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IMMUNITY IN LEPIDOPTERAN INSECTS

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Abstract

Lepidopteran insects provide important model systems for innate immunity of insects, particularly for cell biology of hemocytes and biochemical analyses of plasma proteins. Caterpillars are also among the most serious agricultural pests, and understanding of their immune systems has potential practical significance. An early response to infection in lepidopteran larvae is the activation of hemocyte adhesion, leading to phagocytosis, nodule formation, or encapsulation. Plasmatocytes and granular cells are the hemocyte types involved in these responses. Infectious microorganisms are recognized by binding of hemolymph plasma proteins to microbial surface components. This “pattern recognition” triggers phagocytosis and nodule formation, activation of prophenoloxidase and melanization and the synthesis of antimicrobial proteins that are secreted into the hemolymph. Many hemolymph proteins that function in such innate immune responses of insects were first discovered in lepidopterans. Microbial proteinases and nucleic acids released from lysed host cells may also activate lepidopteran immune responses. Hemolymph antimicrobial peptides and proteins can reach high concentrations and may have activity against a broad spectrum of microorganisms, contributing significantly to clearing of infections. Serine proteinase cascade pathways triggered by microbial components interacting with pattern recognition proteins stimulate activation of the cytokine Spätzle, which initiates the Toll pathway for expression of antimicrobial peptides. Aproteinase cascade also results in proteolytic activation of phenoloxidase and production of melanin coatings that trap and kill parasites and pathogens. The proteinases in hemolymph are regulated by specific inhibitors, including members of the serpin superfamily. New developments in lepidopteran functional genomics should lead to much more complete understanding of the immune systems of this insect group.

INTRODUCTION

Moth larvae have proven to be extremely useful for experiments providing insights on the innate immune systems of insects. Many hemolymph proteins with immune functions were first studied in caterpillars by biochemical methods.[1–11] Lepidopteran larvae have also been important for experiments aimed at characterizing immune functions of insect hemocytes.[12]

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Much of the research on lepidopteran immunity has made use of large moth species, including the tobacco hornworm, *Manduca sexta* and wild silkmoths such as *Hyalophora cecropia*. The domestic silkmoth, *Bombyx mori* has also provided significant discoveries about immunity in moths and with the advantage of a sequenced genome,[13] it continues to serve as an important model organism. The wax moth, *Galleria mellonella*, was one of the earliest species used for research in insect immunity[14,15] and is the subject of much current research. In addition to these laboratory model species, research on immunity is also underway to investigate the immune systems of moths whose larvae are among the most destructive agricultural pests worldwide, particularly including species in the family Noctuidae. In comparison, there has been relatively little research on immunity in butterflies, perhaps because they can be more difficult to rear or due to lower agricultural impact.

In this review, we describe selected developments in research on innate immunity in lepidopteran insects, with an emphasis on aspects discovered through biochemical and cell biological approaches and potential new insights that may be gained from functional genomics methods. Several recent reviews with a focus on lepidopteran immunity are available.[16–23]

HEMOCYTES

With a large hemolymph volume in many lepidopteran larvae and pupae, it is possible to collect 10^4–10^6 hemocytes from a single individual, making feasible the use of cell biological techniques such as flow cytometry and cell sorting that have advanced mammalian immunology. The most abundant hemocyte types typically described in Lepidopteran larvae are granular cells and plasmatocytes, which are capable of adhesion and phagocytosis.[12] Nonadherant hemocyte types include oenocytoids, which synthesize prophenoloxidase (proPO), and spherule cells, whose functions are poorly understood. Monoclonal antibodies are very useful reagents for distinguishing lepidopteran hemocyte populations based on antigenicity rather than morphology,[24–26] which can vary considerably, especially for plasmatocytes. Two subpopulations of plasmatocytes have been distinguished based on monoclonal antibody markers in *Pseudoplusia includens*[26] and in *M. sexta*,[27] which may indicate different stages in differentiation or functional specialization. The composition of hemocyte populations varies through larval development.[28,29] Hematopoesis in lepidopteran larvae[28,31] and the embryonic origin granular cells and plasmatocytes[30] have been described.

Granular cells and plasmatocytes function in immunity through responses that involve adherence of cells to foreign surfaces or to other hemocytes.[22,32] Both granular cells and plasmatocytes can be phagocytic and in different lepidopteran species either cell type may be the predominant contributor to phagocytosis as a defense.[12] A hyperphagocytic cell type, very large cells capable of phagocytosing 500 bacteria, has been described in *M. sexta*.[33] These cells are morphologically similar to a neuroglian-positive subpopulation of plasmatocytes, which can act as a focus for attachment of hemocytes to foreign surfaces[34] and perhaps are the same hemocyte type.

Adhesion of granular cells and plasmatocytes leads to two related responses to infection: nodule formation, in which hemocytes cluster to entrap aggregated microorganisms, and encapsulation, in which hemocytes form a multi-layered cellular capsule around a larger eukaryotic parasite.[12] Adhesion of hemocytes to injured body wall also probably helps to seal wounds to prevent bleeding.[32] Cytokines have been identified in lepidopterans, which promote these hemocyte functions. A hemocyte chemotactic peptide from *Pseudaletia separata* stimulates directed movement and aggregation of hemocytes.[35] This peptide is structurally similar to lepidopteran cytokines called ENF peptides, which have multiple biological activities, including the stimulation of plasmatocyte adhesion and spreading, reduced bleeding and the stimulation of oenocytoid lysis to release proPO.[36–40] RNAi results indicate that the *M. sexta* plasmatocyte spreading peptide from this family promotes hemocyte nodule formation as a protective response to bacterial infection.[41] The active ENF peptides are produced by proteolytic processing of a larger protein present in hemolymph.[36,37] In *M. sexta* hemolymph, a serine proteinase with trypsin-like specificity is responsible for this activation, but has not yet been identified due to its instability (Kanost et al, unpublished results). Eicosanoids such as prostaglandins can also simulate hemocytes to aggregate to form nodules.[42,43] Prostaglandins can also elicit the lysis of oenocytoids, releasing proPO into the plasma.[44] ProPO is activated by plasma proteinases and participates in formation of melanin, which coats nodules and encapsulated objects. This response is discussed in more detail below.

