran species, and there is no apparent hemolin ortholog in the *Drosophila* genome. Hemolin synthesis by fat body is strongly induced by bacterial challenge in *Hyalophora cecropia* and *M. sexta* (Rasmuson and Boman, 1979; Ladendorff and Kanost, 1990, 1991; Wang et al., 1995), and a bacteria-induced hemolin gene has been identified in the fall webworm, *Hyphantria cunea* (Shin et al., 1998). Hemolin is also synthesized in embryos (Bettencourt et al., 2000) and during metamorphosis, in the absence of infection (Yu and Kanost, 1999; Trenczek, 1998; Lanz-Mendoza and Faye, 1999).

Hemolin binds to hemocytes and bacteria (Ladendorff and Kanost, 1991; Zhao and Kanost, 1996; Lanz-Mendoza et al., 1996). These properties suggest that hemolin might function as a pathogen recognition protein and modulate hemocytic immune responses (Kanost and Zhao, 1996; Lanz-Mendoza et al., 1996; Daffre and Faye, 1997; Lanz-Mendoza and Faye, 1999). Hemolin from *H. cecropia* interacts with bacterial LPS (Daffre and Faye, 1997), and it binds to hemocytes in a calcium-dependent manner (Bettencourt et al., 1999). The ability of hemolin to interact with bacteria and the surface of hemocytes suggests that it might function as an opsonin to increase the efficiency of phagocytosis (Kanost and Zhao, 1996). The horseshoe-shape arrangement of the Ig domains in the structure of *H. cecropia* hemolin suggested a mechanism for homophilic binding of hemolin molecules on the surface of hemocytes and microorganisms (Su et al., 1998).

M. sexta hemolin binds to Gram-negative and Gram-positive bacteria and can cause these microorganisms to aggregate. Hemolin binds to LPS from Gram-negative bacteria (Daffre and Faye, 1997; Yu and Kanost, 2002) and to lipoteichoic acid, a major component of the surface of Gram-positive bacteria (Yu and Kanost, 2002). Our results suggest that hemolin has two binding sites for LPS: one interacting with carbohydrate moieties in the O-antigen and outer core regions of LPS and the other interacting with the phosphate groups in its lipid A component (Yu and Kanost, 2002). Binding of *M. sexta* hemolin to lipid A was completely blocked by free phosphate (Yu and Kanost, 2002), which suggests that the site occupied by phosphate in the crystal structure of *H. cecropia* hemolin (Su et al., 1998) might help in binding to lipid A. Interaction with phosphate groups might also be important in binding of hemolin to lipoteichoic acids, which are poly(glycerophosphate) chains linked to membrane phospholipids (Fischer et al., 1990). The binding properties of hemolin to glycolipids that are widely present on Gram-negative and Gram-positive bacteria suggest that it functions as a pattern recognition receptor with broad specificity in the defense against bacterial infection. Further research is needed to investigate how binding of hemolin to bacteria might stimulate responses that lead to bacterial killing.

3. Peptidoglycan recognition protein

Peptidoglycan is present in almost all the prokaryotic organisms and is especially abundant in Gram-positive bacterial cell walls (Sharon, 1975). It is a potent elicitor of innate immune systems in insects and mammals. Peptidoglycan is a heteropolymer of a repeating disaccharide of *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid cross-linked by short peptides (Rosenthal and Dziarski, 1994; Sharon, 1975). In *M. sexta* and *Bombyx mori*, peptidoglycan induces antibacterial protein expression

(Dunn et al., 1985; Kanost et al., 1988; Iketani and Morishima, 1993; Morishima et al., 1995) and activates the protease cascade that leads to phenoloxidase activation (Yoshida and Ashida, 1986; Yoshida et al., 1986; Jiang, Yu and Kanost, unpublished results). In mammals, peptidoglycan stimulates the release of cytokines and chemokines from macrophages, lymphocytes, and other cells, which are involved in inflammation and immunity (Wang et al., 2000; De Kimpe et al., 1995; Rosenthal and Dziarski, 1994).