Hemocyte attachment during encapsulation and nodulation is mediated by cell surface adhesion proteins. Lepidopteran hemocytes have cell surface integrins, which function as adhesion molecules.[45–50] Plasmatocytes of *M. sexta* express a specific integrin that is required for efficient encapsulation.[47,50] The adhesive properties of this hemocyte-specific integrin derive at least in part from binding to neuroglian and a tetraspanin on neighboring hemocytes.[49,51]

RECOGNITION OF MICROORGANISMS

Plasma proteins that bind to components on the surface of microorganisms are a key component of the innate immune system of insects. Such proteins stimulate responses including phagocytosis and activation of proteinase signaling cascades. Some of these “pattern recognition” proteins were first discovered in silkworms and then found to occur in immune systems of other insect groups, whereas others appear to be unique to lepidopterans.

Hemolin is a 48 kDa plasma protein first identified in *Hyalophora cecropia*[52,53] and *Manduca sexta*.[54,55] Hemolin is composed of four I-set immunoglobulin (Ig) domains commonly found in cell adhesion proteins of vertebrates and invertebrates. Hemolin also exists in other lepidopteran species including *B. mori, Hyphantria cunea*, *Lymantria dispar*, *Antheraea pernyi*, *Antheraea mylitta*, *Plutella xylostella*, *Samia cynthia*.[13,56–61] Hemolin has not been identified in isects from any other order, suggesting that it may have evolved after the split of the Lepidoptera from other insect groups. Hemolin shares structural features with neuroglian,[62] a transmembrane Ig-domain protein located on the surface of *Manduca* glial and neuronal cells[63] and the developing embryonic prothoracic gland,[64] as well as a subpopulation of *M. sexta* plasmatocytes.[34,51] Hemolin expression is induced by bacteria

or their surface components injected into the hemocoel. In *H. cecropia*, this transcriptional activation is controlled via an intronic enhancer that contains a κB motif.[65] Developmental and hormonal signals (e.g., 20-hydroxyecdysone) also affect hemolin production.[58,66,67] It has been speculated that hemolin may have an antiviral function.[68] Baculovirus exposure up-regulates hemolin transcription in *A. pernyi*,[69] but not in *B. mori*, *Helicoverpa zea*, or *Heliothis virescens*.[13,70] A possible role for hemolin in antiviral responses remains to be established. On the other hand, several lines of evidence support the idea that hemolin functions in immune responses to bacterial infection. Hemolin binds to bacterial lipopolysaccharide (LPS) and lipoteichoic acid[71,72] and associates with hemocytes.[55,73,74] The horseshoe-shape structure of hemolin suggests that one or more of its Ig domains may interact through domain swapping with Ig domains of cell adhesion molecules such as neuroglian on hemocyte surfaces.[75] Interaction of hemolin with molecules on the surface of bacteria and with hemocyte membranes suggests that it may bring microorganisms to hemocyte surfaces, promoting phagocytosis or nodule formation. RNAi knockdown of hemolin expression in *M. sexta* larvae significantly reduced phagocytosis and nodulation of *E. coli*.[76]

Peptidoglycan recognition proteins (PGRPs) associate with bacterial peptidoglycans through a conserved domain homologous to T4 bacteriophage lysozyme.[77] PGRPs were first discovered in lepidopterans. *B. mori* PGRP-S1 was isolated from plasma of silkworm larvae as a 19 kDa protein that binds to *Micrococcus luteus* peptidoglycan and triggers the proPO activation system.[8] Molecular cloning of PGRPs from *Trichoplusia ni*,[10] *B. mori*,[13,78] *M. sexta*,[79–81] *G. mellonella*,[82] *S. cynthia*,[83,84] *P. xylostella*,[60] and *Ostrinia nubilalis*[85] suggests that multiple PGRPs are present in every lepidopteran species. In addition to binding to peptidoglycan, these proteins may hydrolyze peptidoglycan if the residues for Zn^{2+}-binding and amidase activity are present.[86] The *B. mori* genome has six short (S) and six long (L) PGRP genes: S3 through S6 are putative amidases; L1 and L4 possess a transmembrane region; L6 is likely cytosolic.[13] Constitutive expression of PGRP-S1 is up-regulated in larvae after injection of *Enterobacter cloacae*.[78] *E. coli* or *Bacillus subtilis* treatment increases the mRNA levels for PGRP-S1, -S2 and -S5, whereas *Staphylococcus aureus* injection enhances transcription of *B. mori* PGRP-S1, -S5, -L1, but not -S2.[13] Microbe-induced transcription of PGRPs also occurs in other lepidopteran species.[10,79,82,84] The *T. ni*, *S. cynthia* and *M. sexta* PGRPs bind to *Bacillus meso*-diaminopimelic acid-type peptidoglycans and *M. luteus* Lys-type peptidoglycans.[10,83,87] This *M. sexta* PGRP can stimulate proPO activation.[88] Knockdown of *M. sexta* PGRP1 synthesis increased larval susceptibility to infection by *Photorhabdus luminescens*.[89,90] So far, the level of knowledge on structures and functions of moth PGRPs is significantly less than for *Drosophila* PGRPs.[91,92] Furthermore, molecular details of how peptidoglycan binding by PGRPs promotes the activation of serine proteinases involved in proPO and spätzle activation are unknown.

Insect β-1,3-glucan recognition proteins (βGRPs) and Gram-negative bacteria binding proteins (GNBPs) are a family of ~55 kDa plasma proteins with an amino-terminal glucan-binding domain and a carboxyl-terminal region similar to β-1,3-glucanases. *B. mori* βGRP1, *M. sexta* βGRP1 and βGRP2 and *Plodia interpunctella* βGRP bind to β-1,3-glucans and to bacteria and stimulate the proPO activation cascade.[9,93–97] The amino-terminal domain

of *B. mori* βGRP1 adopts an Ig-like β-sandwich fold and residues have been identified that may form a glucan-binding site.[98] *M. sexta* βGRP1 and βGRP2 gene expression in fat body is differentially regulated: βGRP1 is constitutively expressed, whereas βGRP2 transcripts become highly abundant in the early wandering stage prior to pupation or after an immune challenge.[94,95] *M. sexta* βGRP2 is also present in cuticle of wandering stage larvae.[95] Binding of βGRP1 or βGRP2 to curdlan and *M. sexta* hemolymph proteinase-14 precursor (proHP14) stimulates autoactivation of HP14 to initiate an immune proteinase cascade leading to proPO activation.[99,100] *B. mori* GNBP binds to *E. cloacae* but not *Bacillus licheniformis* or curdlan.[101] An orthologous *M. sexta* GNBP recognizes LPS and laminarin (a β-1,3-glucan with β-1,6 branches) and initiates melanization (Y. Wang and H. Jiang, unpublished results). An active glucanase related in sequence to the GRPs was isolated from midgut extract of *Helicoverpa armigera* larvae. It hydrolyzes β-1,3-glucan but not β-1,4-glucan or glucans with mixed β-1,3 and β-1,4 linkages[102] and probably functions as a digestive enzyme.

C-type lectins (CTLs) from animals are a large group of carbohydrate-recognition molecules that bind ligands in a calcium-dependent manner.[103] *B. mori* LPS-binding protein (LBP or CTL20),[104,105] *Hyphantria cunea* Hdd15,[106] and *M. sexta* immulectin-1[107] were among the first C-type lectins identified in plasma of lepidopteran larvae. They all contain two carbohydrate-recognition domains. CTLs with this dual-domain structure also include: *M. sexta* immulectins-2, -3 and -4,[108–110] *B. mori* MBP (CTL10),[111] immulectin (CTL11),[112] LEL-1 and -2,[113] CTL19, CTL21,[13] and *H. armigera* Ha-lectin.[114] GenBank currently contains more than 30 additional similar CTL cDNA sequences from eleven other lepidopteran species in five families. The only other dual-domain CTL identified in any other insect species is TcCTL3[115] from a beetle, *Tribolium castaneum*. Hence, such tandem domain CTL genes appear to be fairly unique to Lepidoptera and their emergence and expansion may have occurred early in evolution of this insect group. Expression of at least some of these CTL genes is induced microbial infection.[106,108–110,114]

Many of the lepidopteran CTLs bind to bacterial LPS and some to lipoteichoic acid.[105,106,108–110] They can cause agglutination of bacteria and yeast,[107,109–111] presumably due to each of the molecule's two carbohydrate-binding domains binding to carbohydrates on the surface of adjacent microbial cells. This activity most often requires the presence of calcium. Such clustering of microorganisms may result in more efficient clearance of pathogens by hemocytes through nodule formation.[105] Experiments have demonstrated that *M. sexta* immulectin-2 enhances clearance of *Serratia marcesens*,[116] and suppression of immulectin-2 expression by RNA interference reduced larval survival of a *Photorhabdus* infection.[90] In addition, the immulectins can promote proPO activation and melanin deposition at the surface of objects encapsulated by hemocytes,[107,108,110,117,118] which may also promote killing of pathogens and parasites.

Lipophorins are insect hemolymph proteins that transport lipids between tissues.[119] The lipophorin particle contains two protein subunits, apolipophorin-I and apophorin-II. When the neutral lipid load is high, an exchangeable plasma protein, apolipophorin-III (apoLpIII) also associates with low density lipophorin to cover hydrophobic surfaces. In lepidopterans, apoLpIII concentration in hemolymph is generally much lower in larvae than in adults,

where it functions in transporting lipids from fat body to flight muscles during prolonged flight. Lipophorins and apoLpIII have been implicated in several defense mechanisms.[32] Lipophorin appears to be involved in hemolymph clotting in at least some species,[20] and may in this way participate in physical trapping of invading microorganisms. There may also be an association of proteins from the melanization cascade with lipophorin or other clotting components, which could contribute to defense.[120–122] The affinity of lipophorin and apoLpIII for hydrophobic ligands is consistent with their reported binding of bacterial LPS and lipoteichoic acids,[123–128] and the partitioning of these microbial membrane lipids into complexes with lipophorin or apoLpIII may contribute to reducing their toxicity to insect hosts. Lipophorin and apoLpIII have been reported to stimulate other humoral or cellular immune responses in *G. mellonella*,[129–133] and research toward understanding molecular mechanisms underlying such observations is needed.

Pathogenic bacteria and fungi produce proteinases to utilize lepidopteran host proteins as a source of nutrients and to degrade immunity-related defense molecules such as antimicrobial peptides. Thermolysin-like metalloproteinases associated with entomopathogenic bacteria and fungi are essential virulence factors.[134–137] However, the presence of microbial proteinases may also be recognized as a signal of infection and stimulate immune defenses in the host. Evidence for sensing of microbial proteinases and their regulation during innate immune responses has been reported from *G. mellonella*.[138] Thermolysin is a potent activator of the serine proteinase cascade that controls proPO activation leading to melanization. Thus, this virulence factor also directly triggers an immune response. In addition, the activity of microbial metalloproteinases within the body of *G. mellonella* generates peptide fragments that strongly elicit the synthesis of antimicrobial peptides.[139] The immune-stimulatory peptides were identified as collagen IV fragments containing the RGD/RGE motif, which bind to integrins of immune-compentent hemocytes.[140] The stimulation of two innate immune responses, proPO activation and antimicrobial peptide synthesis, in response to microbial metalloproteinases provides evidence that lepidopteran innate immune responses include reaction to danger-associated molecules produced by microbial virulence factors.[141] Furthermore, *G. mellonella* hemolymph contains an inducible metalloproteinase inhibitor (IMPI), the only specific peptidic inhibitor of thermolysin-like metalloproteinases reported to date from any animal.[142] The IMPI gene encodes two distinct metalloproteinase inhibitors that putatively contribute to the regulation of metalloproteinases associated with invading pathogens.[143,144]