A 19 kDa peptidoglycan recognition protein (PGRP), which initiates the phenoloxidase cascade, was purified from *B. mori* (Yoshida et al., 1996). It specifically binds to peptidoglycan but not to β-1,3-glucan or chitin. The primary structure of *B. mori* PGRP is similar to that of bacteriophage T7 lysozyme, which hydrolyzes peptidoglycan. However, PGRP differs at positions corresponding to the catalytic amino acid residues of lysozyme, consistent with the observation that PGRP lacks lysozyme activity (Ochiai and Ashida, 1999). *B. mori* PGRP is mainly expressed in fat body and epidermal cells (Ochiai and Ashida, 1999). Immune challenge results in the up-regulation of PGRP mRNA, although it is constitutively expressed at a low level even before the infection. PGRP was also cloned from another lepidopteran insect, *Trichoplusia ni*, and from mouse, rat, and human (Kang et al., 1998), suggesting that it is an ancient molecule that evolved for detection of bacteria in innate immune systems of animals. In *Drosophila melanogaster*, 12 PGRP genes were identified on three chromosomes and distributed at eight chromosomal loci (Werner et al., 2000).

We have isolated a PGRP cDNA from *M. sexta* and have begun to study its expression and function. The *M. sexta* PGRP is a 19 kDa protein that is similar in sequence to the PGRPs isolated from other insect species (Fig. 1). *M. sexta* PGRP is 54 and 61% identical in sequence to PGRP from *B. mori* and *T. ni*, respectively. *M. sexta* PGRP also shares 24% identity with bacteriophage T3 and T7 lysozymes. *M. sexta* PGRP mRNA is constitutively expressed at a low level in fat body of naive insects. After bacterial challenge, PGRP mRNA level increases (Zhu and Kanost, unpublished results), corresponding with elevated PGRP concentration detected in plasma (Fig. 2).

4. β-1,3-Glucan recognition proteins

β-1,3-glucans are key structural components of fungal cell walls. Pattern recognition proteins, which bind to β-1,3-glucans (β-1,3-glucan recognition proteins, βGRP) have been identified in several arthropod groups. We have purified proteins and cloned cDNAs for two βGRPs from *M. sexta* (Table 1) (Ma and Kanost, 2000; Ma, Jiang and Kanost, unpublished results) and one from another lepidopteran insect, *Plodia interpunctella* (Fabrick, Baker and Kanost, unpublished results). The arthropod βGRPs contain a carboxyl-terminal glucanase-like domain with sequence similar to β-1,3-glucanases from bacteria and a sea urchin (Yahata et al., 1990; Bachman and McClay, 1996), but they lack glucanase activity due to amino acid substitutions in key residues of the catalytic site. *B. mori* βGRP (Ochiai and Ashida, 1988, 2000), *M. sexta* βGRP (Ma and Kanost, 2000), and horseshoe crab factor G subunit α (Seki et al., 1994) have a restricted specificity and high binding affinity for β-1,3-glucans, whereas some other members of this protein family (Gram-negative bacteria-binding proteins, GNBPs) can also bind to Gram-negative bacteria through an interaction with LPS (Lee et al., 1996, 2000; Kim et al., 2000; Beschin et al., 1998). In addition to their carboxyl-terminal glucanase-like domain, members of the βGRP/GNBP family contain a unique amino-terminal domain that is approximately 100 amino acid residues long. In βGRP from *B. mori* and βGRP-1 from *M. sexta*, this amino-terminal domain was shown to have strong binding affinity for β-1,3-glucans (Ochiai and Ashida, 2000; Ma and Kanost, 2000).

βGRPs and GNBPs have been shown to be involved in innate immune responses. βGRPs from *B. mori* (Ochiai and Ashida, 1988, 2000), *M. sexta* (Ma and Kanost, 2000), and *Blaberus craniifer* (Söderhäll et al., 1988), LPS- and glucan-binding protein (LGBP) from *Pacifastacus leniusculus* (Lee et al., 2000), and coelomic cytolytic factor 1 (CCF-1) from *Eisenia foetida* (Beschin et al., 1998) participate in activation of the protease cascade that leads to phenoloxidase activation. Overexpression of DGNBP-1 in a *Drosophila* cell line enhanced antimicrobial gene expression induced by LPS- and β-1,3-glucans (Kim et al., 2000). In the mosquito, *Anopheles gambiae*, GNBP mRNA level was elevated both locally in the midgut and systemically after infection of the mosquito with *Plasmodium berghei*, a parasite that enters the mosquito through the midgut (Richman et al., 1997).