Nucleic acids released from damaged or necrotic cells form another danger signal to enhance insect immune responses. Injection of synthetic oligonucleotides induced attacin expression in *B. mori* larvae.[145] Co-injection of purified host nucleic acids with heat-inactivated *P. luminescens* into *G. mellonella* larvae synergistically elevated the level of antimicrobial activity, reduced the total number of hemocytes (a consequence of the attachment of immune-competent cells to tissues during cellular responses) and prolonged the survival of insects infected by *P. luminescens*.[146] DNA and RNA released from damaged cells may interact with lipophorin to trigger clot formation and entrap invading pathogens in hemolymph. In *Pseudaletia separata*, nucleic acids as well as cytoplasmic proteins (*e.g.*, proPO) are released from oenocytoids through cell lysis induced by the growth-blocking peptide.[40]

ANTIMICROBIAL PEPTIDES AND PROTEINS

Many insect antimicrobial plasma proteins were first identified and isolated from lepidopteran hemolymph. Expression of antimicrobial peptides and proteins is often induced by microbial infection, with strongest expression usually occurring in fat body, although hemocytes also contribute to the pool of antimicrobial peptides secreted into hemolymph plasma.[147] Antimicrobial peptides are also expressed in the midgut of lepidopteran prepupae and secreted into the lumen, perhaps as a prophylaxis against infection during metamorphosis.[148] Antimicrobial peptides can also be expressed in extraembryonic tissues of lepidopteran eggs, providing protection against infection for the developing embryo.[149]

Lysozyme, the first antimicrobial protein reported from insects, was identified more than forty years ago in *G. mellonella*[150] and, like other insect lysozymes, shares structural similarity with C-type (chicken) lysozyme.[151] Its activity against Gram-positive bacteria has been attributed to its ability to degrade cell wall peptidoglycan by hydrolysis of the β-1,4 linkages between N-acetylglucosamine and N-acetylmuramic acid residues.[2] Besides moderate activity against Gram-negative bacteria,[152–154] lepidopteran lysozyme also exhibits antifungal activity.[155] Lyozyme also appears to negatively regulate activation of proPO in *M. sexta*.[156]

Insects produce a variety of amphipathic peptides with antimicrobial activity attributed to their ability to damage cell membranes of pathogens. The first antimicrobial peptide from insects, isolated from the hemolymph of the silkmoth *H. cecropia*, was named cecropin.[4] Families of cecropin genes have now been found in many lepidopteran (and dipteran) species. Thirteen cecropin genes were identified in the *B. mori* genome.[13] Cecropins are typically ~4 kDa basic peptides, which have an amphipathic α-helical structure. The moricins constitute another group of amphipathic α-helical antimicrobial peptides,[157,158] first discovered in *B. mori*.[159] Nine moricin genes are present in the *B. mori* genome.[13] Eight moricin homologs identified in *G. mellonella* have activity against Gram-negative and Gram-positive bacteria, as well as against yeast and filamentous fungi.[160]

Lepidoptera possess glycine-rich AMPs (attacins and gloverins) and proline-rich AMPs (lebocins). *H. cecropia* has two 20 kDa attacin isoforms, an acidic and a basic attacin, with 80% sequence identity.[5,161] The *B. mori* genome also contains two attacin genes,[13] and attacin cDNAs have now been cloned from many lepidopteran species. Treatment of *E. coli* with *H. cecropia* attacins leads to an increase in outer-membrane permeability, preceding any increase in inner-membrane permeability by at least one generation time. Inhibition of outer-membrane protein synthesis is achieved on the transcriptional level and triggered by binding of attacin to the cell surface without entering the inner membrane or the cytoplasm. Primary binding occurs on LPS, explaining why basic attacin is more active against *E. coli* than the acidic form. Another family of proline-rich AMPs, the gloverins, appears to exert a similar mechanism of inhibition of outer-membrane protein synthesis.[162] Expression and evolution of four *B. mori* gloverin genes have been investigated,[163,164] and gloverins have been studied in several other lepidopteran species,[16,165–168] but no homologs have been identified to date in insects from other orders. A family of proline-rich AMPs called lebocins has been characterized in lepidopterans.[16,147,169–173] A somewhat puzzling aspect of this

family is that the 3.5 kDa active lebocin peptide is processed from a larger precursor and in some members of the family, the amino-terminal pro-region of the protein is conserved in sequence, but the carboxyl-terminal sequence corresponding to the original antimicrobial peptide is not, suggesting that the pro-region may have a function not yet discovered.

AMPs stabilized by intramolecular disulfide bonds are widely distributed in insects. Those with three or four disulfide bonds are commonly referred to as insect defensins because of overall structural similarities to mammalian α and β defensins.[174] Insect defensins can be grouped into peptides with an α-helix/β-sheet mixed structure and peptides forming triple-stranded antiparallel β-sheets. Defensin-like AMPs with antibacterial and antifungal activities from several lepidopteran species have been investigated.[175–180] Two cysteine-rich defensin-like peptides from *G. mellonella* specifically inhibit growth of filamentous fungi.[179,180] A group of atypical defensin-like peptide named x-tox identified in *G. mellonella* and two *Spodoptera* species is characterized by imperfectly conserved tandem repeats of cysteine-stabilized αβ motifs, the structural scaffold characteristic of invertebrate defensins and scorpion toxins.[181–183] They are induced upon activation of the immune system but lack detectable antimicrobial activity, suggesting that they may have an immune function yet to be discovered.

EXTRACELLUAR AND INTRACELLULAR SIGNAL TRANSDUCTION STIMULATING ANTIMICROBIAL PEPTIDE SYNTHESIS

Genetic investigations in *Drosophila* have revealed three major immune signaling pathways (Toll, Imd-JNK and JAK-STAT) that are conserved in mammals.[184] Genomic analyses suggest similar pathways exist in other holometabolous insects, including *B. mori*.[13] Additional bioinformatic and experimental evidence described below supports the existence of functional Toll and Imd pathways in lepidopterans.

There are fourteen Toll-like receptor genes in the silkworm genome: six in group A with *Drosophila* Toll and eight in group B with *Drosophila* 18-wheeler.[13] *BmToll* and *BmToll2* are group B genes highly expressed in ovary and their transcripts become more abundant in fat body after injection of microorganisms,[185] suggesting possible involvement in embryonic development and immune response. *BmToll3*, *BmToll4*, *BmToll9* and *BmToll10* mRNA levels in fat body also increase after injection of some microbes. In *M. sexta*, a Toll-like receptor is present in hemocytes, fat body, epidermis, midgut and Malpighian tubules.[186]. Its mRNA level increased in hemocytes, but not in fat body, after injection of microorganisms.

Spätzle, the ligand which activates Toll, has been identified and functionally characterized in *B. mori* and *M. sexta*.[187,188] Spätzle is synthesized as an inactive precursor, proSpätzle, which is secreted as a disulfide-linked homodimer into the hemolymph and requires proteolytic processing to form the active Toll ligand. Expression of proSpätzle is significantly greater in *M. sexta* hemocytes than in fat body.[188] In *B. mori*, expression was detected primarily in fat body and midgut, but hemocytes were not tested.[187] *B. mori* and *M. sexta* proSpätzle proteins are only ~20% identical to *Drosophila* proSpätzle, but have slightly greater similarity (~26% identity) in the carboxyl-terminal 108 residues corresponding to the active form of *Drosophila* Spätzle known to bind to Toll. Recombinant

B. mori and *M. sexta* Spätzle were active when injected into larvae, inducing fat body expression of attacins, cecropins, gloverin, moricin and lebocin in *B. mori*[187] and attacin, cecropin, moricin and hemolin in *M. sexta*, with corresponding strong induction of plasma antimicrobial activity.[188] Howevever, injection of proSpätzle had little effect, indicating the need for proteolytic activation of this cytokine in response to infection.

Hemolymph proteinase-8 (HP8) is a clip-domain proteinase demonstrated to activate proSpätzle in *M. sexta* by specific cleavage to produce the carboxyl-terminal 108 residue fragment, as a disulfide-linked homodimer.[188] The *Drosophila* proteinases most similar to HP8 are Easter and Spätzle processing enzyme, both of which function to activate proSpätzle.[189] Injection of active HP8 into larvae stimulates expression of attacin, cecropin, gloverin and moricin and elevates plasma antibacterial activity, consistent with a role for HP8 as an activator of proSpätzle.[189] HP8 is present in plasma as a zymogen, proHP8 and is activated by another clip-domain proteinase, HP6, an apparent ortholog of *Drosophila* Persephone. Injection of recombinant HP6 also promoted expression of antimicrobial peptides in larvae.[189] ProHP6 is activated in plasma exposed to bacteria or the β-1,3-glucan curdlan, but a hemolymph proteinase responsible for activation of HP6 has not been identified yet. It is apparent that recognition of microbial pattern molecules triggers activation of a proteinase cascade to generate the cytokine Spätzle, leading to expression of a suite of antimicrobial peptides as an innate immune response in this moth (Fig. 1).

Two rel-family transcription factors participate in immunity-related gene expression in *B. mori*.[190–192] *BmRel* encodes, via alternative splicing, RelA and RelB, which are orthologous to *Drosophila* Dorsal. *BmRelish* also encodes two splicing isoforms, Relish1 and Relish2. RelA activates the lebocin-4 gene strongly and an attacin gene weakly.[190] RelB, lacking the first 52 residues of RelA, activates the attacin gene strongly and other genes to a lesser extent. The Rel homology domain in RelA and RelB binds specifically to κB sites in attacin and lebocin-4 genes. In transgenic silkworms whose *BmRel* expression is knocked down, expression of antibacterial peptide genes fails to be induced by *M. luteus*. Knockdown of *BmRelish* expression abolishes antimicrobial peptide production elicited by *E. coli*.[191] Intact Relish1 and Relish2 do not activate promoters of *B. mori* attacin, cecropin B1, lebocin-3 and lebocin-4 genes. Removal of the ankyrin repeats in Relish1 is necessary for its transcriptional activation of antibacterial peptide genes. However, Relish2, which lacks the repeats and the transactivation domain, serves as a dominant negative factor to suppress the function of active Relish1. Relish1 binds to the κB sites in attacin and cecropin B1 genes, while the sites for activating lebocin-4 promotor differ between Relish1 active form and RelA. *Meso*-diaminopimelic acid- and Lys-type peptidoglycans can stimulate differential expression of antimicrobial peptide genes in the silkworm, which is affected by the level, binding affinity and transactivation activity of Relishes and Rels.[192] Relish1 is inhibited by its own ankyrin repeats and RelA and RelB are negatively regulated by *B. mori* Cactus.[193] Cactus interacts with the DNA-binding domain in the Rels but not with Relish1 or Relish2. Taken together, these data strongly suggest that the Toll and Imd pathways are functional in the silkworm to regulate immunity-related gene expression.