5. Immulectins

C-type lectins, a superfamily of calcium-dependent carbohydrate-binding proteins, function in pathogen recognition, cellular interactions, and innate immunity in mammals (Weis et al., 1998; Vasta et al., 1999). For example, mammalian serum mannose-binding protein (also called mannose-binding lectin, or MBL) activates the complement system through the lectin-mediated pathway (Matsushita, 1996; Turner, 1996). Activation of the complement system by MBL is associated with C1r/C1s-like serine proteinases, called MBL-associated serine proteinases (MASPs) (Matsushita and Fujita, 1992; Thiel et al., 1997). Binding of carbohydrate

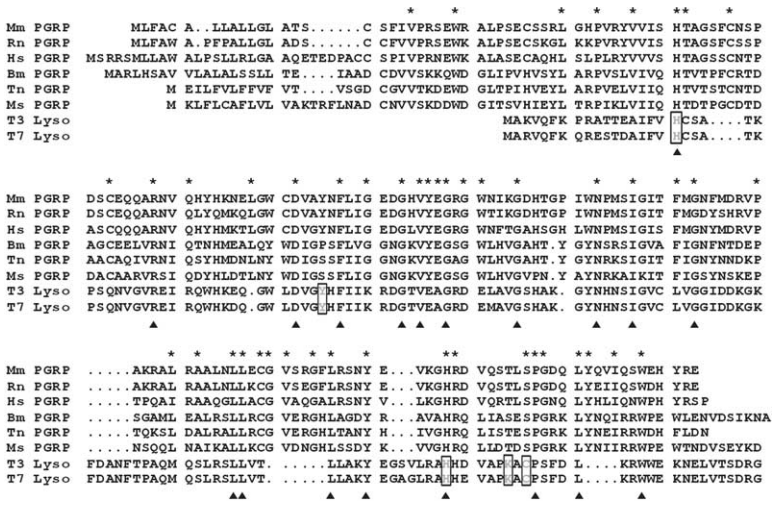

Fig. 1. Alignment of PGRP sequences. The sequences are from mouse (Mm, accession number AF076482), rat (Rn, accession number AF154114), human (Hs, accession number AF076483), *B. mori* (Bm, accession number AB016249), *T. ni* (Tn, accession number AF076481), *M. sexta* (Ms, accession number AF413068), and lysozymes from bacteriophage T3 (accession number P20331) and T7 (accession number P00806). Amino acid residues identical in all six PGRP sequences are labeled with ∗ above the alignment. Residues, which are also conserved in T3 and T7 lysozymes are labeled with a triangle. The five active site residues of the bacteriophage lysozymes (His-17, Tyr-46, His-122, Lys-128, and Cys-130) are indicated by boxes.

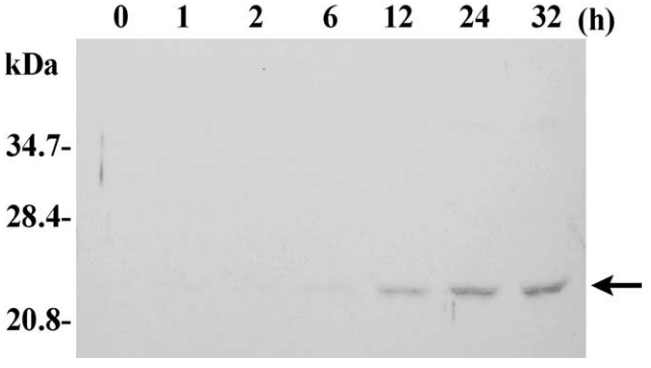

Fig. 2. Bacteria-induced increase in *M. sexta* PGRP concentration in plasma. Day two, fifth instar larvae were injected with *Micrococcus lysodeikticus*. Hemolymph collected at different times after injection was analyzed by immunoblotting, using rabbit antisera to recombinant PGRP. The numbers above the sample lanes indicate post-injection times in hours. The position of PGRP is indicated by an arrow.

ligands to MBL oligomers activates the associated MASPs, thus triggering activation of the complement pathway (Matsushita, 1996; Turner, 1996). Because the lectin pathway does not require antibody–antigen interaction, it may have preceded the evolution of the classical Ig-mediated pathway. MBL also functions directly as an opsonin to increase the efficiency of phagocytosis of bacteria (Kawasaki et al., 1989; Kuhlman et al., 1989).

C-type lectins are also present in insects. A group of 19 genes belonging to this superfamily has been identified in *D. melanogaster* (Theopold et al., 1999), but their functions remain to be determined. However, lectins in plasma of lepidopterans and cockroaches have been shown to participate in innate immune responses including phagocytosis (Jomori and Natori, 1992), hemocyte nodule formation (Koizumi et al., 1997, 1999), and activation of prophenoloxidase (Chen et al., 1995; Yu et al., 1999; Yu and Kanost, 2000).

We have used carbohydrate affinity chromatography to purify four C-type lectins from plasma of bacteria-challenged *M. sexta* larvae (Table 1). Each of these proteins, named immulectins (IML), contains two tandem C-type lectin carbohydrate recognition domains (Yu et al., 1999; Yu and Kanost, 2000). This type of structure has also been found in LPS-binding lectins from *B. mori* (Koizumi et al., 1999) and the fall webworm, *H. cunea* (Shin et al., 1998, 2000). In this respect, the lepidopteran lectins differ from most animal C-type lectins, which contain only a single carbohydrate recognition domain.

The four *M. sexta* IMLs apparently function as pattern recognition receptors involved in prophenoloxidase activation (Yu et al., 1999; Yu and Kanost, 2000) and encapsulation (Yu and Kanost, 2002). Among these, we know the most about IML-2. *M. sexta* IML-2 is constitutively expressed at a low level in the fat body, and its synthesis is induced after injection of Gram-negative bacteria or LPS but not by Gram-positive bacteria or yeast (Yu and Kanost, 2000). IML-2 binds specifically to LPS and apparently contains two distinct binding sites, perhaps corresponding to its two domains. IML-2 complexed with LPS stimulates activation of prophenoloxidase in plasma (Yu and Kanost, 2000).

Furthermore, injection of IML-2 antibody inhibited clearance from the hemolymph of a Gram-negative bacterial pathogen, *Serratia marcescens*, which resulted in decreased survival of larvae (Yu and Kanost, 2002). These results suggest that IML-2 is involved in clearing

and killing of bacterial pathogens. IML-1, although present at much lower concentration than IML-2 (Table 1), is less specific in its binding properties and can bind to Gram-positive and Gram-negative bacteria, and to yeast and cause these microorganisms to aggregate (Yu et al., 1999). IML-3 and IML-4 are known to bind to *N*-acetylgalactosamine and glucose (Yu and Kanost, 2002), but their binding specificity has not yet been fully investigated.

The four IML genes differ in their patterns of regulation (Table 1). IML-1 is not detectable in plasma of most naive insects. Its mRNA level increases after microbial challenge, and IML-1 protein concentration in plasma increases to approximately 1 μg/ml. In contrast, IML-2 is expressed constitutively at a low level, and increases dramatically after Gram-negative bacterial challenge to reach the highest concentration of the four IMLs. IML-3 and IML-4 are expressed constitutively and do not change significantly in concentration in response to infection.

6. Conclusions and questions for future research

We are beginning to understand some of the pattern recognition proteins in the plasma of *M. sexta* and have identified four classes of proteins (hemolin, PGRP, βGRP, IML) that bind to polysaccharides on the surface of bacteria or fungi. An interesting feature of this group of proteins is the redundancy in their ligand binding specificity. That is, more than one protein binds to different classes of microbial polysaccharides. Each protein may stimulate a different (although perhaps overlapping) set of immune responses. A challenge for future research will be to determine how binding of these pattern recognition proteins to microorganisms results in the stimulation of protective responses. In the case of opsonization, the proteins could simply link bacteria to the surface of hemocytes. Stimulation of other responses, such as activation of a proteinase cascade in phenoloxidase activation, may be much more complex. We hypothesize that binding of βGRP or IML to a microbial surface causes a conformational change that permits a new interaction between the pattern recognition protein and some other plasma protein(s), including the first proteinase in the cascade. Resulting protein–protein interactions might then lead to an auto-activation of the initial proteinase zymogen. Similarly, the mechanisms by which mannose-binding protein activates mammalian serine proteinases in the complement system are still not well understood. Additional biochemical studies, using purified recombinant proteins to reconstitute cascade pathways, should increase our understanding of these systems in insects. With the discovery of similar proteins in *Drosophila*, it is likely that genetic studies will become increasingly informative with regard to the functions of pattern recognition proteins in hemolymph. RNA interference technology may eventually be useful for identifying the effects of knocking out expression of pattern recognition proteins in other insect species. Finally, although we now know several proteins that could serve to recognize bacterial and fungal pathogens, the molecular mechanism by which insects recognize other groups of parasites such as protozoans, nematodes, or insect parasitoids remains an area in which we have little knowledge and should be the target of future investigations.

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