PROPHENOLOXIDASE ACTIVATION SYSTEM

PO-catalyzed quinone and melanin formation is a universal response in arthropods for killing and entrapping pathogens or parasites.[194,195] PO in insect hemolymph has tyrosinase-like activities, including *o*-hydroxylation of monophenols and oxidation of *o*-diphenols to quinones.[18] Tyrosine, DOPA and dopamine are substrates in insect hemolymph that contribute to PO-catalyzed quinone formation and subsequent melanin synthesis. Tyrosine hydroxylase and dopa decarboxylase are upregulated in fat body after injection of bacteria and probably contribute to provision of hemolymph dopamine for the innate immune response.[80,196–198] Oxidation of dopamine by PO leads to production of 5,6-dihydroxyindole, which has antimicrobial activity toward bacteria and fungi.[199] PO is produced as a zymogen that requires a specific proteolytic cleavage to gain activity. This regulatory mechanism protects the host insect from potentially harmful effects of the reactive chemicals produced by PO, as the enzyme is activated only when elicited by wounding or infection. Understanding of insect PO and its activation was pioneered through detailed biochemical investigations with *B. mori*,[11] and the *M. sexta* model system is providing new insights into lepidopteran PO function and regulation.[17,19]

ProPO from *B. mori* and *M. sexta* exists in plasma as a heterodimer of two related subunits, each ~80 kDa.[200,201] Insect proPO sequences are related to arthropod hemocyanins, and copper-binding motifs in the two groups of proteins are conserved.[202] ProPO is synthesized constitutively by oenocytoids.[197,201,203] ProPOs lack secretion signal peptides and are released from oenocytoids by lysis of the cells.[40,44] Activation of the proPO zymogen requires cleavage of a conserved Arg-Phe bond about 50 residues from the amino-terminus.[11,18] The crystal structure of the *M. sexta* proPO heterodimer suggests that the proteolysis between Arg51 and Phe52 induces a conformational change to dislodge a specific Phe residue in each subunit and open up the active site for substrate binding.[204] The active site contains a canonical type-3 di-nuclear copper center, with each copper ion coordinated by three conserved His residues. Glu395 of the subunit-2 may act as a general base to deprotonate monophenols, a key step in the *o*-hydroxylation of tyrosine by PO.

Extracellular serine proteinase pathways have evolved in animals to stimulate rapid responses to tissue damage, pathogen invasion, or physiological cues.[205] A few eliciting molecules, via specific recognition and cascade amplification, lead to sequential proteolytic activation of a large number of pathway components within minutes. This type of proteinase pathway results in activation of proPO in response to infection. Many of the proteinases that function in such cascade pathways in arthropods contain a carboxyl-terminal serine proteinase domain similar trypsin or chymotrypsin and one or two amino-terminal clip domains, which have likely regulatory functions.[206] The proteinases from lepidopterans known to activate proPO and most of the proteinases upstream in the activation pathway are clip domain proteinases. Fifteen clip domain proteinase genes were identified in the *B. mori* genome,[13] and fourteen such enzymes are expressed in fat body or hemocytes of *M. sexta*.[207]

In lepidopterans, proPO activating proteinases (PAPs) have been well characterized in *M. sexta*[208–211] and *B. mori*.[212] They are present in hemolymph as zymogens at low

concentration in naïve larvae, and their expression in fat body is upregulated in response to injection of bacteria. *M. sexta* PAP1 contains a single clip domain, whereas PAP2 and PAP3 and *B. mori* proPO activating enzyme each contain two clip domains. The solution structure of the region of PAP2 containing the dual clip domains suggests a potential proPO-binding site, a bacteria-interacting region and a surface for activator/adaptor docking in each domain.[213] Purified *M. sexta* PAP1, PAP2 and PAP3 do not efficiently generate active PO activity, even after a significant amount of proPO is cleaved at Arg51, without the presence of protein cofactors from hemolymph, identified as serine proteinase homologs (SPHs). SPHs also contain clip domains but lack proteinase activity due to substitution of the active site Ser residue with Gly.[214,215] *M. sexta* SPH1 and SPH2 also require proteolytic processing to gain function, which leads to their assembly into the active, high M_r cofactor required in the reaction with proPO and PAP to generate high levels of PO activity.[215,216] This interesting interaction, which does not seem to be required for the silkworm proPO activating enzyme,[212] is not well understood and requires further investigation.

The *M. sexta* proPO activation system includes at least four other serine proteinases (Fig. 1). An initiatiing hemolymph proteinase (HP14) contains five low-density lipoprotein receptor class A repeats, one Sushi domain, one Wonton domain and one proteinase catalytic domain.[217] Adding recombinant proHP14 to larval plasma greatly enhances proPO activation in response to *M. luteus*. The HP14 proenzyme, with its first domain truncated, was isolated from plasma of larvae injected with bacteria.[99] After incubation with β-1,3-glucan and βGRP1 or βGRP2, the proHP14 was converted to a two-chain active form, which significantly enhanced plasma proPO activation. The activation of proHP14 results from an autoactivation cleavage after Leu387, occurring when proHP14 interacts with β-1,3-glucan and βGRP. Characterization of individual domains and truncation mutants of HP14 showed that the amino-terminal regulatory region of HP14 participates in the specific binding of microbial polysaccharides and βGRP1.[100] Proteins orthologous to *M. sexta* HP14 also function at the top of proteinase cascades in immune responses of *Drosophila* and a beetle, *Tenebrio molitor*.[218,219]

HP14 activates a clip domain proteinase HP21, which can then activate proPAP2 and proPAP3,[220,221] resulting in activation of proPO. ProPAP1, which differs from proPAP2 and proPAP3 in having only one clip domain, is activated by HP6.[189] HP6 also functions in the proSpätzle activation pathway,[188,189] providing cross-talk between these two immune cascades in *M. sexta*. Addition of active PAP1 to hemolymph stimulates the proteolytic activation of HP6, HP8, SPH1 and SPH2.[222] PAP1 directly activates proSPH2, but processing of the other precursors is probably indirect, depending on other plasma factors. Consequently, a minute amount of PAP1 added to plasma from naïve larvae stimulates a remarkably high level of PO activity in a short period of time, as a result of a positive feedback loop (Fig. 1). Some gaps in this pathway still need to be filled. To date, it is not clear which HP generates active HP6, leading to both PAP1-mediated melanization and HP8-mediated Toll pathway activation. The proteinase which activates SPH1 has not been identified and the possible involvement of HPs in Gram-negative bacteria-induced defense responses is not yet well understood.

INHIBITORY REGULATION OF HEMOLYMPH PROTEINASES BY SERPINS

Immune responses can produce molecules that are harmful to the host. Serine proteinases and the molecules they activate have potentially toxic effects. Proteinase inhibitors of different families can exist constitutively at relatively high levels in plasma of naïve insects and may also be produced in response to physiological or pathological stimuli.[223] Serpins are ~50 kDa proteins, many of which are irreversible inhibitors and key modulators of immune proteinase pathways.[224] Serpins occur as plasma proteins in vertebrates and invertebrates. They have been purified and studied by molecular cloning from lepidopteran insects including *B. mori*,[225,226] *M. sexta*,[7,227–232] *H. cunea*,[56] *Mythimna unipuncta*,[233] and *Mamestra configurata*.[234] The *B. mori* genome contains 34 serpin genes, which have been analyzed with regard to molecular evolution of this gene family.[13,235] Serpin biochemical and physiological functions in lepidopterans have been characterized most extensively in *M. sexta*.[17,236]

The *M. sexta* serpin-1gene encodes twelve protein isoforms, each having the same amino-terminal 336 residues and a variable region consisting of the carboxyl-terminal ~40 residues, including the reactive center loop that interacts with a serine proteinase during an inhibition reaction. The variable region is produced by mutually exclusive alternative splicing of twelve different versions of the ninth exon of the gene,[237,238] resulting in a group of serpin proteins with diverse inhibitory selectivity.[227] Serpin genes that employ alternative splicing at the same position to generate multiple serpin isoforms have been studied in other moth species. These include *B. mori* serpin genes 1 (3 isoforms) and 28 (4 isoforms),[235] *M. configurata* serpin-1 (9 isoforms),[239] and *Choristoneura fumiferana* serpin-1 (at least 4 isoforms).[240] This mechanism for expanding serpin functional diversity, first discovered in Lepidoptera, been observed in other insect orders and in nematodes.[241]

Physiological functions have been identified for a few of the *M. sexta* serpin-1 isoforms. Serpin-1A, -1E and -1J can inhibit HP8, and serpin-1J appears to be a physiologically relevant regulator of HP8 activity during immune responses[87] (An, Ragan, Kanost, unpublished results). Serpin-1J also inhibits all three PAPs to regulate proPO activation[210,216] (Jiang, unpublished data). Serpin-1I can inhibit HP14.[99] Serpin-1K was identified in hemolymph in a compex with a midgut chymotrypsin,[87] suggesting a potential role for serpin-1 proteins in protection from digestive proteinases that escape into the hemocoel.

A putative orthologous group of serpins including *M. sexta* serpin-3,[229] *B. mori* serpin-3,[235] and an *H. cunea* serpin[56] are synthesized in response to infection and form a clade with *Drosophila* serpin-27A and *Anopheles gambiae* and *Aedes aegypti* serpin-2, which have immune regulatory functions.[235] *M. sexta* serpin-3 contains a reactive site sequence (Asn-Lys-Phe-Gly) highly similar to the proteolytic activation site (Asn-Arg-Phe-Gly) in both proPO subunits. By mimicking these natural substrates, serpin-3 acts as an efficient inhibitor of all three PAPs.[229] *M. sexta* serpin-4 and serpin-5[230] are closely related to each other and form a clade with *B. mori* serpins 4, 5, 7, 8, 14, 31 and 32.[235] Serpin-4 suppresses proPO activation by inhibiting HPs upstream of the PAPs, such as HP1, HP6 and HP21, while serpin-5 forms complexes with HP1 and HP6.[231] *M. sexta* and *B. mori* serpin-6 are apparent

orthologs. *M. sexta* serpin-6 can inhibit PAP3 to block proPO activation and it also inhibits HP8 to potentially regulate the Toll pathway.[242]

LEPIDOPTERAN IMMUNE RESPONSES TO DIFFERENT TYPES OF INFECTION

Herbivorous lepidopteran caterpillars consume enormous amounts of plant diet and are capable of increasing their body weight up to 20% per day. Plant leaves harbor microbial communities, which enter the alimentary canal with the ingested food. The midgut of caterpillars can sense bacterial contamination of the diet and trigger immune responses, which are accompanied with life history tradeoffs.[243,244] Bacteria can also naturally enter and infect lepidopterans through wounds. Phagocytosis by granular hemocytes or plasmatocytes, depending on the species is probably the earliest response,[245] and when numbers of bacteria are relatively low, can efficiently clear infection, particularly with bacteria of low virulence. Larger numbers of bacteria lead to hemocyte aggregation and formation of hemocyte nodules, probably aided by plasma pattern recognition proteins that agglutinate bacteria. Activation of lepidopteran hemocytes to become adhesive involves cytokines from the ENF family[245] and eicosanoid signaling.[42] Hemocyte nodules often become melanized, as products of PO polymerize to form a melanin coat around the aggregated hemocytes and bacteria. This response also generates quinones and reactive oxygen species that may help to kill the entrapped bacteria. The humoral response, synthesis of antibacterial peptides, occurs more slowly than the initial hemocyte response, requiring several hours before significant concentrations of antimicrobial molecules accumulate. This broad-spectrum antibacterial activity, comprised of a mixture of different antibacterial peptides, is an effective protective response that can last up to a few days.

Entomopathogenic fungi can invade insect hosts directly via their sclerotized chitinous integument. Penetration and lateral growth within the inner part of the integument is achieved by joint action of physical pressure and secreted enzymes among which proteinases play a predominant role.[246] Most, if not all, entomopathogenic fungi develop in the hemocoel as cells known as protoplasts or hyphal bodies, which lack a fully developed cell wall. The absence of typical fungal cell wall components such as β-1,3-glucan may allow these fungi to evade the host immune surveillance. However, hyphal bodies of the entomopathogenic fungus *Metarhizium anisopliae* are ingested by plasmatocytes in *G. mellonella* during an early phase of infection,[247] even though they lack β-1,3-glucan on their surface. Ingested hyphal bodies are not killed, but propagate and grow within phagocytic vacuoles of the plasmatocytes, which are likely occupied as a vehicle for dispersal within the hemocoel. Survival of hyphal bodies within the hemocytes as well as overcoming of multicellular encapsulation have been attributed to fungal secondary metabolites (toxins), such as destruxins and cyclosporins which suppress cellular and humoral responses within the infected hosts.[248,249] Similar to bacteria, fungal cells are recognized, phagocytosed or, if too large or too numerous, encapasulated by hemocytes in the hemcoel of Lepidoptera.[250] Eicosanoids have been implicated as mediators in cellular antifungal defense.[251] The lepidopteran antifungal response also encompasses proPO activation, production of reactive oxygen species,[252] and synthesis of potent antifungal peptides including cecropins[253] and

gallerimycin.[254] However, the humoral responses upon infection with parasitic fungi such as *Beauveria bassiana* is different from that observed after challenge with bacteria.[255]

Larger eukaryotic parasites such as nematodes and parasitic wasps provoke hemocytic encapsulation and melanization,[245] but little is understood about molecular mechanisms for recognizing such parasites as foreign.[256] Successful parasites are able to disrupt or suppress the host insect's immune response. Entomopathogenic nematodes have a mutualistic relationship with virulent bacterial pathogens of insects, in which the bacteria produce virulence factors that disable cellular and humoral immune responses of their insect host.[257] Parasitoid wasps that use lepidopteran larvae as hosts inject venom and accessory fluid components that disrupt the host immune system. In braconid and ichneumonid parasitoid wasps, this adaptation includes the injection of polydnaviruses, which infect host hemocytes and express immunity-disrupting proteins, but do not replicate.[258] Among these are gene products which cause apoptosis of hemocytes, disrupt signal transduction pathways in the humoral immune response and block melanization.[259–261]

Some aspects of immunity to viral infection in lepidopterans are now becoming understood.[262] Baculoviruses are the most commonly studied viral pathogens of these insects. These viruses enter the larva by first infecting the gut epithelial cells. A response that can protect caterpillars from these infections is apoptosis of infected midgut cells. Infected cells die before viral replication can be completed, thus preventing spread of the virus to other cells or tissues.[263] Baculoviruses encode gene products that inhibit caspases responsible for initiating apoptosis, allowing the infection cycle to proceed.[263] Hemocytic encapsulation of infected tracheal cells is another immune response to baculoviruses that has been observed in lepidopterans.[262] In addition, hemolymph PO is correlated with virucidal activity toward baculoviruses,[264] and thus, the proPO activation cascade may help protect against baculoviral infection.

CONCLUSION

Lepidopteran insects have some important advantages as model systems for immunological research, including a depth of knowledge developed so far and the availability of large hemolymph samples from individual insects for studies of hemocytes and plasma proteins. With the recent exception of *B. mori*, studies on moths and butterflies have been hampered by a lack of genomic information, which would facilitate proteomics investigations and also lead to more ready identification of candidate genes for experimental study. This situation is likely to change dramatically in the next few years, as it is anticipated that genome sequences for several additional lepidopteran species will soon become available. Transgenic technology for silkworms is now well developed and may yield new fundamental information on immunity and perhaps strains with improved disease resistance. Furthermore, more complete understanding of lepidopteran immune responses could lead to future developments of enhanced strategies for regulating insect pest populations through use of specific pathogens and parasites.

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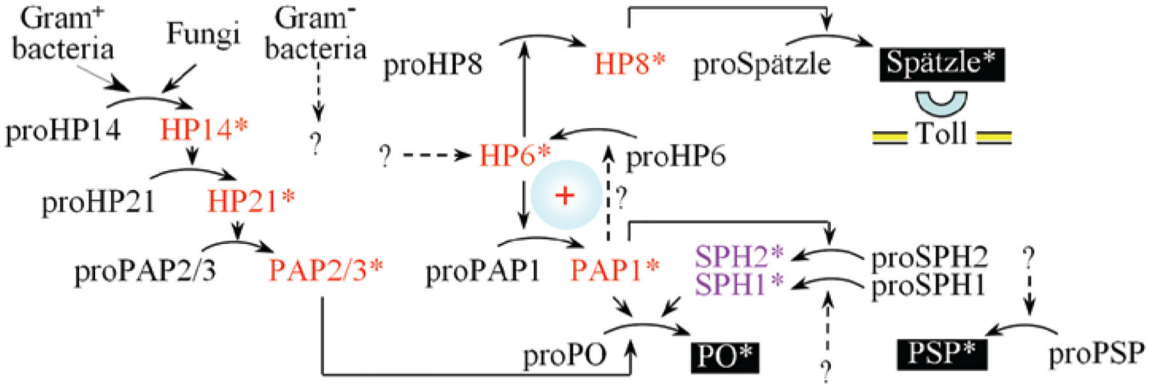

Figure 1.

A current model of the hemolymph proteinase system in *M. sexta* larvae. An initiation proteinase precursor, proHP14, is autoactivated in response to Gram-positive bacterial or fungal infection. HP14 activates proHP21; HP21 activates proPAP2 or proPAP3; PAP2 or PAP3 then cleaves proPO to form active PO in the presence of SPH1 and SPH2. Activation of proPO can also be catalyzed by PAP1 when the high M_r SPH complex is present simultaneously. PAP1 also activates proSPH2 directly and can indirectly lead to proHP6 activation. HP6, whose direct activator is unknown, cleaves proPAP1 and proHP8. PAP1 and HP6 form a positive feedback loop, in which PAP1 indirectly stimulates activation of HP6. HP8 activates Spätzle to induce antimicrobial peptide synthesis via Toll receptor. Active proteins, including HPs, PAPs, SPHs, PO, Spätzle and plasmatocyte-spreading peptide (PSP), are labeled "*" and unknown HPs are marked with "?".